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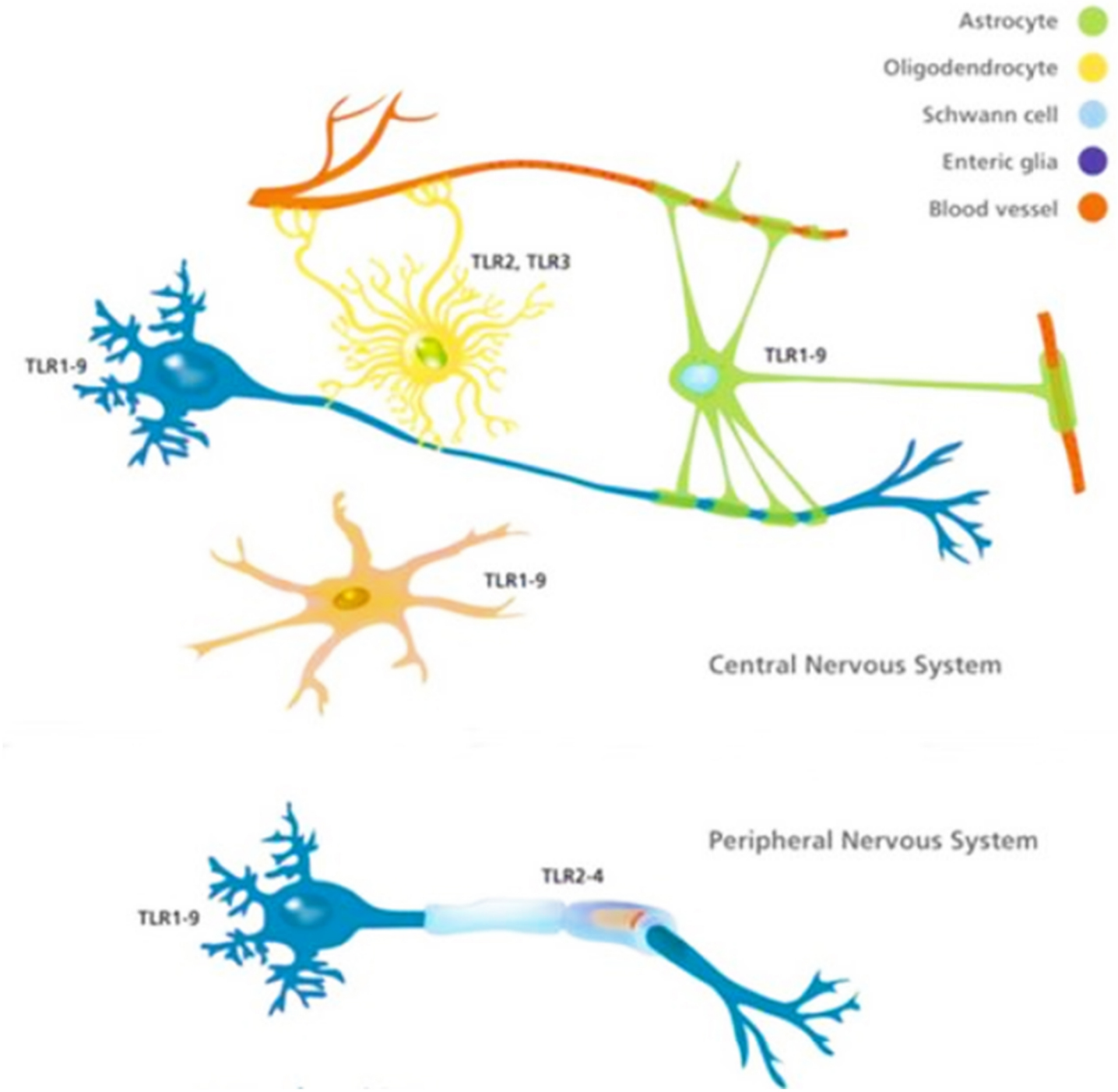

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Neuroimmunology and Neuroinflammation

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Guidelines

China guidelines for the diagnosis and treatment of myasthenia gravis

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Dr. Li is the Director of Department of Neurology in Tang Du Hospital of the Fourth Military Medical University. He graduated from the Fourth Military Medical University in 1985. He has been engaged in the prevention and treatment of myasthenia gravis in Department of Neurosciences in University of Oxford and Case Western Reserve University.

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Being dedicated in researches of clinical Neurology, Dr. Li is proficient in neuroimmunology diseases such as the prevention and treatment of myasthenia gravis. Dr. Li had 9 research projects supported by Natural Science Foundation of China (NSFC), and 1 research project supported by Innovative Research Team Foundation of Ministry of Education. He has published more than 200 articles, 40 of which were published in journals indexed by SCI.

Myasthenia gravis (MG) is neuromuscular disorder induced neurotransmission defects at the neuromuscular junctions. MG is an autoimmune disease in which the autologous immune system, including the corresponding antibodies, immune cells and complement systems, attacks the cholinergic receptor (AChRs) of the postsynaptic membrane, resulting in weakness of skeletal muscle. A rare portion of MG cases is mediated with antibodies specific to muscle specific kinase (MuSK) and low-density lipoprotein receptor related protein 4 (LRP4). The major clinical symptoms of MG are presented as weakness of skeletal muscle, fatigue prone, which are worsened after exercise. Adequate rest and treatment with cholinesterase inhibitors could significantly relieve and reduce the symptoms. The average onset rate is about 8-20 cases per 100,000 people.[1] MG is not age specific but there is a higher onset rate in females than males at the population before 40 of age. Such gender preference is reversed after 50-year-old. At the age category of 40-50, there is no gender preference.

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CLINICAL SYMPTOMS AND CLASSIFICATIONS

Clinical symptoms

Systemic skeletal muscle may be affected. However, at the early stage of MG, there may be firstly the muscle weakness of extraocular, throat and limbs. Seemingly, the skeletal muscles innervated by the cranial nerves are more susceptible than those innervated by spinal nerves. The weakness of skeletal muscles initiates at a group of muscles first and gradually spreads to other groups of muscles and eventually the whole system. For some patients of MG, they may rapidly experience weakness of all skeletal muscles and even myasthenic crisis.

The skeletal muscle weakness of MG is volatile with ease of fatigue, which are worsening towards the end of the day. The weakness and fatigability is progressively worsening during physical activity,

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which is improved after adequate rest. The initial symptom is the ptosis either symmetrically or asymmetrically and diplopia which are observed in more than 80% of patients.[2] In certain cases, alternative or bilaterial ptosis and nystagmus are also observed. Patients suffer also from lagophthalmos. However, pupil size remains normal.

Due to the weakness of the muscles involved in swallowing, there is dysphagia, difficulty of chewing and velopharyngeal insufficiency. For the symptoms of vocality, patients of MG have dysarthria and hypophonia, some of who may also have nasality. Weakness of facial muscle leads to hanging jaw sign, shallow nasolabial fold, leaky cheek blowing, snarling expression when smiling and sleepy or sad expression. Weakness of cervical muscle, patients could not hold their upright easily. Although it is usually not the primary symptoms of MG, limb movement is highly affected spreading from the proximal ends. In certain cases, the respiratory muscle is under myasthenic crisis which leads to dyspnea. Assisted ventilation is necessarily needed to sustain life.[3,4,5]

Classification: modified Osserman scale

Class I: ptosis and diplopia without other muscle weakness elsewhere for 2 years.

Class II: generalized symptoms with more than one set of weak muscle: (1) mild generalized form; Weakness in limbs with or without ocular signs, but without prominent bulbar signs. Patients could live independently; (2) moderate generalized or faciopharyngeal form; symptoms as in II A but with bulbar signs. Patients could live independently.

Class III: severe acute generalized form; acute onset and rapid development. Faciopharyngeal symptoms were observed in the first few weeks to months followed by respiratory insufficiency with or without ocular signs. Patients could live independently.

Class IV: severe chronic generalized form; unapparent signs at early stage due to the slow pathological progress. All symptoms stated in Class I, II and III are developing within 2 years.

Class V: muscle atrophy form; severe development with also muscle atrophy within 6 months.

EXAMINATION

Methyl sulfate neostigmine test

Intramuscular administration of 1.0-1.5 mg methyl sulfate neostigmine for adult. Atropine (0.5 mg) could be applied intramuscularly if there are any muscarinic cholinergic receptor-mediated side effects. For children, neostigmine should be reduced to 0.02-0.03 mg/kg, while total amount should not be more than 1.0 mg. Test should be performed at the muscle with significant symptoms and referred to the clinical absolute score for MG. The baseline of muscle tone should be firstly recorded, which is repeated every 10 min for 1 h. The relative score, as the diagnostic value, should hence be pooled by the absolute score of the trial with most significant improvement according to the following formula:

Relative score = (Baseline-absolute score of each trial)/Baseline × 100%

There is usually less than 25% of negative diagnosis while more than 60% for positive diagnosis, and 25-60% are as suspicious positive cases. Further tests are needed to confirm the negative diagnosis.

Electromyography

Repetitive nerve stimulation

Repetitive nerve stimulation (RNS) is an electrical stimulation to nerves, such as facial, accessory, axillary and ulnar nerves, for MG diagnosis, with repetitive and high-powered low frequency (2-5 Hz) signal. Compound muscle action potential (CMAP) over the testing muscles will be recorded.

The duration of stimulation is about 3 s. The decrement of CMAP of MG is measured by comparing the CMAP value of the fourth or fifth stimuli to that of the first stimulus. Diagnosis will be concluded as positive when there is more than 10% reduction. MG patients on acetylcholinesterase inhibitor medication should not receive this test until 12-18 h of washing out. For diagnosing presynaptic lesions, frequency of RNS should be increased to 10-20 Hz. Increment with more than 100% should be classified as abnormal.

Single fiber EMG

Single fiber EMG (SFEMG) is to measure the variable latency of the single axon innervation to the muscle fibers, known as Jitter. The variable latency is usually about 15-35 s, of which more than 55 ms will be classified as increased variability of latency. Two or more variable latency in every 20 Jitters of a single set of muscle will be classified as abnormal. Any block during SFEMG should also be classified as abnormal. Despite of the significant sensitivity, SFEMG is not specific for MG, which is mainly for Class I MG and those cases without positive outcomes in RNS test. Furthermore, SFEMG is not affected by any acetylcholinesterase inhibitors.

Serum antibody tests

AChR

For diagnosing MG with the specific antibodies, positive results could be detected in about 50-60% patients with ptosis and other ocular sign, while about 85-90% patients with generalized sign would have positive outcomes. Based on also the medical history of muscle weakness, positive diagnosis is enough to confirm MG. Further tests are needed if it is a negative outcome.

Anti-MuSK

Anti-MuSK could be detected in some patients with generalized sign of muscle weakness who have negative outcomes in the test of AChR antibody, while other patients may present positive outcomes of low-density LRP-4 antibody or other antibodies without known antigens at the neuromuscular junctions. Negative outcomes could be due to the low levels or weak affinity of these antibodies, resulting in undetectable outcome. It should also be noted that the ratio of anti-MuSK positive result in Western population is usually higher that that in Asian population.

Anti-striated muscle antibodies

Anti-striated muscle antibodies include anti-titin, anti-RyR and *etc.* This type of antibodies is usually detected in those MG patients with severe signs, desensitization of conventional therapies of MG and with also thymoma. This test is not for MG diagnosis but it strong indicates the opportunity of thymoma and other related transformations.

Thymus imaging

There are approximately 20-25% MG patients suffer also from thymic tumors while 80% have abnormal condition of thymus. Amongst the MG patients with thymic tumors, 20-25% of them present MG symptoms.[7] Ninety-four percent thymic tumors could be positive diagnosed by mediastinal CT. However, some cases are only diagnosed by advanced CT scan or MRIs.

DIAGNOSIS

Bases of diagnosis

Clinical symptoms

There is patchy distribution of weakness on certain particular striated muscle, exhibiting volatility and ease of fatigue. These MG symptoms are usually worsened across the day and after activities, which would be relieved after rest. In majority, ocular muscle is the common and first victim.

Pharmacological response

Positive outcome in neostigmine test.

EMG tests

Ten percent decrement of CMAP and 2 or more variable latency (> 55 ms) in SFEMG with or without any blocks.

Antibody tests

Test of anti-AChR in blood sample of generalized form of MG is positive. Anti-MuSK and LRP-4 would only be detected in some rare cases of MG.

With all clinical symptoms, and positive outcomes of pharmacological and electrophysiological tests, it can be diagnosed as MG. When it is available, blood test of anti-AChR or other related antibodies could be adopted for further confirmation. Furthermore, other unknown diseases may interfere the diagnosis.

Differential diagnoses

Ocular MG

Miller-Fisher syndrome: it is a derivative of Guillain-Barré syndrome, which is with acute paralysis of extraocular muscle, ataxia and loss of tendon reflexes. EMG results indicate there is delay of neurotransmission. Furthermore, in the analysis of cerebrospinal fluid, there is protein-cell separation. In some cases, GQ1b antibody is detectable.

Chronic progressive external ophthalmoplegia (CPEO): CPEO is one of the mitochondrial myopathies, patients of which experienced symmetrical, bilateral and progressive ptosis, paralysis of extraocular muscle, myogenic lesions and lactic acidosis. Some patients may experience also weakness of proximal limbs and delay of peripheral nerve transmission. Muscle biopsy and gene tests are needed for further confirmation.

Oculopharyngeal muscular dystrophy (OPMD): OPMD is a progressive muscular dystrophy, the patients of which experience progressive ptosis and weakness of the extraocular muscles. There is also a slight elevation of the serum level of creatine kinase. EMG diagnosis of OPMD shows myogenic lesion. Muscle biopsy and gene tests are needed for further confirmation.

Orbital Lesions: this condition could be due to orbital tumors, abscess and inflammatory pseudotumors. Patients experienced paralysis of extraocular muscle, conjunctival hyperemia, exophthalmos and edema in eyelids. This could be confirmed by orbital MRI, CT and ultrasonic scans.

Graves' disease: this is a thyroid-related autoimmune disease, patients of which experience hyperthyroidism or hypothyroidism, eyelid lag and weakness of extraocular muscle. In orbital CT scan, extraocular muscle is swelling. There is positive detection of TSH receptor antibody, namely TRAb.

Meige syndrome: this is an extrapyramidal disorder, patients of which experience unilateral or bilateral blepharospasm, reduction of eye fission, non-rhythmic tonic spasms at face, jaw and tongue. Dopamine receptors antagonists or local administration of type A botulinum toxin can improve these symptoms.

Generalized MG

Guillain-Barré syndrome: this is an immune-mediated acute inflammatory peripheral neuropathy, patients of which experience flaccid and weakness of limbs, reduction or loss of tendon reflexes. By EMG, the motor neuronal function presents as increment of conduction latency, slower innervation velocity, blockade and discrete and abnormal waveform.

Chronic inflammatory demyelinating polyneuropathy (CIDP): CIDP is an immune-mediated disorder of the peripheral nervous system, the patients of which experience flaccid and weakness of extremities, hypoesthesia, reduction or loss of deep tendon reflexes. There is a reduction of conduction velocities in motor and sensory neurons, abnormal and block waveform. There is also protein-cell separation in CSF. Diagnosis could be confirmed by biopsy of PNS tissue.

Lambert-Eaton syndrome: this is an autoimmune disorder mediated with the antibodies attacking the presynaptic voltage-gated calcium channels, the patients of which experience muscle weakness and fatigue of proximal limbs, muscle tone enhancement after brief activity but weakness after sustain activities, autonomic nervous system sign, such as dry mouth, orthostatic hypotension, slow gastrointestinal motility, pupil dilation, *etc*. In EMG test, low frequency repetitive stimulation induces small amplitudes of CMAP but increased amplitudes in high frequency repetitive stimulation. This disorder commonly happens with certain malignancies, particularly small cell lung cancer.

Progressive spinal muscular atrophy (PSMA): PSMA is a rare type of motor neuron disease, patients of which experience flaccid, weakness and atrophy of extremities, muscle fasciculations, reduction or loss of deep tendon reflexes. EMG result shows that there is denervation. At resting stage, there is fibrillating potentials and positive peak waves, and even fasciculation potentials in certain cases. Duration of potentials of motor units is broaden, volatility and number of multiphase wave increase during mild muscle contraction. However, the potentials decrease in amplitude and present as single or mixed phases during maximal contraction. Moreover, the conduction velocity of sensory neuron is normal.

Polymyositis: this is a multifactorial inflammation in interstitial area of skeletal muscle, patients of which experience progressive flaccid and weakness of muscle and pain. EMG indicates myogenic lesion and cardial level of creatine kinase is significantly elevated. This disorder could be confirmed by biopsy, which is curable by corticosteroids.

Botulism: botulinum toxin damages the presynaptic membrane of neuromuscular junctions, leading to extraocular muscle paralysis, dilation of pupil but retarded light reflex, weaknesses in swallowing and chewing, dysarthria and symmetrical flaccid paralysis of limbs. If respiratory muscles are affected, there could be Lambert-Eaton myasthenic syndrome liked autonomous signs. There is no significant decrement of EMG in the low frequency repetitive nerve stimulation. However, there is increment of amplitude or no changes in high frequency repetitive nerve stimulation, which is dependent on the severity of poisoning. Diagnosis could be confirmed by isolating and identifying the botulinum toxin in the consumed food.

Metabolic myopathy: This is a disorder led by the compromised muscle metabolism, lipid metabolism or lesion of mitochrondria, patients of which experience flaccid and weakness of limbs and fatigue, reduction or loss of deep tendon reflexes. There is myogenic lesion in EMG and normal or slight elevation of cardiac enzymes levels. Diagnosis could be confirmed by muscle biopsy and gene tests.

TREATMENT OF MG

Therapeutic approaches

Cholinesterase inhibitors

Such inhibitors are the first-line drugs for MG

treatment, which mainly ameliorate the clinical symptoms, particularly for the initial treatment of newly diagnosed patients of MG and as a single agent for long-term treatment of cases of mild MG,[8] although it is not recommended. Dose should be individualized and combined with other immunosuppressive drugs. Pyridostigmine bromide is the most commonly used cholinesterase inhibitor for MG treatment. Side effects include nausea, diarrhea, stomach cramp, bradycardia and increase of oral and respiratory secretions. In China, the maximum oral dosage per day is 480 mg and three to four times.

Immunosuppressive drugs

Glucocorticoids: it is a potent anti-inflammatory and immunosuppressive agent, efficient in MG treatment with significant improvement in 70-80% cases.[9] The commonly used glucocorticoids for MG treatment include prednisone, methylprednisolone and dexamethasone. The indications are as followed: 0.5-1.0 mg/kg/day or 20 mg/day prednisone at morning. According to the glucocorticoid dose conversion, 5.0 mg prednisone is equal to 4 mg methylprednisolone and 0.75 mg dexamethasone. Dosage should be increased by 5.0 mg every three days till 60-80 mg. Improvement could be observed in 2 weeks, which will be significant at 6-8 weeks after treatment. For severe cases, with the adequate communication between physician and patients, patients can receive corticosteroid therapy under mechanical ventilation. Corticosteroid therapy will be achieved by continuous intravenous perfusion of 1,000 mg/day methylprednisolone for 3 days. Afterward, dose should be decreased to 500 mg/day for 2 days. During this, methylprednisolone could be replaced by dexamethasone (10-20 mg/day) for 1 week. After corticosteroid therapy, patients should take prednisone or methylprednisolone at morning as aforementioned. Dosage of prednisone and methylprednisolone should be fine adjusted or individualized according to patients' conditions. If MG conditions are improved, dosage could be gradually reduced after 4-16 weeks. Typically, prednisone could be reduced by 5-10 mg every 2-4 weeks, and then 5 mg every 4-8 weeks when dose is or lower than 20 mg. According to different cases, patients could receive the lowest optimal dose every other day. Too vigorous reduction of drug will worsen the MG conditions.

In adult generalized MG and certain ocular MG cases, glucocorticoids should be reduced or terminated if there are any fluctuation or aggravation. In order to provide an optimal therapy, it is recommended to co-administer other immunosuppressants, such as azathioprine, cyclosporine A or tacrolimus.

Methylprednisolone has a more rapid therapeutic effect in MG treatment than prednisone for 1.25 fold, as the former needs not to be activated in liver. Furthermore, methylprednisolone has a higher immuosuppressive effect (18 fold) than prednisone since the former has a higher affinity to corresponding receptors. Such property produces lesser side effect and steady concentration, which is more suitable for those MG patients with compromised hepatic function.

Intensive monitoring is necessary for treatment with glucocorticoids. There are approximately 40-50% MG patients experiencing transient aggravation and possibly myasthenic crisis. Thus, extra cautions are used for those with severe symptoms and higher risk of myasthenic crisis. It is also highly recommended to administer calcium and bisphosphonates agents for preventing osteoporosis and antacid drugs for preventing gastrointestinal complications. There is also chance to develop steroid myopathy. Chronic usage of glucocorticoids will increase appetite, body weight and central obesity, hypertension, high blood glucose, cataract, glaucoma, endocrine disorders, mental disorders, osteoporosis, osteonecrosis and other alimentary disorders.

Azathioprine: this is the first line agent for MG for both ocular and generalized forms. Azathioprine could also be co-prescribed with glucocortioid so that in short term, the dosage of glucocorticoid could be reduced. At the initial stage of treatment, glucocorticoid and azathioprine provide a better treatment than single use of glucocorticoid or azathioprine. For MG patients who are older than 3 year-old and at teenages, azathioprine could be co-prescribed when therapies with cholinesterase inhibitors and glucocorticoid are not desirable. Azathioprine should be administered in a low dose and gradually increased as this agent could elevate the hepatic enzyme activities and inhibit the marrow function. Effect will be seen 3 to 6 months after administration while the peak effect will reach after 1 to 2 years. About 70-90% MG patients are significantly improved after this treatment.

Instruction is as followed: 1-2 mg/kg/day for children and 2-3 mg/kg/day for adult; spilted into 2-3 times oral dose per day. Chronic usage is allowed until adverse effect and intelarance are seen. About 7-10 days after azathioprine administration, blood test and hepatic functions of patients should be monitored. Side effects includes specific flu symptoms, reduciton of white blood cells and plalete, alimentary symptoms, eaken hepatic function and loss of hair. Chronic users of azathioprine should take blood test every 2 weeks, tests of hepatic and renal functions every 4 weeks. If possible, gene screening of purine methyltransferase

deficient should be performed to reduce the risk of irrevesible marrow lesion.

Cyclosporin A: this is an immunosuppressant for generalized and ocular MG, which starts to effect 3-6 months after administration. This is mainly used when glucocorticoid and azathioprine are not effective. Cyclosporin A could also be used with glucocorticoid for improving the MG with a decreasing blood level of AchR antibody. Cyclosporin A could be used in long term with a similar effect as azathioprine but with lesser side effect. Usually cyclosporin A was taken in orally in 2-4 mg/kg/day. The blood level of cyclosporin A was monitored in order to adjust the dose. The main side effect includes hypertension, tremor, renal dysfunction, muscle ache, gingival hyperplasia and flu-like symptoms. Blood test, hepatic and renal functions are regularly checked every month.

Tacrolimus (FK-506): This is also a potent immunosuppressant used for those patients not susceptible to glucocorticoid and other immunosuppressant, particularly those RyR antibody positive. This is also used with glucocorticoid at the early stage of treatment to reduce the usage of glucocorticoid and corresponding side effect. The therapeutic effect of FK-506 onset rapidly and significant effect should be observed around 2 weeks after administration. FK-506 is usually prescribed at 3.0 mg/day for oral dose. Blood level of FK-506 should be monitored in order to adjust the dose. MG patients with rapid metabolism should receive large dose till a significant effect observed. Side effects include alimentary symptoms, numbness, tremor, headache, hypertension, hyperglycemia, hyperkalemia, hypomagnesimia and renal failure. If no significant side effect, FK-506 could be chronically used. Blood glucose, hepatic and renal functions should be monitored every month.

Cyclophosphamide: this agent will be used when other immunosuppressants fail to provide any therapeutic effect on to severe cases MG or MG with thymus tumor. Cyclophosphamide should also be administered with glucocorticoid, which could be reduced in dose 6-12 months after treatment started. For adult, 400-800 mg cyclophosphamide per week should be intravenously perfused. Patients could also receive 100 mg/day in two oral doses till the total amount reaches 10-20 g (for some cases, patients need 30 g). For children, 3-5 mg/kg/day (totally amount should not exceed 100 mg) in two oral doses was administered. When there is improvement of symptoms, dose could be reduced to 2 mg/kg/day. Extra caution should be paid for children patient receiving cyclophosphamide. Side effects includes reduction of white blood cells count, loss of hair, nausea, vomit, diarrhea, hemorrhagic cystitis and long term suppression of bone marrow mediated cancer risk.

Mycophenolate mofetil (MMF): MMF is not the first line agents for MG but it is also used with glucocorticoid at the early stage of treatment but not azathioprine. Usually, it will be administered at rate of 0.5-1 g and twice a day. When compared to azathioprine and cyclosporine, MMF is safer and with less hepatic and renal side effect. Common side effect includes alimentary symptoms, nausea, vomit and diarrhea. For MG patients taken MMF, their whole blood count will be performed once a week at the first month, twice a month at the second and third months and once a month after 3 months. If there is a reduction of neutrophils, patients should stop taking any MMF.

Anti-human CD20 monoclonal antibody (Rituximab): Rituximab is proven to be effective in treatment of autoimmune diseases.[10] In treatment of MG, rituximab is suitable for patients, particularly those with MuSK positive, who have no significant improvement in treatment of glucocorticoid and traditional immunosuppressants. As single agent of MG treatment, recommended dose for adult is in rate of 375 mg/m^2 (i.v.) once a week. The treatment course is 22 days and agent is totally administered for 4 times.

Treatment with rituximab should be performed with facility of resuscitation. When there are any respiratory symptoms or hypotension, patients receiving this treatment should be monitored for 24 hours. Treatment has to be terminated when there are any adverse effects such as dyspnea, bronchospasm and hypoxemia. Other side effects include fever, chills, bronchospasm, leukopenia, thrombocytopenia and progressive multifocal leukoencephalopathy. It is also crucial to monitor any syndromes of cytokines release.

During this treatment, hepatic and renal functions, blood and urine biochemistries have to be monitored regularly. Treatment should be immediately terminated if there is any immunosuppression mediated side effects. For patients with HBsAg positive and compromised hepatic function, nucleotides (NAs) should be administered 2-4 weeks before treatment.

Intravenous administration of g-globulin

This is for acute situation and pre-operative treatment for MG patients, usually combined with immunosuppressants and glucocorticoid.[11] g-globulin will be intravenously perfused at rate of

400 mg/kg/day for 5 days. Effect of this treatment will onset in 5-10 days and last for 2 months. As similar as the plasmapheresis discussed below, side effects are less but both procedures cannot be combined. For moderate and severe MG patients, repetitive treatment of this could not maximize the therapeutic effect. Side effects include headache, aceptic meningistis, flu signs and renal dysfunction.

Plasmapheresis

This is mainly for acute cases of MG, myathenic crisis and pre-operative treatment for thymectomy.[12] This is also used for cases without further improvement after chronic treatment with immunosuppressants. Plasmapheresis should be performed every other day in the first week, totally 3 times. If there is no significant improvement, procedure should be continued once a week for 5-7 weeks. Each treatment introduces 1,500 mL health human plasma and 500 mL 706 supplement. Significant effect will onset 2 days after the first or second treatment lasting for 1-2 months. Side effects include hypotension, low blood calcium, infection and hemorrhage. Plasmapheresis should be performed in aseptic environment. Termination should be used if there is any complication. MG patients with infection and receiving perfusion of g-globulin should not receive this procedure.

Thymectomy

It is crucial to perform thymectomy for the MG patients with thymus tumor, which could eliminate risks of invasion and proliferation.[7] Thymectomy could also improve the MG signs of patients. However, in certain cases, the MG condition would be worsened. For mild MG (Osserman class I), thymectomy could not have any improvement. However, for Osserman class II to IV, particularly those with AChR antibody positive, thymectomy provides a significant improvement. MG signs would be usually reduced 2-24 months after the operation and medication could be also reduced. Although some MG patients will recover totally after thymectomy, some will experience MG reoccurrence in a few years. Generally thymectomy is beneficial for MG with abnormal thymus glands. Such operation is suitable for patients older than 18 year-old. For severe cases with non-magliant thymus tumor, treatments, such as perfusion of g-globulin, will be firstly recommended than surgery when MG signs have been slightly improved, which could also prevent post-operation myathenic crisis.

Thymus radiotherapy

The sophistication of radiological techniques makes this as a popular therapy for MG.[13] This approach is suitable for those MG patients who experience invasive thymus hyperplasia, reoccurrence of MG and not sensitive to other medication. Daily treatment dose is 1 to 2 Gy and 5 times per week. The total amount is 50-60 Gy.

Table 1: Differential diagnoses of myasthenic and cholinergic crises

	Myasthenic crisis	Cholinergic crisis
Heart rate	Tachycardia	Bradycardia
Muscle	Weak	Weak and fasciculation
Pupil	Normal or dilated	Constricted
Skin	Faint and cold	Warm and flushing
Secretion	Normal	Increase
Neostigmine test	MG improved	MG aggravated weakness

MG: myasthenia gravis

Others

Respiratory muscle training and other strength training in mild case of MG could improve the muscle strength. It is highly recommended that patients should control weight and limit the daytime activity. Seasonal flu shot is also beneficial in therapy.

Therapies for different types of MG

Ocular MG: although it is more prevalent in children under 10 year old and adult above 40, this could be seen in any age groups. 80% patients of MG experience first with ocular MG which could be controlled by individualized doses of AChE inhibitors. For better treatment, AChE inhibitors could be combined with glucocorticoid and methylprednisolone. In recent review literatures, oral dose of glucocorticoid, e.g. prednisone, is better in treat of ocular MG than only AChE inhibitors and more effective in preventing the transformation to generalized forms of MG. However, randomized and blinded clinical trials are needed to confirm this. In order to have better treatment, it is also recommended to apply immunosuppressants and glucocorticoid. Thus, glucocorticoid induced side effect could be reduced.

Generalized form: as AChE inhibitors are not effective enough to control the MG symptoms, treatment should combine with glucocorticoid and other immunosuppressants, e.g. azathioprine, cyclosporine, tacrolimus and MMF. Some cases of generalized MG need methylprednisolone, 40-50% of which may be worsened during treatment and needed endotracheal intubation or tracheotomy. High dose of g-globulin could be used when methylprednisolone fails to provide any effect. Thymectomy should be performed early for those with abnormalities of thymus glands, such as thymus tumor and thymus hyperplasia.

Medication could usually be reduced after operation. For some cases, no more MG signs will be seen post-operation. For children, AChE inhibitors, glucocorticoid and g-globulin are beneficial for generalized form of MG. Otherwise, with cautions, patients could be treated by immunosuppressants and thymectomy.

MG crisis:[14,15] it is the compromised respiratory muscle leading to severe difficulty in breathing, which has to be supported by artificial respiration, such as positive pressure respiration, endotreacheal intubation and tracheotomy, and monitoring the oxygen saturation and partial pressure of carbon dioxide. MG crisis could be classified as in Table 1. For myasthenic crisis, dose of AChE could be increased within the safe window till there is any improvement. Overdose of AChE could be reversed by atropine or methylprednisolone. For some cases, it is also practical to apply high dose of g-globulin and plasmapheresis. For cholinergic crisis, treatment with AChE inhibitors should be reduced or terminated and should not resume and increase gradually until 5-7 days. Atropine or combined with methylprednisolone, plasmapheresis and g-globulin could also be adopted. Nowadays, AChE inhibitors should be limited at not more than 480 mg per day. Thus, cholinergic crisis is uncommon. If respiratory failure is found in blood gas analysis (in both type I and II), endotreacheal intubation and positive pressure respiration should be immediately applied. Artificial respiration of MG patients should have extra care to prevent lung infection and adjustment of the auxiliary breathing mode for earlier independent breathing.

MG at pregnancy:[16] it is still not very clear that how pregnancy affects MG. For most cases, pregnancy will not aggravate MG and affect the labor time and route. AChE inhibitors and glucocorticoid are relatively safe for fetuses but other immunosuppressants may affect the embryonic development which should be terminated if pregnant. Teratogenic drugs, e.g. methotrexate and MMF, should not be used. It is also recommended for MG patients to take caution of contraception.

MG with MuSK antibody positive: generally, AChE, glucocorticoid and immunosuppressants are not effective for MG with AChR antibody negative but MuSK positive. Up to date, there is no special and effective treatment for this type of MG. plasmapheresis could relieve the MG signs for short term. There is a case report that anti-CD20 monoclonal antibody is therapeutic potent to this type of MG.[9] Multiple thymectomy is also beneficial for this type of MG.

MG with other complications

Some MG patients could suffer from also other disorders, such as Graves diseases, polymyositis, multiple sclerosis, Sjogren's syndrome, periodic paralysis, Hashimoto 's disease, rheumatoid arthritis, systemic lupus erythematosus, Guillain-Barré syndrome, aplastic anemia. In some MG cases, cardiac muscle is also the victim, presenting abnormal EEG and arrhymia. Therefore, it is recommended to pay also attention to such condtions other than MG.

Precautions for MG treatment

There are certain contraindication for MG patients, including steroids, antibiotics (e.g. Aminoglycoside), antifungal drugs (e.g. amphotericin), cardiovascular drugs (e.g. lidocaine, quinidine, β-blockers, verapamil and *etc.*), antiepileptic drugs (e.g. Phenytoin, ethosuximide), antipsychotics (e.g. chlorpromazine, lithium carbonate, diazepam, clonazepam), anesthesia (e.g. morphine and meperidine) and anti-rheumatic drugs (e.g. penicillamine and chloroquine).

It is also not recommended to do soapsuds enema. Plenty of rest, staying warm, steady emotion are also important for recovery from MG.

Prognosis

Ten-twenty percent of MG patients in ocular form will spontaneously heal, while 20-30% only experience extraocular MG. For the rest, more than 85% will gradually spread the signs to medulla oblongata and skeletal muscle, developing generalized form in 3 years. The pathogenesis of MG in about two-third of patients will develop to severe level within one year. 20% of MG patients will develop MG crisis within 1 year. MG signs and symptoms will be aggravated in certain conditions such as upper respiratory tract infection, diarrhea, thyroid disease, pregnancy, fever, trauma and medications affect the neuromuscular junctions.

Before the prevalent use of immunosuppressants for MG treatment, the mortality rate of MG is 30%. With also the development of mechanical ventilation and intensive care technique, nowadays the mortality (due to directly MG or indirectly other complication) decreases to below 5%.

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Review

The conflict on posttreatment Lyme disease syndrome: a clinical mini review

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ABSTRACT

Is *Borrelia burgdorferi* responsible for the persistence of symptoms after the standard successful course of antibiotics in Lyme disease patients? This highly controversial issue, concerning the underlying mechanism of posttreatment Lyme disease syndrome (PTLDS), still seems to be a matter of intense conflict of opinion. PTLDS is the manifestation of nonspecific symptoms including fatigue, musculoskeletal pain, dysesthesias, and neurocognitive deterioration after the standard antimicrobial therapy administered to patients suffering from Lyme disease. In this article, we review the conflicting views and published highlights of recent human studies regarding PTLDS.

Key words: Antibiotic therapy; duration of therapy; Lyme disease; nonspecific symptoms; posttreatment Lyme disease syndrome

INTRODUCTION

There is no fundamentally widely accepted definition of posttreatment Lyme disease syndrome (PTLDS). This has led to confusion and controversies and to a lack of data on its incidence, prevalence, and pathogenesis. The most accepted definition is that PTLDS is the manifestation of nonspecific signs and symptoms such as fatigue, muscle pain, arthropathy, neuropathy, and cognitive dysfunction after the standard course of antibiotics that are administered to patients between 10 and 28 days depending on disease stage and severity. It is expected that this syndrome persists for at least 6 months. Additionally, all indicated known diagnostic workup regarding neuroborreliosis has to be negative.[1,2] A sufficient amount of data shows that patients with PTLDS have reduced life functioning than those without the syndrome,[3] or even when compared to patients with other chronic diseases.[4] Intuitively, the presence of PTLDS after recommended treatment is associated with significantly increased health care costs.[5]

NOT TO TREAT PTLDS

The Infectious Diseases Society of America (IDSA) reported that Lyme disease is not always properly diagnosed or treated and that some patients may continue to experience prolonged Lyme disease symptoms even after an intense chemotherapeutic regimen. The diagnosis of so-called "chronic Lyme disease", implying an ongoing infection, is not supported by scientific evidence and the treatment based on long-term chemotherapy is not recommended. Standard courses of antibiotics, between 10 and 28 days depending on the manifestation of Lyme disease, have been proven effective to cure the infection. These chronic symptoms may be due to persisting inflammatory responses to bacterial debris by genetically predisposed individuals after the resolution of the infection, as well as due to joint damages caused by the initial infection.[1] Some already treated patients

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rarely develop a facial nerve palsy or meningitis.[1,2,6] Cranial neuritis, in most cases, appears to be benign, and it is attributed not to a persistent infection but to residual, irreversible neurologic damage. Conversely, if Lyme meningitis was developed shortly after the completion of a course of oral antimicrobial therapy, the patient undergoes another cycle of treatment with either ceftriaxone or with a similar parenteral antibiotic.[6] The presence of such symptoms during the first several weeks to months after treatment most often appears to be due to a slow resolution of the inflammatory process associated with a highly prolonged or disseminated *Borrelia burgdorferi* infection. However, there is no scientific evidence that *Borrelia burgdorferi* persists in such patients.[1,2] Another study on patients with refractory late Lyme arthritis showed that these symptoms may persist for several years, but the incidence and severity of the symptoms do decrease over time, and the estimated number of individuals who continue to have recurrences is reduced by 10-20% each year.[7]

The use of antibiotic regimen for a long time is not recommended, in fact, it does not improve patient outcome. Instead, it can also promote the development of drug-resistant infections. Valid placebo-controlled randomized trials do not support long-term treatment for Lyme disease and have failed to demonstrate any benefit over placebo. In fact, these randomized clinical studies have shown that approximately one-third of patients benefit from placebo.[8,9] Additionally, there is no clear evidence supporting the hypothesis that Lyme disease is a chronic, actively infectious disease requiring ongoing antibiotic therapy.[2,10,11]

TO TREAT PTLDS

In 2014, the International Lyme and Associated Diseases Society (ILADS) published its own treatment guidelines[12] for the management of Lyme disease patients, after adopting the GRADE scheme.[13] Among others, ILADS guidelines address the issue of antibiotic retreatment in patients with persistent symptoms. After performing an individualized risk-benefit assessment, the initiation of a 4-6 weeks antibiotic regimen is recommended in previously treated Lyme disease patients. This is then followed by a reassessment which will determine whether modifications or discontinuation of the treatment is necessary. Even longer treatments may be chosen.[12]

Furthermore, ILADS is critical in interpreting the results of the 4 randomized control trials (RCTs),[8,9,14] based on which the IDSA and other authorities support the idea that there is no infectious mechanism underlying PTLDS. The 4 RCTs did not provide any positive results after antibiotic re-administration suggesting that this retreatment was not specific nor sustainable. In addition, in some cases, retreatment was associated with adverse events.[8,9,14] By analyzing these conclusions, ILADS raises issues on the bias, precision, consistency, and generalization of the results. Therefore, it can be concluded that current evidence supports persistent infection, although other mechanisms may coexist. In addressing this issue, ILADS also suggests that the potential benefits of retreatment are sufficient to support those physicians who wish to treat but cannot mandate retreatment.[12]

In 2012, two critical analyses of the 4 RCTs[8,9,14] were published. A first biostatistical review concluded that all primary outcomes in Klempner[8] and Krupp.[9] trials, except for fatigue in the Krupp trial, were likely underpowered.[15] In the same year, a reappraisal of US clinical trials highlighted the limited generalization of the results and the reduced likelihood of identifying significant treatment effects. This specific study concludes that antibiotic retreatment is potentially beneficial at least in a fraction of the PTLDS group. Thus, the recommendation of not re-administering antimicrobials should be carefully reconsidered. Additionally, it suggests that immune dysregulation as a contributor to pathogenesis should be taken into account in future studies.[16]

Interestingly, brain abnormalities were detected in chronic Lyme patients using neuroimaging based on single photon emission computed tomography. The authors concluded that the use of antibiotics with intracellular activity resulted in an increased resolution or improvement of clinical symptoms detected by imaging in 70% of patients over a 1-2 years period.[17]

COMMENT

The consequences of the lack of a worldwide accepted definitive diagnosis and the lack of an established treatment regimen include poor patient health, discomfort, additional expensive diagnostic testing, lack of health care effectiveness, and deterioration of the doctor-patient relationship.[18] Currently, PTLDS is the paradigm of this scenario.

In this situation, three challenging questions need to be addressed by the scientific community: First, how do we precisely define PTLDS? Second, how do we diagnose PTLDS? And third, is PTLDS a fully treatable condition?

There is a common believe that in order to define PTLDS, an expert panel and subsequently a consensus report seems to be the best solution. To address the

second and third questions, we need to consider the basic principles of pathogenesis and pathophysiology.

Postinfectious autoimmunity vs. persistent spirochetal infection still represents an open question. The hypothesis is that PTLDS may be a result of chronic *Borrelia burgdorferi* infection in combination with other tick-borne coinfections, and the mechanisms of "stealth pathology" utilized by the Lyme spirochete in evading the host immune response establishing infection in diverse both have been reported.[19-21] Additionally, it has been suggested that *borrelia* wall-deficient forms and biofilm formation may play a role in chronic infection.[19] Biofilms are polysaccharide-based structures which protect bacteria and thus promote persistence while their contribution to chronic infection pathogenesis is yet to be evaluated. Further studies on the underlying mechanism in the biofilm process would potentially facilitate the development of antibiotics that may counteract this phenomenon.[19] While clinical testing for Lyme disease remains critical, the use of proteomics and more novel tests are necessary.[19-21] Recently, a human study focusing on Xenodiagnosis to detect *Borrelia burgdorferi* infection has been published showing promising results regarding pathogenesis and diagnosis.[22] Is this the future? Whenever any new diagnostic test is developed, it must be compared to existing diagnostic methods to ensure that it is comparable to specificity and sensitivity before it can be widely implemented.

Analysis of the cerebrospinal fluid (CSF) in PTLDS patients may represent a solution.[1,2,19] The CSF analysis in chronic Lyme encephalomyelitis, a different nosological entity of PTLDS, is constantly showing a mild hyperproteinuria and lymphocytic pleocytosis. In chronic Lyme encephalomyelitis, cerebral magnetic resonance imaging is usually abnormal, showing subcortical or brainstem multiple sclerosis-like, inflammatory lesions. Meningeal gadolinium enhancement is sometimes the only result.[23]

The corticosteroids in neuroborreliosis are not widely recommended. There are no prospective trials that have addressed this question. The need for corticosteroids arises frequently in patients with facial nerve palsy, as some guidelines recommend for treatment of idiopathic facial nerve palsy, but others do not recommend the use of corticosteroids.[2,24] In literature, it has been reported that patients with Lyme arthritis who received steroids are more difficult to cure;[2,25] of note, steroids may well have been used in these patients due to a probably more intense disease or relevant complications. Available recommendations regarding nonspecific neurological symptoms do not exist. Thus, their management has to be assessed according to the best medical practice.

CONCLUSION

Nowadays, there are valid reasons to opt for long-term antibiotic therapy. However, it is critical to focus on the well-designed clinical trials in order to evaluate if a therapeutic intervention has an actual, beneficial effect in contrast to a resolution of symptoms which might spontaneously occur over time. The need for additional research to determine safe and effective treatments must be widely recognized by the scientific community to resolve this long controversy.

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Case Report

Artery of Percheron occlusion: role of diffusion-weighted imaging in the early diagnosis

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ABSTRACT

Bilateral thalamic infarcts have a low frequency among different subtypes of strokes. Since it does not involve a particular vascular territory, it therefore usually involves the occlusion of the artery of Percheron (AOP). Here we report a 79-year-old right-handed Parkinsonian female patient, who was found unresponsive in bed. On examination, the patient was drowsy with a Glasgow Coma Score (GCS) of 10/15 (E2M5V3). She had absent doll's eye response with anisocoric pupils and intermittent vertical gaze palsy. Although the patient had no apparent motor deficits, she was in a state of persistent somnolence with memory impairment and lack of initiative. Diffusion-weighted magnetic resonance imaging (MRI) of the brain showed focal areas of restricted diffusion in the medial part of the thalami bilaterally and the rostral part of mid-brain (right > left) (bilateral paramedian thalamic with mid-brain pattern), suggestive of a hyper-acute infarct in the territory of AOP. The patient was anticoagulated with 40 mg subcutaneous low molecular weight heparin and was started on double anti-platelets along with supportive measures. The level of consciousness is improved at a slow rate to a GCS of 12/15 (E4M5V3). The patient had marked abulia with periods of drowsiness interspersed with periods of restlessness and uttering of abnormal sounds, but she was able to execute simple commands. In conclusion, occlusion of the AOP is a rare cause of coma in elderly patients. Diffusion-weighted MRI is the imaging modality of choice for early diagnosis. Early diagnosis of AOP occlusion may lead to favorable outcomes.

Key words: Artery of Percheron; bilateral thalamic infarcts; coma; diffusion-weighted imaging; thalamic dementia

INTRODUCTION

Bilateral thalamic infarcts have a low frequency among different subtypes of strokes. These infarcts involve the medial aspects of the thalamus in a relatively symmetrical pattern with or without simultaneous involvement of the rostral mid-brain bilaterally. Since it does not involve any of the particular vascular territories, this usually points out to the occlusion of the artery of Percheron (AOP).[1] AOP is a solitary arterial trunk which arises from one of the proximal segments of the posterior cerebral artery and supplies the thalamus and certain structures of the rostral midbrain. The low sensitivity of computed tomography (CT) makes AOP infarction diagnosis difficult. Diffusion-weighted magnetic resonance imaging (MRI) is the imaging modality of choice.[1] However, AOP is rarely visualized on magnetic resonance angiography, and lack of visualization does not exclude its presence.[1] The incidence of AOP occlusion is rare, and early diagnosis

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is challenging. It varies 0.1-2% in all ischemic strokes and 4-18% in thalamic infarction.[2] Here, we report a case of bilateral thalamic infarct due to occlusion of the AOP.

CASE REPORT

A 79-year-old right-handed Parkinsonian female was found unresponsive in her bed at home. She was last seen normal approximately 8 h prior to her admission. There was no recent history of fever, headache, seizure, trauma, and known exposure to toxic substances. There was no history of any memory impairment or dementia. On examination, the patient was drowsy with a Glasgow Coma Score (GCS) of 10/15 (E2M5V3). She had absent doll's eye response with anisocoric pupils and intermittent vertical gaze palsy. The deep tendon reflexes were present and symmetric. Babinski sign was present bilaterally. Although the patient had no apparent motor deficits, she was in a state of persistent somnolence with memory impairment and lack of initiative.

Investigations

Laboratory findings including blood glucose, complete blood count, serum electrolytes, liver and renal function tests, thyroid function tests, arterial blood gas, and ammonia were unremarkable. Electrocardiogram showed normal sinus rhythm.

Imaging

The initial CT showed no obvious brain lesion. MRI of the brain showed focal areas of restricted diffusion [Figures 1 and 2] in the medial part of the thalami bilaterally (bilateral paramedian thalamic with mid-brain pattern), and in the rostral part of mid-brain (right > left). Echo-planar two-dimensional perfusion imaging revealed areas of decreased perfusion in the areas of restricted diffusion [Figure 3]. There were V-shaped hyper-intense signal areas in the pial surface of the midbrain adjacent to the interpeduncular fossa, and therefore, no abnormal signs in this region on the T2-weighted scan [Figure 4]. These imaging findings were suggestive of a hyper-acute infarct. MRI data demonstrated patent basilar tip and posterior cerebral arteries [Figure 5]. Hence, the possibility of hyper-acute infarct in the territory of AOP was considered.

Treatment

The patient was anticoagulated with 40 mg subcutaneous low molecular weight heparin. The level of consciousness is improved to a GCS of 12/15 (E4M5V3). The patient had marked abulia with periods of drowsiness interspersed with periods of restlessness and uttering of abnormal sounds, but she was able to execute simple commands. The patient is currently under our follow-up. She is on anticoagulation. Her consciousness is gradually improved. However, her memory impairment was still persisting as her mini mental state examination score was 23 of 30.

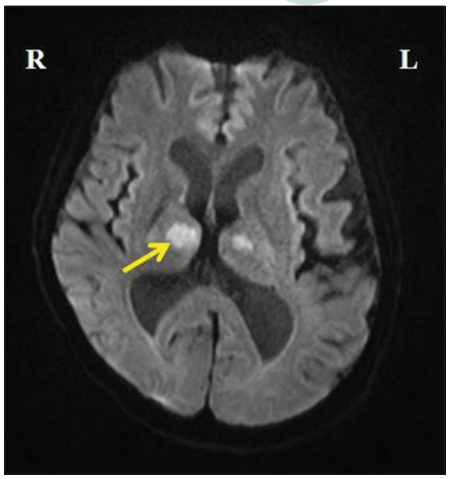

Figure 1: Magnetic resonance imaging of the brain (T1-weighted sequence) showing focal areas of restricted diffusion in the medial part of the thalami bilaterally and in the rostral part of mid-brain (right > left)

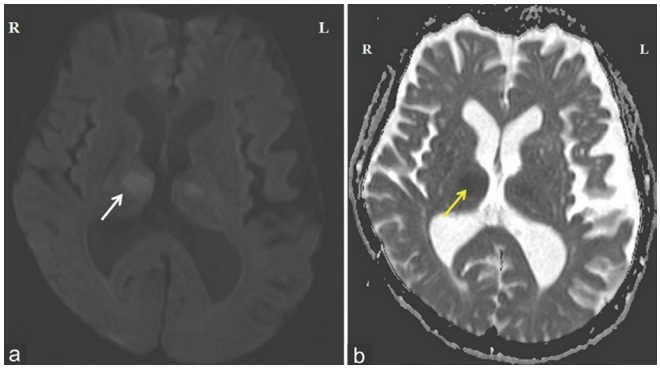

Figure 2: Magnetic resonance imaging of the brain showing focal areas of restricted diffusion in the medial part of the thalami bilaterally and in the rostral part of mid-brain (right > left). (a) Diffusion-weighted imaging sequence; (b) apparent diffusion co-efficient sequence

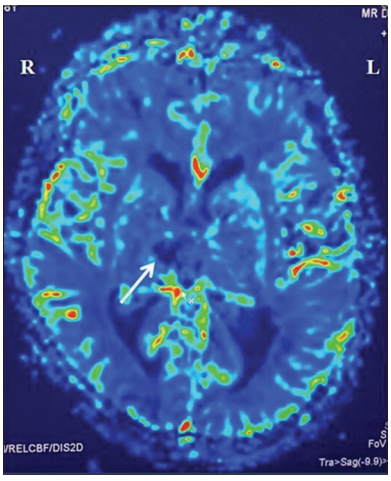

Figure 3: Echo-planar two-dimensional perfusion imaging revealing areas of decreased perfusion in the areas of restricted diffusion

DISCUSSION

Our case illustrates the importance of considering ischemic stroke in the AOP territory as one of the differential diagnosis of acute disturbance of consciousness in the elderly. Bithalamic paramedian infarcts due to occlusion of AOP presents with vertical gaze palsy (65%), memory

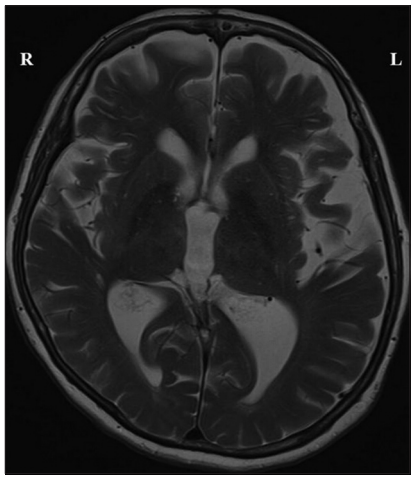

Figure 4: Magnetic resonance imaging brain T2-weighted sequences showing V-shaped hyper-intense signal areas in the pial surface of the midbrain adjacent to the interpeduncular fossa without any abnormal signs in this region

impairment (58%), confusion (53%), and coma (42%).[2] Vertical gaze palsy is due to the disruption of the cortical input that traverses the thalamus to reach the rostral interstitial medial longitudinal fasciculus.[3] Memory impairment, confusion, and coma classically seen in bilateral thalamic infarction, often called together as "thalamic dementia" is explained by the involvement of the reticular activating system and the disrupted connections between the thalamus and the cortex.[4] Thalamic dementia does not develop in case of unilateral lesions.

These patients must be differentiated from those with "top of the basilar artery" syndrome and deep cerebral venous thrombosis (DCVT).[5,6] "Top of the basilar artery" syndrome tends to involve the superior cerebellar artery and posterior cerebral artery territories. MRI showing patent basilar tip and posterior cerebral arteries exclude this diagnosis in our patient. MRI pattern does not confine to a typical arterial territory in DCVT.

Percheron described four normal variations of the neurovascular anatomy of the thalamus and the midbrain.[2] The medial part of the thalamus is supplied from the posterior circulation via the perforating thalamic arteries, which are also known as the paramedian arteries.[2] Occlusion of AOP causes a bilateral paramedian thalamic and rostral midbrain infarction. Most of the AOP infarction is due to small vessel occlusion or cardiac embolism.[5]

Successful tissue plasminogen activator therapy for AOP occlusion is reported in literature,[7] but our patient was outside the treatment time window on the initial

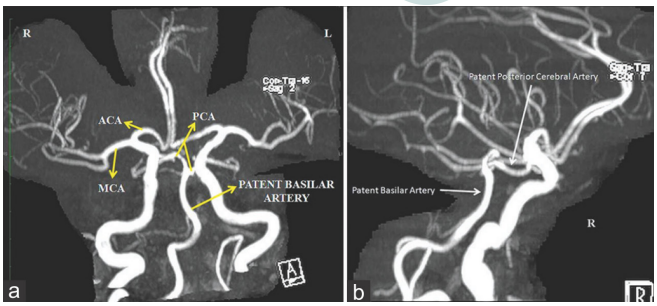

Figure 5: Magnetic resonance angiography showing patent basilar tip and posterior cerebral arteries. (a) Anterior view; (b) lateral view right side

presentation. Long-term anticoagulant therapy is the current treatment strategy suggested for AOP occlusion.[7]

In comparison to ischemic lesions of other cortical-subcortical structures, thalamic stroke has a lower mortality rate and a better prognosis as far as the recovery of motor deficits is concerned. On the contrary, the neuropsychological deficits in terms of memory, cognition, emotional response and behavior tend to persist, and interfere with the social and professional life of the patient.

In conclusion, occlusion of the AOP is a rare cause of coma in elderly patients. Diffusion-weighted MRI is the imaging modality of choice for early diagnosis. Early diagnosis of AOP occlusion may lead to favorable outcomes.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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Case Report

Central nervous system blastomycosis presenting as a year-long chronic headache

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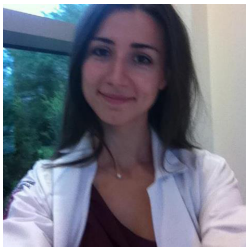

Dr. Elena Grebenciucova is currently a senior neurology resident at the University of Chicago Medical Center. Her interests lie in the field of neuroimmunology and neuro-infectious disorders. This year, she will continue serving patients with neuroinflammatory diseases in a new capacity, as a Neuroimmunology fellow at the University of Pennsylvania.

ABSTRACT

This case describes a posterior fossa mass due to blastomycotic infection in a non-immunocompromised 41-year-old male presenting with a chronic headache for over one year. Given the risk of herniation, no lumbar puncture could be performed. A full work-up found no evidence of systemic infection. Surgical resection helped identify the mass as a blastomycotic abscess. Magnetic resonance imaging characteristics of the mass were helpful in the identification of the mass as a fungal abscess.

Key words: CNS blastomycosis; blastomycotic abscess; chronic headache; posterior fossa mass; fungal abscess

INTRODUCTION

Blastomycosis isolated to the central nervous system (CNS) is exceedingly rare. In the absence of other more common systems' involvement (such as lungs), blastomycosis is rarely considered high on the differential. This case describes a nonspecific presentation of a blastomycotic abscess as a chronic headache and discusses the difficulties associated with the prompt diagnosis of this disease. The scope of this case is to demonstrate that when presenting as a posterior fossa mass, blastomycotic abscesses have a vast differential and ultimately require a biopsy or resection for the correct diagnosis. MRI imaging may be helpful in helping distinguish fungal abscesses from their neoplastic or bacterial counterparts.

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CASE REPORT

A 41 years old male from rural Illinois with a history of hypertension and alcohol abuse presented for evaluation of 16 months of progressive dull occipital headaches, unresponsive to pain medications. Over the prior 6 months the patient developed nausea with rapid head movements as well as dysarthria and right hand clumsiness. Non-contrast computed tomography (CT) of the brain demonstrated an ill-defined mixed density lesion in the cerebellum [Figure 1]. Magnetic resonance imaging (MRI) revealed a 3.5 cm × 2.6 cm intra-axial mass in the midline superior cerebellum abutting the tentorium and compressing the 4th ventricle. The mass was mildly hyperintense on T1-weighted images and heterogeneous but predominately hypointense on T2/FLAIR [Figure 2]. It demonstrated significant internal susceptibility on susceptibility weighted images compatible with vascularity and/or hemorrhage. There

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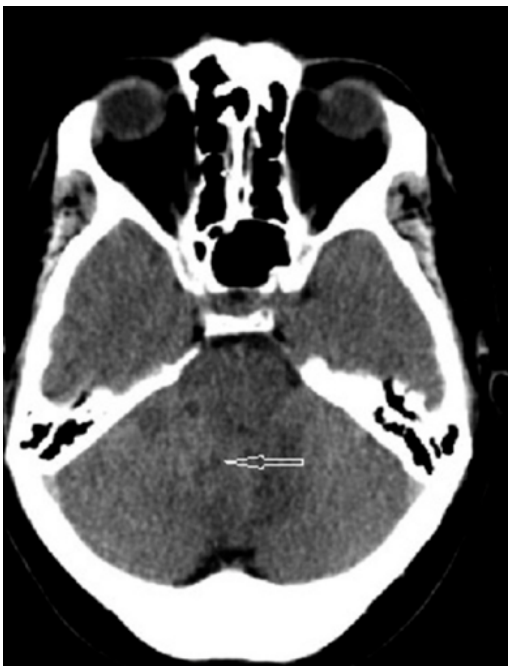

Figure 1: Axial head computed tomography image revealing a predominantly hypodense mass lesion in the cerebellum, centered at the vermis. The lesion compresses the fourth ventricle (arrow pointing to the mass)

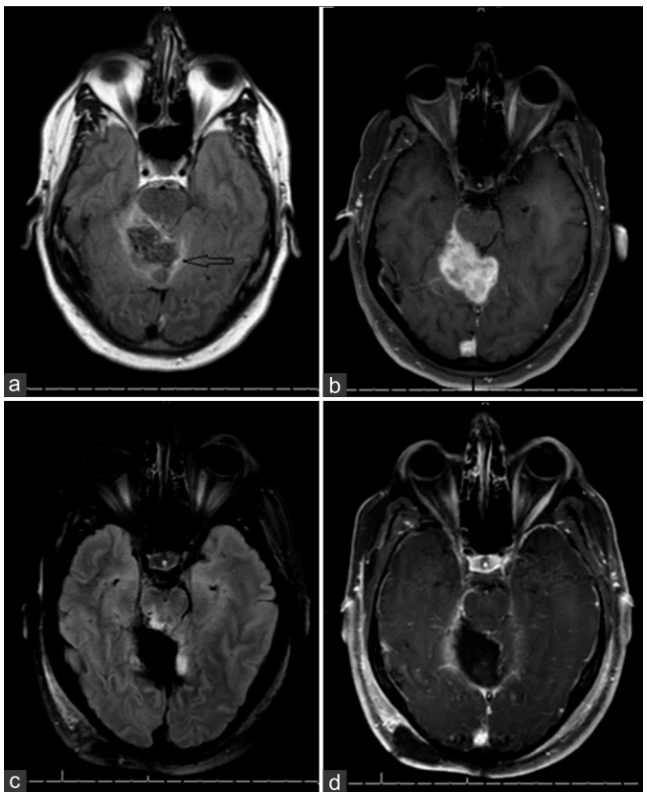

Figure 2: Axial magnetic resonance imaging images of fluid-attenuated inversion recovery (a) and T1 postcontrast (b) at initial presentation and then postoperatively (c and d) revealing complete radiographic resection of the lesion

was avid internal enhancement of the lesion on post-contrast images. Additionally there was a thin tract of contrast enhancement extending from the lesion along the right lateral margin of the brainstem. There was mild edema of the surrounding cerebellum.

Differential diagnosis of the posterior fossa mass in a 41 years old male included neoplastic (high- or low-grade gliomas, medulloblastoma, hemangioblastoma, lymphoma and infectious etiologies. Pilocytic astrocytomas (WHO grade I) and medulloblastomas (WHO grade IV) occur more frequently in the pediatric population. CNS lymphomas occur more frequently in the elderly or HIV+ population. Hemangioblastomas often occur in association with Von Hippel-Lindau syndrome. Other than the suspicion of alcohol abuse, patient was otherwise not immunocompromised. He was HIV negative, lacked peripheral leukocytosis, fevers, and had an unremarkable CT of the chest. Given the location, the size, and the mass effect of the lesion, no lumbar puncture could be pursued due to the risk of herniation. The patient underwent a suboccipital craniotomy achieving a complete resection. Pathology showed granulomas with fungal organisms. Periodic acid-Schieff and Giemsa (GMS) stains revealed rounded yeast forms consistent with *Blastomycosis dermatitidis* [Figure 3]. Post-operative cerebrospinal fluid (CSF) revealed > 1,000 White blood cells with neutrophilic predominance, normal glucose at 64 and high protein at 300.

DISCUSSION

Blastomycosis is a pyogranulomatous infection that occurs predominantly by inhalation of the microconidia of the dimorphic fungus *B. dermatitidis*. The fungus is prevalent in the areas along the Mississipi and Ohio river basins as well as Midwestern regions bordering the Great Lakes. About 91% of infections are pulmonary with subsequent dissemination to the skin, bone or genitourinary system. CNS involvement occurs only rarely 5-10% of cases.[1] Isolated CNS blastomycosis is rare. Infection occurs in both immunocompetent and immunosuppressed hosts. For example, in a case series of 22 patients with CNS blastomycosis, only 12 patients were immunocompromised (i.e. HIV, chronic steroid use, anti-tumor necrosis factor therapy for more than 6 months).[2] Clinical and experimental evidence (predominantly animal studies) suggests that chronic alcohol consumption significantly alters many lines of immune system and predisposes alcoholics to an increased risk of infection, increased morbidity, and mortality.[3]

CNS blastomycotic infection can present as either acute or indolent meningoencephalitis. At times, the only symptoms are intractable headaches. In a recent study that evaluated outcomes of 16 patients with CNS blastomycosis, the most frequent symptoms at presentation were headaches or a focal neurologic deficit in 63 and 56% of patients respectively.[4] Other manifestations include leptomeningeal involvement, single or multiple abscesses intracranially or in the spinal cord, as well as in the epidural space causing cord compression. On the MRI these may present as a single or multiple lesions

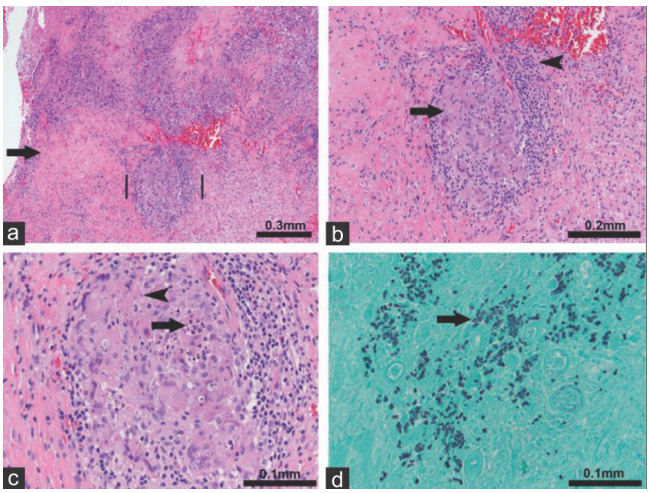

Figure 3: Histologic analysis of the biopsy sample. (a) Histologic sections show a background of reactive fibrosis (arrow) with nodular inflammatory cell infiltrates (between vertical lines). Higher magnification (×10); (b) chronic inflammatory cells: small mononuclear cells (arrow head) and nodular aggregates of pale pink "epithelioid" histiocytes (arrow) imparting a granulomatous appearance (×20); (c) Small spherical organisms are seen (arrow head) and focally a few neutrophils are present in the center of the granuloma (arrow, ×40); (d) GMS staining confirms the presence of fungal organisms that morphologically appear as small dark stained round yeast forms (arrow). *Blastomyces dermatitidis* (×40).

producing mass effect (particularly in the cerebellum), diffuse leptomeningeal enhancement, cerebritis or obstructive hydrocephalus. Restricted diffusion is frequently one of the earliest MRI findings with fungal abscesses. This occurs due to an increased cellularity and viscosity of the pus associated with the infection and frequently precedes gadolinium enhancement. Reduced diffusion signal may frequently be heterogeneous. In smaller lesions, it may be punctate. When compared with fungal infections, bacterial abscesses tend to have a highly restricting homogeneous center. In contrast to their marked diffusion abnormality, fungal abscesses may demonstrate only a weak ring enhancement. This is thought to be secondary to a weak peripheral immune response. A combination of ring enhancement and diffusion signal can help differentiate fungal abscesses from bacterial abscesses or brain metastases. Brain metastases tend to have a thicker ring enhancement and a reduced diffusion in the necrotic center. Brain metastases frequently have a thicker ring enhancement, but typically have no reduced diffusion in the necrotic center.[5]

Definitive diagnosis is established either by isolation of the fungus from a culture or direct visualization on the histological slides. Isolation from the CSF is uncommon. In a case series of 22 patients with CNS blastomycosis, CSF cultures were positive only in 2 patients.[2] Serologic testing is generally considered not to be useful in blastomycosis due to high cross-reactivity with other endemic mycoses. Antigen testing may be positive in the urine and serum. PCR is rarely used and typically not commercially available.

Thus, most cases require a biopsy and a histopathologic examination of the tissue to arrive at the correct diagnosis. The case described above had negative serology, CSF culture and required a tissue sample obtained during resection to diagnose it as a blastomycosis abscess.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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Review

Neuro-oncogenesis and the adult human sub-ventricular zone in high grade glioma

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Dr. Sara G. M. Piccirillo graduated from the University of Milan in Medical Biotechnology in 2003 and gained her Ph.D. in Translational and Molecular Medicine at the University of Milan-Bicocca in 2008. Dr. Piccirillo is currently the Research Associate at the Department of Clinical Neurosciences of Cambridge University in UK. Since 2014, she is also the Visiting Senior Scientist at the University of Texas Southwestern Medical Center in Dallas in USA.

ABSTRACT

The last fifteen years have seen the application of the cancer stem cell hypothesis to tumors of the central nervous system, in particular to high grade glioma (HGG), the most aggressive and common brain cancer in adults. Seminal studies have shown that cancer stem cells (alternatively named tumor-initiating cells) are capable of self-renew and multipotency, similar to their normal counterpart. More importantly they give rise to tumors that closely mimic the phenotype and genotype of human HGG. The identification of neurogenic niches in adult rodent and human brain has further reinforced the hypothesis that HGG might derive from the malignant transformation occurring in these areas, especially in the sub-ventricular zone (SVZ), the largest and most well characterised stem cell niche. Following from evidence of animal model studies supporting this hypothesis, recently we investigated the role of the SVZ in neuro-oncogenesis using tissue material derived from HGG patients. We also described response to conventional chemo-therapies of cancer stem cells isolated from the SVZ and the tumor mass (T) of the same patients and reconstructed tumor evolution. In this review, such findings will be discussed in the context of the current literature on the biology of the SVZ in the normal and disease brain.

Key words: High grade glioma; tumor-initiating cells; sub-ventricular zone; tumor development

INTRODUCTION

High grade glioma (HGG) are aggressive and lethal brain tumors whose prognosis remains dismal despite advances in neurosurgical techniques and combination of radio- and chemo-therapy. The recent years have seen two major directions of investigation: firstly, the evidence from stem cell biology showing that cancer stem-like populations exist in HGG and other brain tumors and secondly, the application of high-resolution genomics to study HGG genetic heterogeneity. However, the existence of cancer stem cells in tumors does not prove *per se* that the disease originates from normal stem cells.

In the brain, the sub-ventricular zone (SVZ) is a germinal niche where neurogenesis persists throughout adulthood. In the last twenty years, seminal studies have described the cellular organisation and functional properties of this niche, mainly composed of neural stem, precursor cells and migrating neuroblasts. Given

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the capacity of stem cells to self-renew and generate differentiating cells while also maintaining their pool, it has been proposed that SVZ stem cells could play a role in tumorigenesis. This hypothesis has been supported by studies using genetically-engineered animal models where the key genetic alterations of HGG occur only in neural stem/precursor cells of the SVZ.

The advent of high-resolution genomic techniques gave us the unique opportunity to overcome the challenges associated with studies in the human brain of HGG where only a small amount of tumor tissue is available and longitudinal studies to assess tumor development are not possible. We developed a real-time fluorescence-guidedtiple-sampling (FGMS) strategy based on 5-aminolevulinic acid to identify cancer stem cells in different tumor regions[1] and we used this approach to describe the extent of spatial genetic intra- tumor heterogeneity in HGG[1,2] and to reconstruct tumorigenesis.[2] In parallel, we derived cancer stem cells from the tumor mass and the SVZ of the same patients and we showed that drug-resistant cells are present in this niche.[3] These findings have implications for the development of new therapeutic approaches targeting the SVZ.

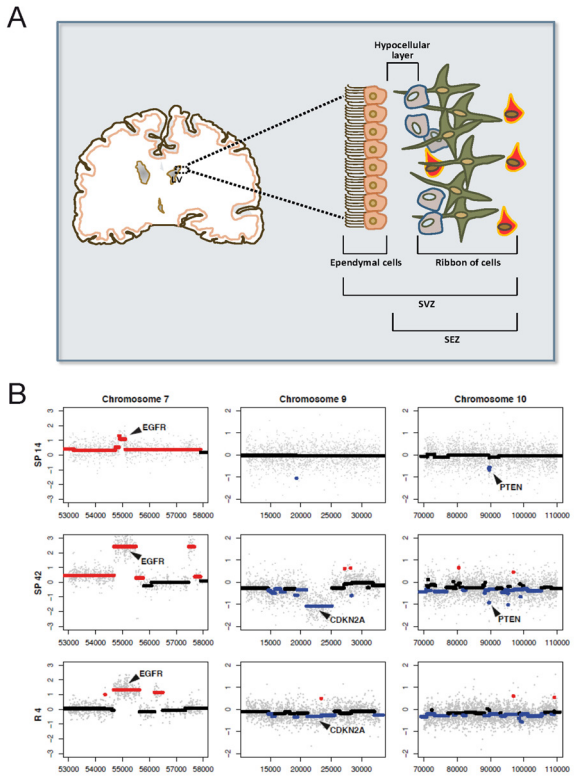

Figure 1: The anatomy of SVZ and the genetic alterations in HGG patients. A: Anatomical structure of the human brain SEZ and SVZ; B: copy number aberrations of three SVZ samples from different patients share hallmarks of HGG such as amplification of EGFR and loss/deletion of PTEN. SP42 and R4 display also loss of CDKN2A, another hallmark of the disease. SP14 also exhibits amplification of AKT3 whereas SP42 shows amplification of CDK6 and MET as well. This clearly confirms the aberrant nature of SVZ cells. HGG: high grade glioma; SEZ: sub-ependymal zone; SVZ: sub-ventricular zone

THE SVZ IN THE ADULT HUMAN BRAIN

The identification of neurogenic niches in rodents[4] has challenged the long-standing notion that the mammalian brain was a quiescent organ characterized by lack of neurogenesis postnatally.[5] In the adult mammalian brain, neurogenesis occurs in 2 germinal regions: the SVZ[6] and the subgranular layer (SGL) of the dentate gyrus of the hippocampus.[7] Several works on the cellular organisation of the SVZ in rodents have revealed the existence of neural stem cells that express the astrocytic marker glial fibrillary acidic protein (GFAP) and give rise to neurons. When compared to the SGL, the SVZ represents the most abundant source of neurons.[8-11] More recently, studies on the adult human brain have shown that the SVZ retains the same functional properties of the rodent brain, but the GFAP+ve cells are organised in a ribbon.[12,13] However, important differences exist between the human and rodent SVZ: (1) in humans, the SVZ is positioned in the wall of the lateral ventricles and is characterized by 4 layers. SVZ astrocytes are organised in ribbons separated from the ependymal layer by a hypocellular gap, that is a reminiscence of the neuronal formation and migration occurring at embryonic stages[14] [Figure 1]. Interestingly, the terms SVZ and SEZ have been used interchangeably, however they describe specifically these layers with the inclusion or not of the ependymal layer [Figure 1]; (2) the number of actively proliferating cells in human SVZ is very low in comparison to rodents;[12,15] and (3) the evidence of the existence of neural stem cells *in vivo* is still missing in humans, whereas it is well established in rodents.

Accumulating evidence points out to the influence of pathological conditions on neurogenesis. These include infections, inflammations, stroke, epilepsy, tumors and neurodegenerative disorders.[16,17] For instance, in Huntington's disease an increase in cell proliferation and neurogenesis occur in the SVZ of disease brains.[18] Extending our understanding of the biology of the human SVZ might lead to the identification of novel therapeutic interventions against the large spectrum of diseases affecting the brain.

THE SVZ AS INFLAMMATORY RESERVOIR

In HGG, the onset of malignant transformation can be seen as a traumatic event that can initiate inflammation. This can then persist during the subsequent phases of tumor growth: promotion and progression.[19] Inflammatory cells, particularly tumor-associated macrophages and microglia, are abundant in HGG and pro-inflammatory genes are overexpressed in the tumor core.[20,21] Most importantly, in HGG inflammation promotes radioresistance.[22] However, so far no study

has investigated the presence and role of inflammatory cells in the SVZ of brain tumor patients.

It has been shown that inflammatory processes involving interleukin-6 (IL-6) are initiated after neonatal CNS after injury and that both IL-6 and another member of the same cytokine family, i.e. leukemia inhibitory factor, contribute to the expansion of neural stem and progenitor cells in the SVZ after injury[22,23] by activating the JAK/STAT pathway.[24] Despite the high expression of IL-6 in HGG and its promotion of tumor growth[25] and invasion,[26] the role of this cytokine is not fully understood. In a mouse model of astrocytomas with inactivation of the IL-6 gene locus, tumor formation is suppressed suggesting that IL-6 is required for glioma growth.[27] Additional studies are required to elucidate the functional role of IL-6 in the SVZ of HGG patients. This might extend our knowledge about its role in promoting malignancy and sustaining neural stem cell self-renewal.

THE SVZ AND THE CANCER STEM CELL HYPOTHESIS

The idea that cancers derive from stem cells is not entirely new.[28] In recent years evidence supporting this concept has been provided by several works on non-solid and solid cancers. Intriguingly, the concept of a stem cell hierarchy inside a tumor found confirmation in several pathologies, from leukaemia to solid cancers (i.e. breast, brain, colon cancers)[29,30] with the hematopoietic system providing the best example in both chronic myeloid leukaemia and multiple myeloma.[31]

However, the initial evidence for the existence of cancer stem cells in several tumors has been followed by the consistent observation that these cells hijack functional properties of normal stem cells. In particular, it has been thought that virtually all cancer cell lines available can be turned into cancer stem cells by changing the growth medium and by exposing them to mitogenic stimuli. More importantly, the functional similarities with normal stem cells has also led to speculate that if cancer cells resemble stem cell features then the tumor itself might originate from the malignant transformation of normal stem cells of that particular tissue, therefore cancer stem cells might represent the tumor "cell of origin". However, "cancer stem cell" and "cell of origin" represent two different concepts[32] that are often confused and used interchangeably.

In HGG, there is evidence that the tumor derives from stem/precursor cells in genetically-engineered animal models of the disease (see next section "The SVZ as an oncogenic niche"). Histological studies on HGG patients revealed a mixture of cell morphologies including virtually all the spectrum of differentiating cells, from highly immature[33,34] to terminally differentiated cells.[35,36] This has further suggested that a stem cell hierarchy might operate in HGG and might be responsible for its highly heterogeneous phenotype.[31]

However, more recent studies have pointed out that the capacity of tumor cells to mimic the functional properties of stem cells is a "plastic" process that can be influenced by extrinsic factors (for instance, a more permissive microenvironment characterized by high immunosuppression[37,38]) or by instrinsic factors (for instance, transcription factor that can induce a stem cell transcriptional program in tumor cells[39]), thus suggesting that cancer stem cells are the result of an aberrant program of cell plasticity.[40]

THE SVZ AS AN ONCOGENIC NICHE

The importance of the SVZ as a potential oncogenic niche stems from an initial study in the 40s' suggesting that brain tumors with ventricular walls contact might originate from the embryonic rests present in the SVZ.[41] This was followed by studies in the 60s' showing that mitosis occurs in the sub-ependymal layer of rodent and primate brain[5,42] and in the 70s' with the intraventricular injections of oncogenic viruses.[43,44] More recently, other studies took advantage of the development of genetically-modified viruses and animal models. Interestingly, in mice it was initially shown that undifferentiated (precursor) cells can be more easily transformed when compared to cells that are terminally differentiated,[45,46] thus corroborating the hypothesis that neural stem/precursor cells might represent the target of malignant transformation. In addition to the above findings, a subsequent study comparing cultures of astrocytes *vs.* neurosphere precursor cells has shown that dedifferentiation of astrocytes (promoted by EGFR activation) makes these cells susceptible to malignant transformation similarly to neural stem cells, by combining loss of critical tumor suppressors, i.e. p16Ink4a/p19Arf.[47]

Following the characterization of the adult brain SVZ as stem cell niche in rodents and humans[8,12,48] and the identification of "cancer stem cells",[49] animal models have been extensively developed in order to understand if the SVZ can be a source of brain tumors.[31]

In HGG, it has been shown that neurogenic regions are susceptible to malignant transformation, in particular following stereotactic infusion of growth factors, such as PDGF, in the SVZ.[50,51] Similarly, using genetically-engineered mouse models, it has also been demostrated that HGG can be driven by tumor suppressor inactivation in neural stem/progenitor cells[52,53] and that a subpopulation of stem-like/Nestin(+ve) cells is

responsible for tumor re-initiation following chemo-therapy.[54] In support of these findings it was also noted that p53 mutations preferentially occur in the SVZ.[55]

Collectively, these results raise the question on whether cancer stem cells directly derive from SVZ stem cells. Although mouse model studies have indicated that this is the case, these findings have been severely hampered by a limited representation of the aberrant genetic landscape of HGG and the use of markers that poorly discriminate between stem cells and precursor cells.[32] More recently, the same question has been addressed by using a transgenic cell-labelling system known as mosaic analysis with double markers.[56] Using this model, it has been proposed that the cells of origin in HGG are oligodendrocyte precursor cells, thus challenging the notion that HGG may originate from transformation and expansion of the neural stem cell pool.

Although the debate about the cell of origin in HGG is still open, the above studies have helped define the potential targets of malignant transformation that

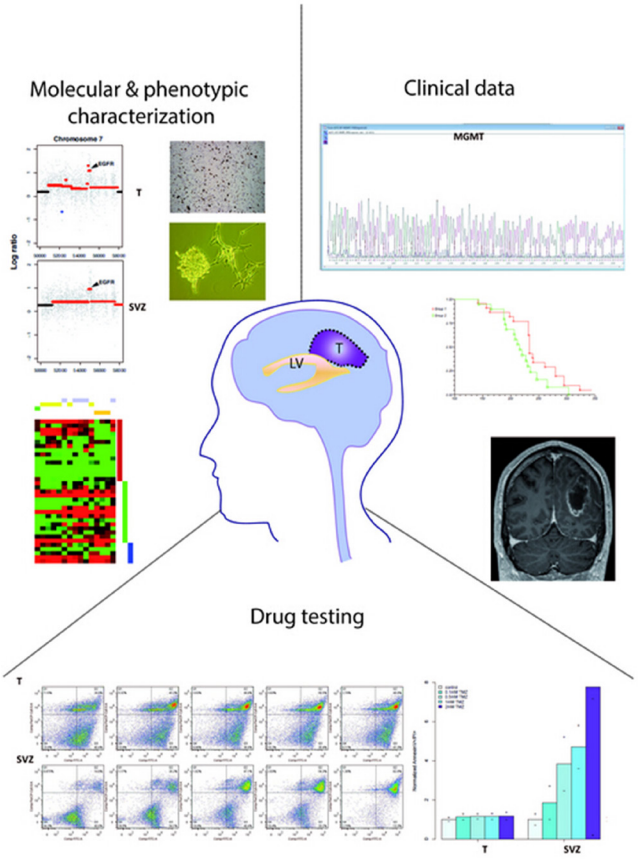

Figure 2: Drug treatments of cancer stem cells derived from SVZ and T combined with molecular and phenotypic characterization of corresponding tissues can help classify HGG patients and develop personalized therapeutic approaches. A better understanding of drug resistance can be achieved by a systematic comparison of drug screening analyses between cancer stem cells isolated from the SVZ and T of the same HGG patient. This will be integrated with molecular profiling of the matched SVZ and T tissues, clinical data and the phenotypic characterization of the derived cells. This approach has the potential to impact on clinical decisions as the molecular/phenotypic characterization and phylogenetic reconstruction may allow a personalized therapeutic approach based on a better understanding of tumor heterogeneity and potentially may lead to the identification of novel targets in the SVZ and in the T. LV: lateral ventricle; T: tumor mass; HGG: high grade glioma; SVZ: sub-ventricular zone

can be further investigated to elucidate the process of oncogenesis in HGG patients. The limited availability of tissue samples and the clinical complex scenario at the time of surgery make it difficult to reconstruct the initial steps of tumor development and alternative methods are needed. Given the critical functional role of the SVZ in the adult human brain, it has been speculated that this niche might play a role in neuro-oncogenesis. This has been the focus of our recent study on HGG patients.[3]

THE SVZ AS A SOURCE OF TUMOR CELLS IN HGG PATIENTS

The identification of cancer stem cells from human HGG has represented a novel tool to develop therapeutic strategies[57,58] and these cells have been proposed as a model that more closely represents the human disease.[59] We took advantage of these findings to objectively interrogate primary HGG in humans using a neurosurgical techniques based on FGMS. In the clinic fluorescence-guided resection has resulted in enhanced cytoreduction and improved progression-free survival in patients in a randomized Phase III trial.[60] We have adapted this technology to allow the objective identification of tumor tissue based on combining fluorescence emission and neuroanatomical landmarks and we have recently demonstrated that this technique can be successfully employed to characterize cancer stem cells derived from fluorescent and non-fluorescent material in HGG patients.[1]

Quite unexpectedly, we observed for the first time that fluorescent material is present in the SVZ of 42 out of 65 HGG patients who underwent surgery using fluorescence-guided resection and we isolated tissue from the tumor mass and the SVZ. Using these samples we reported that the SVZ contains malignant cells that contribute to tumor growth.[3] This has never been demonstrated in humans, but similar observations have been reported in mouse models of HGG.[46,53-55,61]

Importantly, the phylogenetic relationship between SVZ and tumor in these patients identifies the SVZ as a reservoir of tumor cells (either early tumor clones or late-emergent clones that develop during HGG growth) that need to be therapeutically targeted. Thus, we investigated responses to chemo-therapeutic agents using cancer stem cells from SVZ and T of the same patients. Surprisingly, we found that such cells respond differently to therapies, which represent the standard of care for HGG patients. Our data also suggest that a large fraction of cells is resistant to chemo-therapy even at supra-maximal doses[3] providing a possible explanation for the treatment failure seen in HGG patients.

In this context, cancer stem cells isolated from SVZ could be used for drug screening to develop new therapeutic strategies aimed at understanding their mechanisms of resistance [Figure 2]. Targeting the SVZ will require extensive characterization of the phenotype(s) of these cells as well as their studies to assess the response to radiation. Interestingly, it has been shown that irradiation of the SVZ in HGG patients improves progression-free survival.[62]

The involvement of the SVZ in HGG also prompt to the need of classifying tumors according to their location in the brain and integrating these data with molecular and phenotypic analysis and clinical information [Figure 2]. A previous work showed that HGG involving the SVZ give rise to recurrences far from the primary tumor site, contributing to the hypothesis that this is due to migrating neural precursors.[63] However, another study suggested that there is no evidence of a "stem cell signature" in HGG with involvement of SVZ in comparison to those with no involvement of this region.[64] This might be reconciled considering that, as suggested by animal model studies, HGG originated in the SVZ grow along white matter tracts and macroscopically do not show involvement of the SVZ.[52] The combination of studies on the adult human SVZ in HGG patients and animal models might shed new light on the functional role of this region in neuro-oncogenesis.

CONCLUSION

With the life expectancy reduced of twenty years, on average, brain tumors represent the most lethal cancer in adults.[65] Among these, HGG is the most aggressive form and among glioma has the poorest prognosis. The function role of the largest neurogenic niche in the brain (i.e. the SVZ) and its presence in the adult human brain, raised the possibility that this area might play a role in the oncogenic process leading to HGG.

Our study revealed that two types of evolutionary trajectories can be observed in patients: the first sees the SVZ playing a role in the growth of the tumor as early clone, whereas in the second the SVZ represents a late emerging clone that suggests infiltration of this area following tumor growth. These results are extremely important as they provide insights about the cell of origin of human HGG but also might impact on treatment strategies. In this respect, our drug treatment data clearly show distinct patterns of response in the same HGG and the existence of chemo-resistant tumor cells in the SVZ. However, the response of SVZ tumor cells to radiation is still to be explored. Interestingly, previous studies have reported that OPC are sensitive to radiation[66] and more recently it has been shown that OPC-like glioma Olig2+ cells respond better to radiation than HGG cells[67] identified by CD44.[68]

At the same time, other questions need to be addressed: (1) what is the role of the other well characterized neurogenic region in the adult brain (i.e. the SGZ); could this be also source of neural precursors responsible for initiate HGG and other brain tumors in patients? (2) what are the evolutionary trajectories in those patients where the HGG lies in the cortical mantle and does not show any contact with the SVZ by using FGMS? We have just started to scratch the surface of what seems to be a complicated dynamic process of HGG evolution that involves the SVZ, in addition to more recent publications by us[69] and others[70] deciphering the genomic architecture of the T. There is hope that these findings and more studies on the role of SVZ in brain tumors might impact on improving patient survival and leading to personalized treatments.

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Conflicts of interest

There are no conflicts of interest.

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Review

Neuronal toll-like receptors and neuro-immunity in Parkinson's disease, Alzheimer's disease and stroke

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ABSTRACT

Toll-like receptors (TLRs) are part of the innate immune system and can initiate an immune response upon exposure to harmful microorganisms. Neuronal TLRs are considered to be part of an established framework of interactions between the immune system and the nervous system, the major sensing systems in mammals. TLRs in the nervous system and neuronal TLRs are suspected to be important during inflammation and neurodegenerative diseases. The aim of this review is to offer an overview of the current knowledge about TLRs in neurodegenerative pathologies, with a focus on Parkinson's disease. More research focusing on the role of TLRs in health and disease of the nervous system is needed and remains to be explored.

Key words: Neuron; toll-like receptor; Parkinson's disease; Alzheimer's disease; stroke; neurodegeneration; neurodevelopment; infection

INTRODUCTION

Publications were first selected about toll-like receptors (TLRs) on neurons, and on TLRs for which functional information in neurons was available. Publications were also selected for their focus on neurodegenerative diseases. The last literature search was performed on April 14th 2015. This review aims to offer an overview of the current knowledge about TLRs in the nervous system and to show the relevance of these receptors in neurodegenerative pathologies, with a focus on Parkinson's disease (PD).

TLRs are the mammalian orthologue of Drosophila Melanogaster's toll receptor discovered in 1988.[1] TLRs are part of the innate immune system and belong

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to the pattern recognition receptors [Figure 1]. They can recognize both small molecular motifs conserved across microbes (pathogen-associated molecular pattern or PAMP) [Figure 2], and endogenous molecules generated during inflammation or tissue damage (damage associated molecular pattern or DAMP).[2-5] TLRs can initiate an acute inflammatory reaction and subsequently can coordinate the activation of the adaptive immune system. To date, thirteen TLRs are known, of which ten (TLR1-10) have been described in humans.[6] The cell surface TLRs recognize PAMPs that are mainly constituent of the bacterial cell wall or are expressed on the bacterial cell surface, such as lipopeptides and peptidoglycal (TLR1/TLR2, TLR2/TLR6, TLR2/TLR10), lipopolysaccharide (LPS) (TLR4) and flagellin (TLR5). In contrast, the intracellular TLRs mainly recognize microbial nucleic acid including viral double-strand RNAs (TLR3), single-strand RNAs (TLR7 and TLR8) and CpG ODN (TLR9).[7] TLRs can employ two second messenger pathways; the myeloid differentiation primary response gene 88 (MyD88) pathway, activating nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), or the TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway, activating interferon regulatory factor 3 (IRF3) [Figure 2]. NF-κB controls DNA transcription resulting in the production of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)1β and IL6.[7,8] IRF3 is an interferon (IFN) regulatory factor leading to the production of antiviral type I IFN.[2,7]

The presence of TLRs on immune cells and epithelial cells is well known, but their expression is not restricted to these cell types. Glial cells and neurons express TLRs in both the peripheral nervous system (PNS) and the central nervous system (CNS) [Figure 3], allowing neurons to act as immune cells.[9-15] More specifically, in the CNS neurons, astrocytes and microglial cells express TLR1-9, whereas oligodendrocytes express only TLR2 and TLR3.[16-20] Peripheral neurons also express TLR1-9 and enteric glial cell express TLR1-5, TLR7 and TLR9.[13,14,21-23] Neuronal TLR signaling pathways do not necessarily employ NF-κB[24-26] and may involve the glycogen synthase kinase 3β (GSK3β), jun-N-terminal kinase (JNK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathways.[27-29] Interaction between neurons and the immune system has already been reported, setting the scene for neurons acting as immune cells.[30-34] It has been reported that neuronal TLRs are involved in the development and homeostasis of the nervous system, and notably in several neurodegenerative diseases.[35,36] Both TLR2 and TLR4 are involved in neuronal apoptosis, development and survival in the context

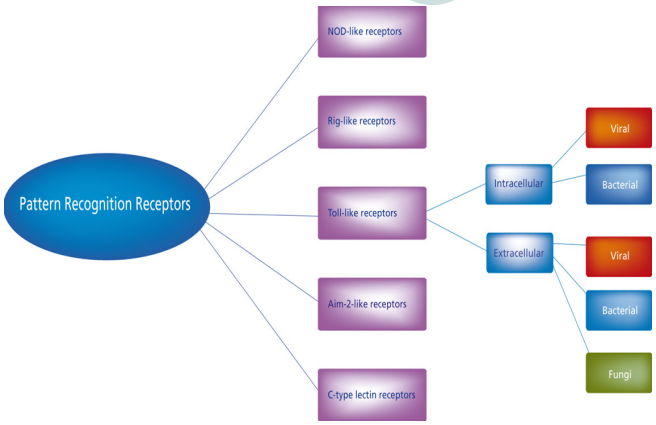

Figure 1: Toll like receptors are part of the innate immune system and belong to the pattern recognition receptors

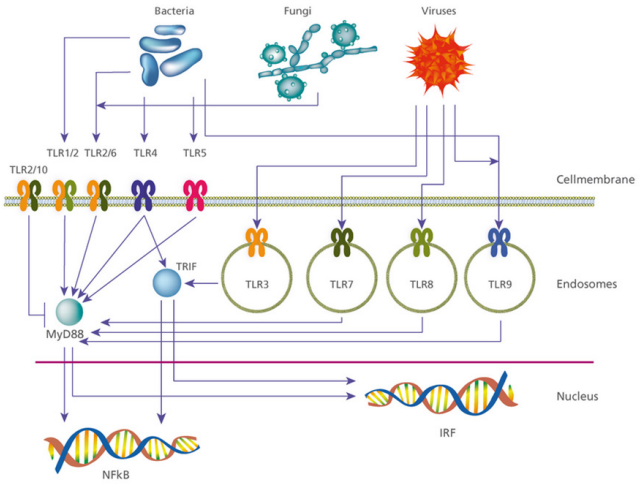

Figure 2: Different pathogens activate different TLRs. TLRs signal through two different pathways using myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon β, leading to activation of NF-kB and IRF respectively NF-kB leads to DNA transcription and cytokine production, while IRF leads to interferon production. TLRs: toll like receptors; NF-kB: nuclear factor κ-light-chain-enhancer of activated B cells; IRF: interferon regulatory factor

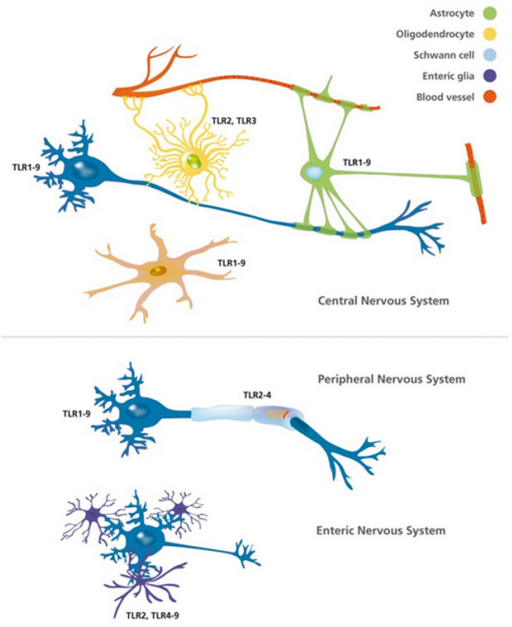

Figure 3: TLRs are differentially expressed by neurons and glial cells of the central, peripheral and enteric nervous system. TLRs: toll like receptors

of opioids exposure, ischemia/stroke, viral infections and Alzheimer's disease (AD),[28,29,37-43] via GSK3β and JNK.[28,29] TLR3 and TLR8 negatively regulate neuronal development and axonal growth and are involved in fighting viral infections,[9,16,24,25,27,44-46] through PI3K pathway.[27] Other information also indicates a role for TLRs in the nervous system during disease. TLR1-5, TLR8 and TLR9 are overexpreesed in PD and multiple systems atrophy (MSA) patients and in animal models of PD, AD, MSA and amyotrophic lateral sclerosis.[47-53] Of these receptors, TLR2 and TLR4 are of special interest in PD, as will become clear in the sections dedicated to these TLRs. The current knowledge about TLR2 and TLR4 in PD has focused heavily on microglia, and not so much on neuronal TLRs. To better understand the potential importance of neuronal TLRs in neurodegeneration and specifically in PD, AD and stroke the current knowledge about the function of neuronal TLRs in neuronal development and neurodegenerative diseases will be discussed here.

NERVOUS SYSTEM TLR EXPRESSION

This section will cover the expression of TLRs on neurons and glial cells [Figure 3]. To discuss the function of TLRs on neurons it is important to know whether neurons express TLRs. For the convenience of the reader, the information about expression has been organized based on location in the nervous system, and stage of life. To discuss the function of TLRs in the nervous system it is also necessary to first address whether glial cells express TLRs, because of their biological relevance for the functioning of the CNS and PNS.

Neuronal TLR expression

TLR expression on primary neurons has been found in several species, amongst which are humans,[10,12,13,15,16,45] mice,[13,14,26,28,43,54,55] and rats.[10,27,56,57] Neuronal TLRs are present in both parts of the nervous system; the CNS[12,26,28] and the PNS.[10,13,14,43,54,57] Expression of TLRs by neurons has been confirmed at mRNA level,[14,21,24,28,43] and protein level.[10,13,24,26,28,43,54,57,58] Most results on neuronal TLRs come from experiments in embryonic neurons because these are easier to culture than neurons from adult animals. Nonetheless, neurons express TLRs throughout life, starting at the embryonic stage,[14,24,26,28,43,58] followed by the postnatal,[54] and the young stages,[10,56] and finally in the adult stage of life.[10,12,13,21,58,55,57] Taken together, these data offer many opportunities to study neuronal TLRs for a wide range of research questions, since it is reported that TLRs are expressed throughout the nervous system and throughout the lifespan of animals.

TLR expression has also been found in several neuronal cell lines. The teratocarcinoma derived human post-mitotic dopaminergic neuronal cell line NT2-N expresses TLR3.[9,59-61] TLR3 is also expressed by human neuroblastoma cell lines (including noradrenergic cell lines, CHP-212 and SK-NSH; a noradrenergic and gamma-aminobutyric cell line, SH-SY5Y; SH-SY5Y and a noradrenergic and dopaminergic cell line, BE(2)-C) and primary human neuroblastic cells.[27,44,45,59] The rat neuroblastoma cell line B103 expresses TLR2.[38] The expression of TLR1-9 has been reported in the human CHP-212 neuroblastoma cell line.[16] Although cell lines are further removed from their original biological environment than primary cells, they do offer a more readily available source of material for further research towards understanding the functions of neuronal TLRs.

Glial TLR expression

The importance of glial TLRs in neurodegeneration is indisputable.[19,32,53,62-65] Glial cells come in many shapes and sizes, and have a critical role in the CNS and PNS, such as immune surveillance, the regulation of chemical environment and the production of the myelin sheath, which makes them biologically relevant to the functioning of neurons.

Glial cells are a heterogeneous group of cells all of them expressing TLRs. Microglia act as immune surveillance of the CNS and express TLR1-9 in both humans and mice.[19,20] Astrocytes regulate the chemical environment of neurons, and they express TLR1-9 in mice and TLR1, TLR3-5 and TLR9 in humans, while TLR2 and TLR6-8 were not detected in human astrocytes.[17,18] Oligodendrocytes and Schwann cells respectively produce the myelin sheath around neuronal axons in the CNS and PNS. Schwann cells express TLR1-9 in mice,[14,66] while only TLR2 has been studied and detected in humans,[67] whereas TLR4 has been studied and detected in rats.[68,69] Human oligodendrocytes only express TLR2 and TLR3, while TLR1 and TLR4-9 were undetectable.[20] In glial cells of the enteric nervous system TLR2 -9 have been found, while TLR1 is absent.[13,22,70]

NEURONAL TLR FUNCTIONALITY

Here we provide a comprehensive overview of the current knowledge about TLR functionality in the nervous system in relation to neurodegeneration. Specific information about neurons, microglia, and other cell types is included when appropriate.

TLR2

TLR2 is a TLR family member that is able to recognize bacterial lipopolypeptides and peptidoglycans.

TLR2 forms heterodimers with TLR1 and TLR6 and mediates the host response to Gram-positive bacteria and yeast infections via stimulation of NF-κB signaling pathway.[71] Recently, it has been demonstrated that TLR2 can also form a heterodimer with TLR10 acting as an inhibitory receptor with immune suppressing effects.[72]

PD

Clinical studies have shown that TLR2 expression is increased in PD. In particular, one study revealed specific increase in microglial TLR2 in the substantia nigra and the hippocampus in the early stages of the disease, but not during the late stages,[73] while another study showed an increase in TLR2 in the striatum of advanced PD patients.[74] These results indicate that expression of TLR2 in either early or advanced PD could be region-specific, and that TLR2 is not necessarily expressed in all regions at the same time. The involvement of TLR2 in PD might be two-dimensional: microglial activation of TLR2 can induce neurotoxicity or TLR2 can be important for the clearance of α-synuclein, thus being neuroprotective.

In support of this function is the evidence that TLR2 polymorphism tends to be associated with an increased risk of PD. This polymorphism results in altered TLR2 promoter transcriptional activity leading to lower expression of TLR2.[75,76] Taken together, these findings are indirect indications of a possible role of TLR2 in the pathology of PD.

Overexpression of human α-synuclein in mice resulted in microglial activation and an increase in TLR2 expression.[74] Microglia seem to form a crucial link between TLR2 and PD pathology; an idea that is supported by results from cell culture studies: exposure to α-synuclein activates cultured microglia and increases their TLR2 expression;[49,77] it also changes the response of microglial cells to TLR1/2 stimulation by increasing their inflammatory response.[78]

It can be speculated that α-synuclein triggers neuroinflammation through microglial TLR2, initiating a positive feedback loop by increasing TLR2 expression on the microglia, resulting in neurodegeneration and disease progression. However, it is not yet clear why this process would be limited to the early disease stage in the substantia nigra and the hippocampus, while only occurring later in the striatum, or whether and how neuronal TLR2 participates in this process.

AD

TLR2 expression was found on microglia surrounding amyloid β (Aβ) plaques in post-mortem brain sections and in an AD mouse model, raising the question whether and how TLR2 might be involved in AD pathology.[47,79] Injecting Aβ in the hippocampus of wild type (WT) mice increases TLR2 expression in microglia; Aβ protein was unable to induce a microglia-dependent inflammatory response in the cortex of TLR2 deficient mice.[80,81] The interaction between Aβ and TLR2 also affects behavior; TLR2 deficient mice showed more pronounced cognitive impairments, which correlated with increased levels of Aβ protein.[81] It remains to be demonstrated whether there is a direct binding between TLR2 and the Aβ protein.

AD-related damage to neurons is at least partially dependent on microglial TLR2, since the effects of Aβ protein and TLR1/2 ligand on neuronal viability are additive and dependent on microglia, and the microglial inflammatory and phagocytic response to Aβ protein is TLR2-dependent.[80,82,83] Aβ protein- and TLR1/2 ligand-induced microglial-mediated neuronal death is likely conferred through the release of inflammatory mediators.[84] There is also indication for the involvement of neuronal TLR2 in AD. Neuronal TLR2 was upregulated when neuronal cultures were exposed to hydroxynonenal (HNE), an AD-related lipid peroxidation product, but not when exposed to Aβ protein.[40] HNE exposure also resulted in an increase in both phosphorylated JNK and cleaved caspase 3; however these effects were abolished by TLR4 knock-out. Therefore, the functional consequence of TLR2 upregulation in neurons by HNE is not yet known.

In summary, microglial TLR2 is key in the neuroinflammatory response of AD pathology, but is also responsible for the clearance of Aβ protein, while neuronal TLR2 might also play a part in this neuroinflammatory environment. So TLR2 can have either a beneficial or detrimental role in AD.

Stroke

Neuronal TLR2 was studied in the cerebral ischemia/reperfusion (I/R) animal model of stroke. Cortical and hippocampal neurons of WT mice subjected to I/R injury showed transient TLR2 protein upregulation, although upregulation in the cortex may not have been exclusively neuronal.[29,37,39] $TLR2^{-/-}$ mice exposed to I/R showed less brain damage, smaller infarct volumes and less neurological deficits than WT mice, and $TLR2^{-/-}$ mice and mice treated with TLR2 antibody showed less inflammatory cell accumulation and reduced neuronal loss.[29,39,42] From a treatment perspective it is interesting that the anti-inflammatory agent baicalin, used for the treatment of stroke, reduced TLR2 expression in

hippocampal neurons after I/R injury.[37] However, before suggesting a potential role of TLR2 in the treatment of stroke, clinical studies are needed to determine whether TLR2 is viable as a marker or target for treatment in stroke.

Animal models show that TLR2 is relevant in relation to stroke, however, they might obscure the specific importance of neuronal TLR2 in the context of glial cells. In order to isolate and study neuronal TLR2 in a stroke model, cultured neurons were exposed to glucose deprivation, a model of stroke.[29] Increased cell death was found in WT neurons, while TLR2$^{-/-}$ neurons were resistant to glucose deprivation induced cell death. In a neuronal cell line oxygen-glucose deprivation resulted in TLR2 upregulation and in an increase of non-apoptotic cell death.[85] These *in vitro* data confirm that stroke can result in neurodegeneration through the activation of neuronal TLR2, making neuronal TLR2 a potential player in brain damage after I/R injury in mice, independent from the influence of glial TLR2.

Information on TLR2 in the brain of patients with stroke is sorely missing and should be sought in future research, starting with investigating expression patterns in different brain regions. Also, a major focus of research should aim at distinguishing neuronal TLR2 from glial TLR2.

TLR3

TLR3 recognizes double stranded RNA associated with viral infection, and host RNA. Ligand binding induces the production of anti-viral mediators like the type I interferons (IFNs), such as IFN-α and -β production by leukocytes. These IFNs stimulate macrophages and natural killer cells to elicit an anti-viral response.[86]

TLR3 has not been studied in direct relationship to neurodegenerative diseases, but work has been performed on the effect of TLR3 in the development of the nervous system. TLR3 expression decreases in the embryonic CNS during neurogenesis.[58] Intrathecal injection of TLR3 agonist polyinosine: polycytidylic acid in postnatal day 4 mice resulted in sensory-motor deficits, neuroanatomical defects and fewer axons in the spinal cord, which was associated with neurodegeneration.[24] The role for TLR3 in this study was demonstrated by the fact that no anatomical or behavioral problems were found in TLR3$^{-/-}$ mice treated with polyinosine: polycytidylic acid.[24] It seems that TLR3 is involved in the proper development of the CNS in early fetal life, because the receptor is differentially expressed at different embryonic stages. After birth, stimulation of TLR3 results in neurodegeneration. The decrease in expression of TLR3 during neurogenesis, as found in the embryonic brain, is also found in cultured neural progenitor cells (NPCs), making NPCs more sensitive to TLR3-mediated inhibition of proliferation than mature neurons.[58] Despite this reported decrease in TLR3 expression during neurogenesis, neurons do express functional TLR3.[24] In primary neurons, TLR3 stimulation inhibits neurite outgrowth and causes irreversible growth cone collapse, without affecting cell survival.[24] Different results were found in the high TLR3-expressing neuroblastoma cell line SK-N-AS, where exposure to a TLR3 ligand resulted in growth inhibition and apoptosis.[44] The difference in results on cell viability could be due to the use of different cell types (dorsal root ganglia,[24] NPCs,[58] and cell lines[44]), thus revealing the limits of cell culture as a model of biological processes.

Although all *in vivo* data are obtained from early life studies and interpretation of these data in the context of neurodegeneration must be done carefully, extrapolating these results leads to the hypothesis that stimulation of neuronal TLR3 could be detrimental in neurodegenerative diseases, especially in the context of viral infections. TLR3 is a viral sensing innate immune receptor. It is known that viral infections like influenza can cause neurodegeneration[87] and that viruses are linked to neurodegenerative diseases:[88] specifically hepatitis C virus, Eppstein-Barr virus and human immunodeficiency virus (HIV) have been associated with PD.[89-91] The involvement of neuronal TLRs during viral infections is discussed in more detail in a later part of this review.

TLR4

TLR4 detects LPS derived from Gram-negative bacteria and host-derived signaling molecules such as heat shock proteins, and extracellular matrix proteins, after which the innate immune system is activated, leading to an inflammatory response.[92-94]

PD

The expression of TLR4 is increased in PD and MSA post-mortem brain tissue, suggesting clinical relevance to TLR4 in PD and neurodegeneration in general.[50,74] Animal experiments have been used to further elucidate the role of TLR4 in PD. TLR4$^{-/-}$ mice were more vulnerable to dopaminergic neuronal loss and motor problems induced by α-synuclein overexpression, but less vulnerable to the induction of PD symptoms by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment.[95,96] Furthermore, TLR4 and α-synuclein are both necessary for LPS-induced neurodegeneration in mice.[97,98] Therefore, mouse models support the importance of TLR4 in PD, but make no suggestion

as to the impact of TLR4 on disease development, since TLR4 was both protective and harmful in these models. Since TLR4 is suggested to be protective in the context of α-synuclein overexpression, TLR4 seems likely to be protective in the context of PD, since α-synuclein misfolding and aggregation is a hallmark of the disease. The harmful contribution of TLR4 to MPTP-induced PD seems to suggest that TLR4 might have a negative impact on the health of a subgroup of PD patients who suffer from toxin-induced PD (for instance after exposure to MPTP or rotenone). Therefore, TLR4 appears mostly protective in the context of PD, but it might be harmful in the context of toxin-induced PD.

Similar to TLR2, microglia form a link between TLR4 and PD. Microglia are necessary for LPS-induced degeneration of rat cortical neurons in cell culture since these neurons themselves do not express TLR4, and nitric oxide and superoxide seem to be at least partially responsible for microglial-induced neurodegeneration.[97,98] On the other hand, microglial TLR4 is necessary for the neuroprotective endocytosis of α-synuclein.[96] Microglia activated with α-synuclein downregulate TLR4, disabling any neuroinflammatory positive feedback loop, but also reducing the ability of the microglia to take up α-synuclein from their environment.[77] These results are contradictive and whether TLR4 is protective or injurious in PD is still a matter of debate. The balance between the contribution of microglial TLR4 to neuroinflammation and the endocytosis of α-synuclein might eventually determine whether this receptor is protective or harmful to the surrounding neurons. TLR4 is a promising target for future PD research. The role of TLR4 during PD can be both beneficial and harmful, and the factors determining the outcome need to be investigated in more detail. It seems that neuronal TLR4 could be protective, but the role of microglial TLR4 is still not fully understood. Resolving this debate could potentially lead to approaches aimed at turning harmful TLR4 responses into protective ones.

AD

One of the first lines of evidence suggesting that TLR4 might be involved in AD pathology comes from two studies where a TLR4 polymorphism was found to be protective against late onset Alzheimer's disease in the Italian population and glial cells surrounding Aβ plaques showed increased TLR4 expression in post mortem brain tissue.[99,100] In a genetic AD mouse model TLR4 knock-out reduced the expression of TNFα and chemokine (C-C motif) ligand 4 (also known as macrophage inflammatory protein-1β) in cortex homogenate, while increasing the amount of activated microglia, activated astrocytes, and Aβ protein in the brain.[101,102] Effects of TLR4 knock-out on behavior or disease progression have not been reported. The increase of Aβ protein in the brain of TLR4$^{-/-}$ animals could be due to a lack of TLR4-mediated Aβ clearance, potentially by microglia.[102,103]

Cell culture data support a pro-inflammatory role for microglia, and implicate microglia-induced inflammation in neuronal degeneration. Mouse microglia initiate an inflammatory and phagocytic response to aggregated Aβ through TLR4, resulting in microglia-mediated neuronal death.[82,99] Microglia need TLR4 to initiate LPS-stimulated Aβ uptake, in fact they trigger a stronger inflammatory response to Aβ in combination with LPS.[84,102]

Neurons themselves respond to Aβ and AD-related peroxidation product HNE through TLR4, resulting in apoptosis.[40] Since little is known about the role of neuronal TLR4 in AD, it is important to start exploring the function of this receptor in animal models and in patient tissue, in a way that differentiates glial-mediated TLR4 responses from neuronal responses, for instance by selective knock-down of TLR4 in neurons in AD mouse models. Collectively, it seems that TLR4 induces an immune response in AD through pro-inflammatory cytokines, aimed at the removal of Aβ by microglial uptake, but also phagocytosis of neurons by microglia. Insufficient removal of Aβ results in an increase of Aβ in the extracellular space, the subsequent activation of microglia and astrocytes, and neuronal apoptosis. In light of this hypothesis, it is also interesting to note the similarity in the role of microglial TLR4 in AD and PD, where this receptor is responsible for the uptake of disease-specific aggregated protein and initiation of neuroinflammation, possibly causing neuronal death.

Stroke

TLR4 has both beneficial and detrimental effects in stroke models. Neurons of I/R treated mice show TLR4 upregulation, a first clue that TLR4 is involved in stroke-induced brain damage.[29,37] Paradoxically, mice treated with low dose systemic LPS two days before I/R injury had smaller infarct sizes and less neuroinflammation in the brain, while TLR4$^{-/-}$ mice had less stroke-induced brain damage and less neurological deficits after I/R treatment.[29,104] Although these results do not suggest a beneficial or harmful role of TLR4 in stroke, the data are not mutually exclusive. TLR4 stimulation before stroke seems to be protective, while TLR4 stimulation during stroke seems detrimental. This hypothesis is supported by *in vitro* experiments. Increased TLR4 activity does not increase neuronal death, and TLR4 stimulation

can be beneficial to neuronal survival at low concentrations.[43,105,54,106] On the other hand, glucose deprivation increased TLR4 expression and cell death in neuronal cultures while TLR4$^{-/-}$ neurons were less susceptible to glucose deprivation induced cell death.[29,37] In agreement with the *in vivo* results, it seems that TLR4 stimulation per se is not harmful to neurons and it might even be beneficial. During stroke TLR4 has a negative impact on neuronal survival, possibly in part through glucose deprivation, but most likely also as the result of a more profound inflammatory process.[107] In relation to the opposing effects of TLR4 in PD, it is curious that TLR4 can have either protective or detrimental effects in the context of stroke.

TLR8

In innate immune cells TLR8 functions as an endosomal receptor that recognizes viral single-stranded RNA. Stimulation of TLR8 induces the activation of the My88 signaling pathway leading to an anti-viral response.[108]

The role of TLR8 in PD and AD has not yet been studied in detail, and is therefore unknown. In stroke patients a higher level of TLR8 mRNA expression in whole blood sample was positively correlated with poor patient outcome after three months, larger infarct volume and greater inflammatory response.[109] Similar results were found in a mouse stroke model: diseased animals had increased TLR8 mRNA expression in their brain and systemic administration of a TLR8 agonist before ischemic insult increased infarct size and neurological problems.[110] The neuronal damage in stroke patients and mice could be mediated by neuronal TLR8, since TLR8 stimulation of neurons results in fewer and shorter neurites and apoptosis and slightly but significantly increases oxygen-glucose deprivation induced cell death, while TLR8 silencing reduced oxygen-glucose deprivation induced cell death.[26,110] Interestingly, neurodevelopmental research has shown that TLR8 is differentially expressed in the embryonic and postnatal brain in mice.[25,26] In the mouse brain TLR8 expression increases between embryonic day 12 and postnatal day 1, and decreases between postnatal day 7 and adulthood.[26] During early embryonic development TLR8 expression is high in postmitotic migrating cells, but not in the periventricular proliferative area.[26] During late embryonic development, TLR8 was restricted to axonal tracts (including the olfactory nerve fiber layer, cortical intermediate zone, internal capsule, anterior commissure, fimbria of hippocampus and optic chiasm).[26] Postnatal expression is diffuse throughout the brain and located in soma.[26] This indicates a potential role of TLR8 in brain development. Considering how little is known about its function in the nervous system, specifically on neurons, both the fields of neurodevelopment and neurodegeneration have much to explore with regards to TLR8. This TLR could be considered critical to study the role of TLRs on neurons in neurodegeneration, and PD in particular.

NEURONAL TLRS AND VIRAL INFECTIONS

In the previous sections we have discussed that TLRs play an important role in microglial-mediated neuroinflammation of neurodegenerative diseases. In this context it is extremely relevant to investigate further the TLR-induced immune-like functions of neurons and to understand the role of neurons in neuroinflammation. Viral infections offer useful conditions to study TLR-mediated neuronal immune functions, because neurons respond to viral infections by upregulating TLR and secreting IFN.

Neurons upregulate TLR3 and TLR4 mRNA in response to HIV and adenovirus infection, they also upregulate IFN-β mRNA in response to Sendai virus, and increase IFN production after TLR3 and TLR8 stimulation.[16,27,45,111] TLR3 and TLR8 are virus-sensing receptors and virus-infected cells use IFNs to signals to the neighboring cells that an infection is ongoing and to induce an immune response from nearby immune cells (or glial cells). All together, such findings suggest that neurons can act as immune cells. These neuronal TLR-mediated immune responses seem to be protective to the neurons themselves. Stimulation of TLR3 on neuronal cell lines inhibits HIV replication through IFN-λ, and TLR3 and TLR8 stimulation of a neuronal cell line results in lower susceptibility to herpes simplex virus-1 potentially through IFN-α. Moreover, TLR3$^{-/-}$ primary neurons showed increased infection when exposed to West Nile virus compared to WT neurons which was not due to changes in IFN-α of IFN-β production.[16,45,46] The protective effect of TLR3 during viral infection of the CNS has been confirmed in mice: infection of TLR3$^{-/-}$ mice with West Nile virus resulted in a higher viral burden in neurons and increased mortality compared to WT mice.[46] The viral-sensing receptors TLR3 and TLR8 are able to initiate a protective immune-like response in neurons upon viral challenge. Unfortunately, it is not clear whether TLR2 or TLR4 have a similar potential to protect neurons against pathogenic (bacterial) attack, and what the resulting immune response would be. Therefore, it is interesting to investigate a wide range of possible immune-like responses in neuronal cultures exposed to endogenous and exogenous TLR2 and TLR4 stimuli.

CONCLUSION

This review summarizes the relevance of TLRs in the

nervous system, and especially in neurodegenerative pathologies. Current literature shows that several neuronal TLRs are involved in the development of the nervous system and in neurodegenerative diseases. Neuronal TLRs are important for NPC proliferation, axonal growth, cell survival and in defense against viral infections. The capacity of TLR-stimulated neurons to respond as immune-like cells (production of cytokines and induction of apoptosis) is of special interest for neurodegenerative diseases, since microglial-mediated neuroinflammation is a feature of neurodegenerative diseases. These results raise the question whether neurons are active contributing to neuroinflammatory degenerative process such as PD. TLR2, TLR3, TLR4 and TLR8 are all important for neuronal function and are implicated in PD, AD and stroke. This suggests that these TLRs should be investigated further in PD and other diseases as the first innate immune receptors on neurons.

Neuronal TLR2 has divergent functions in the nervous system. It responds to tissue damage during stroke, and allows neurons to respond to the neuroinflammatory environment of AD pathology. It is interesting to analyze the involvement of neuronal TLR2 in neurodegenerative diseases other than stroke and AD. Elucidating the role of neuronal TLR2 in PD is very attractive, since microglial TLR2 has already been described in PD pathology.[49,73,77,78] We hypothesize that α-synuclein triggers neuroinflammation through microglial TLR2, initiating a positive feedback loop by increasing TLR2 expression on the microglia, resulting in neurodegeneration and disease progression. The contribution of neuronal TLR2 in this process is yet unknown, and could be evaluated by studying PD mouse models with a specific knock-out of TLR2 in neurons, and by studying the immune response of neurons activated with TLR2 stimuli in culture.

Neuronal TLR3 regulates cortical development and neurogenesis and is able to initiate immune-like responses in response to viral infections. This provides an interesting perspective to explore the function of neuronal TLR3 in neurodegenerative diseases, since we hypothesize that neuronal TLR3 will be detrimental in this context, and because viral infections could cause PD through the development of encephalopathy.[112] Expression patterns of TLR3 in brain tissue of early and late PD would shed light on whether TLR3 is indeed an interesting candidate for future PD research, and what role neuronal TLR3 might play in disease development.

TLR4 is a very promising target for future PD research. The effect of TLR4 during PD can be both beneficial and harmful, but the factors determining the outcome are yet unknown. It is conceivable that neuronal TLR4 could be protective, but the role of microglial TLR4 is still in uncertain. One explanation for the confounding function of TLR4 in PD needs to be sought in the interaction between TLR4 stimulation and the stimulation of other receptors. Such interactions are known to occur for TLR4, in fact neuronal TLR4 interacts with the transient receptor potential cation channel V1 receptor to transduce itch signals and possibly to transduce pain caused by bacterial infections.[55,113] These data open up a new scenario of research in PD, particularly using specific neuronal TLR4 deficient animals, especially if this knock-out can be initiated before, during and after PD initiation, since the beneficial or detrimental effects of TLR4 in stroke seem to be dependent on the timing of TLR4 stimulation in relation to stroke. Similarly, the study of the effects of TLR4 stimulation in neuronal cultures in combination with (microglial) immune signals known to be important in PD pathology represents also another research direction.

TLR8 influences neuronal growth and survival and is also important for the initiation of the immune response during neuronal viral infections. The implications for neurodegenerative diseases are manifold, since viral infections have been linked to PD and a better understanding of the mechanisms underlying neuronal survival could help to reduce neuronal death.[112] Since so little is known about TLR8, further investigation about the role of this receptor represents an opportunity for future PD research. Neuronal TLRs are an emerging research area, which will have implications for neurodevelopmental, neurodegenerative and neuroinflammatory research. To date neuronal TLR2-4 and neuronal TLR8 are known to be promising candidates for future studies. Elucidation of the function of other neuronal TLRs requires further research that would lead to a better understanding of the interaction between the nervous system and the immune system.

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Conflicts of interest

J. Garssen is employee at Nutricia research, Utrecht, the Netherlands.

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Original Article

Morphological and behavioural variation in CNS innate defence cell microglia is development and age sensitive

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Payal Ghosh is a Junior Research Fellow holding the prestigious UGC-NET fellowship from MHRD, Government of India and working with Dr. Anirban Ghosh for over three 3 years. She is involved to find out the colonization and morpho-functional pattern of microglia with its cellular adaptability throughout the developmental continuum in rodent model.

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ABSTRACT

Aim: Microglia, the innate defence cells in central nervous system (CNS), alters their shapes and function with age. We observed and identified these morphological changes and functional association throughout the developmental gradient until adulthood in rat brain. **Methods:** Early and late embryonic stages, neonates and adult brains of albino rats were sectioned for routine Haematoxylin Eosin (HE) staining and specialized silver-gold staining to show distribution and morphological variation *in situ*. Isolated microglia from different age groups was subjected to scanning electron microscopy (SEM) for observing ultrastructural shapes of microglial cells. The Viability of isolated cells was measured by trypan blue staining and their cellular identity by immuno-staining for CD11b. Finally, phagocytic limitations of the cells in normally developing brain were assessed by carbon particle ingestion and oxidative burst through nitroblue tetrazolium assay to investigate microglial age-sensitivity behavioural response. **Results:** HE staining spotted overall cellular distribution in the brain and cells with monocytic appearance among the other CNS cells. On contrary, silver-gold staining showed variable morphologies of microglia in various age groups and also showed the appearance of ramified microglia in adult. Nearly 90% of isolated cells were viable and positive for CD11b. SEM showed variable shapes of amoeboid and ramified forms. Immunofluoresence confirms microglial identity. Functionally, microglia showed an age dependent baseline phagocytic capacity in normal condition which changes with developmental phase and age with most active phagocytic behaviour around perinatal phase. **Conclusion:** In normally developing brains, microglia shows variability in morphology and baseline phagocytic activity that changes with age. These results may represent the normal physiology of CNS development and function.

Key words: Microglia; central nervous system; development; phagocytosis; nitroblue tetrazolium assay

INTRODUCTION

Among the ectodermal allies in brain there are some mesodermal aliens spread throughout the tissue. They are myeloid/monocytic lineage cells and acting as the sentinel of this delicate organ on behalf of the innate immunity of the body. Although there is a consensus about the myeloid origin of microglia, much controversy remains regarding the precise nature of microglial progenitors. Many authors claim that microglial cells arise early during development from progenitors in

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the embryonic yolk sac that are then seeded in the rudimentary brain and persist there into adulthood.[1] Another school of scientists thought that their entry in brain from blood also occurs at some points in embryonic and early natal phases, which also gradually populates into entire brain and designated as microglia.[2-5] Microglia function like a hybrids of glia and leukocytes and thus express a variety of cytokine receptors as well as producing cytokines themselves.[6] Microglia forms an extremely stable population in brain and comprise up to 20% of total glial population forming an immune accessory network.[7,8] Microglia can adapt according to the central nervous system (CNS) microenvironment, monitor the CNS integrity and also act under the strict control of neurobiochemical environment. Recent studies indicate that they function in maintaining normal tissue homeostasis in brain at the resting state through scanning their territorial domains.[9-11] They phagocytose cellular debris, contribute to restructuring neuronal circuits and triggering repair, which are assumed to be related with development.[12-16] Depending on specific environmental context microglia play a dual role of neuroprotection or neurotoxic.[12,17-19]

There are few studies which showed that microglial morphology changes with age, but most of the studies have dealt with their changes in neuropathological conditions.[20] Microglial transformation from ramified to amoeboid affects their functional modifications. The effector role of the cells are mostly studied in disease models or subjects,[21] But seldom has any attempt been made to evaluate the baseline physiological response of the cells in a normally developing brain beginning at birth. However, recent findings showing their role in neuronal circuit development and maintaining tissue homeostasis indicates that they have a basal physiological function from developing embryo to growing adult.[11,13] In the present study our attempt is to find that basal morphological and behavioural variation of microglia from embryo to growing brain up to its maturation, excluding aging brain. With identifying *in situ* distribution and morphological transformation, we also isolated them to assess the morphological differences and functional deviation in terms of phagocytosis.

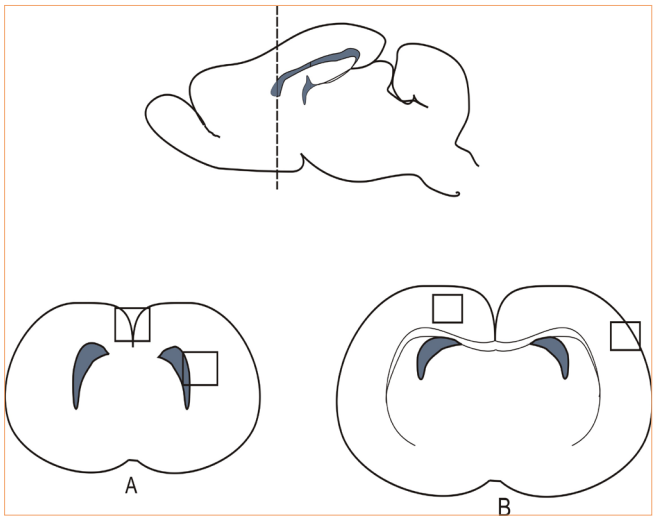

Figure 1: Graphical representation of a rat brain showing the sagittal and coronal view of the section planes. Dotted line in the sagittal view shows the plane of section used for histology and filled grey areas are showing lateral and 3rd ventricles. Diagram marked A shows the coronal view of late embryonic and neonatal pups where square boxes are showing the sectioned areas adjacent to lateral ventricles and neocortex at the cleavage of two hemispheres which are the areas represented in the photomicrographs. Diagram B shows the coronal view of adult rats where square boxes are showing the regions of cerebral cortex represented here

METHODS

Animal and grouping

The Sprague-Dawley rats were maintained for the experiment as approved by institutional animal ethical committee (Approval No. -AG/CP/IAEC-WBSU/2011-12/5) and according to the animal experiment procedures strictly followed the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised in 1985). The animals were fed with pellet diet, or equivalent, and water *ad libitum*, 12 h light and dark cycle were maintained, examined and weighed at regular interval throughout the experimental period. Reproductively matured male and receptive female were set for breeding at a rate of 1:2 respectively, examined for confirmation of mating usually made by visualising the copulatory plug, after that pregnant mothers were separated and pregnancy days were counted to obtain the required embryos.[22] Neonates were maintained with their respective mothers in one cage as they were at waning age. The groups of animals maintained were (1) early embryo (ED 10 ± 1); (2) late embryo (ED 18 ± 2); (3) neonate (D 5 ± 1); (4) young adult (D45 ± 5) and (5) mature adult (D 240 ± 10).

Histological sectioning of brain tissue and haematoxylin-eosin staining

The rats were deeply anaesthetized with sodium pentobarbital (50 mg/kg body weight). The whole brain was dissected out, initially placed in ice cold Phosphate Buffer Saline (PBS) and then postfixed in 10% buffered formalin (NICE, India) for overnight at 4 °C. After fixation, foetal, postnatal and adult brains were washed in PBS, dehydrated through graded alcohol (30%, 50%, 70%, 95% and absolute alcohol) and embedded in paraffin (MERCK, India) through histokinate processing. From this block coronal sections of brain were cut at 5-7 μm thickness with a microtome (WESWOXTM OPTIK Rotary Microtome, Model-MT-1090A, India). The sagittal and coronal sections [Figure 1] show the schematic positions of the rat brain cerebral cortex adjacent to the ventricular margin and inner and outer cortex which are used and represented in the study. The sections were then routinely stained with haematoxylin-eosin (HE)

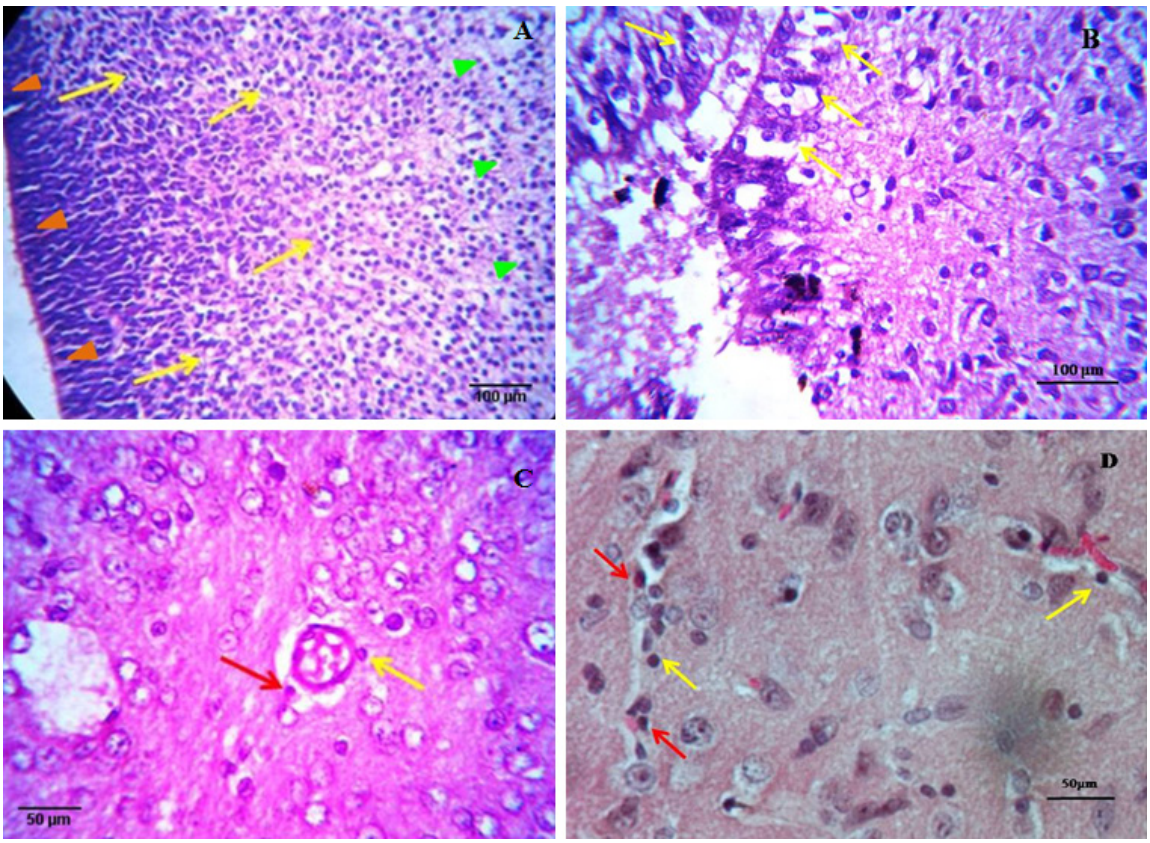

Figure 2: Haematoxylin-eosin stained brain sections of early embryo (×40) (A), late embryo (×40) (B), neonate (×40) (C) and young adult (×100) (D). (A) Different neuroglia precursor cells and a huge number of predicted myeloid cells of forming ventricular margin radiate towards outer cortex and colonize there. Migration was indicated by arrows (yellow), ventricular margins by orange arrowheads and outer cortex by green arrowheads; (B) a distinct band of cells with a definite organisation appears at the margin (indicated by arrows) but immediately after that diffused cell matrix with scattered cells is observed; (C) in case of neonates along with oligodendrocytes, astrocytes, neuronal cells, a prominent blood capillary with extravasation (indicated by red arrow) of amoeboid monocytic cells out of the BBB is visible. A cell also tethered to the margin of the capillary (indicated by yellow arrow); (D) gathering (indicated by red arrows) and infiltration (indicated by yellow arrows) of blood vessel containing leukocytes into the brain parenchyma to form a stable population of monocytic cells. Perivascular macrophage/microglia are also observed

to identify and observe the distribution of overall cell population in brain. Selected fields were documented by Olympus DSC (12 Megapixel) camera through Olympus CH20i Microscope and processed by Image J software (NIH, USA).

Silver gold staining of brain tissue

Histological sectioning of brain tissues [Figure 1] were then subjected to a specialized silver carbonate staining first introduced by del Rio Hortega (1918) and gold toning by Penfield (1937), modified by McCarter (1939).[23,24] Briefly, 10 µm thick brain sections were initially deparaffinised by using xylene (MERCK, India) and placed in aqueous ammonia solution, then passed through Globus' hydrobromic acid (MERCK, India) solution and washed. The slides were then rinsed into 50% aqueous solution of silver carbonate derived from silver nitrate (FINAR, India) and sodium carbonate (CDH, New Delhi) for 1 h. Sections were then passed in formalin and washed and placed in aqueous gold chloride (HIMEDIA, India) to counter-stain. After that, slides were rinsed thoroughly in dH_2O and fixed in sodium thiosulfate (MERCK, India) solution, washed, dehydrated in alcohol and mounted in DPX (MERCK, India) to observe under the microscope. The morphological changes of microglia *in situ* from embryonic to adult normal brains were documented with the microscope Nikon Eclipse TS 100, using CCD Camera (DS-Fi2-U3) and analysed by NIS Elements BR software (Nikon Corporation, Japan).

Isolation of microglia from different age groups of brains

Microglia were isolated and characterized as described previously with slight modification.[25] Briefly, after heart perfusion of anesthetized rats, dissected brain tissues were placed under a binocular stereomicroscope (Magnus MS-24) to peel off major blood vessels and capillaries. The whole brain was mechanically dissociated, lightly homogenized and enzymatically digested for 30 min at 37 ºC by 5-15 U type II collagenase (Sigma-Aldrich, USA) and 500 U DNase I (Sigma-Aldrich, USA).[26] Then the suspension are washed and resuspended in ice cold PBS and passed through stainless steel sieve of porosity about 80 µm to make single cell suspension. The cell suspensions were then allowed to adhere on glass petri-dish (DURAN, Czech Republic) for 1 h in 5% CO_2 humified environment at 37 ºC (CO_2 Incubator Galaxy 48S, New Brunswick, Germany). The adherent cells were then recovered with Trypsin-EDTA (MP Biomedicals,

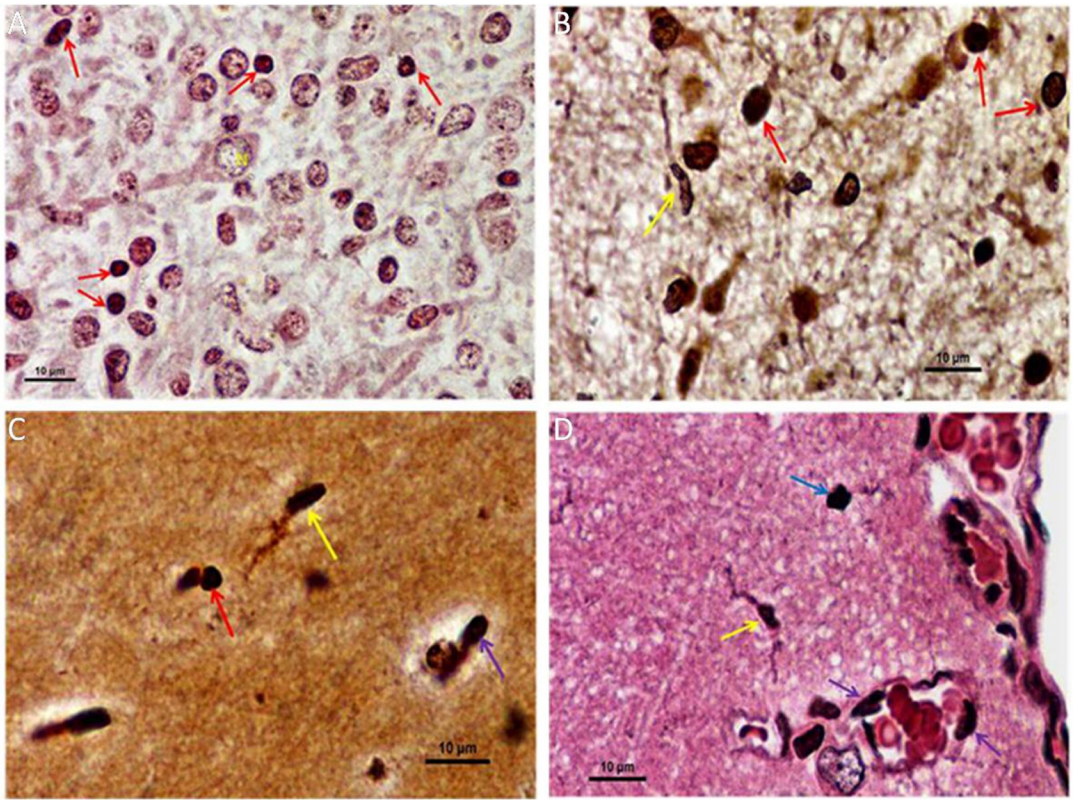

Figure 3: Silver gold staining of brain tissue showing morphological variation of microglia/macrophage in both developing and adult rat brain (×100, Oil immersion). (A) In late embryo, densely stained myelo-monocytic lineage cells (indicated by red arrows) along with other neuroglial cells and a distinct neuronal cell body are observed in brain tissue matrix. "N" represents nucleus of the neuron; (B) in neonates, mostly amoeboid microglial cells (indicated by red arrows) are evenly distributed throughout the matrix and a distinct ramified microglia (indicated by yellow arrow) is also appeared; (C) whereas in young adult, ramified microglia with their slender ramifications (indicated by yellow arrow) are clearly visible and cells with amoeboid morphology (indicated by red arrow) are also observed. In addition, the entry of a deeply stained cell into the brain matrix from a capillary (indicated by violet arrow) is visible; (D) in late adult, both irregular shaped amoeboid microglia (indicated by blue arrow) and ramified microglia with their projections (indicated by yellow arrow) are clearly found in cortical regions. From blood capillary or vasculature the entry of myeloid cells in brain parenchyma is visible by the presence of deeply stained cells at the margin of capillary (indicated by violet arrow), among them a few cells just entering into the tissue matrix and resides there

USA) solution followed by addition of 1X RPMI-1640 media with 10% fetal bovine serum (FBS) is used to neutralize the reaction of trypsin-EDTA solution[27] and there after centrifuged, supernatant was discarded and pellet was washed. The resulting cell suspensions were then laid on 20-70% Percoll gradient at 2,000 rpm for 25 min yielding highly enriched microglia at the interface. Cells were recovered, washed and suspended in RPMI-1640 (MP Biomedicals, USA) containing 1% penicillin-streptomycin (P/S, MP Biomedicals, USA) and their viability was immediately measured by trypan blue exclusion (10 µL trypan with 10 µL cell suspended in media) in Neubauer Improved Chamber (Marienfeld, Germany). In cases of embryo and neonates, the whole brain of 5-6 pups or embryos were pooled together and treated with half-dilution of enzyme concentration as for adult rat and some modification in centrifugation. The morphological variation among different groups viability of isolated cells were documented and photographed by Nikon Microscope Nikon Eclipse TS 100, using CCD Camera (DS-Fi2-U3) and NIS Elements BR software (Nikon Corporation, Japan).

Characterisation of microglial cells by immunofluoresence with CD11b marker

Isolated cells were cultured in RPMI 1640 media (contains 10% FBS + 1% P/S) for 3 days in a humified CO2 incubator at 5% CO_2 level at 37 ºC. After a PBS wash cells were mildly fixed with 2% paraformaldehyde solution for 10 min at 4 °C temperature, washed and stained with FITC conjugated fluorescein anti-CD11b antibody (eBioscience, USA) diluted in 1% FBS (Gibco®, Life technologies, USA) in PBS (1:500) and incubated for 1 h in a dark humified chamber. After washing, the cells were observed under inverted fluorescence microscope (Nikon Eclipse TS 100), photographed using a CCD Camera (DS-Fi2-U3), and analysed by NIS Elements BR software (Nikon Corporation, Japan).

Ultrastructure of microglia in scanning electron microscopy

The cells immediately isolated from brain tissue were fixed in 2.5% glutaraldehyde for 4 h at 4 °C followed by washing in PBS and gradually dehydrated in graded alcohol and finally brought to 100% acetone. Dehydration were followed by critical-point drying, spread on 1 cm^2 grease-free glass slide placed on metallic stub with conducting silver paint. The cells were laid on a glass platform and then coated with gold-palladium alloy of 100Å-200Å thickness in a diode sputtering system. Then the samples were observed under Scanning Electron Microscope (HITACHI, S530, Japan) at 15 KV beam

Table 1: Age dependent cellular viability (in percentage) as obtained in culture

Groups	% of viable cells
Early embryo (ED 10 ± 1)	87 ± 3
Late embryo (ED 18 ± 2)	86 ± 2
Neonates (D 5 ± 1)	90 ± 3
Young adult (D 45 ± 5)	92 ± 3

voltage. The specimens were scanned and photographed.

Phagocytosis and measurement of oxidative burst of microglia by nitroblue tetrazolium chloride reduction assay

Phagocytic properties of the isolated cells from different age groups were observed and quantified parallel by incubating cells with ultrafine carbon particles (SRL, India) and nitroblue tetrazolium chloride (NBT) salt (Sigma, USA). NBT reduction assays with carbon particle ingestion, were performed to visualize phagocytosis and measure the oxidative burst of phagocytic cells. For both early and late embryos, a pool of cells was made from a single mother's foetus or pups. In case of early embryos from a single mother, an average of 7 embryos were obtained from which cells were isolated. The same process was done for another 2 pregnant mothers (n = 3 pregnant mother foetus). For late embryos, an average of 6 embryos were isolated from a single mother for an experiment, which was conducted in triplicate (n = 3 pregnant mother foetus). In the case of neonates, 3 sets, each containing an average of 6 pups, were taken to execute the experiment (n = 3). Whereas in case of young adults as the volume of brain and number of cells obtained from each adult rat was sufficiently higher 3 individuals (n = 3) were used for three sets of experiments. In each age group, cell numbers were diluted to 10^7 cells/mL. The isolated cells along with 0.1% NBT and ultrafine carbon particle were allowed to incubate at 37 °C overnight. The reaction was stopped with 0.1 N chilled HCl. A sample of cells from each reaction mixture was transferred to a Neubauer's chamber to observe under a phase contrast microscope (Nikon Eclipse TS 100) and documented using a CCD Camera (DS-Fi2-U3) and analysed with NIS Elements BR software (Nikon Corporation, Japan). The remainder of each reaction mixture was centrifuged, supernatant removed, pellets resuspended in pyridine, and boiled for 10 min, which converted the extract into blue by reducing NBT into formazan, which can be measured colorimetrically at 540 nm. The OD value is directly proportional to the level of phagocytosis or, more precisely, the reactive oxygen species (ROS) generation,[21] by different developmental age groups.

Statistical analysis

Microsoft Excel (Redmond, WA) was used to compute and tabulate experimental results. For statistical interpretation, one-way ANOVAs, followed by Tukey-Kramer *post-hoc* tests, were performed, and the results were analysed using OriginPro 8 Software and graphically represented. Data were expressed as mean ± SD and a P value < 0.05 was considered significant.

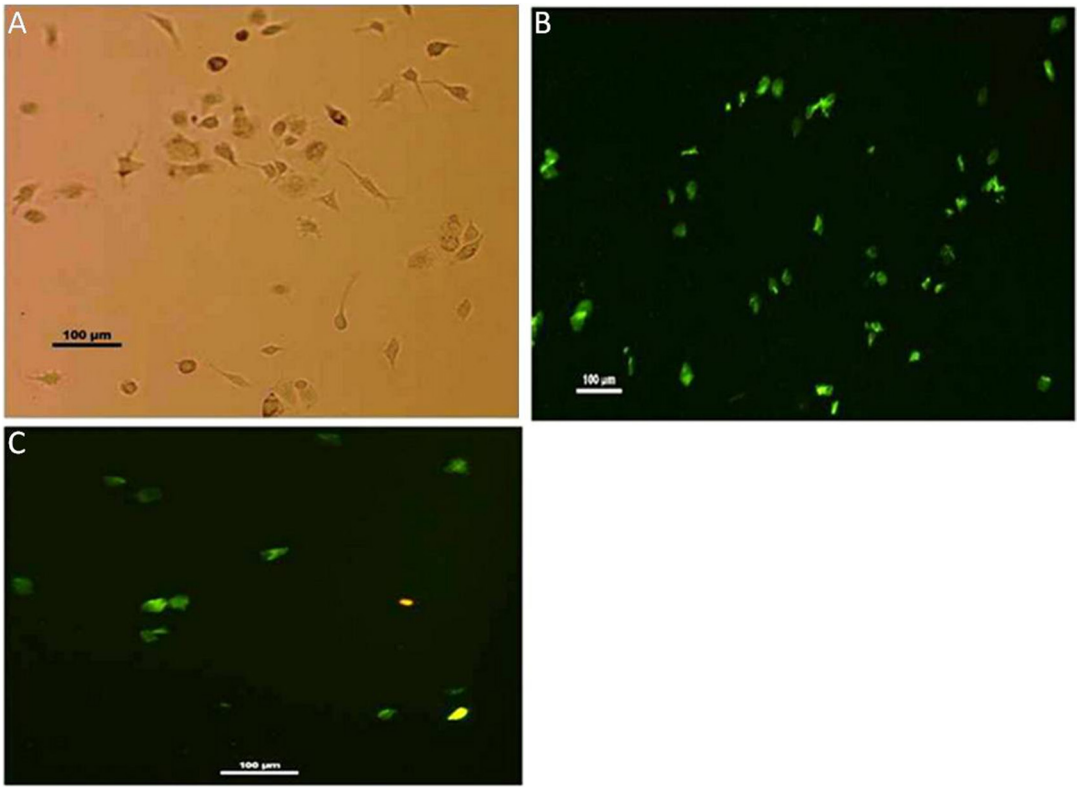

Figure 4: Isolated microglial cells. (A) Microglia isolated from rats shows varied morphology i.e. both irregular and elongated cells with extended pseudopod. (×10); (B) immunostaining of isolated cells from adult rat brain showing CD11b+ (positivity) prominently with morphological variations, i.e., both irregular and elongated cells are observed (×10); (C) whereas in case of neonates most of CD11b+ cells are showing irregular morphology (×10)

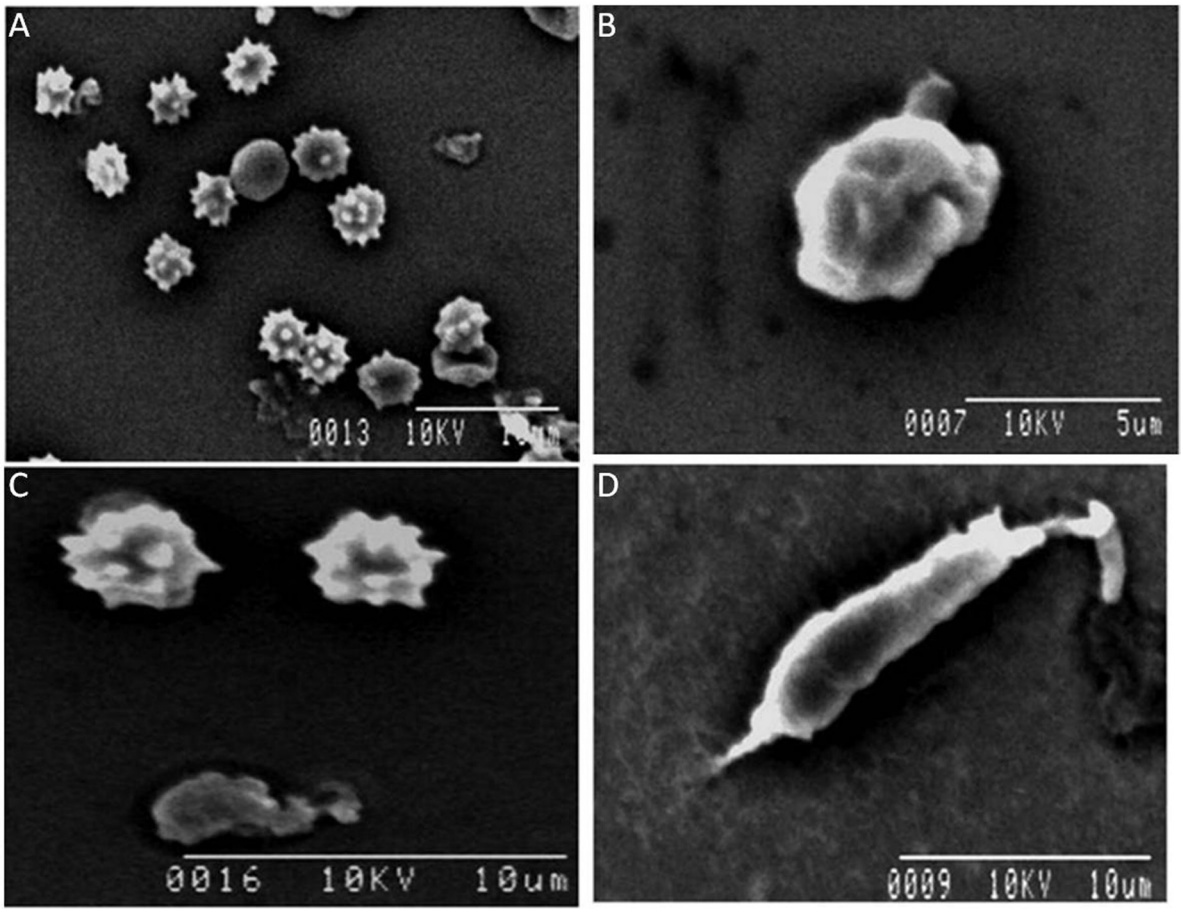

Figure 5: Scanning electron micrograph of isolated microglia cells from brain tissue. (A) Microglia recovered from early embryonic developing brain show many of them retain spines bearing surfaces which radiate out of the cell body ; (B) a few cells remain amoeboid in shape with a ruffled surface membrane and short stubby projections in late embryo; (C) in neonates, the presence of both amoeboid cells with spines bearing surfaces and also extended structure of amoeboid microglia cell; (D) ramified or "resting" microglia isolated from adult brain with extended pseudopodia are seen

RESULTS

Colonization pattern of neuroglial cells from embryo to adult

Staining of brain tissue of different ages with normal HE staining shows a changing cellular distribution in the brain across time. In the early embryo, huge numbers of presumptive neuroglial and myeloid cells were found to migrate from the inner ventricular margin to the outer cortex region. Different glial progenitor cells, along with neuronal precursor cells, colonize the brain parenchyma from the predictive neuroglial stem cell line at the margin of the forming ventricle [Figure 2A]. In the case of a late embryo, normal HE staining shows a remarkably different cellularity near the ventricle. A cellular band is distinct at the margin, but immediately after that, a diffused cell matrix starts with scattered cells. Variations among the cells are more prominent, indicating further differentiation occurring in the cells [Figure 2B]. An example of a section from a neonate of 4-5 days clearly shows perivascular monocyte cells and a cell extravasating out of a blood capillary, hence, across the blood brain barrier (BBB), indicating blood capillaries as important sources of myeloid/monocytic cells that colonize the brain [Figure 2C]. In a section from an adult, normal HE staining reveals brain parenchyma with a blood vessel containing leukocytes, many of which are present at the margin of the capillary and tethered to the endothelium and therefore extravaseting at the perivascular spaces, along with other neuroglial cells in the matrix [Figure 2D]. Therefore, normal HE staining shows overall cellular distribution, colonization, and differentiation patterns of various brain cells that change remarkably from embryo to adult.

Variable morphological forms of microglia in developing through adult brain

Silver-gold staining of brain tissue, *in situ*, can differentiate myeloid lineage cells, including microglia/macrophages, from neuronal and glial populations by its characteristic dark staining relative to background.[28,29] As a result, changes in shape from "amoeboid" in the embryo to "ramified" in the adult, is documented. In early embryos, the developing brain is saturated with presumptive cells that stain darkly and are hard to distinguish between neuro-glial and microglial precursors, and hence, were omitted. In late embryos [Figure 3A], the deeply stained myelo-monocytic lineage cells, or presumptive microglia, appear prominently in comparison with other neuroglial cells. A distinct and prominent neuronal cell body, along with a dendritic

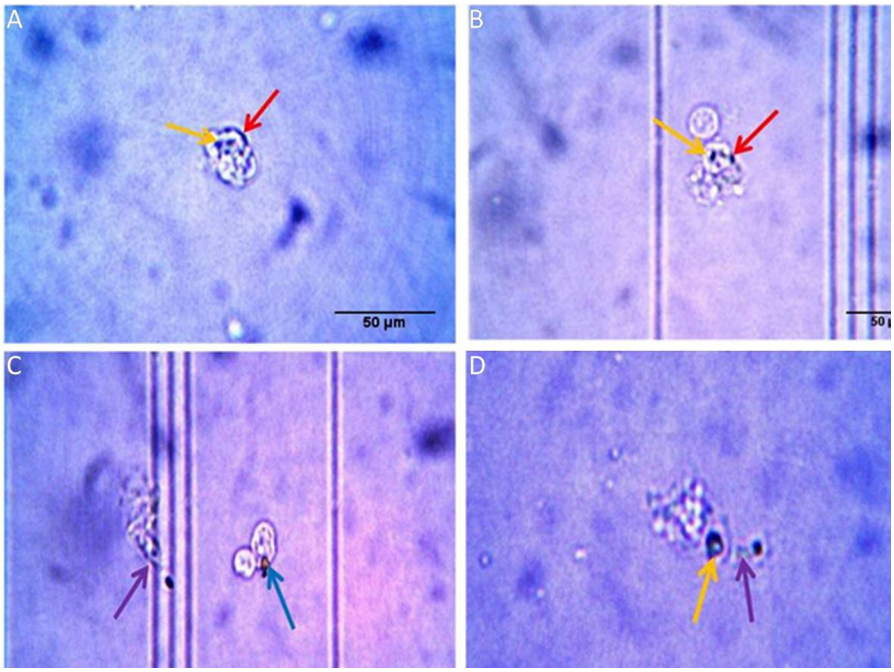

Figure 6: Microglial ingestion of carbon particle in (A) early embryo, (B) late embryo, (C) neonates and (D) adult. In case of both embryonic stages a few carbon particles were attached to the cell (indicated by red arrows) whereas some particles become engulfed by the cell (indicated by yellow arrows). In neonates, a phagocytic vacuole (indicated by blue arrow) with carbon particle is observed along with a cell which engulfs a carbon particle by extending its pseudopodia (indicated by violet arrow). In an adult, an irregular shaped cell shows the same pattern of phagocytotic movement towards a carbon particle; the cell engulfs one particle and also extends a projection towards another carbon particle (indicated by yellow & violet arrows respectively)

projection, is also observed. In neonates [Figure 3B], microglia cells are found evenly dispersed throughout the brain matrix, along with other neuro-glial cells. Both amoeboid and distinctly ramified microglia, with their long tactile projections, has now appeared. Furthermore, amoeboid microglia predominates in neonatal brain. In the case of 3 months old rat brain or young adult [Figure 3C], the so called ramified microglia with their slender projections, elongated cell body, and nucleus are clearly visible and appear high in number. Similarly, cells of amoeboid morphology are also observed. In the mature adult [Figure 3D], microglia, with their ramifications, are found distinctively throughout brain, along with other amoeboid forms. Here, ramified microglia are prominent in the cortical region along with an irregular shaped amoeboid form and the morphological differences are distinctly prominent. At the same time, it is seen that some deeply stained cells tethering with the margin of blood capillary or vasculature, while others just seem to enter in to the tissue matrix. In brief silver-gold staining shows that during pre and postnatal phase, amoeboid microglia are present predominantly, and a few ramified form of microglia are detected in postnatal phase. Whereas, in adult, both forms of microglia were observed and a blood capillary distinctly enabling the entry of myeloid cells into brain parenchyma.

Identification, characterisation and measurement of cell viability of isolated microglia

Microglia isolated from rat brain are identified with their CD11b positivity when immunostained with anti-CD11b-FITC conjugated antibody. Isolated groups from different groups show irregular shapes and variations. Representative figure of phase contrast of microglia isolated from young adult shows both amoeboid and ramified microglia with slender projections [Figure 4A]. These morphological variations are also supported by immunostaining. In the case of an adult, CD11b+ cells show both irregular and elongated morphology [Figure 4B], whereas isolated cells from neonates show rounded/irregular structures [Figure 4C]. Trypan blue-staining, separate from immuno-staining, was used to discriminate between viable (unstained) and non-viable cells (blue) not shown, was done to determine the yields of isolated, viable cells (in percentage) from different age groups [Table 1].

Evaluation of the ultrastructural changes of microglial cells isolated from brain tissue

Morphological variations of microglia become prominent when isolated microglia from different groups is studied ultrastructurally. Microglial cells isolated from neonates show rounded/irregular morphology, whereas those isolated from adult brain appear as irregular, elongated morphology with variations. These morphological variations among different age groups are also supported by further ultrastructural analysis. Cellular architectures of microglia isolated from brain tissue are determined by scanning electron microscopy (SEM). SEM studies reveal the population of microglial cells in developing early embryonic brain. Most of the isolated cells are irregularly round with serrated surface, typically monocyte/macrophage morphology in this stage of

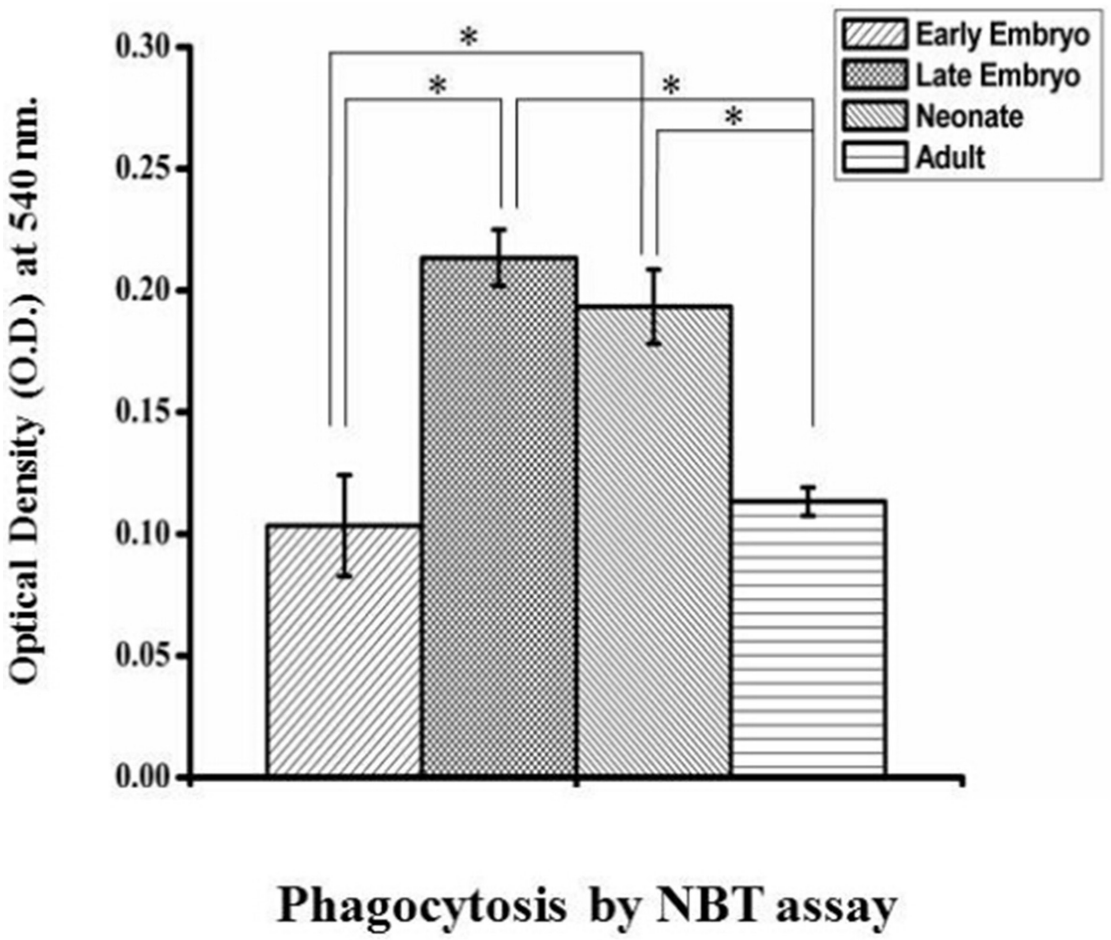

Figure 7: Quantification of phagocytosis using a colorimetric NBT assay. ROS generation by cells from different age groups in normal conditions was measured by using the colorimetric NBT assay done in triplicate set. Data are statistically interpreted by using one-way ANOVA followed by Tukey-Kramer test used for post-hoc multiple comparisons where $P < 0.05$ is considered significant. Significant difference associated with phagocytic ability present between different age groups where P value is less than 0.05 indicated by * symbol in graph. Graphical representation shows there is a significant increase of phagocytic ability in both late embryo and neonates as compared to the early embryo (indicated by * mark) whereas in case of adult the variation is insignificant with early embryo. Significant difference in phagocytic potential between these two groups (late embryo and neonates) with the adult is also indicated by * symbol

developing brain [Figure 5A]. This pattern [Figure 5B] is gradually changing where the amoeboid cells appear with extended pseudopodia or cellular projections in the late embryonic phase. Figure 5C reveals the presence of both amoeboid microglia and the extended structure of a microglial cell. Both of them are showing active phagocytic forms. When an animal develops from infant to adulthood its microglial structure changes from rounded to elongated ramified forms. In an adult [Figure 5D], a cell shows an elongated structure with projection and a few slender filopodia that are prominent at the edge of the cell. This is the ramified, or so called resting, form that is characteristic of microglial populations observed in adult brains.

Phagocytosis by microglia isolated from different age groups of animals

Carbon particle ingestion shows the phagocytic activity in all stages from early embryo to adult. Carbon particle ingested or adhere to the cell is found in both embryos [Figure 6A and B]. In neonates, phagocytic vacuole engulfing carbon particle in one cell and pseudopodial extension phagocytising carbon particle by another is visible [Figure 6C]. In case of adult, cell shows engulfed carbon particles along with a pseudopodial projection that entangle another carbon particle to draw and engulf [Figure 6D]. Measurement of phagocytosis in terms of oxidative burst is done in different age groups without any external stimulus and the animals maintained in normal condition. So the values of phagocytosis of different age groups reflect their baseline phagocytic capacity in normal condition. The OD value is directly proportional to their phagocytic capacity. This shows a prominent age dependent trend of phagocytic capacity. In early embryonic stage when they enter and populate in huge number to the brain parenchyma, they show less phagocytic potential. But it shows high phagocytic capacity at the late embryonic stage and continues for few days just after birth. Afterwards, during resting stage in stable adult brain this activity reaches to a basal level which is comparable to the early embryonic stage [Figure 7]. Statistical interpretation of the data by one way ANOVA, followed by Tukey-Kramer test used for post-hoc multiple comparisons, shows that there is a

significant variation in phagocytic capacity between early embryo and both late embryo and neonates in phagocytic potential or oxidative burst in normal condition. But the difference between late embryo and neonates is insignificant. In contrast, there is a significant difference in phagocytosis between these two groups (late embryo and neonates) with the adult. Thus, it shows the trend of high phagocytic capacity just before and after birth, while early adulthood microglial cells become inactive in terms of phagocytosis, therefore, transformed into a resting or surveillance stage.

DISCUSSION

Our present study revealed a shift of microglial structure, distribution, and function across pre- and postnatal stages of development in the rat brain under normal physiology. This transverse analysis of microglial activities in brains from embryos to adults shows that microglia are capable of dynamically adjusting their position, shape, and function as the developmental requirements of the CNS change. If an age related structure-function model of microglia has been determined, deviations from it may be diagnostic for some CNS disorders. However, this study is limited to the developmental maturation of the rat brain, mostly up to young adulthood, which excludes the aging brain.

Our study shows significant differences in the morphology of microglial cells throughout the developmental/age phases, along with their functional attributes. Initially, by HE staining, round shaped macrophage/myeloid lineage cells, in both embryonic and infant stages, were found in brain parenchyma, whereas in adults, these cells showed conversion into elongated structures. Throughout the developing brain parenchyma, a particular colonizing pattern of blood-borne myeloid cells was observed as they migrated from the inner ventricular margin to the outer cortex region. In late embryonic and infant stages, few cells were observed to enter the brain from blood vascular fenestrations. However, in adults, normal HE staining revealed the presence of monocytes, many of which were present at the margin and tethered to the endothelium, in the perivascular space infiltrating from the capillary into deep brain parenchyma. Thus, while transitioning to adulthood, cells of the myeloid lineage stabilize their positions in the CNS and develop their morphological attributes. Furthermore, although the general notion of microglial populations being fixed in normal adult brains,[7,8,30,31] the present micrographs indicate a potential avenue for blood-borne, myeloid-monocytic cells to enter the brain parenchyma.

Ultrastructural study by SEM of isolated cells also revealed the structural changes. Microglia recovered from brains of different developmental ages show distinctive trends in morphology. Many of them retain spine bearing surfaces that radiate from the cell body, a feature also seen in micrographs of isolated cells. A few cells remain amoeboid in shape with a ruffled surface membrane and short stubby projections. Ramified or "resting" microglia with extended pseudopodia, isolated from adult brains, were also found, which in turn gives us information about the amoeboid to ramified transition throughout the various developmental phases or ages.[32,33] With the maturation from embryo to adult, the shift from amoeboid to ramified morphology, is supported prominently in the silver-gold and SEM studies. The microglia/macrophage specific CD11b marker expression, determined by immunofluorescence, in isolated cells confirms their lineage. Phagocytosis of ultrafine carbon particles by isolated monocytic/myeloid-lineage cells occurred at all stages, from early embryo to adult, but the efficacy varied among the different groups. The concept of a microglial dichotomy with M1 and M2 phenotypes[34] may be examined with this model. The NBT assay, for estimating ROS generation, documented mild phagocytic activity in the early embryo and adult and much higher phagocytic activity in late embryos and neonates. This difference of phagocytic activity during the perinatal period is functionally related with the developmental organization of brain tissue. In that stage, neuronal positioning and extension, formation of neuronal circuitry, abrogation of wrong connections and reformation of proper contacts, and synaptic pruning are at their highest levels to develop a properly functional CNS system.[13,15,35,36] Thus, there are enough phagocytic activities to trim and clean the forming CNS during the perinatal phase.[12,35,37] Furthermore, phagocytosis at a controlled pace has an active and important role in developing and maintaining proper organization and integrity of CNS tissue for the lifetime of the animal. Hence, our study showed that the functionally linked increase in levels of basal phagocytic activity in microglial cells isolated from brains, before and after birth, were present mostly in cells showing an amoeboid morphology. The overall information gathered from our study is a morphological, functional relationship of brain macrophage/microglia in normally developing rat brains with a significant pattern of colonization in both early and late embryo, neonates, and even in adults. This study documents the spacio-temporal activities of versatile, immune-competent brain cell sunder normal physiological conditions from development to maturity. These baseline activities may be used as a reference frame for detecting and analysing morphological, functional anomalies of microglia in deformities and disease.

In summary, our results show an age-dependent variation of morphological and behavioural functioning

of microglia, which act as sentinels of the CNS. Normal HE staining and specialised silver-gold staining of brain tissue show morphologic variations of these cells in both pre and postnatal stages of the developing rat brain, which are further supported with an ultrastructural, SEM study. The microglial identity of the isolated cells was confirmed with a CD11b marker. The cells in the normally developing brain also show an age dependent variation in their baseline phagocytic capacity, which is higher in the perinatal phase and assumed to be related with the normal physiology of CNS development and function. Therefore, changes in age sensitive microglial morphology and phagocytic activity from the embryonic to the adult brain could be an important reference when considering any neuropathological conditions with microglial involvement.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

Infective endocarditis with brain lesions misdiagnosed as viral encephalitis

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ABSTRACT

Infective endocarditis (IE) is caused by infection of the endocardial surface of heart. It typically affects one or more heart valves, the mural endocardium, or a septal defect. In recent years, many IE patients suffered from atypical initial symptoms. Here, in this case report, a 12-year-old patient was initially diagnosed as encephalitis. However, it was later noticed that this was a misdiagnosis for the following reasons: the echocardiography showed a vegetation attached to his mitral valves; the cranial magnetic resonance imaging showed lesions that were consistent with a cardioembolic distribution. The final diagnosis was IE.

Key words: Antiplatelet agents, embolic events, infective endocarditis

INTRODUCTION

Infective endocarditis (IE) is caused by infection of endocardial surface of heart. It may affect one or more heart valves, the mural endocardium or a septal defect.[1] Embolic events are serious complications, and it is estimated that they occur in 10% to 50% in IE patients.[2] Embolic stroke is among the most notable and life-threatening ones. It interferes with patient normal activities and can cause death.[3] However, as IE clinical symptoms have become atypical and the morphology and location of embolic intracranial lesions are in diverse forms, it may easily lead to misdiagnosis.[4] Here, we describe a case of cerebral embolism with atypical IE symptoms.

CASE REPORT

The patient is a 12-year-old male without any previous disease. On November 28, 2014, he got a fever with the body temperature of 39.5 °C. He no longer had fever after infusion. Three days later, he suddenly suffered from headache, along with nausea and vomiting. Subsequently, he had an episode of generalised tonic-clonic seizure. These symptoms lasted several minutes. Then he came to his senses, but with low weak voice and slow responses. His cranial magnetic resonance imaging (MRI) was performed in the referring hospital. This revealed the presence of multiple lesions in bilateral cerebellar hemisphere, the right thalamus and occipital lobe. Cerebrospinal fluid was acellular with normal protein and glucose. He was diagnosed with viral encephalitis and treated with intravenous acyclovir and mannitol. On December 4, the patient suddenly developed a left limbs weakness. At that stage he was then transferred to our clinic.

In the physical examination, we observed that he was having a heart murmur, slow response, low weak voice, slow light reflex in left pupil and left hemiplegia. There were no meningeal signs. Routine blood examination showed mild anemia (hemoglobin 122 g/L, normal 130-175 g/L). Upon his admission, the following laboratory tests gave a negative result: blood biochemistry analysis, coagulation profile, myocardial

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enzymes, thyroid function, erythrocyte sedimentation rate, anti-streptolysin *O* test, rheumatoid factor, high-sensitivity C-reactive protein, blood cultures, and autoantibody series (such as antinuclear antibodies, ds-DNA and so on). The test results of pathogens (bacteria, viruses and treponema pallidum) were also negative in blood. Routine electroencephalogram showed there was no spike or slow waves. Another test on cerebrospinal fluid (CSF) showed no obvious abnormalities. His magnetic resonance angiography revealed that intracranial arteries were normal. His previous MRI showed that all lesions were distributed in the posterior circulation. After reading his MRI report, the consensus was to perform diffusion weighted imaging (DWI). We found hyper-intensity within the areas of lesions [Figure 1]. Carotid artery ultrasound revealed no abnormalities. Transthoracic echocardiography confirmed there was a vegetation (10 mm × 4 mm) attached to mitral valves [Figure 2]. These imaging tests were consistent with IE and cerebral embolism (caused by IE).[5] The patient refused a heart operation, so he was treated with 1.6 million units of penicillin G sodium for 4 weeks. After 6 months of follow-up, the patient significantly improved and was back to normal life. His re-examination of transthoracic echocardiography showed there was no mitral valves vegetation.

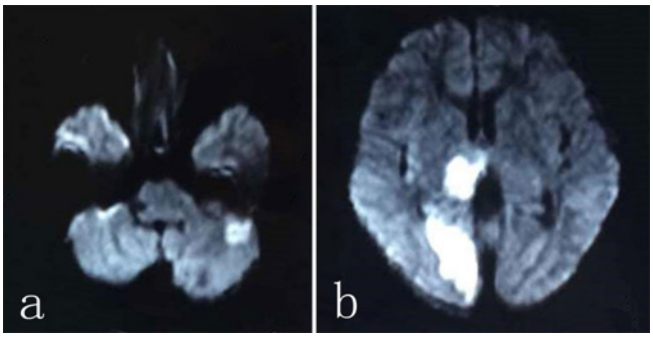

Figure 1: Diffusion weighted imaging and found enhancement within multiple lesions in (a) bilateral cerebellar hemisphere; (b) the right thalamus and occipital lobe

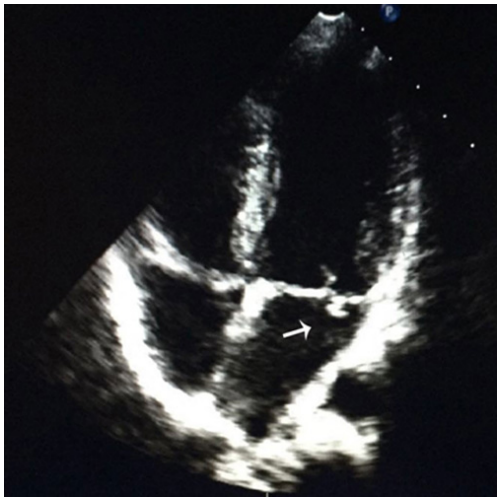

Figure 2: Transthoracic echocardiography showed there was a vegetation (10 mm × 4 mm) attached to mitral valves

DISCUSSION

Clinical manifestations of IE have a variety of symptoms and signs. These include fever, arterial embolic phenomena (cerebral embolism, renal embolism, pulmonary embolism, *etc.*), heart murmur, clubbing of fingers and toes, and other symptoms. Laboratory examinations may show leukocytosis, anemia, rapid erythrocyte sedimentation rate, positive blood culture, as well as vegetations and other powerful identifiers in echocardiography.[5] In recent years, however, many atypical IE patients had complications as their initial symptoms. For example, some studies showed that about one-third of IE patients developed stroke.[6]

Our patient also got atypical IE features: considering his symptoms, it is quite natural to associate fever with headache, vomiting and epileptic seizures. He seemed to respond well to the initial treatment of intravenous acyclovir and mannitol. For this reason, he was diagnosed as encephalitis. However, negative results of CSF test were not in favour of this conclusion. After reconsidering the whole course of disease, it was hypothesized that all his symptoms were part of a basilar syndrome. In fact, except heart murmur or nervous system manifestations, there was no other sign. The echocardiography of the patient ultimately confirmed there was a vegetation (10 mm × 4 mm) attached to mitral valves. This is a strong predictive factor of embolic events.[7] Besides, from the MRI results, we noticed there were lesions in bilateral cerebellar hemisphere of his brain. This was not among the commonest locations for herpes simplex encephalitis (in fact, characteristic changes are in the temporal lobes) and there was some evidence to support cardioembolism. Thus, it is likely that an event of cardiac embolism has taken place, since many areas of intracranial arteries were affected, especially bilateral lesions (or lesions in both anterior and posterior circulation).[8] For our patient, his DWI results were in agreement with our diagnosis, in fact all the lesions were distributed in multiple areas in posterior circulation. This is consistent with cardioembolic lesions in IE patients.[9] Additionally, continual variant symptoms occurred as expected in cardioembolism.

Based on the patient's clinical manifestations, it was very likely that streptococci caused his infective endocarditis. For this reason, penicillin was chosen as first line treatment. However, blood culture and anti-streptolysin *O* test were both negative. It is worth noting here that there might be several explanations

to support our results. Firstly, negative blood-culture occurs in 2.5-31% of all cases of IE, so the negative results are not totally unexpected.[1] Secondly, anti-streptolysin *O* test is a test for activity of Lancefield's Group A streptococci (or occasionally Groups C and G). The intensity of the response varies with the duration of activity of the stimulus. The maximum response does not generally develop before 5 or 6 weeks, and it may be partially suppressed by antibiotic therapy.[10] In this case, after the initial symptom characterized by fever, infusion was given to lower patient temperature. However, the patient does not recollect which infusion was administered. While antibiotics abuse is a common phenomenon in China, we hypothesize that such infusion may be some sort of antibiotic therapy. The patient was hospitalized in our clinic within 2 weeks after the fever, and it is also possible that the response may not develop to a maximum level. All the factors listed here may have led to negative blood tests, but the effect of penicillin cannot be excluded. Regarding the use of anti-platelet agents, it is suggested that this treatment can be prolonged in absence of bleeding.[11] Aspirin is classical anti-platelet agent but may cause Reye syndrome in children.[12,13] Considering that the patient was only 12 years old it was agreed to treat him with clopidogrel 1 mg/kg daily, instead of aspirin. Of note, there was no sign of recurrence after six months of follow-up.

In conclusion, here we report a patient with embolic stroke as a consequence of endocarditis. Atypical IE manifestation is likely to cause misdiagnosis and clinicians should be cautious when considering its symptoms. We hope that the case presented here will also help clinicians to diagnose IE and its complications, as well as carry out early treatments to reduce morbidity and mortality.

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Conflicts of interest

There are no conflicts of interest.

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Review

Immunotherapeutic strategies for glioma treatment

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ABSTRACT

Glioblastoma is the most common and malignant primary brain tumor. Despite intensive clinical investigation and several novel therapeutic approaches, the median survival continues to remain poor and it is usually in the range of fifteen months. Immunotherapy is a beacon of hope for cancer treatment and offers a different approach against glioma. Various approaches have been used, such as dendritic cell based vaccines, peptide vaccines, T-cell-based therapies and immune checkpoint blockade with promising results. This paper provided an overview of the results of the most exciting immune therapeutic strategies for the treatment of gliomas.

Key words: Glioma; immunotherapy; vaccines

INTRODUCTION

Glioblastoma (GBM) is by far the most common type of primary brain tumor in adults. This devastating disease is usually incurable and virtually all GBM patients succumb despite treatments that consist of surgery, radiotherapy and chemotherapy. The median survival time remains in the range of 15 months.[1,2] GBM is an heterogenous tumor and there is great variability regarding response to treatment and outcome. Verhaak *et al.*[3] developed a molecular classification of GBM into Classical, Mesenchymal, Proneural and Neural subtypes based on gene expression. Epidermal Growth Factor Receptor amplification and the absence of *p53* mutations characterize the Classical subtype, whereas the Mesenchymal subtype is characterized by deletions or mutation of the gene and the Proneural subtype is characterized by alterations of Platelet Derived Growth Factor A and point mutations in cytosolic isocitrate dehydrogenase. A clinical significance was also reported, concluding that therapeutic approaches need to be GBM subtype-specific.[3]

Immunotherapy is an attractive treatment option that involves the stimulation of patient's immune system against cancer cells with high specificity and minimal toxicity.[4] In the late 1800s, William B. Coley, a pioneer in immunotherapy, was the first who injected a mixture of live streptococcus bacilli and subsequently heat-killed streptococcus into sarcomas and induced regression of these tumors.[5] GBM cases of increased survival after bacterial infection have been documented, whereas patients with neutrophil to lymphocyte ratio in the blood that exceeded 4.7 differ significantly from those with neutrophil to lymphocyte ratio lower than 4.7 and were associated with worse survival.[6,7] Nevertheless, GBM can evade by several mechanisms immune surveillance, such

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as loss of major histocompatibility complex (MHC) antigen expression that prevent their recognition by the $CD8^+$ T cells.[8] $CD8^+$ T cell cytotoxic activity has been considered key for tumor eradication and these cells have been detected in GBM tissue. Furthermore, the tumor secretes factors that suppress T-cell proliferation and dendritic cells maturation. Increased ratio of CD3 and $CD8^+$ to $FoxP3^+$ T cell correlated significantly with patient's survival in primary GBM.[9]

Further suppression of the immune system in GBM patients can be caused by systematic corticosteroid treatment that is used for the reduction of vasogenic oedema and as a consequence of chemo-radiotherapy. The present review summarizes all major progresses that have been made in immunotherapeutic treatments against gliomas, such as dendritic cells based therapies, vaccines (such as EGFRvIII and IDH1), tumor specific targets, T cell engineering and immune checkpoint inhibitors.

DENDRITIC CELLS THERAPIES

Dendritic cells (DC) are professional antigen presenting cells (APC) and have been reported to be a promising treatment method against glioma. DCs can be subdivided into myeloid DC (mDCs) and plasmacytoid DC (pDCs). Dendritic cells can be extracted from blood and can be incubated with GBM cells. After antigen presentations, DC can be injected back to patients as immunotherapy. However, this approach requires firstly, tumor tissue collected during surgery and secondly, several weeks for vaccination preparation. Side effects of this approach are minimal and several studies have shown that DCs are capable of inducing immune response.[10,11] Prins *et al.*[12] compared the safety, feasibility, and immune responses of malignant glioma patients that were treated with DC pulsed with autologous tumor lysate or with synthetic glioma-associated antigens. The results showed that DCs pulsed with autologous tumor lysates produced a better anti-tumor immune response.[12] When autologous DCs transfected with autologous tumor stem cell-mRNA they induced an immune response against the patient's GBM stem cells. The vaccinated patients had significantly better progression-free survival.[13] mDC proved to be superior to pDC in producing a robust antitumor T cell response resulting in tumor eradication and better long-term survival in mice.[14] Recently, Mitchell *et al.*[15] showed that pre-conditioning the site of vaccination with a recall antigen such as tetanus/diphtheria toxoid can significantly increase the efficacy of DC vaccination.

Apart from loading DC with regular antigens, Xu *et al.*[16] used cancer stem-like cells (CSC) as sources of antigens for DC vaccination, given that these cells express increased levels of MHCs and tumor associated antigens. This vaccination induced antigen-specific Th1 immune response and, when tested in 9 L CSCs brain tumor model, it resulted in robust antitumor T-cell immunity and a significant survival benefit.[16] Another interesting approach is the possibility of preloading DC and $CD14^+$ cells with chemotherapeutic drugs before immunotherapy. In a recent study, both $CD14^+$ and DCs were incubated with paclitaxel for 24 h. The cells loaded the drug and this was subsequently released in the conditioned medium. Growth inhibition was observed when this medium was used to culture U87MG cells.[17] Of note, U87MG is a commercial cell line that has been expanded *in vitro* for many passages, thus its utility in defining therapeutic approaches for glioma is questionable.

VACCINES

The rational for vaccines lies in the presentation of tumor associated antigens in the immune system. Several ways exist to provide antigens for vaccine administration, one of which is autologous DC as previously described. Recently, a vaccine called Gliovac (ERC 1671), was prepared using autologous antigens that derived from excised tumor tissue and were combined with allogeneic antigens from glioma tissue resected from other GBM patients. This vaccine was capable of triggering powerful polyclonal immune reactions. When administered in 9 recurrent GBM patients, that were treated with surgery, radiotherapy, temozolomide and bevacizumab, the vaccine showed minimal toxicity and enhanced overall survival that reached 77% at 40 weeks.[18] Vaccination with rindopepimut, composed of the EGFRvIII peptide sequence conjugated to the immunogenic carrier protein keyhole limpet hemocyanin, showed promise in a phase II study. The median progression-free survival and overall survival was 14.2 and 26 months in vaccinated patients compared to 6.4 and 15.2 months in controls, respectively.[19] A Phase III clinical trial is now underway. One major disadvantage of peptide vaccines is that a different treatment strategy is usually required when tumor recurs. Sampson *et al.*[20] showed that the EGFRvIII-targeted peptide vaccine triggered loss of the EGFRvIII expression in 82% of patients at the time of tumor recurrence.

The R132H mutation is a tumorigenic mutation and can be found in the majority of low grade gliomas, secondary GBMs and rarely on primary GBMs or gliosarcomas. Paradoxically, the presence of mutation is a favorable prognostic marker, even when assessed in comparison with the O6-methylguanine-DNA methyltransferase promoter status.[21] A vaccine of peptides encompassing

the mutated region showed great promise for the treatment of (R132H)-mutated tumors.[22] In an intracranial glioma model, the R132H mutation could be effectively targeted by the immune system: the results of this study demonstrated a significant increase in survival of treated mice compared to controls and 25% of the mice were cured. After evaluating the $CD8^+$ T cell response in spleen there was a significant difference for the immunized mice compared to controls.[23]

Heat shock proteins (HSP) are evolutionary conserved family of proteins that serve as molecular chaperones and inhibit non specific protein aggregation.[24] HSP can be recognized and activate APC cells which in turn present them on major histocompatibility complexes I (MHC I) and II (MHC II).[25] Various HSP have been utilized for this purpose. Peptides bound to HSP-96 proved safe in a phase I trial and resulted in a 47 weeks median survival after surgical excision in the 11/12 patients that responded to the vaccination.[26] In a phase II study of 41 patients, that received complete excision of recurrrent GBM and vaccination, the median overall survival reached 42.6 weeks.[27] The HSP47 was also utilized as a glioma associated antigen. In 26.9% of GBM patients there was a positive cytotoxic T lymphocyte response that resulted in a significant better progression-free and overall survival than negative responders.[28] Recently, a vaccine composed of the recombinant mycobacterial HSP65 with mouse glioma 261 (GL261) tissue lysate increased the survival of mice bearing GL261 gliomas by enhancing the ratios of brain-infiltrating Th17 cells subset and inflammatory cells.[29] Of note, although efficient for studies based on anti-glioma therapeutic modalities, GL261 cells exhibit moderate immunogenicity.[30]

TUMOR SPECIFIC TARGETS

Monoclonal antibodies constitute an attractive type of biological therapy. The selection of tumor antigens suitable for antibody targeting and therapy requires firstly, the target antigen to be confined in the tumor and secondly, to be absent or to have a very low expression on normal tissue. Antigens involved in angiogenesis are a suitable target of monoclonal antibodies, in fact GBM is a highly vascular tumor that expresses high levels of the pro-angiogenic vascular endothelial growth factor (VEGF) and VEGF receptors.[31] Hypoxic conditions that are present in this tumor further increase VEGF production. Bevacizumab is a humanized monoclonal immunoglobulin G1 antibody that neutralizes the biological activity of human VEGF-A and inhibits its binding to vascular growth factor receptor 1 (VGFR-1) and VGFR-2 on tumor endothelial cells.[32] Such agent has been approved for the treatment of recurrent GBM and has been also used in combination with cytotoxic agents such as irinotecan with favorable results.[33]

Overexpression and/or amplification can be found in up to 40% of primary GBM. Several EGFR mutations have been reported, the most frequent being EGFRvIII that occurs in 25-64% of cases.[34] Cetuximab is a chimeric monoclonal antibody against EGFRvIII with high affinity. In a phase II study, involving patients with recurrent GBM, cetuximab combined with bevacizumab and irinotecan was safe, except from skin toxicity and displayed encouraging response rates. Nevertheless, the combination treatment does not seem to be more effective in comparison to bevacizumab and irinotecan alone.[35] Recently, an antibody drug conjugate, named AMG 595 was tested. AMG 595 is composed of maytansinoid DM1, which are potent microtubule-targeted compounds blocking the proliferation of cells at mitosis. After conjugation to an anti-EGFRvIII antibody, it was observed that the drug inhibited the proliferation of U251 cells and induced tumor mitotic arrest in xenografts expressing EGFRvIII.[36]

Cancer stem cells display a high tumorigenic potential and treatment resistance, given their low proliferation rate, eventually resulting in GBM recurrence. Targeting GBM stem cells is an attractive treatment strategy and various approaches have been tested. The AC133 epitope expressed on the CD133 glycoprotein has been used as a marker to identify stem cells. Recently, a recombinant specific antibody that binds both to AC133 and to T cells (via the CD3 receptor) has been developed. This agent suppressed the outgrowth of $AC133^+$ subcutaneous GBM xenografts.[37] Nevertheless, it is important to note that CD133 as well as other markers such as CD15, do not discriminate between tumorigenic and non-tumorigenic cells, thus questioning their use in glioma to identify CSCs.[38,39]

The highly immunosuppressive tumor microenvironment is considered to be a significant barrier to successful immunotherapy. The fibrinogen-like protein 2 (FGL2) that can be found in malignant cells has been reported to act as an immune-suppressor in GBM, permitting the tumor to grow by suppressing tumor-targeted immune responses. Mice treated with an anti-FGL2 antibody had a median survival of 27 days compared with 17 days as the median survival of mice injected with an isotype control antibody.[40]

T CELL ENGINEERING

Chimeric Antigen Receptor (CAR) cells are cytotoxic

T lymphocytes (CTL) engineered to express tumor antigen-specific proteins. Such targets are the EGFRvIII, human epidermal growth factor receptor 2, erythropoietin-producing hepatocellular carcinoma A2 (EphA2) and the interleukin-13 receptor alpha2 (IL13Rα2). Recently, it has been suggested that EGFRvIII-directed CAR T cells are able to suppress tumors of EGFRvIII (+) GBM in xenogeneic subcutaneous and orthotopic models.[41] The EphA2 has been found increased in the majority of GBM specimens and cell lines and at very low levels in the normal brain.[42] Chow *et al.*[43] developed EphA2-specific T cells that resulted in regression of glioma xenografts and better survival. The IL13Rα2 is a cell surface receptor which is not significantly expressed in normal brain but over-expressed in a subset of high-grade gliomas. Similarly, in a trial evaluating an engineered chimeric antigen receptor, autologous primary human $CD8^+$ cytotoxic T lymphocytes targeting IL13Rα2 were tested for the treatment of recurrent GBM. The intracranial administration was safe and promising in a pilot study of 3 patients.[44]

IMMUNE CHECKPOINT INHIBITORS

Immune checkpoint inhibitors against regulatory pathways in T cells provide a gateway to development of new treatments for several cancer types.[45] This has been explored in several tumors, by testing antibodies to cytotoxic T lymphocyte antigen-4 (CTLA-4), i.e. an important immunosuppressive receptor, inhibition of indoleamine 2,3-dioxygenase 1 (IDO) and blocking antibodies targeting either the receptor of the programmed death 1 (PD-1) checkpoint or its major ligand. For instance, in a murine GL261 glioma model, a long-term survival in at least 50% of treated animals was achieved by combining radiotherapy with anti-CTLA-4 antibodies and anti-4-1BB, that drives the proliferation of $CD8^+$ T cells.[46] Moreover, using a syngeneic intracranial mouse glioma model, Wainwright *et al.*[47] reported that simultaneous blockage of CTLA-4, IDO and PD-L1 results in long term survival of all mice.

OTHER APPROACHES

Another interesting approach is based on macrophages which have the ability to cross the intact blood brain barrier. Baek *et al.*[48] showed that macrophages loaded with gold nanoshells could infiltrated into glioma spheroids and after near-infrared light laser irradiation there was complete growth inhibition in an irradiance-dependent manner. Recently, allogeneic natural killer (NK) cells against patient-derived GBM *in vitro* and *in vivo* have been tested with promising results. Killer Ig-like receptor (KIR) 2DS2 positive NK cell subsets displayed a functional activation advantage and resulted in greater cytokine production, propensity for degranulation and greater persistence *in vivo* compared with KIR2DS2 negative NK cells.[49] In order to enhance the killing capability of cytotoxic lymphocytes, another approach was based on the modulation of microvilli and filopodia that are characteristic of glioma cells. These structures physically prevent cytolytic lymphocytes from eliminating glioma cells. In particular, knocking-down Fascin-1, an important scaffolding protein that is involved in the microvilli and filopodia formation, resulted in increased lymphocyte cytotoxicity and inhibition of cell proliferation and invasion.[50] Recently, the intratumoral administration of an oncolytic adenovirus, the AdCMVdelta24, led to an increased number of Interferon gamma-producing $CD8^+$ T cells and a decrease in the tumor-infiltrating regulatory T cells in a mouse model.[51]

Interestingly, it has been suggested that radiotherapy complement immunotherapies; in fact, irradiated cancer cells release peptides that can activate DC. Furthermore, radiotherapy in combination with immune checkpoint inhibitors such as (anti-CTLA-4 and/or anti-PD-L1) may stimulate $CD8^+$ T cell-mediated anti-tumor immunity.[52]

CONCLUSION

GBM is an extremely heterogeneous tumor, comprised of both differentiated and stem cells.[53] This is also highlighted in the recent gene expression-based molecular classification of GBM into four subtypes, namely Proneural, Neural, Classical and Mesenchymal.[3] Thus, a multifaceted approach combining several treatment strategies might be eventually required to achieve better results. Recent data seem to suggest that immunotherapy constitutes a promising treatment strategy for malignant gliomas despite several limitations such as the modest Class I MHC expression and the absence of Class II MHC expression in tumor cells. Further combinatorial treatments that involve the current standard therapies and immunotherapeutic approaches are under way and hopefully they will lead to more promising results.

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Conflicts of interest

There are no conflicts of interest.

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Original Article

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Assessment of health-related quality of life in patients with multiple sclerosis living in the Fars province of Iran

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ABSTRACT

Aim: The aim of this study is to determine the health-related quality of life (HRQoL) among patients with multiple sclerosis (MS) living in the Fars province of Iran. **Methods:** A total of 100 patients with clinically definite MS who were referred to a clinic affiliated with Shiraz University of Medical Sciences were eligible to participate in this study. The HRQoL was evaluated using a Persian version of the Medical Outcomes software. Data were analyzed using descriptive statistics, MANOVA, ANOVA and an independent *t*-test. **Results:** Patient variables in this sample included the following: 80% of the participants were female, 68% of the participants were married, 30% of the participants had completed primary school, 38% of the participants had completed high school and 32% of the participants attained a university degree. No significant difference among HRQoL scores attributable to these variables were observed among participants in this study. The overall mean scores for the physical and mental components of the HRQoL were 59.48 ± 24.63 and 49.26 ± 23.15, respectively. The paired *t*-test showed that when compared with a normal sample, the patients in this study had mental component scores of the HRQoL that were significantly lower than physical component scores ($t = 5.72$, df = 99, $P < 0.001$). **Conclusion:** The HRQoL scores among patients with MS are significantly lower than those among members of the healthy population, especially with respect to the mental component of the test. Therefore, close consideration of mental and physical problems and appropriate management of MS can improve quality of life in these patients.

Key words: Health-related quality of life; multiple sclerosis; medical outcomes software

INTRODUCTION

Multiple sclerosis (MS) is a progressive disease affecting the central nervous system that causes immune-mediated damage to the myelin sheath, which results in physical and cognitive impairments. Multiple sclerosis is a complex disease with different signs and symptoms. These signs and symptoms depend on the extent and location of the nerve damage. The disease pattern is mixed and punctuated by periodic attacks with partial recovery exhibited between the attacks.[1] The Multiple Sclerosis International Federation Report suggests that the number of patients with MS has increased from 30 to 33 per 100,000 between 2008 and 2013 with the prevalence of the disease among women twice that of men.[2]

MS is a chronic progressive disease without cure that begins during early adulthood. Patients with MS live with this disease for a long period of time, and it negatively influences their social, economic and emotional well-being.[2] Health-related quality of life (HRQoL) is a multidimensional concept that includes physical, social, and emotional aspects of life and is an essential indicator to evaluate the impact of therapeutic plans on the lives of patients with MS.[3] Earlier research has also documented the impact of gender differences and education level on HRQoL scores.[4,5] This previous research has suggested that there is a significant positive correlation between

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demographic factors, such as marital status, level of education and employment status, with HRQoL in patients with chronic diseases.[6,7] For example, employed patients have reported better emotional well-being and married patients have reported better sexual function than that of patients who were unemployed or unmarried, respectively.[6]

HRQoL measures a patient's level of satisfaction with life.[3] Knowing patient outcomes and level of satisfaction is essential for the success of treatments provided by a health-care provider. The aim of this descriptive-comparative study is to assess the HRQoL in patients with MS.

METHODS

A total of 100 patients with MS, aged between 16 and 65 years, were selected for participation in this study. The 2010 revision of the McDonald criteria[8] was used to confirm a diagnosis of MS. The participants were selected from a pool of all MS outpatients who were referred to the medical clinics at Shiraz University of Medical Sciences during 2012 using the convenient non-probability sampling method. Patients who received high doses of methylprednisolone administered as pulse therapy during the past 3 months as well as patients with chronic co-morbid diseases such as cancer, diabetes, epilepsy, renal failure, and heart disease, or major psychological problems, such as psychosis, were excluded from participation in the study. The severity of illness was measured using the Expanded Disability Status Scale (EDSS).[9] The EDSS scores for 90% of our patients were fewer than 5, and the mean score was 2.41 ± 1.91 when measured during the assessment of HRQoL.

A research assistant explained the confidentiality, objectives and procedures of the study to each patient before participants gave their oral consent to volunteer in the study. The study protocol complied with ethical codes issued by the Psychology and Counseling Organization of the Islamic Republic of Iran.

Measurements

All the patients in this study completed a demographic form and the Medical Outcomes Study Short-Form (SF-36). The SF-36 is a universal self-report questionnaire used to evaluate the effect of medical treatments on quality of life (QoL).[10] The SF-36 comprises 36 items, which measure eight subscales of HRQoL, including: (1) physical functioning; (2) role limitations due to physical health problems; (3) bodily pain; (4) general health perceptions; (5) vitality; (6) social functioning; (7) role limitations due to emotional problems; (8) general mental health. These subscales can be divided into 2 main categories: physical or mental components. The scores in every subscale and each main category ranged from 0 to 100. Patients with the lowest scores had a worse QoL. The SF-36 was translated into Persian in 2005.[11] The Persian version of the SF-36 possesses good psychometric properties, and it has good internal consistency (between 0.65 and 0.90) as well as adequate Cronbach's alpha reliability. The results of the comparison of the known groups, convergent validity and principal-component-factor-analysis showed that the Persian iteration of the SF-36 has sufficient validity. The authors of the present study concluded that the Persian iteration of the SF-36 can be used during clinical practice and research.[11] SPSS software version 16 (SPSS Inc, Chicago, IL) was used for the statistical analysis of the data. Descriptive statistics were used to summarize the basic features of the collected data. Group differences were assessed using MANOVA, ANOVA and an independent *t*-test. A $P \leq 0.05$ was considered statistically significant.

RESULTS

Included in this study were 80 women and 20 men with a mean age of 35.1 ± 9.5 years. In this study, 62% of the patients had relapsing-remitting MS, 25% had secondary progressive MS, 9% had primary progressive MS and 4% had a clinically isolated syndrome. The mean duration of disease between the first diagnosis of MS and participation in the study was 6.4 ± 3.8 years. In this study, 80% of the patients were women, 68% of the patients were married, 30% of the patients were employed, 30% of the patients had completed primary school, 38% of the patients had completed high school and 32% of the patients had attained a university degree. The mean and standard deviation of the total SF-36 score, the physical component and the mental component were calculated for all participants. The mean scores for the full test, the physical component and the mental component were 57.53 ± 23.27, 59.48 ± 24.63 and 49.26 ± 23.15, respectively. Table 1 shows the descriptive data of the eight subscales of HRQoL in this study and a previous study conducted in Iran.[11] The first row of Table 1 shows the mean (SD) of the current study, and the second row shows the results of the SF-36 gathered from 4,163 individuals who were randomly selected in 2005 from the general population of Tehran.[11] To compare the current study results with the Iranian normal population results, we computed the 95% confidence interval (CI) for the mean of the scores of each of the eight subscales assessed using the Tehran

Table 1: Mean (SD) of current sample and normative data

	Physical functioning	Role limitations because of physical health problems	Bodily pain	General health perceptions	Vitality	Social functioning	Role limitations because of emotional problems	General mental health
Current study	64.27 (33.45)	51.75 (42.23)	66.22 (31.04)	55.71 (24.76)	50.45 (23.79)	63.66 (29.74)	48.66 (43.27)	34.27 (15.44)
Tehran normal sample[8]	85.3 (20.8)	70.0 (38.0)	79.4 (25.1)	67.5 (20.4)	65.8 (17.3)	76.0 (24.4)	65.6 (41.4)	67.0 (18.0)
95% CI for Tehran normal sample	66.45-67.54	68.84-71.15	78.63-80.1	66.88-68.11	65.27-66.32	75.25-76.74	64.34-66.85	66.45-67.54

Table 2: Mean (SD) and independent t test for marital status

	Single	Married	t	P
Full Scale	62.31 (18.90)	57.50 (24.46)	0.90	NS
Physical Component	64.13 (23.48)	59.48 (25.18)	0.81	NS
Mental Component	54.21 (17.26)	48.60 (24.74)	1.06	NS
Physical Functioning	71.47 (33.56)	64.64 (32.64)	0.90	NS
Role Limitations Because of Physical Health Problems	59.61 (44.20)	51.83 (41.50)	0.79	NS
Bodily Pain	71.44 (32.07)	64.22 (31.16)	0.99	NS
General Health Perceptions	54.01 (18.55)	57.23 (26.69)	0.56	NS
Vitality	55.38 (15.16)	49.41 (26.30)	1.08	NS
Social functioning	71.97 (23.08)	62.66 (31.02)	1.38	NS
Role Limitations Because of Emotional Problems	52.56 (37.91)	48.03 (45.48)	0.451	NS
General Mental Health	36.93 (11.78)	34.28 (16.39)	0.75	NS

NS: not significant

normal sample and reported these values in the third row of Table 1. The mean for each subscale score lay outside the 95% CI; thus, the averages of the subscales in the current study are significantly lower than those of the normal group.[11] Of note, the subscale with the lowest value in this study is the general mental health subscale.

Table 2 summarizes the scores assessing marriage status. This Table shows that the scores of unmarried patients were higher than those of married patients for every measure except for the general health perception subscale; however, the difference between the overall scores for both groups was not statistically significant. Therefore, the marital status could not significantly affect the SF-36 scores.

The mean (SD) scores of the SF-36 and its subscales for women and men are presented in Table 3. The total average scores among men were higher than those for women except for the general health perception subscale, which may reflect gender differences or differing expectations of health.

Table 3: Average scores for female patients and male patients

	Females	Males
Full Scale	57.14 (22.16)	59.08 (27.87)
Physical Component	59.17 (24.05)	60.71 (27.47)
Mental Component	48.49 (22.0)	52.35 (27.70)
Physical Functioning	63.58 (33.10)	67.04 (35.57)
Role Limitations Because of Physical Health Problems	51.25 (42.26)	53.75 (43.13)
Bodily Pain	64.50 (31.54)	73.00 (28.70)
General Health Perceptions	57.36 (23.59)	49.07 (28.67)
Vitality	49.25 (23.35)	55.25 (25.51)
Social Functioning	63.65 (27.93)	63.68 (36.95)
Role Limitations Because of Emotional Problems	47.08 (43.63)	55.00 (42.26)
General Mental Health	33.97 (14.99)	35.48 (17.48)

A 2 (gender) × 3 (educational levels) factorial MANOVA was performed to examine the effect of gender and educational levels on the eight subscales of the SF-36 as dependent variables. The results from the MANOVA analyzing the eight subscales of SF-36 were statistically significant (Wilkes's lambda = 0.143, F (8, 87) = 65.047, $P < .001$). The MANOVA output for the main effect of gender (male *vs.* female) indicated no significant effect (Wilkes's lambda = 0.90, F (8, 87) = 1.21). No statistically significant differences among the three educational levels as the main effect were observed (Wilkes's lambda = 0.85, F (8, 87) = 0.895). The MANOVA results suggest that the interaction between gender and educational level was not statistically significant (Wilkes's lambda = 0.823, F (8, 87) = 1.11). Therefore, a one-way repeated measures ANOVA with a Greenhouse-Geisser correction was used to detect any potential significant difference between the means of the dependent variables (the eight subscales of the SF-36), which showed that the means of the eight subscales of the SF-36 scores were significantly different [F (4.818, 476.974) = 19.114, $P < 0.000$]. Partial Eta Squared (η^2_p = 0.162) showed that almost 16% of the variance in the score can be accounted for by mean differences. The results from the ANOVA with repeated measures showed that there is an overall significant difference between the means of the subscales. A post-hoc Bonferroni pairwise comparison was used to detect any differences [Table 4]. Table 4 shows that there are

Table 4: Bonferroni pairwise comparison between the means of dependent variables

	Role limitations of physical health problems	Bodily pain	General health perceptions	Vitality	Social functioning	Role limitations of emotional problems	General mental health
Physical functioning	D = 12.52 S = 3.560 *P* = .018	D = 30.00 S = 3.05 *P* = .000	D = 15.60 S = 4.41 *P* = .017	D = 8.56 S = 2.98 *P* = NS*	D = -1.92 S = 3.71 *P* = NS	D = .61 S= 3.05 *P* = NS	D = 13.82 S = 2.74 *P* = .000
Role limitations of physical health problems		D = 17.47 S = 4.03 *P* = .001	D = 3.08 S = 4.53 *P* = NS	D = -3.96 S = 4.09 *P* = NS	D = -14.45 S = 4.06 *P* = .016	D = -11.91 S = 3.81 *P* = NS	D = 1.30 S = 3.79 *P* = NS
Bodily pain			D = -14.39 S = 3.91 *P* = .011	D =-21.43 S = 2.00 *P* = .000	D = -31.92 S = 3.03 *P* = .000	D = -29.38 S = 2.32 *P* = .000	D = -16.17 S = 1.66 *P* = .000
General health perceptions				D = -7.04 S = 4.015 *P* = NS	D = -17.53 S = 4.02 *P* = .001	D = -14.99 S = 3.87 *P* = .005	D = -1.78 S = 3.71 *P* = NS
Vitality					D=-10.490 S = 3.659 *P* = NS	D = -7.953 S = 2.691 *P* = NS	D = 5.260 S = 2.037 *P* = NS
Social functioning						D = 2.53 S = 3.42 *P* = NS	D = 15.75 S = 3.08 *P* = .000
Role limitations of emotional problems							D= 13.212 S = 2.092 *P* = .000

*Not significant

several significant differences between the means.

For example, physical and social functions were not statistically different; however, physical function was statistically different from the general health perceptions and general mental health. The paired *t*-test indicated that there was a significant difference between the average of the physical component and that of the mental component ($t = 5.72$, df = 99, $P < .001$).

DISCUSSION

In this study, our findings suggest that the total HRQoL scores in patients with MS were significantly lower than those of the normal general population in Iran.[11] Similar findings have been previously reported in other countries,[12,13] which also demonstrated lower HRQoL scores in patients with MS when compared with those of healthy persons. Furthermore, HRQoL scores among patients with MS were even lower than those among patients with other chronic diseases such as rheumatoid arthritis and inflammatory bowel disease.[14] Reports from other countries have shown the same results, for example, patients with MS have more frequently reported chronic pain than members of a healthy control group.[15] Alternatively, these reports have documented a higher percent of unemployment and retirement among patients with MS.[16] The authors of a review article in Croatia described patients with MS as having a lower quality of life than that of either a non-patient population or an otherwise unhealthy population.[17] The above mentioned findings can be explained by the chronic and long-standing course of MS as well as the unpredictable and disabling nature of the disease.

Our study also shows that the scores of the mental components of the HRQoL were significantly lower than those of the physical components among the study participants. Of note, it is commonly believed that MS is a progressive and physically disabling sickness, and patients with MS are more likely to display mental and psychological problems. Patients with MS may feel low self-efficacy because they feel that there are many limitations affecting their activities, and they are restricted from participating in social events. Low self-efficacy in a chain of events that can impact work, social life, family relationships, mood and QoL.[18] Common neuropsychiatric disorders experienced by patients with MS are anxiety, depression, cognitive decline, irritability and anger.[19] Some studies have reported that the rate of depression, anxiety and suicide in the patients with MS is higher than that of the general population with other medical conditions.[20] The neuropsychiatric symptoms of MS occur early during the course of the disease. For example, researchers observe the presence of cognitive function impairment in 60% of patients with a disease duration of less than 2 years.[21] Additionally, research suggests that as early as one year following diagnosis with MS, about half of patients exhibit depression, anxiety and distress.[22] In a recent critical review conducted by Ciro *et al.*[23] the authors found several case reports of bipolar disorder clearly preceding MS onset. Some studies examining the HRQoL in the patients with MS have shown that the clinicians are more concerned with the physical problems of the disease whereas the patients mainly believed that their vitality, role limitations, emotional problems, and mental health are essential indicators of disease burden.[24]

Consistent with our findings, other studies have shown that there was no significant difference between men and women with respect to HRQoL;[13,25] however, some studies have reported that women with MS had lower HRQoL scores than men.[26] Casetta *et al.*[27] have studied 370 patients with MS to evaluate gender differences with respect to HRQoL and have reported that the impact of disability is significantly more in men, especially when measured using HRQoL scales that are related to mental well-being.

Our findings show that when compared with married patients, unmarried patients with MS attained better (but not statistically significant) scores in the majority of QoL domains. This finding lies in contrast to results of other studies which have shown that QoL scores are lower in unmarried patients.[28] Whereas the support provided by family members alleviates some physical and mental problems in patients with chronic diseases such as MS, increasing disability and sexual dysfunction in patients who have no supporting family can result in lower HRQoL. Although in our study there was no relationship between age and HRQoL among patients with MS, other studies have reported varied results regarding this issue. Some researchers have reported lower HRQoL scores among older patients;[29] However, other researchers have documented better HRQoL scores among older patients.[30] Lower HRQoL scores among the elderly, healthy coping mechanisms and better adjustment to the disease seem to be responsible for the varied effects of age on HRQoL.[29]

Finally, the findings of the present study show that educational level has no impact on HRQoL. Šabanagić-Hajrić and Alajbegović[5] have reported that educated patients had higher HRQoL than uneducated or less-educated patients; however, Busche *et al.*[31] have shown that high school and college graduates with MS had higher scores in the physical components of the HRQoL. Furthermore, Patti *et al.*[32] have documented that educational level has been an independent predictor of both physical and mental domains of HRQoL.

In conclusion, HRQoL in patients with MS is significantly lower than that of the normal population especially with respect to the mental domain. In the future, should physicians pay closer attention to the cognitive and other neuropsychiatric components of HRQoL as well as the physical components of HRQoL in patients with MS, they could better improve the appropriate management of this disease.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

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Bilateral facial weakness following dengue fever

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ABSTRACT

Dengue, an acute viral disease transmitted by *Aedes* mosquitoes, is highly endemic in many tropical and subtropical areas of the world. Dengue has a wide clinical spectrum, ranging from mild clinical febrile illness to severe life-threatening conditions like dengue hemorrhagic fever and dengue shock syndrome. Neurological complications of dengue infection have been observed more frequently in the recent past. They are widespread and may involve almost all parts of nervous system through various pathogenic mechanisms. We report a case of a 30-year old male who developed bilateral facial weakness after dengue fever.

Key words: Bilateral facial weakness; dengue fever

INTRODUCTION

Dengue is second most common mosquito-borne disease affecting humans after malaria.[1] Around 2.5 billion population is at risk of dengue infection worldwide, and its endemic zone comprises more than 100 countries of the world. It is caused by arbo viruses which belong to the *Flaviviridae* family. Dengue virus 1-4 are the known serotypes of the virus.[1] The clinical presentation of dengue has a wide spectrum, ranging from mild clinical febrile illness to severe life-threatening conditions like dengue hemorrhagic fever and dengue shock syndrome. Recently, virological characteristics of dengue viruses have been changing, resulting in widespread neurological complications.[2]

Neurological manifestations of dengue infection can be grouped into 3 categories: (1) concerned with neurotropism leading to encephalitis, meningitis, myositis, rhabdomyolysis and myelitis; (2) related to the systemic complications of dengue infection that can lead to encephalopathy, stroke (both hemorrhagic and ischemic), hypokalemic paralysis and papilledema; (3) post-infectious leading to acute disseminated encephalomyelitis, encephalomyelitis, myelitis, neuromyelitis optica, optic neuritis, Guillain-Barré syndrome probable Miller-Fisher syndrome, phrenic neuropathy, long thoracic neuropathy, oculomotor palsy, maculopathy and fatigue syndrome.[3] We report a case of a 30-year old male, who developed bilateral facial weakness after dengue fever.

CASE REPORT

A 30-year-old male, without any significant past medical illness presented with difficulty in talking followed by difficulty in eating and drinking. His wife also noticed that he was unable to close his eyes. There was no history of any limb weakness or paraesthesia. He also had fever for two weeks that lasted for 3-4 days. On presentation, he was conscious, alert and followed verbal commands. He was hemodynamically stable and his physical examination was unremarkable. However, following a neurologic examination, a bifacial lower motor neuron weakness was marked. His motor and sensory examination was unremarkable.

MRI scan of his brain with contrast study was concluded as normal. Electrophysiological evaluation of facial nerve revealed normal latency and reduced amplitude. His peripheral nerve conduction study was normal. He was also evaluated for fever and found

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to have dengue IgM antibody positive. Vasculitic markers, serum ACE and lyme serology were negative. He underwent CSF evaluation which revealed cytoalbuminologic dissociation. (Cell count: 10, 100% lymphocytes, Protein: 100, Sugar: 77, PCR for CMV: Negative, PCR for Herpes: Negative). He was managed with low dose steroid and physiotherapy. His facial weakness improved gradually and was discharged.

DISCUSSION

Dengue has been a known clinical entity since 1780.[4] The association of dengue infection and neurological abnormalities was first described by Sanguansermsri and colleagues in 1976, in a patient presenting with encephalopathy.[5] Many neurological symptoms are associated with dengue and have been recognized for over a century. The classic signs of acute infection are headache, dizziness, lightheadedness, insomnia, agitation, irritability and depression. A minority of symptoms manifests as encephalopathy.[6]

Among the neurological manifestations that appear post-dengue, meningoencephalomyelitis, transverse myelitis, post-infectious encephalitis, epilepsy, tremors, Bell's palsy, mononeuropathy and Guillain-Barré syndrome (GBS) stand out.[7] GBS is a rare complication of dengue fever with an incidence of between 0.6 and 1.9 per 100,000.[8] The pathophysiology of these neurological complications can be explained by the occurrence of cerebral edema, cerebral hemorrhage, cerebral anoxia, hyponatremia, liver failure associated with portal-systemic encephalopathy, micro-capillary hemorrhage or release of toxic products.[9]

Bilateral facial weakness may be a component of GBS. However, isolated bifacial weakness has not been reported to our knowledge. Although CSF albumino-cytological dissociation was present in our case, but electrophysiological evaluation and neurological evaluation was normal except bilateral lower motor neuron type facial weakness. Our case calls for special attention because the dengue infection remains a serious public health problem in tropical countries such as India, but little is known about the actual incidence of neurological complication of dengue.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

Disc herniation or ependymoma recurrence?

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ABSTRACT

In this paper, a 41-year-old female with previous history of ependymoma is reported. The patient underwent gross-total resection of the tumor and ventriculo-peritoneal shunt placement, followed by radiotherapy to the posterior fossa and the upper-cervical spinal cord region. Three years later she developed numbness in her right arm, body and leg. Magnetic resonance imaging (MRI) of the entire neuraxis revealed no evidence of tumor recurrence, while a small enhancing area was noted in the left anterolateral spinal cord at the level of the C1-C2 vertebrae and a left posterior-lateral herniated disk in the C5-C6 level which was not present in the earlier MRI at initial diagnosis. Lumbar punctures were negative for malignant cells. The patient's symptoms were first attributed to radiation-induced effect. Follow-up results of brain and the cervical spine MRI were performed which showed disappearance of the small abnormality in the left C2 spinal cord area but persistence of the herniated C5-C6 disk. Thus, the current diagnosis of right-sided numbness due to pressure of the left anterolateral spinothalamic tracts from the herniated C5-C6 disk was made. This is a unique case, in which herniated disk pressuring effects needed to be differentiated from both radiation-induced treatment effect and tumor recurrence.

Key words: Disc herniation; ependymoma recurrence; radiotherapy

INTRODUCTION

Most of the intracranial ependymomas in adults are supratentorial in contrast to the childhood ependymomas that are usually infratentorial.[1] According to the World Health Organization (WHO) they are classified into grades I, II and III.[2] Grade I includes the myxopapillary ependymoma and subependymoma; grade II is the most common variant and grade III is the anaplastic variant. Gross total resection followed by limited-field radiotherapy is the standard form of treatment in uncomplicated cases.[3,4] Craniospinal radiation should be reserved only for cases where there is documented leptomeningeal seeding.[1,5] Radiation necrosis is a rare complication (< 5%) of conventional radiotherapy,[6] but when it does occur it poses a challenge to differentiate from tumor recurrence.[7,8] We report herein a unique case where a herniated disk in a patient with a previously treated ependymoma needed to be differentiated from radiation-induced injury and tumor recurrence.

CASE REPORT

ependymoma presented to the Neurology clinic for persisted numbness in her right arm, body and leg of 10-month duration. The patient was diagnosed 3 years earlier with hydrocephalus due to a fourth ventricular ependymoma, without evidence of cerebrospinal fluid (CSF) seeding in magnetic resonance imaging (MRI) of the brain or the entire spine [Figure 1]. At that time, she underwent gross-total resection of the tumor and ventriculo-peritoneal shunt placement, followed by 6,000 rads radiotherapy to the posterior fossa and the upper-cervical spinal cord region. Approximately 10 months prior to her visit to our clinic, the patient developed unilateral numbness in her right arm, body and leg, worse in the leg than in the arm. MRI of the entire neuraxis revealed no evidence of tumor recurrence. However, a small enhancing area

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was noted in the left anterolateral spinal cord at the level of the C1-C2 vertebrae [Figures 2 and 3]. A left anterior-lateral herniated disk was also noted in the C5-C6 level which was not present in the initial MRI at diagnosis [Figures 2 and 4]. Three lumbar punctures were negative for malignant cells. The possibility of ependymoma recurrence was therefore ruled out and the symptoms were attributed to radiation-induced effect. The patient continued to be followed with frequent MRI. Although the following-up MRI demonstrated progressive reduction of the enhancing abnormality in the upper cervical cord, the patient's symptoms persisted and she presented to our clinic for a second opinion. The neurological examination was unremarkable except for decreased pinprick and temperature sensation in her right side below the C5 dermatome. Additional MRI of the brain (not shown) and the cervical spine were performed which revealed disappearance of the previously noted small abnormality in the left C2 spinal cord area but persistence of the herniated C5-C6 disk [Figures 5 and 6]. The diagnosis of right-sided numbness due to selective pressure of the left anterolateral spinothalamic tracts by the herniated C5-C6 disk was therefore made.

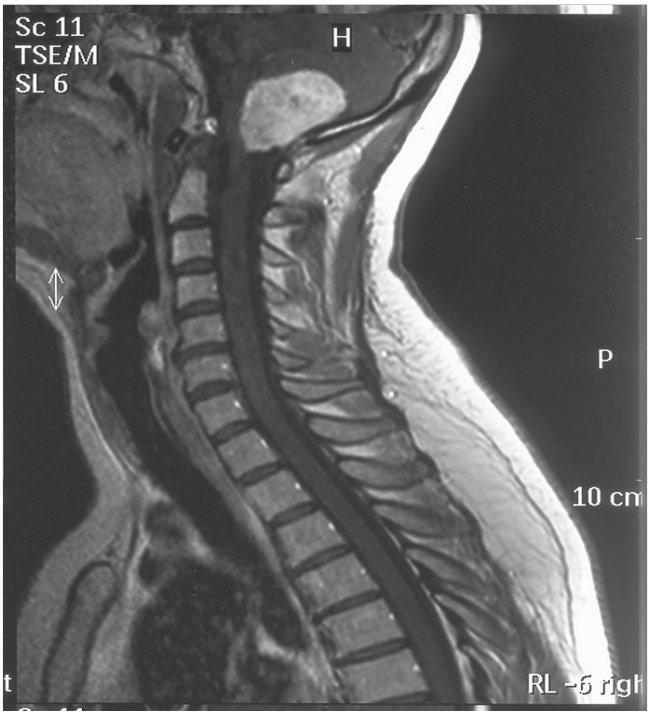

Figure 1: Pre-surgical T1-weigheted sagittal MRI with contrast of the C-spine. The large homogenously enhancing tumor of the posterior fossa is seen but no evidence of leptomeningeal disease or herniated disk at the C5-C6 level. MRI: magnetic resonance imaging

DISCUSSION

Ependymomas in adults are more frequently supratentorial (approximately 2/3 of cases) in contrast to children that are infratentorial.[1] When they are located in the posterior fossa, they can fill the fourth

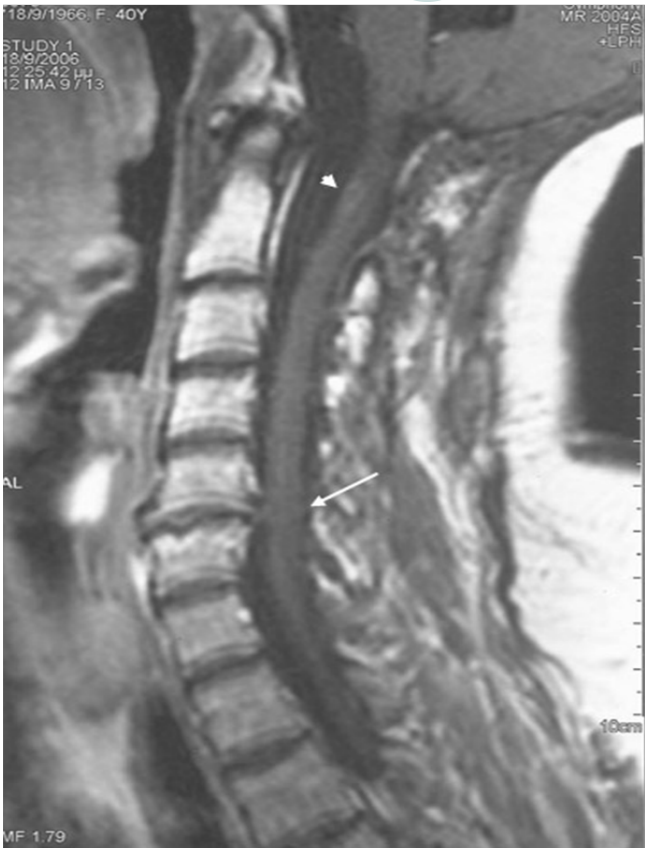

Figure 2: T1-weigheted MRI with contrast of the C-spine when the patient developed right-sided numbness. There is no evidence of tumor recurrence in the posterior fossa but there is an enhancing spinal cord abnormality at the C2 level (arrowhead), and a herniated disk at the C5-C6 level (arrow). MRI: magnetic resonance imaging

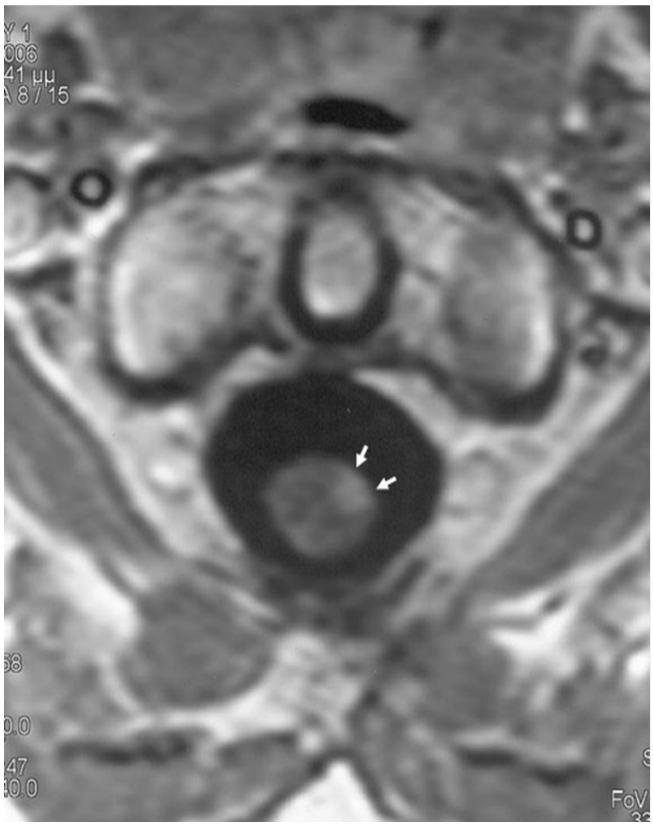

Figure 3: Transverse T1-weigheted MRI section with contrast through the C2 area revealed the small cord abnormality to be located in the left anterolateral region without mass effect, consisted with radiation damage. MRI: magnetic resonance imaging

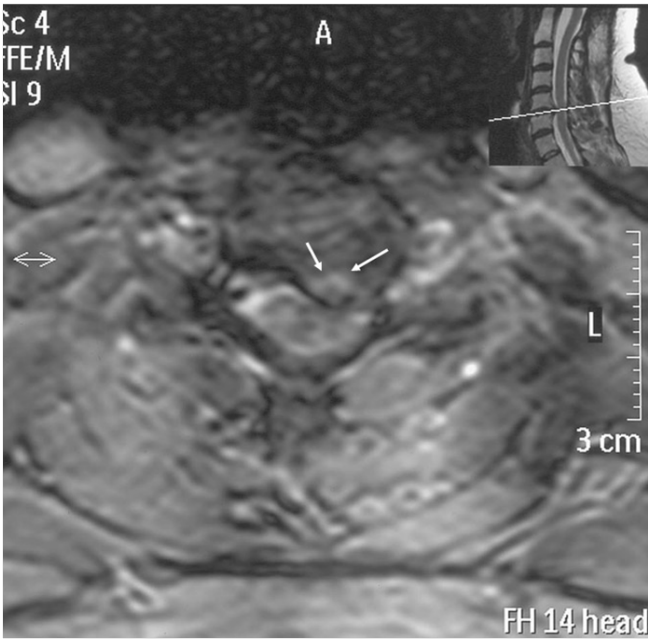

Figure 4: Transverse T2-weigheted MRI section through the C5-C6 area revealed the herniated disk fragment to compress the left anterolateral spinal cord. MRI: magnetic resonance imaging

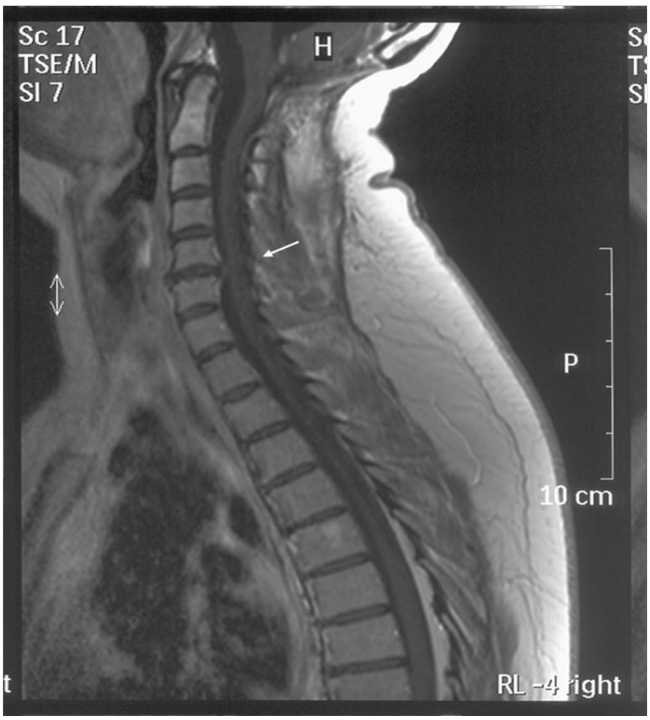

Figure 5: T1-weigheted MRI with contrast of the C-spine during the patient's visit to our clinic demonstrating disappearance of the C2 spinal cord lesion but presence of the herniated disk at the C5-C6 level (arrow). MRI: magnetic resonance imaging

ventricle and cause hydrocephalus, as in our case. Gross-total resection followed by local radiotherapy could be curative if there is no CSF seeding.[3-5] During tumor recurrence, local recurrence is the primary pattern of failure and spinal seeding is uncommon in the absence of local failure.[1] In the present case, a herniated C5-C6 disk compressed only the left anterolateral spinothalamic tracts but not the left C6 nerve root or the corticospinal tracts, resulted in controlateral arm, body and leg sensory changes. This

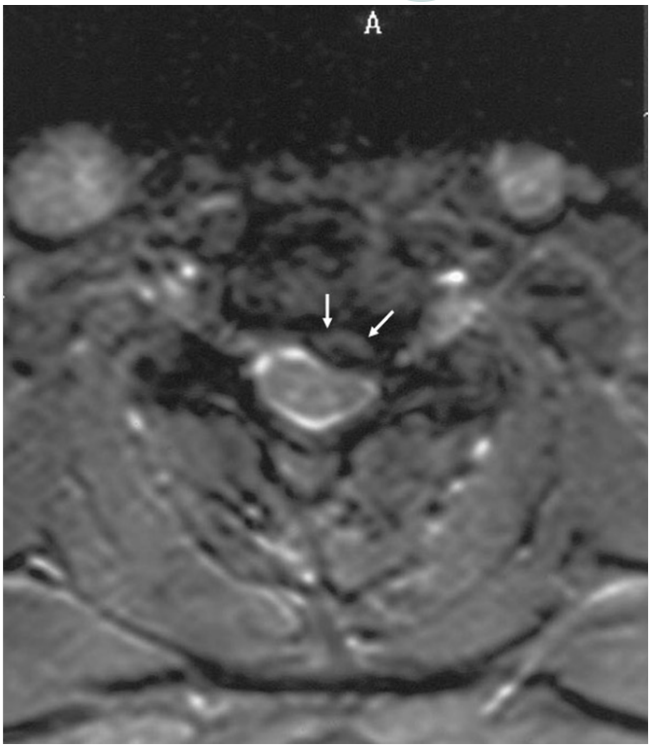

Figure 6: Transverse T2-weigheted MRI section through the C5-C6 area showing unchanged the herniated disk fragment compressing the left anterolateral spinal cord (arrows). MRI: magnetic resonance imaging

is an unusual case since one would expect symptoms primarily related to the nerve root compression in addition to the spinal cord impingement. Furthermore, even in anterolateral disk herniation, the spinal cord was compressed, and corticospinal tract signs should be expected besides the spinothalamic tract signs.

In conclusion, this represents a unique case in which a herniated disk presses only the spinothalamic tracts, and that needed to be differentiated from a temporarily occurred asymptomatic radiation-induced effect, and tumor recurrence. The patient was instructed to wear a soft collar and avoid heavy use of her arms. The patient also received medical treatment with nonsteroidal anti-inflammatory drugs and gabapentin. Laminectomy was not performed due to the significant clinical improvement after the medical therapy, while the patient has improved at the 6-month follow-up evaluation.

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Conflicts of interest
There are no conflicts of interest.

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Topic: Autoimmune neurological diseases associated with autoantibodies specific for synaptic antigens

Neurological diseases associated with autoantibodies targeting the voltage-gated potassium channel complex: immunobiology and clinical characteristics

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ABSTRACT

Voltage-gated potassium channels (VGKCs) represent a group of tetrameric signaling proteins with several functions, including modulation of neuronal excitability and neurotransmitter release. Moreover, VGKCs give a key contribution to the generation of the action potential. VGKCs are complexed with other neuronal proteins, and it is now widely known that serum autoantibodies directed against VGKCs are actually directed against the potassium channel subunits only in a minority of patients. By contrast, these autoantibodies more commonly target three proteins that are complexed with alpha-dendrotoxin-labeled potassium channels in brain extracts. These three proteins are contactin-associated protein-2 (Caspr-2), leucine-rich, glioma inactivated 1 (LGI-1) protein and the protein Tag-1/contactin-2. Neoplasms are detected only in a minority of seropositive patients for VGKC complex-IgG and do not significantly associate with Caspr-2 or LGI-1. Among all the cancers described in association with VGKC complex-IgG, lung carcinoma, thymoma, and hematologic malignancies are the most commonly detected. We will review all the major neurological conditions associated with VGKC complex-IgG. These include Isaacs' syndrome, Morvan syndrome, limbic encephalitis, facio-brachial dystonic seizures, chorea and other movement disorders, epilepsy, psychosis, gastrointestinal neuromuscular diseases, a subacute encephalopathy that mimics Creutzfeldt-Jakob prion disease both clinically and radiologically and autoimmune chronic pain. The vast majority of these conditions are reversible by immunotherapy, and it is becoming increasingly recognized that early diagnosis and detection of VGKC complex-IgG is critical in order to rapidly start the treatment. As a result, VGKC complex-IgG are now part of the investigation of patients with unexplained subacute onset of epilepsy, memory or cognitive problems, or peripheral nerve hyperexcitability syndromes.

Key words: Chronic pain, epilepsy; facio-brachial dystonic seizures; leucine-rich glioma inactivated 1 protein; limbic encephalitis; movement disorders; neuromyotonia; voltage-gated potassium channels

INTRODUCTION

Voltage-gated potassium channels (VGKCs) represent a group of tetrameric signaling proteins with several functions, including modulation of neuronal excitability and neurotransmitter release.[1] Moreover, VGKCs contribute to the generation of the action potential. Neurological autoimmune and paraneoplastic syndromes involve only a small number of VGKCs, notably the "Shaker" type Kv1 channels (Kv1.1, Kv1.2, Kv1.6), sensitive to alpha-dendrotoxin.[2] VGKCs are complexed with other neuronal proteins, and it is now widely known that serum autoantibodies directed against VGKCs are actually directed against the potassium channel subunits only in a minority of patients. In contrast, these autoantibodies more commonly target three proteins that are complexed with alpha-dendrotoxin-labeled potassium channels in brain extracts.[3] These three proteins are contactin-associated protein-2

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(Caspr-2), which is localized at the juxtaparanodes in myelinated axons and associates with Transient axonal glycoprotein 1, postsynaptic density-95/discs large/zonula occludens-1, and the ankyrin-spectrin protein;[4] leucine-rich, glioma inactivated 1 (LGI-1) protein that is most strongly expressed in the hippocampus; and the protein Tag-1/contactin-2, associated with Caspr-2.

Bien *et al.*[5] demonstrated that T-cell cytotoxicity is not a major contributor for the pathogenesis of the neurological syndromes associated with VGKCs, whereas antibody and complement-mediated neuronal cell damage are prevalent.

Neoplasms are detected only in a minority of seropositive patients for VGKC complex-IgG (16% in the experience of Mayo Clinic)[2,4] and do not significantly associate with Caspr-2 or LGI-1. Among the tumors that are believed to be associated with VGKC complex-IgG, lung carcinoma, thymoma and hematologic malignancies are the most commonly described.[2]

We will review all the major neurological conditions associated with VGKC complex-IgG. These include Isaacs' syndrome,[6] Morvan syndrome (MoS),[7] limbic encephalitis (LE),[8,9] facio-brachial dystonic seizures (FBDS),[10,11] chorea and other movement disorders,[12] epilepsy,[13] psychosis,[14] gastrointestinal neuromuscular diseases,[15,16] a subacute encephalopathy that mimics Creutzfeldt-Jakob prion disease both clinically and radiologically[2] and autoimmune chronic pain.[17]

PERIPHERAL NERVE HYPEREXCITABILITY

Motor nerves

Isaacs' syndrome (neuromyotonia) Immune-mediated neuromyotonia, also known as Isaacs' syndrome, is the most severe phenotype of peripheral nerve hyperexcitability. It is characterized by spontaneous and continuous muscular activity resulting from repetitive motor unit action potentials of peripheral origin. The syndrome was described for the first time by Isaacs in 1961.[18] Isaacs also established the peripheral nerve origin of the discharges by documenting the persistence of abnormal electromyographic activity after proximal nerve block. The main clinical features of the neuromyotonia are muscle twitching at rest (visible myokymia), cramps and muscle stiffness, impaired muscle relaxation after voluntary contraction (pseudomyotonia), along with hyperhidrosis. Patients may also suffer from weakness. Other symptoms include myokymia of the limb, trunk,[19] face[20] and tongue[21] muscles. In some patients, hypertrophy of muscles can occur due to continuous muscle activity.[22] The main electromyographic hallmark of the neuromyotonia is the presence of spontaneous, continuous doublet, triplet or multiplet single motor unit discharges, firing at a high intraburst frequency (30-300 Hz).[23] In addition, fibrillation potentials and fasciculations are often present, the former indicating the discharge of a single muscle fibers. About 40% of patients with acquired neuromyotonia have detectable anti-VGKC antibodies[24] and the percentage increases to 80% if there is an associated thymoma. Interestingly, the dysfunction of peripheral nerve VGKCs can be also due to genetic cause, that is, episodic ataxia type I. In fact, episodic ataxia type I is caused by a mutation of the potassium channel gene KCNA1 on chromosome 12.[25]

The acquired, immune-mediated form of the neuromyotonia has been described in association with many autoimmune diseases, such as myasthenia gravis, Addison disease, vitiligo, Hashimoto thyroiditis, pernicious anemia, celiac disease, and rheumatoid arthritis.[26] It is well established that neuromyotonia may also be paraneoplastic. In such cases, the pathophysiology is likely due to cross-reactivity of tumor antigens and VGKCs.[26] Most cases of paraneoplastic neuromyotonia are related to small cell lung carcinoma[27,28] and thymoma,[29,30] but it has also been reported an association with Hodgkin lymphoma,[31] bladder[32] and ovarian carcinoma.[33] Membrane-stabilizing drugs, namely the anticonvulsants carbamazepine, phenytoin, sodium valproic acid, lamotrigine are used for symptomatic relief in patients with Isaacs' syndrome as they usually provide significant improvement of stiffness, muscle spasms, and pain. Their mechanism of action helps reducing neuronal repetitive firing through interaction with VGKCs. If the response is not sufficient, oral corticosteroid may be prescribed, and nonsteroid immunosuppressive drugs such as azathioprine and methotrexate may also be considered as treatment.[34] Plasma exchange often produces clinical improvement lasting about 6 weeks with a significant fall in VGKC antibody titers.[34] Intravenous immunoglobulins are also indicated for severe neuromyotonia, providing short-term relief. In the paraneoplastic form of the neuromyotonia, treatment of malignancy is warranted.[34]

Sensory nerves

Chronic pain

Klein *et al.*[17] found that the 50% of VGKC-complex antibody positive patients experience pain in isolation (28%) or with accompanying neurologic manifestations (72%), not attributable to an alternative cause. VGKC-complex antibodies related pain is

subacute in onset, chronic in course, neuropathic, nociceptive, regional, or diffuse. It is significantly associated with Caspr-2 antibody positivity, but not with LGI-1 antibody,[17] and occurs in isolation or with recognized neurologic manifestations of VGKC-complex autoimmunity.[11,35,36] It is characterized by prominent morbidity and in some cases may require to be treated by narcotics. It has been hypothesized that pain related to VGKC antibodies is due to the hyperexcitability of nociceptive pathways, although such involvement is difficult to be demonstrated, and patients' symptoms are often disproportionate to objectively measured neuropathic dysfunction. It has been demonstrated that VGKCs act synergistically with the potassium/sodium hyperpolarization-activated cyclic nucleotide-gated ion channel 2 (HCN2), that is an inward rectifying channel acting as a regulator of nociceptive pain.[37] VGKC and HCN2 act synergistically to maintain nociceptive afferent sensory neural thresholds,[38,39] and it is reasonable to hypothesize that VGKC-complex antibodies may interfere with their functional activity.

Membrane-stabilizing antiepileptic drugs may have some benefit in patients with VGKC-complex autoimmune chronic pain. Interestingly, 81% of patients described by Klein *et al.*[17] experienced improvement in their pain by immunotherapy, allowing narcotics to be discontinued in some cases.

More recently, Rosch *et al.*[40] reported two cases of ganglioside antibody-negative pediatric Guillain-Barrè syndrome associated with Caspr-2 antibodies. Both patients experienced a full recovery. Thus, Caspr-2 might be a possible autoimmune target in Guillain-Barrè syndrome. Certainly, further studies are needed in order to fully understand the relevance of Caspr-2 as an autoantigen in the pathophysiology of Guillain-Barrè syndrome.

Autonomic nerves

Gastrointestinal neuromuscular disorders described in association with VGPC complex-IgG

Gastrointestinal neuromuscular diseases are characterized by symptoms of intestinal neuromuscular dysfunction.[41] These disorders may be attributable to congenital or, more frequently, acquired conditions. An autoimmune pathophysiology has been proposed to explain acquired gastrointestinal neuromuscular diseases. Interestingly, inflammatory neuropathy is common in patients affected by gastrointestinal neuromuscular diseases and autoantibodies directed against neuronal antigens are present in some patients. VGKC complex-IgG have been detected in the sera of patients with primary and paraneoplastic slow-transit constipation,[15,16] primary achalasia,[42] postinfective irritable bowel syndrome associated with inflammatory enteric neuropathy,[43] chronic intestinal pseudo obstruction,[44] and esophageal and colonic dysfunction secondary to infection by the protozoan parasite trypanosoma cruzi (Chagas' disease).[45] The presence of VGKC-Ab (or other antineuronal antibodies) in the early phases of gastrointestinal neuromuscular diseases still remains contentious. However, if present, early detection, followed by proper immunotherapy could be important in order to prevent the progressive deterioration of gut function.

CENTRAL NERVOUS SYSTEM MANIFESTATIONS

LE

LE is generally characterized by a subacute and progressive onset of episodic memory deficits, disorientation, and recurrent seizures. Additional features are hallucinations, sleep-cycle disturbances, agitation, and delusions. There is histological evidence of mesial temporal lobe inflammation. LE can be associated with several antibodies including anti-Hu,[2] anti-CV2/CRMP5,[46] anti-Ri,[47,48] anti-Ma2,[49,50] and anti-amphiphysin.[51,52] Antibodies targeting neuronal cell surface antigens, such as ion channels and ligand-gated ion channels have been recently identified. VGKC complex-IgG are a good example of the second category of antibodies. VGKC antibodies were first reported in 2001 in two patients affected by reversible LE[53] and then in two series in 2004.[8,9] VGKC-LE has been described in association with antibodies against LGI-1 in 80-90% of patients[54] or Caspr-2 in 5-10%.[55] Very few patients have contactin-2 antibodies, and some patients have no specific target.[56] VGKC-LE is frequently diagnosed in the absence of associated tumors.[57,58] In a recent study, only the 21.4% of the patients showed malignancies.[59] Hyponatremia is a characteristic feature of VGKC-LE. It is present in about 60% of patients, and it is initially resistant to treatment, but it usually resolves as the VGKC complex-IgG titers decline.[9] The serum hyponatremia usually follows a syndrome of inappropriate antidiuretic hormone (SIADH) secretion pattern. Intermittent and episodic hypothermia, along with neuropathic pain, both responsive to immunotherapy, have been reported in patients affected by VGKC-LE.[60] A severe sleep disorder, characterized by insomnia, deep diurnal drowsiness and complete disappearance of rapid eye movement sleep has also been associated with VGKC-LE.[61] Autonomic dysfunctions have been extensively described in VGKC-LE patients. Vincent *et al.*[9] reported sweating and hypersecretion due to an effect of the antibodies on the postganglionic sympathetic neurons. More recently, episodic bradycardia has been recognized as a prodrome

of LGI-1-LE[62] and in all the patients reported, led to pacemaker implantation. Interestingly, in other cell-surface antibody-associated neurological disorders (i.e. N-methyl-D-aspartate receptor antibody encephalitis) bradycardia has been rarely reported.

FBDS represent a typical manifestation that may precede the development of LGI-1 LE.[11] The clinical features of FBDS will be extensively discussed elsewhere in this review. They may be characterized by facial twitching, hand and leg posturing. The antiepileptic drugs do not usually reduce seizure activity. In contrast, early initiation of plasma exchange and immunosuppression help to avoid the development of full-blown LE.

Routine cerebrospinal fluid (CSF) analysis may reveal a mild lymphocytosis in some patients and protein, and glucose levels may be modestly raised or within normal limits. Polymerase chain reaction is obviously negative for herpes simplex virus and other neurotropic viruses while oligoclonal bands may be present, but rarely unmatched with serum bands.[9]

In a recent magnetic resonance imaging (MRI) study on patients affected by VGKC-LE[59] initial MRI findings included unilateral or bilateral amygdala and/or hippocampal enlargement and T2 hyperintensity in 78.6% of patients at some time point during the disease course. Restricted diffusion, mild ill-defined contrast enhancement, and extratemporal findings were also reported. Interestingly, more than a quarter of the patients with initially negative MRI or only unilateral abnormalities then progressed to bilateral involvement, supporting the hypothesis of radiologic progression of the disease. It is still a matter of debate whether these changes reflect persistent inflammation or alternatively they are secondary to recurrent seizures. In fact, patients with VGKC-LE have a very high frequency of epileptic seizures,[58] and this has been hypothesized to be related to the development of mesial temporal sclerosis, often seen in follow-up VGKC-LE patients. Patients with VGKC-LE and high signal in the medial temporal lobes typically develop hippocampal atrophy as the high signal declines.[63,64] It is not clear yet whether the cases of otherwise "cryptogenic" mesial temporal sclerosis are at least partly related to a remote effect of VGKC autoimmunity.

FBDS

FBDS are very brief highly distinctive seizures associated with VGKC-complex antibodies, almost always in the LGI-1 subtype. They carry a high chance of developing VGKC-LE, and their recognition should prompt consideration of immunotherapies in order to prevent the onset of LE. The FBDS was first described in 2008 by Irani *et al.*[10] and then better characterized in 2011.[11] As compared to the initial descriptions, it is now evident that the age of onset of FBDS is broad, varying from 28 to 92 years,[65,66] possibly with a small male prevalence.[11,65] The daily frequency of FBDS is high, ranging from 6 to 360 attacks per day at their peak.[11] Emotions and movements are common triggers of FBDS,[11,65] and a sensory aura or auditory hallucinations may precede them.[65] Every FBDS is characterized by a dystonic posturing of the arm, both proximally and distally, and may involve also the ipsilateral face and less commonly, the trunk and the ipsilateral leg. It is worth noting that events involving the leg alone have been rarely observed.[65] Furthermore, synchronous bilateral dystonia and rapidly alternating events have been reported.[65] Either side can be involved, but FBDS are usually unilateral on any occasion.[11] If FBDS can be classified as tonic seizures, or as a movement disorder, namely a form of dystonia, is still a matter of debate[67] and data to support the former and the latter hypothesis are summarized in Table 1. FBDS are often accompanied by ictal automatisms and may be, followed by fear, agitation and speech arrest.[65] The duration of FBDS was reported to last < 3 s in the early description,[11] however it is now clear that they may last also between 10 and 30 s.[65] Serum sodium levels are often reduced in FBDS patients presenting also with cognitive impairment but are rarely low during the period with facio-brachial dystonic seizures alone.[11,65] If the patient experiences FBDS alone, with no cognitive impairment, routine MRI is unremarkable in the vast majority of cases.[11,65] However, routine MRI showed a high signal change in the putamen in a patient described by Irani *et al.*[65]

Table 1: Data to support the hypothesis of FBDS as tonic seizures, or as a movement disorder

Movement disorders	Epileptic seizures
Loss of consciousness not always noted	The majority of patients experiences loss of awareness, although not during every attack
Electroencephalography: epileptic activity in a minority of patients (24%)	Electroencephalography: focal slowing or epileptiform changes in 24% of cases with FBDS is a significant proportion (very brief duration of the attacks arising from spatially limited or deep foci)
Functional neuroimaging: altered glucose metabolism in different cerebral regions, including basal ganglia	Brief duration and highly stereotyped attacks
Poor response to antiepileptic drugs	LGI-1 antibodies associated with typical medial temporal lobe seizures in the context of limbic encephalitis, often refractory to anticonvulsants
Chorea and other movement disorders associated with VGKC	The frequent ictal presence of automatisms, and fear, agitation and speech arrest after the motor event

FBDS: facio-brachial dystonic seizures; VGKC: voltage-gated potassium channels; LGI-1: leucine-rich, glioma inactivated 1

in 2013 and a gadolinium-enhancing lesion involving the caudate and globus pallidus in a patient that we described in 2013.[68] When cognitive impairment is present, both unilateral and bilateral medial temporal lobe and also caudate signal changes have been described.[11,65] Interestingly, the basal ganglia signal changes are controlateral to the facio-brachial dystonic seizures in all the patient described so far.[65,68] Electroencephalography shows ictal epileptiform activity in a minority of patients with a frontotemporal, frontal, or temporal focus.[11] The LE following FBDS is clinically undistinguishable from the other VGKC-LE with amnesia, confusion, hallucinations, sleep disturbances and other nondystonic seizure types.[11] The initial treatment with antiepileptic drugs is ineffective in the majority of the patients, while the initiation of immunotherapy with corticosteroids, intravenous immunoglobulin, and plasma exchange has proven to be useful in order to reduce the frequency of FBDS.[11,65] The cessation of FBDS after the immunotherapy is associated with a significant reduction in serum VGKC complex/LGI-1 antibody titers.[65] Antiepileptic drugs are also responsible for adverse cutaneous reactions in a high proportion of patients and the involvement of both eyes and mouth has been reported in one patient,[65] while the documented psychiatric side effects of steroids (including paranoia and hypomania) generally improve by tapering steroids. In patients with persisting VGKC-complex/LGI1-antibodies, relapses of FBDS have been described during the steroid tapering period, and all the relapses showed an absolute response, after increasing the corticosteroid dose.

The recognition of FBDS is critical because the response to anti-epileptic drugs is unsatisfactory while response to immunotherapy is excellent, and early initiation of immunosuppressant therapy offers the chance to modify the course of this neurological disorder avoiding the development of full-blown LE.

Chorea and other movement disorders associated with VGPC complex-IgG

Extrapyramidal involvement has been reported in 21% of patients with positive VGKC complex-IgG. The majority of these patients have tremor associated with Parkinsonism, some patients experience tremor only while few patients have chorea.[12] Tofaris *et al.*[69] described two patients in which chorea was the main symptom and anticipated by several weeks the onset of LE. The involvement of the basal ganglia has been extensively demonstrated in patients with LGI-1 antibodies[11,65,68] and it is worth noting that both the patients described by Tofaris *et al.*[69] had residual executive dysfunction despite significant memory improvement after the immunotherapy. These sequelae further suggest the involvement of a subcortical neuronal network in these patients. Furthermore, myoclonus was reported in 29% of patients with positive VGKC complex-IgG.[12] The majority of the patients showed generalized myoclonus, but segmental/multifocal, unilateral and focal cortical myoclonus have also been reported.

Epilepsy

In recent years, a number of studies have reported antibodies targeting neuronal cell surface antigens in patients with epilepsy, including antibodies against the VGKC-complex and the N-methyl-D-aspartate glutamate receptor.[70-72] The role of such antibodies in the pathogenesis of epilepsy has not been fully established,[73] but they have been found more likely to occur in patients with focal epilepsy of undetermined cause rather than in patients with structural/metabolic focal epilepsy.[71] In particular, VGKC-complex antibodies are of interest in epilepsy, as they target VGKC that play an important role in regulating neuronal excitability. Lilleker *et al.*[73] found that in patients with unexplained adult onset epilepsy, VGKC-complex antibodies were positive in the 8% of cases and the 4.2% of the patients had titers higher than 400 pM. Patients with VGKC-complex antibodies titers higher than 400 pM had focal seizures and in 4 out the 6 described cases the seizure focus was diagnosed in the temporal lobe. These patients reported rising abdominal sensation, olfactory hallucination, piloerection, dejà vu, oromasticatory automatism and depersonalization. Two patients had generalized tonic-clonic seizures in addition to focal seizures, and one patient had FBDS. No patient had the clinical syndrome of LE, and no patient was diagnosed with cancer. The sera of all 6 patients were tested for LGI-1 and Caspr-2 antibodies. The serum of the patient who presented with FBDS tested positive for LGI-1 antibodies; one patient was positive for Caspr-2 antibodies; all the other patients were negative for both LGI-1 and Caspr-2 antibodies. Brain MRI was normal in all patients except in one patient showing increased signal in the left hippocampus and amygdala that was attributed to recent seizures. Interestingly, all the patients improved with immunotherapy, although not all of them have been rendered seizure-free. Furthermore, Iorio *et al.*[72] found that patients with neural antibodies not responding to antiepileptic drugs may benefit from immunotherapy. They studied 81 patients (39 patients with epilepsy and other neurological symptoms and/or autoimmune diseases responsive to antiepileptic drugs and 42 patients with AED-resistant epilepsy). Neural autoantibodies were detected in 22% of patients, mostly from the antiepileptic drug-resistant

epilepsy group. Three patients with antiepileptic drug-resistant epilepsy had anti-LGI-1 antibodies. Twelve patients received immunotherapy and 9 (75%) achieved a > 50% reduction in seizure frequency. Interestingly, LGI-1 is also linked to seizures of genetic etiology. Mutations in the LGI-1 gene are responsible for autosomal dominant temporal lobe epilepsy with auditory features (buzzing and tinnitus).[74-76] Indeed, the identification of autoantibodies, such as those targeting VGKC-complex, has changed paradigms in the diagnosis and management of epilepsy and has expanded the phenotypic spectrum of autoimmune disorders. In future, the discovery of new autoantibodies may also further expand the range of the autoimmune epilepsies.

Psychiatric manifestations

Several psychiatric manifestations have been described in patients with VGKC-complex antibodies.[12,14] These are often affective-predominant and include confusion, memory impairment, personality changes, depression, agitation, hallucinations, and anxiety. A clinical improvement has been reported in the majority of the patients who received immunotherapy.[12] Neuropsychiatric presentations are significantly more common in patients with higher autoantibody values, and clinical improvements are more likely in patients treated early. Further studies are needed in order to clarify the exact prevalence of VGKC-complex antibodies in patients from the general population with psychiatric manifestations.

Subacute encephalopathy that mimics Creutzfeldt-Jakob prion disease associated with VGKC complex-IgG

Rossi *et al.*[77] described three patients that were referred with possible prion disease. Their clinical picture was in keeping with autoimmune encephalitis, and they had very high VGKC-complex/LGI-1 antibodies. Otherwise, low titers of neuronal antibodies occurs rarely in suspected patients with sporadic Creutzfeldt-Jakob disease (sCJD) and when present should be interpreted with caution. Atypical features in sCJD, such as FBDS, hyponatremia[36] and autonomic dysfunction, may suggest an autoimmune disorder.[77] A high titer of VGKC-complex Ab (LGI-1 negative) was also identified in a 61-year-old Caucasian man with a novel prion protein (PRNP) gene mutation and Gerstmann-Straüssler-Scheinker disease, but despite 1 year of aggressive immunosuppressive treatment the patient died.[78]

Interestingly, nonspecific markers of neuronal degeneration in CSF such as 14.3.3 and S100B proteins may test positive in patients with VGKC complex-IgG encephalitis, thus being not completely reliable for the definite diagnosis of sCJD.[77]

The demographic, clinical and neuroradiological characteristics of the patients positive for VGKC complex-IgG (except for FBDS, dysautonomia, and hyponatremia) are not distinguishable from CJD[36,77] and most patients fulfilled the World Health Organization diagnostic criteria for sCJD.[36,79] Thus, it is critical to consider autoimmune encephalitis in the differential diagnosis of sCJD in order to promptly test for the relevant antibodies.

MORVAN'S SYNDROME

Morvan's "fibrillary chorea" or MoS was first described by the French physician Augustin Marie Morvan[80] in 1890 in a patient who exhibited myokymia combined with excessive sweating and disordered sleep. It is a rare entity characterized by peripheral and central nervous system (CNS) involvement, specifically, neuromyotonia, hallucinations, delirium, insomnia, and autonomic disturbance [Table 2].[80,81] Peripheral nerve involvement is mainly characterized by neuromyotonia, but neuropathic pain in the feet and/or legs and back, areflexia and a stocking-type sensory loss may also be present.[81] In some cases, insomnia may be severe, amounting to not less than complete lack of sleep (agrypnia) for weeks or months in a row.[82] Common encephalopathic manifestations are spatial and temporal disorientation, confusion, amnesia, hallucinations and agitation. Epileptic seizures, including generalized tonic-clonic seizures and partial seizures consistent with FBDS, are present in about one-third of the cases.[11] Compulsive behaviors, stereotypies, and reduplicative paramnesias can be part of the CNS involvement.[83] Autonomic disturbance includes hyperhidrosis, pruritus, drooling, severe constipation, urinary incontinence, excessive lacrimation, and cardiac arrhythmias.[84] Autonomic system dysfunction has been described in the 93% of MoS patients, being hyperhidrosis and cardiovascular instability the most common manifestations.[81] Weight loss, skin lesions or itching, and hyponatremia due to the SIADH secretion are

Table 2: Symptoms and signs of Morvan's syndrome

Peripheral nervous system	Central nervous system	Autonomic system	Systemic features
Neuromyotonia	Insomnia	Hyperhidrosis	Weight loss
Neuropathic pain	Disorientation/ confusion	Tachycardia	Skin lesions
Areflexia	Amnesia	Blood pressure abnormalities	Hyponatremia
Stocking-type sensory loss	Hallucinations	Drooling	
	Agitation	Constipation	
	Delusions	Urinary incontinence	
	Seizures	Excessive lacrimation	

also possible symptoms. MoS usually presents with a slow, insidious onset over months to years[85] and is almost exclusively seen in males. In about the 90% of cases, it spontaneously goes into remission, while in the remaining 10% of cases leads to death.[86]

Patients with MoS may have an associated underlying tumor, including thymoma, that is the most common, lung cancer,[87] sigmoid cancer,[88] testicular cancer and lymphoma, thus indicating the paraneoplastic nature of the disease.[89] On the other hand, patients without an associated tumor have been also been studied, and they generally experience a good clinic response to immunotherapy.[81] Interestingly, it has been described the occurrence of MoS after scrotal tap and injection of sclerosing agent for the treatment of hydrocele in 5 males.[90] Some MoS cases associated with thymomas and myasthenia gravis have also been reported.[91] VGKC-complex antibody serum levels are increased in the 90% of the patients [Figure 1] and although these are directed against LGI-1, Caspr-2, or commonly both, Caspr-2 antibodies predominate and are always associated with thymoma. Fewer patients have been reported also with contactin-2 antibodies.[81] It is intriguing that low levels of Caspr-2 mRNA have been detected in the human prostate: although Caspr-2 is predominantly expressed in the nervous system, the male reproductive system may be a source of the antigen and MoS onset after scrotal drainage[90] may be a crucial factor to break the immune tolerance. Moreover, thymectomy and thymoma chemotherapy may act as disease triggers, thus suggesting that thymic tumors may also harbor the antigenic targets, in particular, Caspr-2. CSF analysis in MoS usually shows normal protein, glucose, white cell count, and IgG index. Oligoclonal bands may be detected. Marked changes in circadian serum levels of neurohormones have been described, with serum levels of melatonin and prolactin substantially lower than normal and without a circadian rhythm of release.[87] Plasma levels of norepinephrine were found to be high throughout the 24 h period, without the physiological nocturnal decrease.[83,87] Increased serum levels of cortisol were also observed.[87] A negative MRI is a characteristic finding in MoS,[81,88] but frontal T2 hyperintensity in one patient and bilateral hippocampal T2 high signal in another patient have also been reported[81] and in these cases the diagnosis of "LE associated with neuromyotonia" should be more appropriate. MoS treatment is based on immunotherapies, including plasma exchange, intravenous immunoglobulin, corticosteroids, azathioprine, cyclosporine, and cyclophosphamide. In the paraneoplastic forms of MoS, the management of the underlying malignancy is mandatory.

CONCLUSION

The clinical spectrum of the neurological disorders associated with VGKC complex-IgG is rapidly expanding, and new associated conditions have been described in the last years. The vast majority of these disorders are reversible by immunotherapy, and it is becoming increasingly recognized that early diagnosis and detection of VGKC complex-IgG is critical in order to rapidly start the treatment. As a result, VGKC complex-IgG are now part of the investigation of patients with unexplained subacute onset of epilepsy, memory or cognitive problems, or peripheral nerve hyperexcitability syndromes. It is still not fully understood how VGKC complex-IgG could cause such a range of different clinical presentations. The accelerated development that the research on antibody-mediated syndrome has had in the last period has been exciting and has made possible to diagnose and to treat clinical syndromes that would have otherwise been poorly defined. Certainly, the development and validation of experimental models of VGKC autoimmunity will represent the next critical challenge in order to clearly elucidate how the antibodies get into the CNS and understand if also antibody-binding, internalization and loss of the target surface antigens, along with complement activation, are involved in the physiopathology. In fact, there is a need to extend our understanding of the pathophysiological mechanisms of these syndromes in order to improve their diagnosis, and ultimately, to

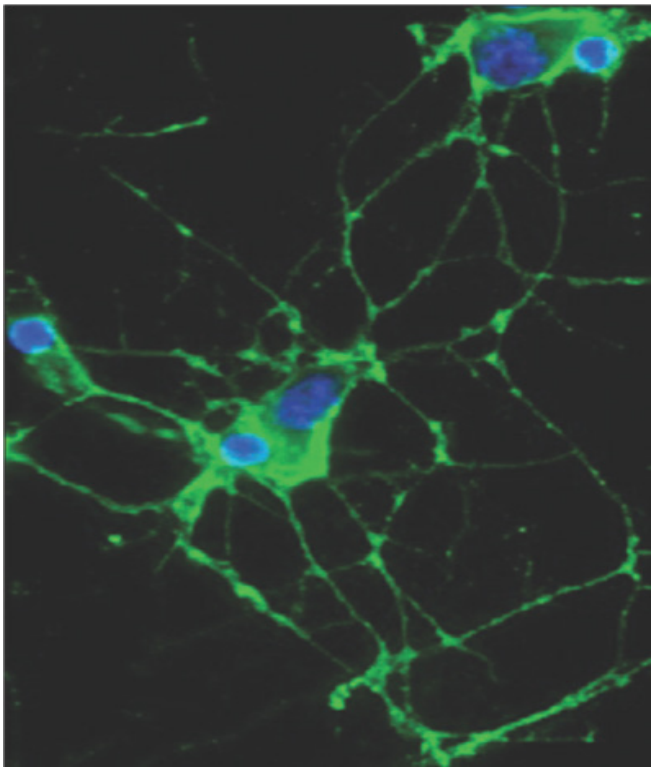

Figure 1: Indirect immunofluorescence showing IgG in the serum of a patient with Morvan's syndrome binding to the surface of a live rat hippocampal neurons

develop more effective targeted therapies.

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There are no conflicts of interest.

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Topic: Autoimmune neurological diseases associated with autoantibodies specific for synaptic antigens

Encephalitis associated with autoantibody binding to the anti-N-methyl-D-aspartate receptor: immunopathogenesis, mechanisms, and clinical characteristics

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ABSTRACT

Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis has been increasingly recognized in recent years. This condition may be the most common cause of antibody-mediated encephalitis worldwide. The majority of patients are young at the time of onset, female, and present with an acute-to-subacute onset of behavioral changes followed by seizure, abnormal movement, autonomic dysfunction, and finally hypoventilation with coma if left untreated. The immunopathogenesis of this disease may be due to antibody-mediated internalization of NMDARs from synapses, which results in the dysfunction of particular brain regions (especially the hippocampus and frontostriatal area). Compared to serum, the cerebrospinal fluid permits the more sensitive detection of anti-NMDAR antibody. Ovarian teratoma may be present in up to 40% of patients but is less frequent in children or late-onset disease (> 45 years old). The severity at the time of disease onset and time to appropriate immunotherapy (high-dose steroid plus plasmapheresis or intravenous immunoglobulin) are independent factors that are associated with good outcomes.

Key words: Abnormal movement; anti-N-methyl-D-aspartate receptors encephalitis; glutamate; immunotherapy; ovarian teratoma; psychiatric symptoms; seizure

INTRODUCTION

The N-methyl-D-aspartate receptors (NMDAR) are glutamatergic ion channels that are widely expressed in both cortical and subcortical areas of the brain. These receptors are essential in memory and behavior. Hyperactivity of NMDAR may be the underlying mechanism of seizure and some types of dyskinesia, and under activity may be related to schizophrenia.[1]

Anti-NMDAR encephalitis has been increasingly recognized in recent years. The exact incidence of anti-NMDAR encephalitis is unknown. Data from a retrospective study indicated that anti-NMDAR encephalitis represented 1% of all young patients (aged 18-35 years) who were admitted to an intensive care unit with encephalitis of an unknown etiology (excluding infectious causes).[2] The data obtained from a population-based prospective study in England showed that 4% of all cases of encephalitis presented anti-NMDAR antibody.[3] In the California Encephalitis Project,[4] anti-NMDAR encephalitis was identified in 4% of patients < 30 years of age with encephalitis of an uncertain etiology, and it was detected 4 times more frequently than herpes simplex virus type 1 encephalitis, West Nile virus, or Varicella zoster virus encephalitis. Anti-NMDAR encephalitis was also the most common cause of antibody-mediated encephalitis. There is no information regarding the incidence of anti-NMDAR encephalitis in Asia. However, in Japan, a condition called acute juvenile female nonherpetic encephalitis (AJFNHE) is almost

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identical to anti-NMDAR encephalitis. A nationwide survey conducted on AJFNHE showed that the annual incidence was 0.33/100,000 people in Japan.[5] One retrospective study in Thailand reported that anti-NMDAR encephalitis represented 5% of all cases of encephalitis.[6] Increases in clinical awareness and laboratory availability have led to a rise in the number of diagnoses and case reports, suggesting that the incidence of the disease might be similar to Western countries.

IMMUNOPATHOGENESIS

Anti-NMDAR antibody can be detected in cerebrospinal fluid (CSF) and/or serum. To be considered pathogenic, the autoantibody should bind to an extracellular antigen (such as an ion channel or neurotransmitter receptor) and cause a functional or structural change. Anti-NMDAR antibody has been shown to bind the NR1 subunit of the NMDAR and reduce the density of NMDARs on the cell membrane.[7] Because NMDARs are protected by the blood-brain barrier (BBB), the antibody somehow enters privileged sites via unknown mechanisms. Disruption of the BBB may be initiated by infection, followed by up-regulation of the major histocompatibility complex or other inflammatory mediators that finally weaken the barrier (some patients develop flu-like symptoms prior to the occurrence of clinical encephalopathy). In some patients, ovarian teratoma is detected at the time of the development of encephalopathy. It has been postulated that the immune response is initiated by this tumor, which expresses NMDAR on its surface.[8] The mechanism underlying immune tolerance is disrupted, potentially by the ectopic expression of NMDARs by the tumor or another mechanism in combination with leakage of the BBB, leading to an attack by the immune system on the NMDAR.[9] It is likely that memory B cells activate T-cells by crossing the BBB and undergoing clonal expansion and differentiation into plasma cells that produce anti-NMDAR. These autoantibodies bind to NMDARs expressed in various areas of the central nervous system and subsequently cause receptor dysfunction.

The acute effect of anti-NMDAR on the NMDAR has been investigated.[10] One study has shown that a decrease in the NMDAR density on the surface of both excitatory and inhibitory hippocampal neurons caused NMDAR hypofunction through immunoglobulin-induced receptor internalization (crosslinking of the receptors).[10] This internalization of receptors resulted in a rapid and selective loss of NMDARs from the neuronal membrane that was titer-dependent and could be reversed after removal of the antibodies.[9] The degree of loss of NMDAR synaptic function may explain the clinical symptoms of patients with this encephalopathy, who initially present with behavioral or cognitive dysfunction with or without seizure, followed by abnormal movements, dysautonomia, and coma with hypoventilation.[11] A recent study using an animal model of anti-NMDAR encephalitis showed that continuous intraventricular infusion of anti-NMDAR from the CSF of patients with anti-NMDAR encephalitis to mice produced progressive memory deficit, anhedonia, and depressive-like behavior.[12] This correlated with the degree of hippocampal binding by anti-NMDAR and decreases in the density of total and synaptic NMDAR clusters as well as total NMDAR protein concentration. After discontinuation of the infusion, the symptoms improved over the course of a week. The reversal of symptoms correlated with decreased hippocampal bound antibody and restoration of NMDAR levels.[12] The clinical symptoms of the frontostriatal syndrome (psychosis, catatonia, and dystonia) and semi-rhythmic movements may be mainly due to the inactivation of GABAergic neurons as a result of the decreased NMDAR function. This may be explained by the hypofunction of NMDARs, causing the alteration of homeostatic synaptic plasticity to adjust the inhibitory tone in a compensatory direction by down-regulation of inhibitory synapses on excitatory neurons.[10] This decreased function also affects dopaminergic, noradrenergic, and cholinergic pathways, which may explain the autonomic dysfunction and effects on the ponto-medullary respiratory network that lead to hypoventilation.[11]

CLINICAL MANIFESTATIONS

Typical manifestations of anti-NMDAR encephalitis are described as classic symptoms of psychotic or cognitive dysfunction, seizure, abnormal movement, and autonomic dysfunction. The symptoms develop in an acute-to-subacute onset that is usually preceded by prodromal and followed by psychotic features.[13] The spectrum of psychiatric features is varied, and more than one feature can be detected individually. Short-term memory problems are common, but they may be under-detected due to the overwhelming psychotic features. The sequence of symptoms is as follows: seizure, hyperkinetic movement disorders, autonomic instability, and then unresponsiveness with hypoventilation. The seizure may develop early in the course of the disease and usually decreases in frequency with disease progression.[13] After 2-3 weeks, patients who have not been treated develop an unresponsive phase. This clinical presentation of the patient includes mutism, akinetic mutism, and unresponsiveness to verbal commands with eye-opening but the loss of eye contact similar to

catatonic schizophrenia. These symptoms alternate with periods of agitation. Some patients develop bizarre and inappropriate behavior such as smiling, echophenomenal (both words and movement), or catalepsy-like symptoms.[11] Dissociative (paradoxical) responses to stimuli (unresponsive to painful stimuli, but resistant to eye opening) are often presented in patients, mimicking a psychogenic condition or malingering. Most patients later develop hyperkinetic abnormal movements, the majority of which are oro-lingual-facial dyskinesia; however, other types of movement may also be observed. During the same period, autonomic instability and hypoventilation also occur. The autonomic manifestations include hyperthermia, tachy-bradycardia, and labile blood pressure. Autonomic dysfunction leads to a prolonged cardiac pause and requires a temporary pacemaker. Hypoventilation can present alone or in association with autonomic instability, which necessitates respiratory support. This phenomenon often occurs during the period of hyperkinetic movement, or it can occur during early stages of symptoms. Within 4 weeks of symptom onset, most patients develop a similar spectrum of symptoms irrespective of their age.[14] The characteristics of classical anti-NMDAR encephalitis progression are summarized in Figure 1. However, the clinical presentation of patients with anti-NMDAR encephalitis varies depending on the individual patient. This review focuses on each symptom of anti-NMDAR encephalitis.

Prodromal symptoms

This viral-like illness usually presents 1-2 weeks before the development of psychiatric symptoms.[13] It is not known whether the symptoms are due to NMDAR dysfunction, the systemic immune response to autoimmune disease or secondary responses to a viral infection which later precipitate autoimmune disease.

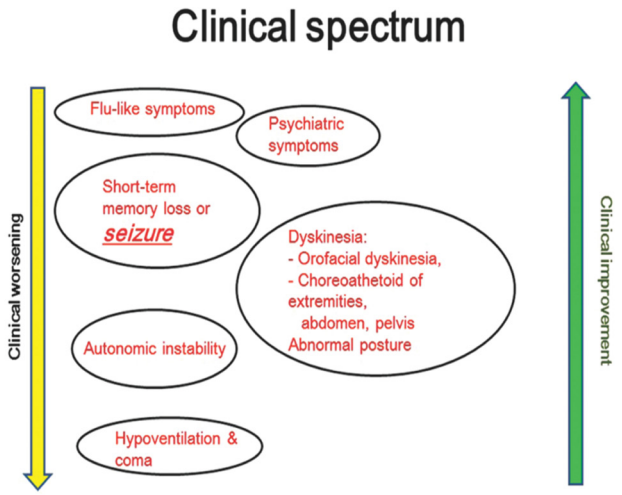

Figure 1: The spectrum of anti-N-methyl-D-aspartate receptor encephalitis showing the common sequence of symptoms with clinical worsening and improvement

Psychiatric symptoms

The psychiatric symptoms of anti-NMDAR encephalitis encompass a broad spectrum that includes anxiety, depression, agitation, abnormal behavior, delusion, hallucination, mania, and frank psychosis.[13] The symptoms usually present at the beginning of the disease, leading to medical attention (mostly by a psychiatrist). It is the most common initial manifestation in both sexes.[15] In younger children, parents may describe the symptoms as temper tantrums, behavioral changes, aggression, and progressive speech deterioration.[16] Staff phobia has also been reported in children or adolescents.[16] Overall, the psychiatric symptoms associated with the initial manifestation or during relapses are the same in both sexes and all ages.[17] Isolated psychiatric symptoms can be observed in up to 4% of patients (either at disease onset or during relapses).[17] These symptoms may be explained by reduced NMDAR synaptic content and disruption of receptor function in discrete regions of the brain. NMDARs are widely expressed throughout the entire brain, and, therefore, the density of receptor expression or the susceptibility of some regions (especially the frontostriatum or hippocampus) to autoantibodies may be the cause of the symptoms.[17]

Cognitive dysfunction

Cognitive dysfunction, especially short-term memory impairment, has been underestimated due to the predominance of psychiatric and speech problems that interfere with the cognitive assessment.[11] There is evidence that IgA antibody subtypes recognizing the NMDAR antibody might be present in patients with progressive cognitive decline.[18] However, a later study suggested that IgA subtypes against NMDAR can be found in the control population and are not related to the neurological disease.[19] The role of NMDAR-IgA remains uncertain.

Seizure

Seizures occur in approximately 70% of adults and are even more common in children.[14] They typically occur after a prodromal period and psychiatric symptoms in adults, but they may be the initial manifestation and occur with greater frequency in children and adult males.[15] This phenomenon may be explained by a reduced influence of hormonal factors or by a selection bias whereby women with initial psychiatric symptoms are more likely to be suspected of this disease compared to men. Up to 5% of patients with anti-NMDAR encephalitis have purely a seizure disorder without prominent neuropsychiatric involvement.[20] The seizure types

can present as generalized, focal, or complex partial seizures. Some cases may progress to status epilepticus or nonconvulsive status epilepticus. Most of these cases are refractory to treatment with standard antiepileptic drugs but may respond well to immunosuppressive drugs. One case report described nonconvulsive status epilepticus lasting for 6 months that was refractory to all immunomodulating therapies but showed marked improvement following removal of an ovarian tumor.[21] Seizures in anti-NMDAR encephalitic patients usually have an extratemporal origin.[20,22]

The most commonly observed (90%) electroencephalogram (EEG) pattern typically shows diffuse slowing or predominantly anterior slowing, but these phenomena do not correlate with the clinical and MRI findings.[13,16,23] One-third (34%) of patients exhibited focal slowing. One case series described a unique EEG pattern of "extreme delta brush" in 30% of the patients in early stages of the disease. This pattern suggests the occurrence of more severe disease (a more prolonged hospitalization).[23]

Abnormal movement

Abnormal movement (mostly hyperkinetic movement) has been described in up to 80% of patients during the course of the disease and may be the initial manifestation in some patients, especially in the pediatric group.[14] However, abnormal movement usually follows psychiatric symptoms or seizure. Some of these symptoms may be difficult to differentiate from seizure clinically, but the EEG does not reveal electrographic seizure during an episode.[24] These abnormal movements do not respond to anti-epileptic or dopamine receptor antagonist drugs. Abnormal movements can alert clinicians to investigate autoimmune processes in cases of suspected viral encephalitis, which do not typically present this feature.[25,26]

Various forms of abnormal movement have been described in anti-NMDAR encephalitis. The majority of these movements are complex uni- or bilateral stereotypic movements, in particular, orofacial dyskinesia.[14,27] The spectrum of abnormal movements includes chorea, choreoathetosis, facial/limb myorhythmia, facial-limb-truncal dystonia, myoclonus, tremor, opsoclonus-myoclonus or ataxia, and opisthotonus.[14,27-29] The distinct abnormal movements observed in anti-NMDAR encephalitis may be due to a dissociated state, in which movement disorder may persist during unconsciousness.[28] This feature may be difficult to differentiate from frontal lobe seizure, but an EEG might provide helpful information.[28] One patient can develop more than one characteristic of abnormal movement during the course of the disease. MR spectroscopy of the basal ganglia and thalamus may show a reduction of the N-acetylaspartate/creatine (Cr) ratio in patients during involuntary movements.[30]

DIAGNOSTIC EVALUATIONS

The CSF profile in cases of anti-NMDAR encephalitis typically shows pleocytosis and mild protein elevations. The normal CSF profile does not exclude immune-mediated disease. The brain MRI may be normal in up to 50% of cases.[13] The EEG typically shows diffuse slow or rhythmic activity. The EEG of anti-NMDAR encephalopathy is characterized by an extreme delta brush, which can be found in up to 30% of cases.[23] For specific antibody testing, it is recommended that both CSF and serum be assessed. In the majority of immune-mediated limbic encephalitis including anti-NMDAR encephalitis, the CSF is more sensitive than the serum, excluding cases of VGKC-complex autoantibody (Lgi1 and Caspr2), in which the serum may be more sensitive than the CSF.[13,31] The NMDAR-IgG can be demonstrated by the presence of immunologic reactivity to mouse brain tissue (especially in the hippocampus area and the granular layer of the cerebellum) or NMDAR-transfected cells [Figure 2]. The antibody titer is higher in the CSF compared to the serum in patients with a poor outcome or the presence of teratoma, and titer changes in the CSF are more likely to be related to clinical relapses than to changes in the serum.[32]

Because ovarian teratoma is found in up to 40% of cases of anti-NMDAR encephalopathy, it is recommended that these patients be screened for this condition. If the initial workup is negative for ovarian teratoma,

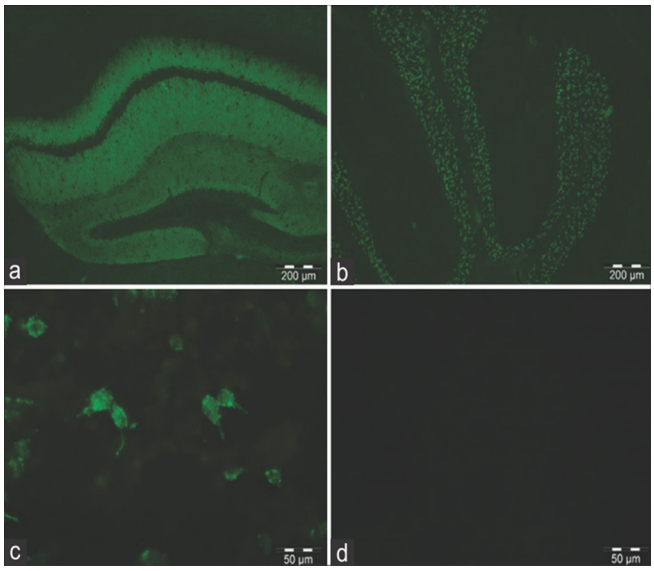

Figure 2: Immunohistochemistry of mouse brain sections showing binding of the N-methyl-D-aspartate receptor (NMDAR)-IgG to the hippocampus. (a) and granular layer of the cerebellum; (b) HEK293 cells expressing NMDAR (GluN1); (c) show antibodies binding to the cell membrane; (d) no reactivity is seen with normal cerebrospinal fluid

periodic screening for at least 2 years is recommended, even for patients who are in recovery.[11] Younger age (< 12 years), older age (> 40 years), and male gender are associated with a reduced risk of tumor.[14]

TREATMENT, CLINICAL RESPONSE AND RECOVERY

A large observational cohort study showed that immunotherapy (high-dose steroid plus either plasmapheresis or intravenous immunoglobulin) and tumor removal, if applicable, yielded beneficial neurological outcomes.[14] Patients in whom a tumor was detected and removed within 4 months of disease onset experienced a more complete and rapid recovery than those without tumor.[13] In patients who did not respond to first-line treatment, second-line therapy (cyclophosphamide or rituximab) resulted in improved outcomes and fewer relapses. Two independent predictors of good outcomes were a lower severity of the disease and an earlier time to appropriate treatment. These determining factors are maintained irrespective of the age of disease onset.[14,33] The recovery process is usually the reverse of the clinical presentation.[11] Autonomic and abnormal movement are usually recovered before other symptoms. Psychiatric features may persist up to several months.[13] The anti-NMDAR titer in the CSF usually decreases in parallel to the clinical response, but the presence of antibody may persist for a long period (up to 15 years) despite normal clinical features.[34] Clinical history and neurological examination, including a neuropsychiatric test, is the most useful indicator of the treatment response,[31] and thus, the periodic detection of the anti-NMDAR titer is not necessary in the context of stable clinical status.

ANTI-NMDAR ENCEPHALITIS IN OLDER AGE

The onset of anti-NMDAR encephalitis at an age of > 45 years has some characteristic features that differ from those of young patients. There is a greater frequency of males compared to females (1:1.2), a reduced frequency of tumor association, fewer seizure episodes, and a greater tendency to present with memory deficit in the late-onset group.[33] If tumors are found, then carcinoma is more likely than teratoma. However, within 4 weeks of onset, the patients develop symptoms that are typical of the disease in young adults. The delay in diagnosis and treatment is longer in the late-onset group. This observation may be due to the wide differential diagnosis in clinical presentation. However, other prognostic factors, including an earlier time to treatment, the use of second-line immunotherapy in the case of first-line drug failure, and younger age are associated with improved outcomes.

PREGNANCY AND DISEASE

There is concern that the anti-NMDAR antibody (subtypes IgG1 and IgG3), which can cross the placenta beginning at 14th week of gestation and up to the time of delivery,[35] may affect the developing brain of the fetus. Factors that may determine outcomes are the gestational age of disease onset, antibody titer of the maternal serum, timing of treatment to deplete maternal pathologic antibody, and the fetal BBB. A few case reports of anti-NMDAR encephalitis during pregnancy showed a positive outcome.[36,37] However, one case report showed evidence for the transplacental transfer of NMDAR antibody.[38] The antibody titer in this infant was the same as in the mother at 2 days after delivery and declined until a negative titer was determined at the age of 1 year. The infant showed a delay in global development and had cortical dysplasia. It remains unknown whether these abnormalities resulted from the effect of the transplacental anti-NMDAR antibody or were an indirect effect of the maternal illness.

OVERLAPPING ANTI-NMDAR ENCEPHALITIS AND DEMYELINATING DISEASE

Many case reports have described the co-existence of anti-NMDAR antibody and demyelinating disease (AQP4-IgG, MOG-IgG).[39] The encephalopathy may precede, follow, or occu simultaneously with a clinical episode of demyelinating disease. Most patients respond to immunotherapy but tend to require more intensive treatment and display more residual deficits. These findings should prompt the awareness of physicians that patients with the demyelinating disease who develop atypical symptoms such as abnormal movements or vice versa should be investigated for other conditions.[39]

CONCLUSION

Anti-NMDAR encephalitis is more common than expected and may be the most common cause of antibody-mediated encephalopathy. This disease should be suspected in children or young adults with acute behavioral problems, seizure, and abnormal movements. Late-onset disease (patients > 45 years old) may present with memory problems. Investigations of anti-NMDAR antibody or other autoantibodies that are present in the CSF and serum are recommended. Patient outcomes depend on the severity of the disease at the time of onset, early immunotherapy, and adequate second-line drugs if the response to first-line therapy fails. Long-term surveillance of ovarian teratoma in young female patients is prudent if the initial workup is negative.

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Conflicts of interest

There are no conflicts of interest.

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Topic: Autoimmune neurological diseases associated with autoantibodies specific for synaptic antigens

Encephalitis associated with autoantibodies binding to γ-aminobutyric acid-A, γ-aminobutyric acid-B and glycine receptors: immunopathogenic mechanisms and clinical characteristics

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ABSTRACT

Recent, discoveries of neural antibodies have facilitated the diagnosis of immune-mediated, immunotherapy-responsive neurologic disorders. Antibodies that target inhibitory central nervous system receptors, such as γ-aminobutyric acid-B, γ-aminobutyric acid-A, and glycine receptors, disrupt inhibitory regulatory synaptic functions, and lead to neuronal hyperexcitability. The myriad of neurologic, manifestations associated with these antibodies includes seizures, encephalopathy, muscle rigidity and stiffness. This article provides a review of the immunopathogenic mechanisms and the clinical and therapeutic implications of autoimmune encephalitis associated with these antibodies that target inhibitory receptors.

Key words: Autoimmune encephalitis; autoimmune epilepsy; limbic encephalitis; neural antibodies

INTRODUCTION

Recent, discoveries of neural antibodies that bind to antigenic targets in the brain have led to a paradigm shift in the clinical approach to patients presenting with encephalopathy,[1-4] cognitive change,[5] and refractory seizures.[4,6] With a wider availability of neural antibody testing, a significant proportion of the patients, who were previously diagnosed with encephalitis of undetermined etiology have been shown to have neurologic symptoms caused by an underlying autoimmune disorder and some of these patients respond favorably to immunosuppressive treatments.[7] Neural antibodies that, target channels or receptors on the neuronal cell surface can interfere with the function of these proteins, leading to altered neuronal excitability, and a myriad of neurologic syndromes that mirror genetic and pharmacologically induced disorders of the target receptors.[8] In this review, we describe the immunopathogenic mechanisms of autoimmune encephalitis associated with antibodies targeting the inhibitory synaptic receptors γ-aminobutyric acid-B ($GABA_B$), γ-aminobutyric acid-A ($GABA_A$), and glycine receptors (GlyRs), together with their clinical and therapeutic implications.

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ANTI-$GABA_B$ RECEPTOR AND ANTI-$GABA_A$ RECEPTOR ENCEPHALITIS

GABA, the main inhibitory neurotransmitter in the brain, binds to metabotropic and ionotropic receptors to regulate neuronal activity. To date, $GABA_B$ receptor ($GABA_BR$) and $GABA_A$ receptor ($GABA_AR$) have been identified as antigenic targets of autoimmunity [Table 1].

Anti-$GABA_B$R encephalitis

The $GABA_BR$ is a metabotropic G-protein-coupled

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receptor on presynaptic, postsynaptic, and extrasynaptic membranes, expressed in both the central and peripheral nervous systems, particularly the hippocampus, thalamus, and cerebellum.[9] This receptor is a heterodimer comprising two subunits: $GABA_{B1}$ and $GABA_{B2}$. Both subunits need to be co-expressed in order to form a functional receptor.[9,10] The extracellular domain of the $GABA_{B1}$ subunit binds to GABA while the $GABA_{B2}$ subunit couples the receptor with the effector G protein.[11] Antibodies to $GABA_BR$ bind to the B1 subunit of the $GABA_BR$,[4] the component that is required for GABA binding and receptor function.

$GABA_BR$ exert inhibitory regulatory effects on synaptic transmission by inhibiting presynaptic voltage-gated calcium channel-mediated neurotransmitter release and by activating postsynaptic potassium channels, resulting in hyperpolarization of neuronal membranes, and inhibition of adenylate cyclase.[11,12] $GABA_BR$ dysfunction is implicated in a variety of neurological disorders such as epilepsy, in which genetic mutations may play a role.[13,14] Pharmacological disruption of the $GABA_BR$ leads to seizures, cognitive deficits, and behavioral changes,[10,15,16] all of which may be seen in autoimmune anti-$GABA_BR$ limbic encephalitis.

Antibodies to $GABA_BR$ were first described in 15 patients with limbic encephalitis, in whom subacute early onset of seizures was a distinctive feature.[4] Seizures were predominantly of temporal lobe onset with secondary generalization, and 3 of the 15 patients developed status epilepticus. Memory impairment, confusion, hallucinations, and behavioral changes consistent with limbic involvement were frequently seen. Electroencephalography (EEG) changes included epileptiform discharges, electrographic ictal activity, and/or temporal lobe slowing.

Magnetic resonance imaging (MRI) brain imaging typically demonstrates unilateral or bilateral increased T2/fluid attenuated inversion recovery signal changes in the medial temporal region, consistent with limbic encephalitis.[4,17] Extratemporal changes in the grey and white matter, cerebellum, basal ganglia, and brainstem have also been reported.[4,17-19] Cerebrospinal fluid (CSF) examination may yield lymphocytic pleocytosis, elevated protein, and oligoclonal bands.[4] As with other types of limbic encephalitis, the EEG, MRI brain, and/or CSF exam may be normal, and should not preclude the diagnosis or presumptive treatment when the clinical presentation is suspicious.

Limbic encephalitis, the most common neurologic manifestation of anti-$GABA_BR$ encephalitis,[4,20,21] typically occurs in the setting of very high $GABA_BR$ antibody titers.[4,20] A widening phenotypic spectrum of anti-$GABA_BR$ disorders including cerebellar ataxia,[17,20,22,23] opsoclonus-myoclonus,[17,18] and brainstem encephalitis[19] is now appreciated. Extralimbic presentations may be explained by the high expression of $GABA_BR$ outside of the hippocampus, including the cerebellum.[9] Other rarer neurological features of anti-$GABA_BR$ encephalitis include chorea, myelopathy, peripheral neuropathy, and myopathy, particularly in patients with lower antibody titers.[4,20] It is uncertain, whether all of these extralimbic neurologic manifestations can be attributed solely to the $GABA_BR$ antibodies or whether the co-existence of other neural autoantibodies may contribute to the increasingly diverse neurological features being reported. Known neural antibody accompaniments to the $GABA_BR$ antibodies include the 65 kDa isoform of glutamate decarboxylase (GAD-65), voltage-gated calcium channels (N-type and P/Q type), voltage-gated potassium-complex (VGKC-complex), and neuronal nuclear and cytoplasmic antibodies [such as antineuronal nuclear autoantibody (ANNA-1), ANNA-3, collapsing response mediator protein-5 IgG, and anti-glial nuclear autoantibody/SOX-1 antibodies].[4,20-22]

In comparison to other cell surface neural antibody associated encephalitis, anti-$GABA_BR$ encephalitis is probably uncommon. In a clinical service laboratory, only 7 of 3,989 (0.2%) patients with suspected autoimmune encephalopathy were found to have the $GABA_BR$ antibody.[20] There is no obvious gender predisposition for this neurologic disorder. A paraneoplastic etiology is diagnosed in approximately half of the patients with $GABA_BR$ antibodies.[4,17,20,21] The neurologic disorder usually precedes the diagnosis of malignancy, and the most frequently encountered tumor is small cell lung cancer.[4,20] Tumors are more likely to be detected in older patients.[4] Although, lung tumors from patients with $GABA_BR$ encephalitis have not been studied for $GABA_BR$ expression, samples of archived small cell lung cancers from patients without encephalitis were found to react with both guinea pig and human $GABA_B$ IgG. This suggests that the $GABA_BR$ could be expressed by small cell lung cancer and could potentially trigger an autoimmune reaction.[4] Other oncologic associations of anti-$GABA_BR$ encephalitis include neuroendocrine lung tumor, multiple myeloma, esophageal carcinoma, malignant melanoma, and carcinoid of the thymus.[4,19,20,22,23]

Neurological improvement has been reported in up to 90% of patients with anti-$GABA_BR$ encephalitis who received immunotherapy and appropriate cancer treatment (if the tumor was detected).[4,17,20,21] As the cases reported so far were retrospectively ascertained, there was heterogeneity in the immunotherapies used. A variety of immunotherapie have been used successfully, including various combinations of first-line agents, corticosteroids, intravenous immunoglobulin (IVIg),

and/or plasma exchange (PLEX).[4,20] Patients who do not respond to these treatments warrant second line and maintenance treatments, such as rituximab, cyclophosphamide, mycophenolate mofetil, and azathioprine.[17,20,23,24] Neurological improvement may be incomplete or not sustained. Despite optimal immunosuppressive treatment, patients with $GABA_BR$ antibodies can deteriorate due to tumor progression, chemotherapy-related complications, and/or treatment-resistant relapses.[4,17,22-24] Further studies to elucidate the optimal treatment regimens are needed. The presence of an underlying small cell lung cancer and the co-existence of other paraneoplastic neural antibodies targeting intracellular (neuronal nuclear and cytoplasmic) antigens have been suggested as poor prognostic indicators.[17,20]

Anti-$GABA_AR$ encephalitis

The $GABA_AR$ is a ligand-gated ion channel located at synaptic and extrasynaptic sites that functions to mediate fast inhibitory synaptic transmission.[25,26] Activation of the $GABA_AR$ triggers opening of intrinsic chloride channels, thereby eliciting an inhibitory postsynaptic potential.[27] Disruption of $GABA_AR$ results in increased neuronal excitability and seizures.[27] Mutations in the α1 and β3 subunits of the $GABA_AR$ gene have been implicated in epilepsy syndromes.[27-29] Benzodiazepine and barbiturate, medications used for the treatment of seizures and status epilepticus, enhance GABAergic inhibition to exert an anticonvulsant effect.[30]

$GABA_ARs$ are pentamers comprising combinations of five subunits that form chloride ion channels. Different combinations of subunits result in functional heterogeneity. Synaptic $GABA_ARs$, which contain the α (α1-3), β, and γ subunits, are responsible for phasic inhibition. By contrast, extrasynaptic and perisynaptic $GABA_ARs$, which are responsible for tonic inhibition, comprise α4 or α6 subunits combined with β and δ subunits.[31] The $GABA_AR$ antibody binds to the α1,β3, or both subunits of the synaptic $GABA_AR$.[32,33] $GABA_AR$ antibodies reduce the density of the $GABA_AR$ at synaptic sites when applied to rat hippocampal neurons, suggesting that antibody binding leads to the relocation of $GABA_AR$ from the synaptic membrane.[32,33] This phenomenon is similar to the loss of synaptic $GABA_AR$ and resultant neuronal hyperexcitability observed in epilepsy and status epilepticus.[27] The combined reinforcing effects of antibody-mediated synaptic $GABA_AR$ relocation, together with the status epilepticus-induced loss of $GABA_AR$, could support a postulated model to explain the severity of seizures in patients with anti-$GABA_AR$ encephalitis.[33]

Recently, 18 patients with autoimmune encephalitis and prominent seizures were described with $GABA_AR$ antibodies, 6 of whom had very high antibody titers.[33] Patients with high titers in both serum and CSF developed a particularly rapid, severe progressive encephalopathy with refractory seizures and/or status epilepticus, for which intensive care admission for pharmacologically-induced coma was required. Other reported clinicalmanifestations in $GABA_AR$ antibody seropositive patients are opsoclonus-myoclonus, affective problems, hallucinations, mutism, aphasia, memory impairment, hemiparesis, chorea, cerebellar ataxia, and Stiff-man syndrome (SMS).[32,33] $GABA_BR$, GAD-65, N-methyl-D-aspartate receptor (NMDAR), leucine-rich, glioma-inactivated 1 and contactin-associated protein-like 2 antibodies frequently co-exist in these patients.[32,33] A propensity for other neurological autoimmune conditions such as myasthenia gravis has also been noted.[32,33]

$GABA_AR$ antibodies are reported in both children and adults (age 2-74 years), but larger cohorts need to be characterized.[33] A low frequency of tumors in seropositive patients has been reported. In the initially published study of 18 patients, only one patient was found to have cancer (Hodgkin lymphoma).[33]Two recent additionally reported cases had invasive thymoma.[32]

The electroencephalograms of patients with anti-$GABA_AR$ encephalitis may demonstrate generalized slowing suggestive of encephalopathy, multifocal ictal and interictal discharges, or status epilepticus.[33] CSF findings range from normal to lymphocytic pleocytosis.[33] Distinctive to $GABA_AR$ antibodies, the majority of patients, especially those with high antibody titers, had extensive temporal and extratemporal MRI brain abnormalities[33] which could be a consequence of autoimmune inflammation in the brain or prolonged ictal activity. The extensive radiologic changes contrast with those of patients with limbic encephalitis associated with other neuronal synaptic and cell surface antibodies, such as NMDAR and VGKC-complex antibodies, in which MRI abnormalities are often confined to the mesial temporal regions.

Despite the severity of their presentation, 80% of the patients reported with anti-$GABA_AR$ encephalitis demonstrate partial or complete recovery with a combination of immunotherapy, antiepileptic drugs, and supportive treatment.[32,33] In severe cases, multiple immunotherapies may be required. Treatment options are the same as with $GABA_BR$ and GlyR antibody mediated disorders. In addition to immunotherapy, early recognition and treatment of epilepsy, as well as supportive treatment (including ventilation support) are pivotal.

GlyR antibody encephalitis

Glycine, a key neurotransmitter for fast postsynaptic

Table 1: Key demographic, clinical, and AI/oncologic associations of the $GABA_BR$, $GABA_AR$, and GlyR-α1 antibodies

	$GABA_BR$	$GABA_AR$	GlyR-α1
Clinical features	Limbic encephalitis (memory impairment, hallucination, confusion, behavior changes) with early and prominent seizuresLess commonly: cerebellar ataxia, opsoclonus-myoclonus, brainstem encephalitis, chorea, myelopathy, peripheral neuropathy, and myopathy	High serum antibodies concentration: rapidly progressive encephalopathy, refractory seizures, and status epilepticusLow serum antibodies concentration: seizures, SMS, opsoclonus-myoclonus, behavioral change, psychosis, confusion, chorea, ataxia, hallucinations, and hemiparesis	Axial/limb spasms, rigidity, and myoclonus (SMS) + brainstem signs (PERM) Optic neuropathy, seizures, cognitive impairment, autonomic disturbance, respiratory failure, and transverse myelitis also reported in isolation or with SMS or PERM
Onset	Mostly subacute/acute	Acute/subacute	Subacute > acute > chronic
Age group	Wide range, children, and adults	Wide range, children, and adults	Wide range, children, and adults
Gender, male:female	1.3:1	2:1	1:1
Inflammatory CSF*	73%	40%	50%, but OCB frequently negative
MRI	65% abnormal (medial temporal > extratemporal changes)	High serum antibodies concentration: 100% multifocal temporal and extratemporal T2/FLAIR hyperintensities	< 30% T2/FLAIR abnormalities in temporal lobes, SC abnormalities rarely reported (short, multifocal, and LETM)
EMG	-	-	60% abnormal (continuous motor activity, stimulus induced motor activity)
Other coexisting neural antibodies	56% (VGCC, AGNA, GAD-65, VGKC-complex, NMDAR, ANNA-1, -2 and -3,CRMP-5 IgG, amphiphysin, BRSK2)	70% (AChR, NMDAR, $GABA_B$, GAD-65, VGKC-complex)	Rare (GAD-65, MOG, NMDAR, aquaporin-4, VGKC-complex)
Associated tumors	62% (SCLC most common, also neuroendocrine lung, malignant melanoma, esophageal, malignant melanoma, thymus anaplastic carcinoid)	15% (invasive thymoma, Hodgkin lymphoma)	Approximately 10% to date (breast cancer, lymphoma (both Hodgkin and NHL), leukemia, lung cancer, melanoma)
Fatalities (%)	36	20	< 10

*Inflammatory CSF includes pleocytosis, high protein, raised IgG index, and/or oligoclonal bands. AChR: acetylcholine receptor; AI: autoimmune; AGNA: anti-glial nuclear autoantibody; ANNA: antineuronal nuclear autoantibody; BRSK2: BR serine/threonine-protein kinase-2; CRMP-5: collapsing response mediator protein 5; CSF: cerebrospinal fluid; EEG: electroencephalogram; EMG: electromyography; FLAIR: fluid attenuated inversion recovery; $GABA_AR$: γ-aminobutyric acid receptor A subunit; $GABA_BR$: γ-aminobutyric acid receptor B subunit; GAD-65: glutamic acid decarboxylase-65; GlyR-α1: glycine receptor alpha-1 subunit; LETM: longitudinally extensive transverse myelitis; MOG: myelin oligodendrocyte glycoprotein; MRI: magnetic resonance imaging; NHL: nonHodgkin lymphoma; NMDAR: N-methyl-D-aspartate receptor; OCB: oligoclonal bands; PERM: progressive encephalomyelitis with rigidity and myoclonus; SC: spinal cord; SMS: stiff-man syndrome; SCLC: small cell lung carcinoma; VGCC: voltage-gated calcium channel; VGKC-complex: voltage-gated potassium channel complex

inhibitory neurons in the CNS, has a complex functional pathway that involves pre- and post-synaptic GlyR interacting with other neurotransmitters (GABA and glutamate), NMDAR, and postsynaptic anchoring proteins like gephyrin. Antibodies directed at any of these targets may affect the glycinergic system, resulting in neurological dysfunction.[34] Whether due to strychnine (a GlyR antagonist) poisoning, genetic mutations of the GlyR gene (hereditary hyperekplexia), or immune-mediated encephalitis, GlyR dysfunction may be associated with severe muscle spasms, stiffness, agitation, seizures, myoclonus, autonomic instability, and/or respiratory failure.[35,36]

GlyRs, pentamers of α1-α4 and β-subunit proteins, are ligand-gated chloride ion channels, widely distributed in the CNS. They are predominantly expressed in the olfactory bulb, retina, hippocampus, brainstem (auditory, visual, vestibular, and sensory nuclei), cerebellum, and spinal cord.[37,38] Glycine binding mediates opening of the GlyR chloride channel, resulting in hyperpolarization of the membrane potential and reduced neuronal excitability. The GlyR antibody targets the α1 subunit of the postsynaptic GlyR and is associated with hyperexcitable neurologic disorders [Table 1].[39] Gephyrin allows multiple GlyR to cluster together on the synaptic membrane. Antibodies to gephyrin, an anchoring protein in the postsynaptic GlyR, have been described only in a single case to date.[40]

Classic neurologic manifestations associated with GlyR antibodies are progressive encephalomyelitis with rigidity and myoclonus (PERM) and SMS.[39,41,42] PERM and SMS were first described as separate clinical entities, but today these 2 conditions are considered to belong to a continuum of CNS hyperexcitability disorders. Patients with PERM and SMS share common features of rigidity, painful spasms, autonomic disturbances, hyperekplexia, and myoclonus. The widespread distribution of hyperexcitability and brainstem involvement classically distinguishes PERM from SMS and associated psychiatric symptoms such as anxiety are more commonly observed in SMS patients.[43-46] The autoimmune nature of these conditions, and specifically the involvement of GlyR antibodies in some cases was not appreciated until recently.[41,42] SMS was initially associated with antibodies to GAD-65 (60-70% of cases),[47,48] gephyrin (1 case),[40] and amphyiphysin (< 5% cases, in the setting of both small cell lung and breast cancers).[48,49] In 2008, Hutchinson *et al.*[41] reported the first case of PERM with GlyR antibodies. It remains unresolved whether all of these

antibodies are truly pathogenic.[50,51] McKeon *et al.*[42] reported that 10 of 81 (12%) patients with SMS spectrum disorders were positive for GlyR antibodies. Interestingly, GlyR seropositivity was associated with better responsiveness to immunotherapy regardless of GAD-65 status, suggesting a pathogenic role. There are no passive transfer animal models of GlyR antibodies to date.[39,42]

The spectrum of GlyR antibody encephalitis manifestations is now widening beyond classic PERM and SMS. Various combinations of psychiatric disturbances, cognitive dysfunction, seizures (focal/generalized epilepsy and new-onset status epilepticus), and movement disorders, autonomic instability with central hypoventilation, pseudobulbar and/or oculomotor dysfunction, steroid responsive optic neuropathy, and transverse myelopathy have now been described with GlyR antibodies.[39,52-54]

The onset of symptoms in GlyR antibody neurologic syndromes is typically acute to subacute. In the largest case series of 52 GlyR antibody positive patients with a variety of presentations, there appears to be no sex predominance and all age groups are vulnerable.[39] Patients frequently have a history of other autoimmune disorders.[39] As with other autoimmune encephalopathies, GlyR antibodies may co-exist with other antibodies, such as NMDAR, GAD-65, VGKC-complex, myelin oligodendrocyte glycoprotein, and aquaporin-4 antibodies although this is rare.[39,42,53,55,56] Tumors are identified in less than 20% of cases (thymoma, lymphoma, breast cancer, small cell lung carcinoma and leukemia).[39,42,57,58]

GlyR antibodies may be detected in both serum and/or the CSF.[39,59] CSF evaluation is possibly more sensitive than serum, therefore testing both is recommended.[42] CSF lymphocytic pleocytosis or raised protein may be seen, and oligoclonal bands were negative in 50-70% of 2 case series recently published.[39,56] Imaging is typically normal. Rarely, MRI temporal lobe T2-weighted abnormality with subsequent hippocampal volume loss is detected, particularly in cases associated with significant seizure activity. EEG may be normal, or show features of focal or generalized ictal activity.[59]

A combination of immunotherapies (corticosteroids, IVIg, PLEX, cyclophosphamide), pharmacological therapies targeting symptoms of motor hyperexcitability and pain (clonazepam, diazepam, baclofen, gabapentin), and anticonvulsants (levetiracetam) are required to control clinical symptoms.[39,42] Eighty percent of patients with GlyR antibodies showed a substantial response to immunotherapy.[39,42] Two cases were reported that responded dramatically to thymectomy in addition to other immunotherapy.[39,60] In the largest case series to date, 6 out of 52 (12%) patients continued to have sporadic relapses whilst on treatment.[39]

CONCLUSION

An increased awareness of the autoimmune mechanisms underlying cases of noninfective encephalitis and/or refractory seizures has led to increased recognition, earlier treatment, and improved outcomes in a subgroup of patients previously considered untreatable. Antibodies targeting the inhibitory receptors $GABA_B$, $GABA_A$, and glycine are three more recently appreciated, but important antibodies to consider in refractory seizure disorders and encephalitis of unclear etiology. A high index of suspicion and an awareness of the expanding clinical spectrum of these antibody-mediated disorders should prompt early neural antibody testing in patients with typical constellations of neurological symptoms, in particular refractory seizure disorders and encephalitis of unclear etiology. Once identified early, these conditions may be responsive to immunotherapy. There are sparse data to recommend one immunotherapeutic regime over another. Large cohort studies of patients with anti-NMDAR encephalitis suggest that first line therapy should comprise corticosteroids, IVIg, and/or PLEX, followed by second line immunotherapy (cyclophosphamide, rituximab, or both) in patients who fail to respond to initial treatment.[61] A practical approach, guided by the literature on autoimmune encephalitis with antibodies against neuronal surface antigens, is suggested in Figure 1. Immunotherapy needs to be complemented by supportive, symptomatic medical therapy. There is a consensus that early treatment confers better outcomes. Age and antibody appropriate tumor screening should be performed in all cases and may be aided by testing for other co-existing neural antibodies.[62]

The neurologic hyperexcitability effects of antibody binding to $GABA_A$, $GABA_B$, and GlyRs (and potentially other receptors in the future) reflect the important functions mediated by these inhibitory neuronal synaptic receptors. More research is needed in order to better understand this novel category of immune-mediated encephalitis. Further studies could focus on immunopathogenic mechanisms of these antibodies in causing disease, as these may be potential targets for directed treatment. To date, the numbers of patients reported with these antibodies remain small, with most cases retrospectively identified. With increasing access to testing for neural antibodies, the clinical spectrum of these autoimmune encephalitides may continue to expand. Systematic studies of prospectively identified, newly diagnosed cases should help to provide data on the long-term course of the disease, prognostic factors, and optimal immunotherapeutic regimes.

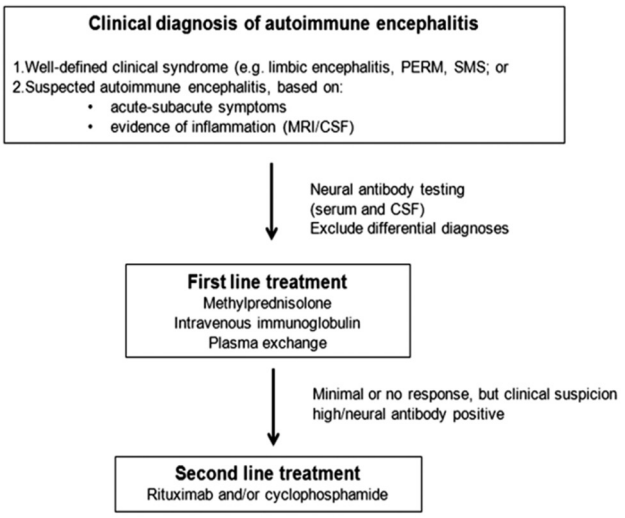

Figure 1: Suggested algorithm for approach to suspected autoimmune encephalitis.[61-63] PERM: progressive encephalomyelitis with rigidity and myoclonus; SMS: stiff-man syndrome; MRI: magnetic resonance imaging; CSF: cerebrospinal fluid

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Conflicts of interest

There are no conflicts of interest.

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Topic: Autoimmune neurological diseases associated with autoantibodies specific for synaptic antigens

Diagnostic algorithms in autoimmune encephalitis

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ABSTRACT

Over the past decade the discovery of novel forms of encephalitis associated with neuronal surface antibodies had changed the paradigms for diagnosing and treating disorders that were previously mischaracterized. Recognition of clinical syndromes, consistent methods of diagnosis, and early targeted immunotherapy can lead to a favorable outcome in diseases that may be associated with significant disability or death if left untreated. Here the conditions associated with neuronal surface antibodies are briefly reviewed, some general aspects of these syndromes are considered and guidelines that could help in the recognition of these disorders are suggested. Furthermore, a diagnostic algorithm to detect and characterize neuronal cell surface autoantibodies is suggested and some of the caveats of serum testing are outlined. Future directions will involve the identification of novel autoantibodies, the standardization of methods to detect and characterize them, as well as evaluation of the most efficacious therapeutic strategies in patients with established diagnosis of autoimmune encephalitis.

Key words: Autoimmune encephalitis; neuronal surface autoantibodies; paraneoplastic syndromes

INTRODUCTION

Anti-neuronal autoimmune encephalitis (AIE) is a complex syndrome resulting from a self-directed response to neuronal antigens. These disorders can be associated with immunoglobulinG (IgG) autoantibodies specific to intracellular neuronal antigens (e.g. Hu, Yo, Ri) and to neuronal surface or synaptic antigens [e.g. N-methyl-d-aspartate receptor (NMDAR), amino-3-hydroxy-5-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), gamma-aminobutyric acid B GABA(B)R]. The first group of AIE typically occurs in the setting of cancer, resulting from an autoimmune reaction against intracellular antigens co-expressed by the cancer and the central nervous system (CNS). The autoantibodies are thought to be not pathogenic but an epiphenomenon, and patients show limited or no response to immunotherapy. Compelling evidence suggests that in the second group of AIE, the binding of the autoantibodies to extracellular antigens directly causes neuronal dysfunction, which can be reversed by antibody-depleting therapies,[1] such as plasmapheresis and intravenous immunoglobulins. In contrast to classical paraneoplastic syndromes, AIE associated with synaptic autoantibodies is often not paraneoplastic and can affect patients of all ages, including children and young adults.[2]

Over the past ten years, the characterization of encephalitis associated with neuronal surface autoantibodies has changed our perspective on their diagnosis and treatment. In these disorders, the autoantibodies are associated with a characteristic phenotype and their detection contributes to the neurological diagnosis. As early treatment speeds recovery, reduces disability and decreases relapses, it is important that the immune pathogenesis of these disorders is promptly recognized.

In this paper a diagnostical gorithm is proposed for a clinical approach to AIE and screening of the associated autoantibodies.

DIAGNOSTIC APPROACH

The diagnosis of AIE should be suspected in patients developing subacute cognitive impairment, psychiatric disturbances, movement disorders or seizures. The diagnosis will be further support by the evidence of CNS inflammation from cerebrospinal fluid (CSF) analysis or magnetic resonance imaging (MRI). Autoantibody testing has a critical role in confirming the diagnosis and in leading

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the search for the presence of an underlying neoplasm [Figure 1].

Clinical presentation

AIE is usually a multistage process. Most of these disorders have a rapid course, developing over a few days or weeks, with behavioral and memory alteration, decreased level of consciousness and seizures. This clinical picture is typical of limbic encephalitis (LE). However, the severity and predominance of some symptoms over others may help the clinician in the diagnosis of different AIE subtypes and may lead the search for specific antibodies [Table 1]. For example, both GABA(B)R and gamma-aminobutyric acid A [GABA(A) R] antibodies are typically associated with refractory seizures,[4,5] patients with leucine-rich glioma-inactivated 1 (LGI1) autoantibodies can present with facio-brachial dystonic seizuresand hyponatremia caused by syndrome of inappropriate antidiuresis (SIAD),[6] while AMPAR-antibodies are frequently found in patients with LE or psychosis.[7] In anti-NMDAR encephalitis, psychiatric disturbances are the most frequent symptoms of onset in women,[8] while seizures are prominent in men.[9]

The detection and characterization of IgLON family member 5 antibodies represents an interesting link between autoimmunity and neurodegeneration. These autoantibodies were found to be associated with sleep disturbances, cognitive impairment, the movement disorder and brainstem symptoms with a chronic progressive course.[10]

In some cases, symptoms may extend beyond CNS: AIE associated with autoantibodies to dipeptidyl-peptidase-like protein-6 may present with diarrhea poorly responsive to symptomatic treatment and significant weight loss that can precede neurological symptoms including brainstem and

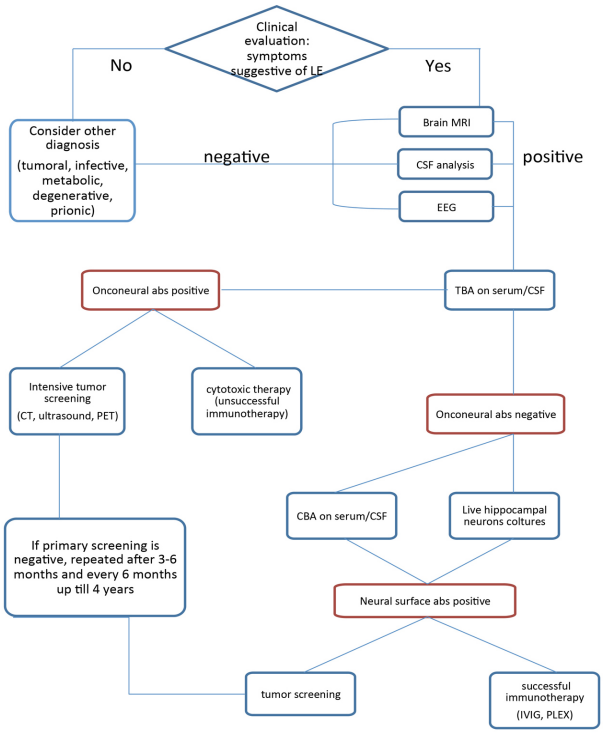

Figure 1: Flowchart summarizing a preferred diagnostic approach to AIE. AIE: Anti-neuronal autoimmune encephalitis; CSF: cerebrospinal fluid; CBA: cell-based assay; EEG: electroencephalogram; TBA: tissue-based assays; CT: computed tomography; PET: positron emission tomography; MRI: magnetic resonance imaging; IVIG: intravenous immunoglobulin; PLEX: plasmapheresis

psychiatric dysfunction.[11]

Ancillary tests

At presentation, about 80% of patients with AIE have a mild-to-moderate CSF lymphocytic pleocytosis (usually < 100 white blood cells/L), 30% have a mild-to-moderate increase inprotein concentration, and 50-60% have oligoclonal bands.[12] In contrast to most autoimmune encephalitides, encephalitis with LGI1-IgGusually occurs with normal or

Table 1: Neuronal surface autoantibodies, associated tumors and clinical syndromes

Antigen	Tumor	Clinical symptoms	Clinical clues
NMDAR	Ovarian teratoma (58%) < 18 years old	Memory impairment, psychosis (mainly in women), seizures (mainly in men), central hypoventilation	Orobuccal dyskinesia; dysautonomia
LGI1	Thymoma (< 10%)	LE	Hyponatremia; faciobrachial dystonic seizures
CASPR2	Thymoma (38%)	Encephalitis/Morvansynd/ neuromyotonia	Peripheral nerve hyperexcitability; neuropathic pain
AMPAR	SCLC, breast, thymoma (60-70%)	LE, psychosis	
GABA(B) R	SCLC (50%)	LE, ataxia	Refractory seizures
GABA(A) R	-	Status epilepticus, seizures, LE	Refractory seizures
mGluR1	Hodgkin and non Hodgkin lymphoma (e.g. cutaneus lymphoma); prostate adenocarcinoma[3]	Cerebell arataxia	
mGluR5	M. Hodgkin	Ophelia syndrome	Memory impairment
DPPX (Kv4.1)	Follicular B cell, lymphoma, CLL	Hallucinations, agitation, myoclonus, tremor, SPS	Diarrhea
IgLON5	-	Brain stem dysfunction, LE	Non-REM and REM-sleep disorder
GlyR	Thymoma	SPS, progressive encephalitis	
Dopamine 2R	-	Basal ganglia encephalitis, Sydenham Chorea	

NMDAR: N-methyl-d-aspartate receptor; LGI1: leucine-rich glioma-inactivated 1; CASPR2:contactin-associated protein-like 2; AMPAR: amino-3-hydroxy-5-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; GABA A/B R: gamma-aminobutyric acid A/B receptor; mGluR1/5: metabotropic glutamate receptor type 1/5; DPPX: dipeptidyl-peptidase-like protein-6; GlyR: Glycine receptor; CLL: chronic lymphatic leukemia; SCLC: small cell lung cancer; LE: limbic encephalitis; SPS: stiff-person syndrome; IgLON5: IgLON family member 5

minimal CSF findings.[13]

The electroencephalogram (EEG) is almost always abnormal in all types of AIE, showing focal or diffuse slow activity that can be associated with focal or multifocal epileptic discharges. Except for a pattern referred to as extreme delta brush, that may occur in patients with anti-NMDAR encephalitis,[14] there are no pathognomonic EEG abnormalities for any AIE subtypes.

MRI of the brain is often diagnostic in patients with LE, usually showing increased Fluid Attenuated Inversion Recovery/T2-weighted (FLAIR/T2) signal involving one or both temporal lobes, without contrast enhancement. Similar findings can, however, occur in patients with herpes simplex encephalitis or medial temporal lobe seizures. In NMDAR encephalitis brain MRI is normal in up to 66% of cases, while the remaining patients may have unspecific cortical or subcortical FLAIR/T2 abnormalities, sometimes involving the posterior fossa or medial temporal regions, often with small areas of demyelination, and more rarely with extensive demyelinating abnormalities.[15]

In patients with GABA(A)R antibodies, brain MRI often shows multifocal cortical-subcortical FLAIR abnormalities.[16]

Detection of autoantibodies

Several techniques are available for intracellular and synaptic antibody detection, for example, tissue-based assays (TBA; in-house or commercially available), cell-based assay (CBA; in-house or commercially available), indirect immunofluorescence on live hippocampal or cortical neurons (in-house) and immunoprecipitation (IP; in-house). In TBA, antibodies in patient serum or CSF are detected by indirect immunofluorescence on a substrate of mouse or rat brain sections. TBA is an excellent screening method, as the antibody target antigen can be suspected from the staining pattern (e.g. neuropil), although it must be confirmed by more specific techniques [Figure 2A]. As regards the detection of onconeural antibodies (e.g. Hu, Ma2, Ri, amphiphysin), commercial immunoblots with recombinant proteins for the most common autoantibodies are widely available. On the other hand, the gold standard for neuronal surface autoantibody detection is CBA, in which cells (e.g. human embryonic kidney 293 cells) expressing the appropriate antigens are incubated with patients' serum/CSF, and antibodies are identified by indirect immunofluorescence [Figure 2C]. This is a highly sensitive technique, but it is time consuming and requires specific facilities and expertise.

Indirect immunofluorescence on live hippocampal or cortical murine neurons is used as a screening method, in some laboratories, for the detection of antibodies binding neuronal plasma membrane proteins [Figure 2B].

The most reliable method of detecting antibodies specific for neuronal plasma membrane antigens involves using a combination of TBA and indirect immunofluorescence on live neuronal cultures as screening following by confirmatory CBA.

Caveats in the diagnosis

Standardized methods of antibody testing are critical to ensure the correct diagnosis of AIE. However there are few studies, mainly in anti-NMDAR encephalitis,[17-19] comparing the sensitivity and specificity of different techniques in serum and CSF samples from patients with AIE. For example, Gresa-Arribas *et al.*[20] examined paired serum

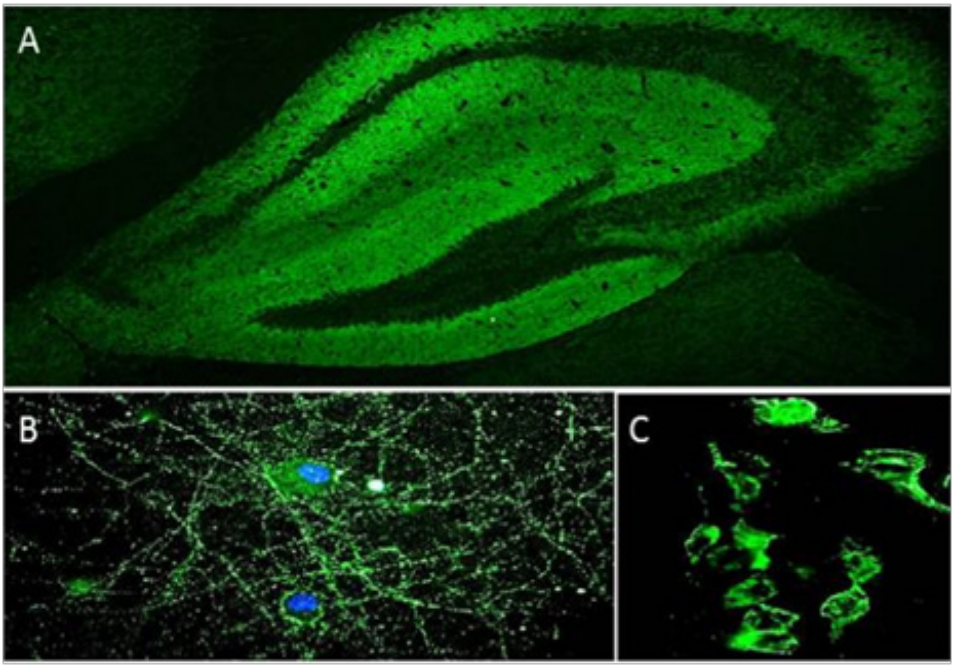

Figure 2: IgG in the CSF from a patient with anti-NMDAR encephalitis bind to the neuropil of the mouse hippocampus (A), to the cell-surface of live, non-permeabilized mouse hippocampal neurons (B) and to the plasma membrane of HEK293 cells expressing NMDAR (C). anti-NMDAR: anti-N-methyl-d-aspartate receptor

and CSF from 250 patients with anti-NMDAR encephalitis and 100 controls tested with three different assays showing that, while autoantibodies are always detected in CSF, serum testing with any type of cell-based assay (live or fixed cells) led to false negative results in at least 13% of the patients. Similarly, in another study,[21] 23% of patients with anti-NMDAR encephalitis tested negative when only serum samples were evaluated by live CBA. Moreover, Dahm *et al*.[22] recently reported that approximately 10% of patients with diverse neuropsychiatric disorders and healthy individuals may have IgA, IgM or IgG autoantibodies to the NR1 subunit of the NMDAR in the peripheral blood. However they found a prevalence of IgA and IgM at low titer, whose detection has no utility for diagnosing NMDAR encephalitis. Using commercial tests, IgA, IgM and IgG anti-NMDAR autoantibodies, at low titer, showed a broader distribution and can be detected in the serum of a considerable percentage of healthy subjects.[23] These data suggest that CSF anti-NMDAR autoantibody titers show a much better correlation with clinical symptoms than blood autoantibody titers. In contrast, LGI1-antibodies seem to be more prevalent in serum, although most patients have also CSF autoantibodies.[13]

In general, these findings suggest that multicenter studies are needed to determine the sensitivity and specificity of the different methods of autoantibody detection. To minimize errors of interpretation and misleading diagnoses, in all suspected cases of AIE both serum and CSF should always be tested for autoantibodies.

Tumor screening

CNS dysfunction sometimes reflects an effective immune response to an underlying neoplasm. Paraneoplastic neurological disorders should be suspected when the onset is subacute, with rapid progression not explained by more common disorders. Neurological symptoms generally precede the diagnosis of cancer that may remain unsuspected and undetectable both clinically and by conventional radiology for long after neurological symptom onset.[24]

Although certain neural-specific autoantibodies frequently associate with distinctive neurological presentations, none of the antibodies is specific for a unique syndrome. Autoantibodies that are highly predictive of cancer, however, are tightly associated with a limited number of tumors that express the antibody-target antigen [Table 1]. Therapeutic intervention in these cases consists of searching for and treating the associated tumor in order to eliminate the disease-triggering stimulus.[25] In addition, the detection of onconeural or neuronal surface antibodies is crucial in the diagnosis of an immune condition, thus in the therapeutic decisions [Figure 1]. In the case of onconeural antibody positivity, the neuronal tissue damage is thought to be initially mediated by T-cells and consequently, an immunosuppressive therapy does not usually improve the clinical symptoms. If, in contrast, cell surface structures are the target of the humoral reaction, early immunosuppression, if applicable, tumor resection is indicated. In these cases substantial recovery is possible.[26] The European Federation of Neurological Societies developed a useful guideline regarding tumor screening in AIE.[27] For screening of the thoracic region, a computed tomography (CT)-thorax is recommended, which if negative is followed by fluorodeoxyglucose-positron emission tomography. Breast cancer is screened for using mammography, followed by MRI. For possible pelvic and gastrointestinal malignancies, ultrasound scanning (US) of the pelvic region followed by CT is recommended in women (especially for ovarian teratoma), while US of the testes should be considered in men under 50 years of age, and colonoscopy in both men and women over 50. If primary screening is negative, it should be repeated after 3-6 months and then every 6 months up till 4 years.

CONCLUSION

Ongoing research on AIE constantly increases the number of novel autoantibodies and expands the spectrum of neurological syndromes, which is crucial in the differential diagnosis. Identification of the specific AIE is important in the complex management of these patients as these disorders may have different co-morbidities or associated tumors. Moreover, the discovery of new AIE has led to unsuspected links with other CNS diseases (e.g. antiepileptic-drug-resistant epilepsy, relapsing encephalitis post-HSV, demyelinating disease) making the diagnosis an interdisciplinary challenge for the treating physicians.[28-30]

Given the abundance of antibodies that have been reported so far, physicians face the dilemma of which antibody to test first, especially if TBA gives inconsistent results. In terms of priority, it is important to consider first NMDAR and voltage-gated potassium channel (VGKC-complex) which comprises LGI1 and contactin-associated protein-like 2 autoantibodies, because they are the most frequent antibodies associated with AIE.

The clinical spectrum of autoimmune encephalitis is broad, but prompt recognition and treatment often leads to excellent outcome. Yet, despite being a potentially reversible neurological condition, no clear guidelines for diagnosis and treatment of AIE exist.

Prospective population-based studies to evaluate the impact of different immunotherapies in AIE as well as to standardize the different diagnostic tests are needed in order to improve the management of these complex disorders.

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Conflicts of interest

There are no conflicts of interest.

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Original Article

Thrombolysis lead to better long-term outcome in Chinese stroke patients

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ABSTRACT

Aim: The rate of thrombolysis in Chinese acute ischemic stroke (AIS) was low and little was known about the long-term outcome. We aimed to compare the prognosis between thrombolysis and ordinary anti-platelet strategies in AIS. **Methods:** Patients, who were consecutively registered in our hospital from January 2005 to June 2012, were retrospectively studied. Inclusion criteria: (1) primary diagnoses of cerebral infarction coded with implantable cardioverter defibrillator-10 I63 to I69; (2) symptoms onset to treatment time (OTT) within 6 h; (3) thrombolysis with alteplase (TROM) or ordinary anti-platelet therapy (ANTP). Exclusion criteria: (1) symptoms and signs diminished rapidly without apparent neurological deficits; (2) no visible lesions on diffusion weighted image in magnetic resonance imaging; (3) cerebral infarction caused by serious metabolic in-balance or infections. The endpoints were defined as favorable (modified Rankin Scale 0-2) or being survival. Proportions of favorable outcome or survival were estimated by Kaplan-Meier curve and Cox regression. **Results:** One hundred and sixty eight cases were analyzed. Ninety one were in TROM and 77 in ANTP. Male accounted for 82 (48.8%) and female 86 (51.2%). The median of age was 74 [interquartile range (IQR) 67-79], national institute of health stroke scale (NIHSS 9) (IQR 5-17) and OTT 3.9 h (IQR 3.0-4.8) respectively. The median length of follow-up was 112 (IQR 63.4-163.8) weeks. By the end of December 31, 2012, 87 patients (51.8%) reached favorable outcome while 81 (48.2%) unfavorable. Forty five (26.8%) cases deceased. Kaplan-Meier curve estimation showed a longer favorable period of time in TROM than those in ANTP (212 weeks 95% confidence interval (CI) 169.5-254.5 *vs.* 126.9 weeks 95% CI 105.2-148.6; Log-Rank test $x^2 = 19.632$, $P = 0.000$), while no significance was seen in survival time (258.0 weeks 95% CI 231.5-284.5 *vs.* 160.8 weeks 95% CI 153.0-168.5; Log-Rank test $x^2 = 2.427$, $P = 0.119$). In Cox regression, thrombolysis showed an independent protective effect for longer period of favorable outcome [202 *vs.* 151 weeks, $P = 0.026$, heart rate (HR) 1.96, 95% CI 1.958-3.540] and longer survival time instead (333 *vs.* 170 weeks, $P = 0.000$, HR 4.322, 95% CI 1.942-9.618). The estimated proportion of favorable outcome in Chinese urban AIS was about 91% for 1 year and 50% for about 3.4 years, while the estimated proportion of survival was about 98.5% for 1 year and 50% for about 5.3 years, respectively. **Conclusion:** Chinese urban AIS patients who underwent thrombolysis with alteplase might have a better long-term outcome than those receiving ordinary anti-platelet therapy.

Key words: Stroke; thrombolytic therapy; Chinese; alteplase; Cox regression

INTRODUCTION

Thrombolysis with alteplase had been proven to be most effective in acute ischemic stroke (AIS) and its long-term good effects were verified by the Third International Stroke Trial 3.[1] In China the rate of thrombolysis of AIS was low, perhaps due to the fear of bleeding or conceptions of no need to treat mild stroke.[2] There is still no controlled study concerning the long-term outcome in Chinese AIS. Even in patients with lacunar infarction, which account for nearly

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Table 1: Main differences of variables between thrombolysis and anti-platelet groups

Variables	TROM (n = 91)	ANTP (n = 77)	Z/x^2	P value
Demographics				
Gender (male), n (%)	39 (23.2)	43 (25.6)	2.815	0.121
Age (years), median (IQR)*	72 (14)	76 (12)	-2.779	0.005
Length (w), median (IQR)*	149.6 (140.9)	90.7 (69.6)	-3.463	0.001
OTT (h), median (IQR)	3.7 (1.5)	4.2 (2.2)	-1.855	0.064
Vital signs				
NIHSS Scale, median (IQR)*	13 (11)	5 (5)	-7.443	0.000
BT (℃), median (IQR)	36.5 (0.6)	36.5 (0.5)	-0.140	0.889
HR (/min), median (IQR)	80 (19)	80 (18)	-0.151	0.880
BPS (mmHg), median (IQR)	154 (35)	150 (41)	-0.997	0.319
BPD (mmHg), median (IQR)	85 (21)	80 (21)	-1.951	0.051
Laboratory tests				
WBC (×10^9/L), median (IQR)	8.66 (3.98)	8.77 (2.27)	-0.347	0.729
PLT (×10^9/L), median (IQR)	218.00 (68.00)	206.70 (65.00)	-0.275	0.783
HCT, median (IQR)	0.39 (0.05)	0.38 (0.08)	-1.653	0.098
INR, median (IQR)	1.02 (0.21)	1.06 (0.11)	-1.132	0.258
APTT (s), median (IQR)	34.40 (5.60)	34.90 (4.53)	-0.939	0.348
FIB (g/L), median (IQR)	3.45 (1.09)	3.45 (0.92)	-1.187	0.235
Hs-CRP (mg/L), median (IQR)	11.33 (17.99)	10.37 (20.01)	-0.672	0.502
GLU (mmol/L),median (IQR)	7.70 (3.40)	7.84 (1.84)	-0.624	0.533
Bicarbonate (mmol/L), median (IQR)	23.50 (3.50)	23.17 (2.40)	-0.178	0.858
TG (mmol/L), median (IQR)	1.05 (0.67)	1.04 (0.71)	-0.516	0.606
CH (mmol/L), median (IQR)	5.04 (1.57)	4.95 (1.11)	-0.196	0.845
TPR (mg/L), median (IQR)*	0.04 (0.17)	0.14 (0.30)	-2.237	0.025
TP (g/L), median (IQR)	64.20 (8.50)	62.73 (5.50)	-1.565	0.118
ALT (iu/L), median (IQR)	19.00 (10.00)	19.48 (9.39)	-0.105	0.916
CR (mmol/L), median (IQR)	80.00 (33.50)	87.00 (33.35)	-1.004	0.315
TOAST classifications				
Atherosclerotic, n (%)	63 (37.5)	56 (33.3)		
Cardiac embolism, n (%)	22 (13.1)	17 (10.1)	0.288	0.866
Small artery, n (%)	6 (3.6)	4 (2.4)		
OCSP classifications*				
Total anterior, n (%)	18 (10.7)	4 (2.4)		
Partial anterior, n (%)	49 (29.2)	48 (28.6)		
Posterior, n (%)	16 (9.5)	18 (10.7)	7.993	0.046
Lacunar, n (%)	8 (4.8)	7 (4.2)		
Hemorrhagic transformations*				
None, n (%)	70 (41.7)	74 (44.0)		
Hemorrhagic Infarction, n (%)	9 (5.4)	3 (1.8)	14.042	0.001
Parenchymal, n (%)	12 (7.1)	0 (0)		
Risk factors				
Hypertension, n (%)*	64 (38.1)	42 (25.0)	4.463	0.038
Diabetes, n (%)	23 (13.7)	17 (10.1)	0.235	0.717
Heart arrhythmia, n (%)*	20 (11.9)	6 (3.6)	6.416	0.017
Heart failure, n (%)*	29 (17.3)	9 (5.4)	9.704	0.003
Smoking, n (%)	24 (14.3)	26 (15.5)	1.090	0.314
Stroke history, n (%)	19 (11.3)	13 (7.7)	0.432	0.559
Family history of stroke, n (%)*	7 (4.2)	0 (0)	6.181	0.016
Outcome				
Favorable, n (%)	44 (26.2)	43 (25.6)	0.938	0.356
Deceased, n (%)	30 (17.9)	15 (8.9)	3.868	0.056

*P < 0.05 (two tailed); TROM: thrombolysis group; ANTP: anti-platelet group; OTT: onset to treatment time; IQR: interquartile range; NIHSS: national institute of health stroke scale; BT: body temperature; HR: heart rate; BPS: systolic blood pressure; BPD: diastolic blood pressure; WBC: white blood cell count; PLT: platelet count; HCT: hematocrit; INR: international normalized ratio; APTT: activated partial thromboplastin time; FIB: fibrinogen; hs-CRP: high sensitivity C reactive protein; GLU: blood glucose; TG: triglyceride; CH: total cholesterol; TRP: troponin; TP: total protein; ALT: aminotransferase; CR: serum creatinine

37% of total AIS and supposed to be "mild", did not reach a favorable end.[3] We aimed to compare the prognosis between thrombolysis and ordinary anti-platelet strategies in Chinese AIS.

METHODS

Our hospital is one of the tertiary teaching institute attached to the Guangzhou Medical University, which is financed by government and located in the central downtown of Guangzhou city, having a total of 1,200 beds and supplies emergency medical services covering 1.5 million residents and admits more than 500 documented stroke patients each year. The number of inhabitants in the city has exceeded 12 million. One of the authors (LN) was ever a collaborator of the imaging-based thrombolysis trial in acute ischemic stroke-II.[4]

We searched the patients who had been consecutively registered in our database from January 2005 to June 2012.

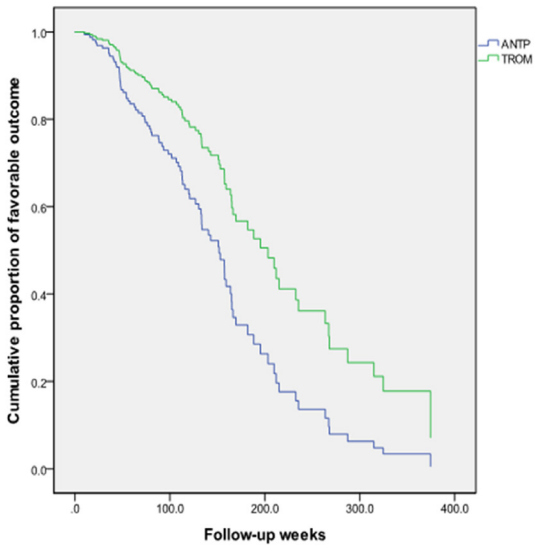

Figure 1: Cox regression. Estimation of favorable outcome proportions between TROM and ANTP. TROM: thrombolysis group; ANTP: anti-platelet group; $P = 0.026$

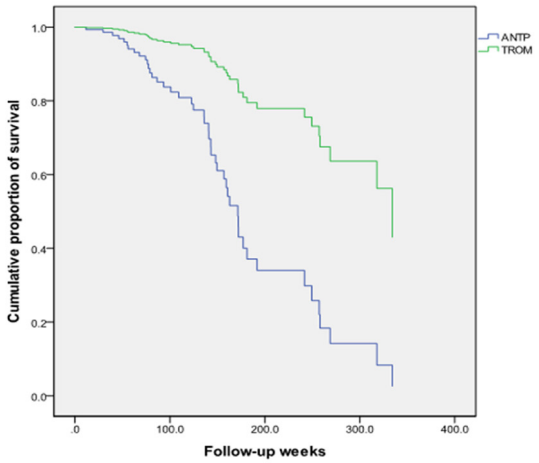

Figure 2: Cox regressions. Estimation of survival proportions between TROM and ANTP. TROM: thrombolysis group; ANTP: anti-platelet group; $P = 0.000$

The survey was approved by The Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University. The inclusion criteria were the following: (1) primary diagnoses of cerebral infarction coded with International Classification of Diseases 10th edition I63 to I69; (2) symptoms onset to treatment time was within 6 h; (3) thrombolysis with alteplase as per NINDS trial protocol or ordinary anti-platelet therapy such as aspirin and clopidegrel. Exclusion criteria included: (1) symptoms and signs diminished rapidly without apparent neurological deficits; (2) no visible lesions on diffusion weighted image in magnetic resonance imaging; (3) cerebral infarction caused by serious metabolic in-balance or infections. Patients were divided into thrombolysis group and ordinary anti-platelet one. The endpoints were defined as favorable (modified Rankin Scale 0-2) or survival. Follow-up were conducted through December, 2012 by structured telephone interview.

Data for variables nearest to the time point of treatment were collected using Microsoft® Office Excel 2003 (Microsoft Corporation, Redmond, WA, USA). The variables included demographics, vital signs, laboratory tests and radiological manifestations. Known risk factors such as cardiac abnormalities, hypertension and diabetes, smoking and prior stroke were included. The time elapse before treatment was recorded. National Institute of Health Stroke Scale (NIHSS) scores were recorded in documents and reviewed by an author (WY) who had passed the NIHSS training course in 2009.

Statistical analysis

For baseline independent variables, quantitative missing values were replaced by linear regression estimates. We used binary correlations to test the collinearities of independent variables and made combinations or reductions under professional considerations. Differences between groups were tested by Mann-Whitney *U*-test, Pearson chi-square and Fisher's exact test. In Kaplan-Meier curve estimation and Cox regression, log rank test and backward step-wise likelihood chi-square test were used respectively. The inclusion and exclusion criterion of stepping probability was 0.05 and 0.10 respectively. $\alpha = 0.05$ (two sided) was considered significant. Data were calculated by IBM® SPSS® statistics version 19.

RESULTS

Of the 2,949 AIS patients screened, one hundred and eighty three met the inclusion and exclusion criteria. Fifteen patients lost follow-up. One hundred and sixty eight individuals entered the final analyses. Ninety one were included in thrombolysis group (TROM) and 77 in anti-platelet (ANTP) one. Male accounted for 82 (48.8%) and female 86 (51.2%). The median of age was 74 [interquartile range (IQR) 67-79], NIHSS 9 (IQR 5-17) and onset to treatment time 3.9 h (IQR 3.0-4.8) respectively. The median length of follow-up was 112 (IQR 63.4-163.8) weeks. Differences of variables between two groups were listed in Table 1. Patients in TROM were younger, had higher NIHSS scales and lower serum troponin level, higher proportion of total anterior cerebral infarction, more hemorrhagic transformations and higher proportions of hypertension, heart abnormalities and family history of stroke. By the end of December 31, 2012, 87 patients (51.8%) reached favorable outcome while 81 (48.2%) unfavorable. Death occurred in 45 (26.8%) cases. No significant differences were detected between TROM and ANTP. In Kaplan-Meier curve estimation, patients in TROM showed a longer favorable period of time than those in ANTP [212 weeks 95% CI 169.5-254.5 *vs.* 126.9 weeks 95% confidence interval (CI) 105.2-148.6; Log-Rank test $x^2 = 19.632$, $P = 0.000$], while no significance was seen in survival time (258.0 weeks 95% CI 231.5-284.5 *vs.* 160.8 weeks 95% CI 153.0-168.5; Log-Rank test $x^2 = 2.427$, $P = 0.119$). After adjusting covariates of age, gender

and NIHSS, international normalized ratio, high sensitivity C reactive protein, heart failure, diabetes and interaction of NIHSS and thrombolysis in Cox regression, thrombolysis in AIS showed an independent protective effect for longer period of favorable outcome [202 *vs.* 151 weeks, $P = 0.026$, heart rate (HR) 1.96, 95% CI 1.958-3.540]. While adjusted for factors of white blood cell count, onset to treatment time and serum bicarbonate level, serum creatinine level and interaction of NIHSS and white blood cell count additionally, thrombolysis itself might be an independent predictor for longer survival instead (333 *vs.* 170 weeks, $P = 0.000$, HR 4.322, 95% CI 1.942-9.618) [Figures 1 and 2]. The estimated proportion of favorable outcome was about 91% for 1 year and 50% for about 3.4 years (4.2 *vs.* 3.1), while the estimated proportion of survival was about 98.5% for 1 year and 50% for about 5.3 years (6.9 *vs.* 3.5).

DISCUSSION

In this hospital-based retrospective cohort study, we found thrombolysis with alteplase might have a protective effect for longer period of time of favorable outcome and survival in Chinese AIS patients. To our knowledge, this is the first observational survey that compared the long-term prognoses of thrombolysis and anti-platelet therapy in mainland China. The overall estimated five-year survival rate was 50%, comparable to published data.[5] Wang *et al.*[6] found one-year survival rate of hospitalized AIS patients was about 89.2% in west China, much lower than that one in our cohort (98.5%). This may due to the fact that they enrolled patients with symptoms onset within 14 days. Much of them might not have received timely thrombolytic or anti-platelet therapies. With time elapsed, stroke might progress and leading to a worse end. Notably, much of patients in their data set were mild stroke (median NIHSS score 5) with high proportion of small artery occlusion (42.9%), while we enrolled more severe patients (median NIHSS score 9) with 70.8% large artery occlusion sub-type. These results might remind the importance of timely and fully managements of mild stroke, which is more common in Chinese population with unfavorable outcome,[3,7] to reach a longer survival.

Compared to that of Gensicke *et al.*[8] in Switzerland, Chinese AIS patients received thrombolytic therapy seemed to have longer 50% survival time (6.9 *vs.* 4.0 years) and good outcome (4.2 *vs.* 3.0 years). We cannot make a conclusion due to the disparities of pre-defined endpoint (mRs 0-1 in Swiss *vs.* 0-2 in CHN) but the stroke severity was comparable (NIHSS 13 *vs.*14), which had been proven to be the most important determinant of stroke mortality.[9] Although Asian ethnic patients in US had higher mortality rate in hospital stay after thrombolysis,[10] and we too previously reported a higher 3-month mortality rate of 18%[11] than that in western countries,[12] further study should be performed to clarity the potential benefits of thrombolysis for long-term survival in Chinese patients, with prospective design and less bias.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Case Report

Herpes zoster internuclear ophthalmoplegia

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ABSTRACT

Internuclear ophthalmoplegia (INO) is caused by a lesion in the medial longitudinal fasciculus. Patients with INO are usually asymptomatic but may have diplopia and oscillopsia. The most common causes of INO are ischemia and demyelination. Occurrence of INO due to infectious etiologies like tuberculosis, AIDS, brucellosis, cysticercosis and syphilis is well known. However, clinical presentation of INO associated with herpes zoster is very rare. The possible pathogenic mechanism for varicella zoster virus (VZV) induced INO could be demyelination or microinfarction in the brainstem. In the present study, a case of 56 years old male with double vision, with a recent history of herpes zoster, has been reported. Clinical examination revealed right INO. VZV IgM antibodies were positive and patient recovered fully after treatment with acyclovir and steroids.

Key words: Demyelination; herpes zoster virus; internuclear ophthalmoplegia; medial longitudinal fasciculus; varicella zoster virus

INTRODUCTION

In Internuclear ophthalmoplegia (INO) there is damage to the medial longitudinal fasciculus (MLF) between the 3rd and 6th cranial nerve nuclei which impairs the transmission of neural impulses to the ipsilateral medial rectus muscle.[1] It is clinically characterized by failure to adduct the ipsilateral medial rectus and nystagmus of the abducting eye. Tuberculosis, brucellosis, cysticercosis, syphilis and multiple sclerosis are the common infectious diseases which are responsible to cause INO in a patient.[2] Herpes zoster is a relatively rare etiology of INO. To the best of our knowledge, only two studies focusing on the association between herpes zoster and INO have been published so far. In agreement to the previous publications; we report here the case of a patient with INO, who also had herpes zoster vasculopathy. The goal of this report is to highlight the rare case of herpes zoster leading to INO.

CASE REPORT

A 56 years old male presented with diplopia in the left gaze was admitted. The diplopia worsened while looking at the distant objects. Occular examination revealed that he had right INO showing restriction of adduction in the right eye with nystagmus on abduction in the left eye. His vertical eye movements and convergence were normal. Pupil and fundus examination were normal. Rest of the neurological examination was also normal. Neck stiffness was not present. He had no fever. Healed herpetic scars were present in the left maxillary region.

Two weeks before the onset of diplopia he was diagnosed with herpes zoster and was under treatment with oral acyclovir. He did not have any other co-morbid illness. A previous history of chicken pox infection at the age of 10 years was reported.

Routine blood examination including complete blood count, renal function test and electrolytes were normal. Chest x-ray and electrocardiogram were also normal. Magnetic resonance images (MRI) scans of the brain with contrast revealed no abnormality. Cerebro-spinal fluid analysis showed pleocytosis and elevated protein with normal sugar level. Serum Varicella zoster IgM antibody was positive.

The patient was treated with intravenous (IV) acyclovir

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and IV methylprednisolone followed by oral prednisolone. He symptomatically improved within two months.

DISCUSSION

In this study, the patient had right INO with a recent history of herpes zoster infection presenting painful rash distribution along V3 branch of the left trigeminal nerve. He possibly had damaged right MLF, without exhibiting significant brainstem lesions in the MRI of the brain. The positive serum herpes zoster IgM antibody report of the patient suggested recent reactivation of the latent VZV. The exact pathogenic mechanism by which Varicella zoster causes INO has not been elucidated, however, some studies assume it is due to multifactorial etiologies. The proposed hypothesis are: demyelination in the brainstem;[3] microinfaractions in the brainstem due to inflammatory meningovasculitis producing small vessel vasculopathy of the supplying cranial nerves.[4]

The herpes zoster particles spread along trigeminal afferent fibers and cause small vessel vasculopathy. Initially the reactivated virus spreads transaxonally to the arterial adventia and then spreads transmurally to the lumen. It causes thickening of intima, disruption of elastic lamina and loss of smooth muscle cells, which leads to occlusion of the involved vessel.[4] It has been found to be associated with disruption of atheromatous plaque and hypercoagulability induced by VZV.[5] It has also been shown that the herpes zoster vasculitis may closely mimic Giant cell arteritis (GCA).[6] Till date, only two studies have reported cases of herpes zoster infection induced INO. Caroll *et al*.[3] suggested that the pathologic process was due to the onset of demyelinating process in the brainstem, whereas, Al-Abdulla *et al*.[7] reported it could be due to herpes zoster vasculopathy that can mimic GCA. The authors reported MRI of the brain of the patient was normal; which was observed in this study as well.

Varicella zoster vasculopathy after primary infection or reactivation may involve large vessels causing unifocal granulomatous arteritis and small vessels causing multifocal vasculopathy.[8] Histopathological studies on arteries with Varicella zoster vasculopathy shows VZV DNA, VZV antigen, herpes virus particles, Cowdry A inclusions and multinucleated giant cells.[9]

In our present study, although the patient had a definite clinical history of recent herpes zoster supported by positive serum VZV IgM antibody, the MRI of the brain with magnetic resonance angiography was found normal. It is possible that microinfarctions due to restriction of the disease inflammation to a small single artery in the brainstem were not detected in MRI scans of the brain.

A spectrum of neurologic complications may follow herpes zoster infection such as motor neuropathies of the cranial and peripheral nervous system, encephalitis, meningoencephalitis, myelitis and Guillain-Barre syndrome. Our patient had presented INO after herpes zoster infection, which is a very rare neurological manifestation of VZV, however, he recovered completely with appropriate treatment. We wanted to highlight this case because of its infrequency of occurrence.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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Case Report

Spontaneous intracranial hypotension complicated with cerebral venous thrombosis and subdural effusion: a case report

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ABSTRACT

Spontaneous intracranial hypotension treatment can be complicated by concomitant cerebral venous thrombosis and subdural hematoma. A 48 years old male, presenting orthostatic headache and neck pain for 1 month displayed sagittal sinus thrombosis and bilateral subdural effusions, as well as extradural fluid collection at T3-T8 level, upon magnetic resonance imaging. Cerebrospinal fluid opening pressure was 50 mmH_2O, and a leak was confirmed at C2-C3 level by computed tomography (CT) myelogram. The presence of subdural hematoma precluded anticoagulation treatments. An autologous epidural blood patch at C2-C3 level under CT guidance improved the patient's condition, remaining free of residual symptoms or recurrence at six-month follow-up.

Key words: Spontaneous intracranial hypotension; cerebral venous thrombosis; subdural effusion; autologous epidural blood patch

INTRODUCTION

Orthostatic headache, low cerebrospinal fluid (CSF) pressure, and noninterrupted diffuse pachymeningeal enhancement observed upon magnetic resonance imaging (MRI) of the brain, characterize spontaneous intracranial hypotension (SIH). The estimated annual incidence of this uncommon disorder is 5 per 100,000, and its cause lies in spontaneous CSF leaks that result in CSF hypovolemia and hypotension (CSF opening pressure < 60 mmH_2O).[1] CSF composition may be normal or show increased protein content and pleocytosis.

SIH cases occasionally present with concomitant subdural effusions and, more rarely, cerebral venous thrombosis (CVT) (2% of patients).[3] The most characteristic brain MRI finding in SIH is diffuse pachymeningeal enhancement, that is caused by an increase in venous blood volume secondary to the loss of CSF pressure.[4] This alteration can lead to subdural hematoma and CVT through two main mechanisms: (1) SIH is associated with rostrocaudal sagging of the brain due to the loss of CSF buoyancy,[4] resulting in a negative intracranial pressure gradient that may damage the venous endothelial lining by stretching the cerebral vessels, and can produce tears in bridging

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veins in the dural border cell layer, causing them to rupture and leading to subdural hematoma; (2) the loss of CSF volume reduces absorption of CSF into the cerebral venous sinuses, resulting in increased blood viscosity in the venous compartment,[5] which could contribute to dural sinus thrombosis in patients with risk factors for thrombosis. The general consensus is that CVT should be treated with heparin, since a meta-analysis concluded that this treatment is safe and is associated with a clinical trend (not statistically significant) of reduction in the risk of death and dependency. Thus, most of the reported SIH patients with CVT have been treated with anticoagulation so far along with bed rest, hydration and epidural blood patches (EBP) [Table 2].[6-14] On the other hand, cases of large subdural hemorrhage require surgical drainage and treatment of the underlying cause of SIH. Most SIH patients without other complications recover after bed rest with foot end elevation, hydration and steroid therapy. Nevertheless, the mainstay of SIH treatment is the application of EBP at the CSF-leak site (injection of 10-20 mL of autologous blood into the spinal epidural space).[15] Relief of symptoms, particularly orthostatic headache, is often dramatic after EBP, and if it fails it can be repeated. On the other hand, direct EBP at the cervical area is challenging due to the narrow space of region and its proximity to important neural structures, therefore, this treatment is not performed in all cervical-leak cases. With this case report we aimed to provide further evidence that EBP is a feasible and efficient treatment for SIH with CSF leak in the cervical area, subdural hematoma and CVT.

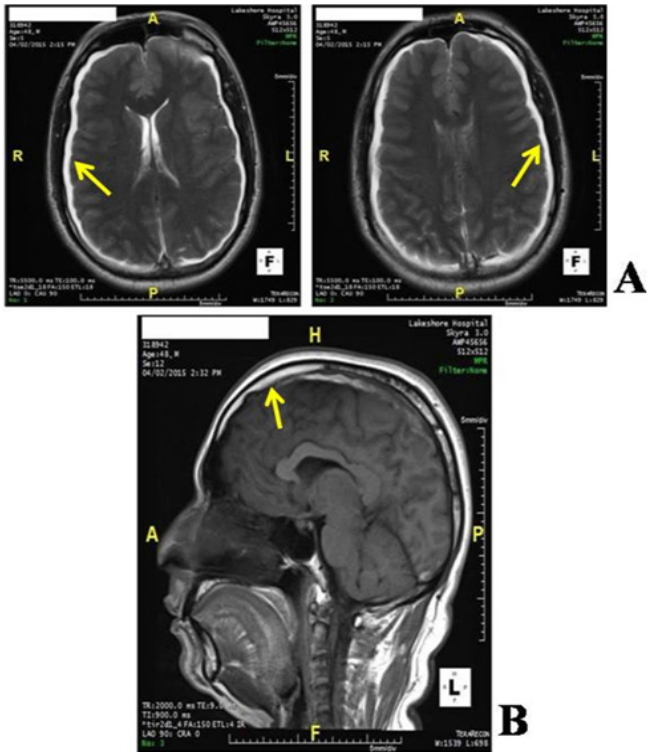

Figure 1: MRI brain. (A) T2 weighted sequences showing bilateral symmetrical fronto-parietal and occipital subdural effusions (as marked by the arrow); (B) T1 weighted sequences showing saggital sinus thrombosis (as marked by the arrow)

CASE REPORT

A 48 years old Indian male presented with headache and neck pain of 1 month duration. The patient had severe occipital headache with visual analogue score of 9. The headache worsened in the upright position and was completely relieved after lying down. The patient was otherwise normal, without any significant past clinical history.

On examination, the patient was conscious, oriented and afebrile, with a pulse rate of 82 beats per minute and blood pressure of 130/80 mmHg. Eye movements were normal in all directions and there was no sign of nystagmus. Both pupils were equal and reactive to light. Cranial nerve and fundus examinations were also normal and no motor weakness or sensory loss was present. Flexor plantar response was positive and bilateral. No signs of meningeal irritation nor focal neurological deficits were found.

T2 weighted sequences from the MRI of the brain showed bilateral symmetrical fronto-parietal and occipital subdural effusions [Figure 1A], while T1 weighted sequences unveiled sagittal sinus thrombosis [Figure 1B]. T2 sequences from the spine MRI revealed elliptical high signal extra axial collection posterior to the spinal cord at T3 to T8 level, which was suggestive of CSF leak [Figure 2].

A lumbar puncture with CSF manometry was performed under aseptic conditions, finding a CSF opening pressure of 50 mmH_2O. CSF content analysis [Table 1] showed elevated proteins [normal CSF protein ranges from 20-40 mg/dL]. CT myelography revealed extradural contrast extravasation at C2-C3 level [Figure 3A].

Vasculitic work-up yielded negative results for anti-nuclear antibody, anti-double stranded DNA antibody, perinuclear anti-neutrophil cytoplasmic antibody, cytoplasmic anti-neutrophil cytoplasmic antibody and anti-phospholipid antibody. Thrombophilia screening resulted also negative, as anti-thrombin, protein C and protein S were normal. Factor V Leiden

Table 1: Cerebrospinal fluid analysis

Parameters	Results
CSF colour	Clear fluid, no xanthochromia, no turbidity
CSF pressure	50 mmH_2O
CSF protein	315 mg/dL
CSF sugar	93 mg/dL
CSF cell count	Occassional RBCs only

CSF: cerebrospinal fluid; RBCs: red blood cells

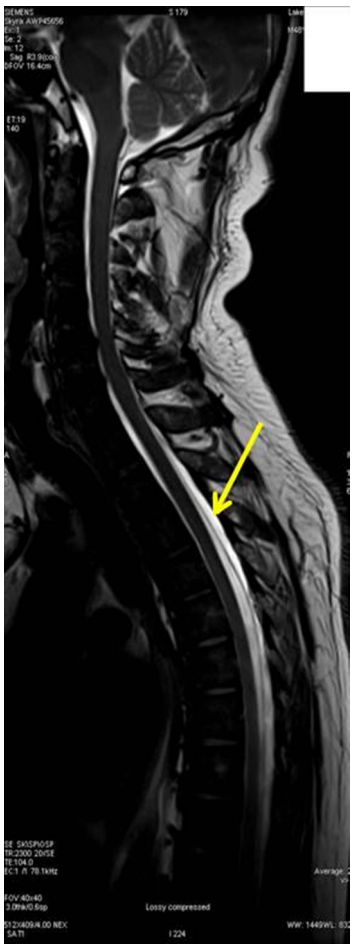

Figure 2: T2 sagittal sequence from the spine MRI showing elliptical high signal extra axial collection posterior to the spinal cordat T3 to T8 level (as marked by the arrow, suggestive of CSF leak)

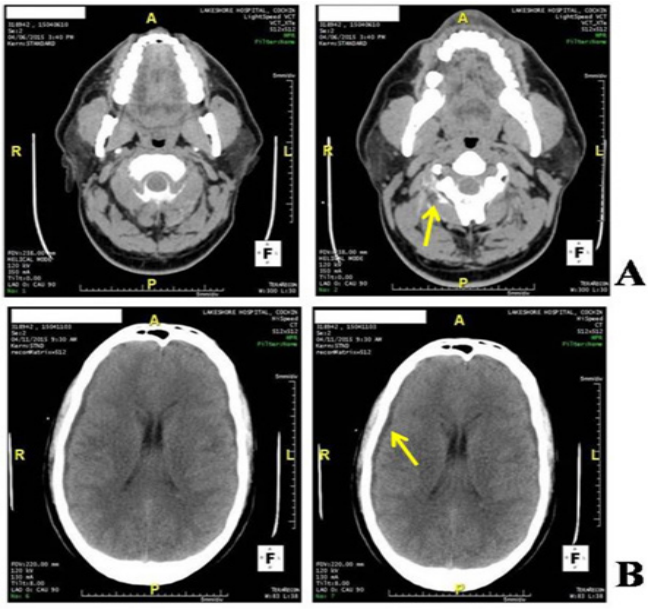

Figure 3: CT. (A) Myelography showing extradural contrast extravasation at C2-C3 level (as marked by the arrow); (B) plain CT brain after epidural blood patch showing mild reduction in the extent of subdural effusions (as marked by the arrow)

mutation was also not detected.

The patient was advised complete bed rest with foot end elevation and to remain adequately hydrated. Anticoagulation for CVT was precluded since he had bilateral subdural effusions and CVT was secondary to SIH. After 24 h of bed rest and adequate hydration, since the patient was very much symptomatic, a cervical autologous epidural blood patch with 10 mL of blood was performed under CT guidance and achieved the resolution of the symptoms within a week without worsening the CVT. Brain CT taken on day 5 following epidural blood patch showed mild reduction in the extent of subdural effusions [Figure 3B]. In addition, the patient showed no residual symptoms or recurrence at six-month follow-up.

DISCUSSION

Literature clearly defines clinical signs, typical MRI findings and treatment options of SIH.[3] In an intact cranium, the total intracranial volume must be constant according to the Monroe-Kellie hypothesis.[4] SIH usually occurs due to spontaneous CSF leaks in the inferior cervical and superior thoracic spine. Mechanical stress, meningeal diverticula and connective tissue diseases have been reported as the potential risk factors for the development of SIH.

The Monroe-Kellie hypothesis states that the decrease in intracranial blood volume is compensated by the dilatation of the cerebral veins.[4] Furthermore, CSF loss reduces the CSF absorption into the cerebral venous sinuses leading to an increase in blood viscosity in the cerebral compartment.

In our patient, epidural blood patch was performed earlier within 24 h of conservative therapy since the patient was very much symptomatic and SIH was complicated with subdural effusions and CVT.

In 2015, Kapoor and Ahmed did a comprehensive electronic literature search to include studies that reported on performance of cervical EBPs in patients with CSF leak at the cervical level.[16] Their review provides Class II level of evidence that cervical EBPs are safe and effective in reliving positional headache due to CSF leak. A total of 15 studies, reporting on 19 patients were included. All patients presented with a headache that increased in the standing position, and improved in the supine position. All patients were identified to have a CSF leak at the cervical level. Eight patients first underwent a lumbar EBP, without complete, long-term relief. All these patients, along with 11 patients who did not undergo a lumbar EPB prior to cervical EBP, reported complete, long-term pain relief. EBPs were mostly done in the prone position, using imaging guidance. An average of 5-8 mL of autologous blood was injected in the epidural space. No major neurological complications were

Table 2: All the reported data of patients with cerebral venous thrombosis and spontaneous intracranial hypotension in whom the site of cerebrospinal fluid leak was demonstrated.

Authors	Year	Age	Gender	OP	Location of CSF leak	AC	EBP
Flemming and Link[6]	2005	31	M	?	Cervical/Thoracic	Yes	No
Kataoka *et al.*[7]	2007	45	M	40	Cervical/Thoracic	Yes	Yes
Albayram *et al.*[8]	2007	45	M	?	Thoracic	Yes	Yes
Wang *et al.*[9]	2007	33	F	80	Cervical	No	Yes
Tan *et al.*[10]	2008	26	F	50	Thoracic	Yes	Yes
Schievink and Maya[11]	2008	32	M	0	Thoracic	Yes	Yes
Schievink and Maya[11]	2008	43	M	40	Thoracic	Yes	Yes
Yoon *et al.*[12]	2011	26	M	50	Cervical	No	Yes
Dangra *et al.*[13]	2011	35	M	?	Cervical	Yes	No
M. C. Garcia-Carreira *et al.*[14]	2014	29	F	30	Thoracic	Yes	Yes
M. C. Garcia-Carreira *et al.*[14]	2014	54	M	20	Thoracic	Yes	Yes
Present Case	2015	48	M	50	Cervical	No	Yes

CSF: cerebrospinal fluid; OP: opening pressure (mm H_2O); AC: anticoagulation; EBP: epidural blood patch; age (years); M: male; F: female.

reported in any patient.

In 2014, Garcia-Carreira *et al.*[14] described two cases of spontaneous intracranial hypotension associated with cerebral venous thrombosis. In one case, extensive cerebral venous thrombosis involved the superior sagittal sinus and multiple cortical cerebral veins. In the other case, only a right frontoparietal cortical vein was involved. When spontaneous intracranial hypotension and cerebral venous thrombosis occur together, it raises difficult practical questions about the treatment of these two conditions. In most reported cases, spontaneous intracranial hypotension was treated conservatively and cerebral venous thrombosis was treated with anticoagulation. Garcia-Carreira *et al.*[14] supported aggressive treatment of the underlying cerebrospinal fluid leak.

Again in 2015, Wang E and Wang D reported a case of successful treatment of a patient with spontaneous intracranial hypotension correlated with MRI finding of cerebrospinal fluid (CSF) leak with extradural collection at the upper cervical spinal level.[17] This patient received two lumbar epidural blood patches without lasting relief. Later on, the radiographic evidence of prominent CSF leak with extradural fluid collection was noted at C1-2 level. The patient was then treated with a cervical epidural blood patch, which provided headache pain relief lasting 6 months. A second cervical epidural blood patch was performed, and the patient was headache free since then.

Table 2 shows all the reported data of patients with cerebral venous thrombosis and spontaneous intracranial hypotension in whom the site of cerebrospinal fluid leak was demonstrated. All these patients who received EBP responded very well and was symptom free during the follow up.

There remain some controversies in treatment of SIH complicated with CVT. Primary conservative management of SIH accompanied by anticoagulation for CVT is usually advised. If the symptoms of SIH persist even after bed rest and adequate hydration, epidural blood patching could be considered. Anticoagulation for CVT can be withheld till the symptoms of SIH get controlled. The mortality of CVT is 5% but with SIH, it can be increased. Therefore firstly SIH should be treated.

In 2013, Güler *et al.*[18] reported a case of cerebral venous thrombosis accompanying with intracranial hypotension. They advocated anticoagulation for this patient only after SIH symptoms resolved. CVT is not reported in any SIH case after the resolution of symptoms. So, in our patient we tried to treat the primary cause, SIH and hence anticoagulation was precluded. We advocate primary treatment of the underlying spinal CSF leak, particularly when symptoms of SIH persist. In our patient, we did an autologous epidural blood patch at the site of CSF leak under CT guidance and the patient improved. In addition, the patient had no residual symptoms or recurrence at six-month follow-up. We think that our case can add additional information to the literature regarding the management of CVT in SIH.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Original Article

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Symptom severity, quality of sleep, and treatment adherence among patients suffering from schizophrenia and depression

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ABSTRACT

Aim: Treatment non-adherence is a common problem in patients suffering from schizophrenia and depression. This study investigated the possible relationships between symptom severity, quality of sleep, and treatment adherence. **Methods:** Thirty outpatients with schizophrenia and 58 outpatients with depression were enrolled in this study. The beck depression Inventory-II, the positive and negative syndrome scale, and the pittsburgh sleep quality index were used to assess symptom severity and quality of sleep, and sleep log data were used to measure treatment adherence. **Results:** The preliminary results showed no significant relationship between symptom severity and treatment adherence or between quality of sleep and treatment adherence in patients with depression. However, a significant positive relationship was found between negative symptoms and treatment adherence and a significant negative relationship between quality of sleep and treatment adherence in patients with schizophrenia. **Conclusion:** The present exploratory study revealed a positive relationship between symptom severity and treatment adherence and a negative relationship between quality of sleep and treatment adherence in patients with schizophrenia, but no significant relationships in patients with depression were found. Future studies are needed in order to gain a better understanding of possible risk factors related to treatment non-adherence.

Key words: Depression; quality of sleep; schizophrenia; symptom severity; treatment adherence

INTRODUCTION

Dropout is a serious problem in treating patients because treatment can only be effective if delivered adequately and completely.[1] To date, very little is known about possible risk factors that might predict patient dropout.[2] Findings by Herman *et al.*[3] indicate that early treatment dropout is an essential factor in determining treatment success and failure because in case of dropout, patients receive little or nothing of the intervention involved and, therefore, cannot

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benefit from therapy. "Adherence to treatment" refers to the extent to which a patient's behavior conforms to the advice of health professionals.[4] Although dropout and adherence to treatment are related issues, there are also important distinctions between these two concepts.[5]

Clinical psychologists have reported that, unfortunately, non-compliance with homework as part of cognitive-behavioral therapy is a common phenomenon in clinical practice.[6] This is problematic because research has shown that homework compliance is positively associated with reduced symptom severity.[7]

Treatment non-adherence is a common problem in patients suffering from depression or schizophrenia.[8] In cases of schizophrenia, poor adherence to medication and psychosocial treatment are prevalent and increase the probability of relapse and re-hospitalization.[9] In their review of 39 studies published in English since 1980 and specifically examining risk factors for medication non-adherence, Lacro *et al.*[10] pointed out that in those studies, the mean rate of non-adherence to medication for patients with schizophrenia was 41%. Moreover, in their systematic review of 103 studies on adherence to treatment by patients with psychosis, Nosé *et al.*[11] reported a mean rate of failure to keep scheduled follow-up appointments of 24% in patients with psychoses. Rates of treatment dropout are reported less often; the figures for this range from 20% to 56% in patients with schizophrenia.[12-14] In patients with depression, the severity of the disorder has been associated with treatment dropout in some studies,[15] but not in all.[16] Agreeing with Last *et al.*[15] Leserman[17] indicated that patients with depression were less likely to adhere to a treatment program than patients without depression and experienced worse outcomes in health.

Schizophrenia and depression are both seemingly dropout-prone disorders,[18,19] but which specific factors can be related to the non-adherence to treatment in patients with either condition is arguable. An association between less severe psychiatric symptoms and better treatment adherence was found,[20] and both the severity of the disease and the patient's attitude towards the prescribed medication were found to be related to adherence in patients with schizophrenia.[21] In this case, the severity was found to correlate negatively with treatment adherence while the patient's attitude towards the prescribed medication was found to correlate positively with treatment adherence.

Also, a role for quality of sleep in treatment adherence of patients with schizophrenia or depression has been suggested.[22,23] Phillips and colleagues,[23] for instance, considered adherence to treatment to be a factor that correlated with sleep disturbance and depression. Their results showed that women with greater sleep disturbances also had a higher level of depressive symptoms and poor adherence to their medication regimen. According to these findings,[23] not only the severity of the disorder but also suffering from sleep disturbances might be related to treatment non-adherence in patients with schizophrenia or depression. A possible role for quality of sleep in treatment adherence is further supported by the fact that both schizophrenia and depression are co-morbid with sleep disturbances.[24,25]

However, to date, not much research has been conducted on the specific factors that can be related to the non-adherence to treatment in patients with schizophrenia or depression. As a result, more research is needed on the possible factors suggested in the literature, symptom severity and quality of sleep, as being related to treatment non-adherence. The aim of the present study was, therefore, to investigate the possible relationships between symptom severities, quality of sleep, and treatment adherence further. Thereby, noting that treatment non-adherence limits the improvements in independent living, employment, and quality of life to a large degree in patients with schizophrenia or depression is important.[26] First, patients with a more severe depression were hypothesized to have worse treatment adherence than patients with a less severe depression. Secondly, patients with schizophrenia who show more positive and negative symptoms were hypothesized to have worse treatment adherence than patients with schizophrenia who show fewer positive and negative symptoms. A final hypothesis was that patients who have a poor quality of sleep have worse treatment adherence than patients who have a good quality of sleep.

METHODS

Setting and participants

The participants in this study consisted of 17 female and 13 male adult patients with schizophrenia with an average age of 41 (SD = 8.80) and 40 female and 18 male adult patients with depression with an average age of 45 (SD = 12.14). All participants were outpatients of the LVR-Klinik Bedburg-Hau in Germany and were diagnosed by their psychiatrist according to the 10th revision of the International Classification of Diseases and Related Health Problems (ICD-10).[27] Note that in Germany, the ICD-10[27] is used instead of the Diagnostic and Statistical Manual

of Mental Disorders, fifth edition (DSM-V).[28] The inclusion criterion for all participants was that they be between 18 and 65 years of age, and the exclusion criteria for the patients with schizophrenia or depression were substance abuse, epilepsy, and other neurological disorders. Finally, all patients took part voluntarily without any inducement and signed the informed consent form.

Material

BDI-II

The Beck Depression Inventory-II (BDI-II)[29] was used to assess depression severity. The BDI-II is a 21-item self-report inventory, and each item is rated on a 4-point scale (i.e. from 0 to 3). Thus, an individual's scoring range would lie between 0 and 63. The higher the total scores on the BDI-II, the more severe the depressive symptoms of the patients are.

PANSS

In addition, the Positive and Negative Syndrome Scale (PANSS)[30] was used to measure the symptom severity of the patients with schizophrenia. The PANSS has to be filled in by a psychiatrist. It consists of three subscales: the positive scale, the negative scale and the general psychopathology scale. The scoring range for an individual is between 7 and 49 for the positive scale, 7 and 49 for the negative scale, and 16 and 112 for the general psychopathology scale; as a result, the total scores on the PANSS are between 30 and 210. The higher the total scores on the PANSS are, the more severe the positive and negative symptoms of the individuals with schizophrenia are.

PSQI

The Pittsburgh Sleep Quality Index (PSQI)[31] was used to measure the subjective quality of sleep of the patients with schizophrenia or depression. The PSQI is a self-report inventory and measures sleep quality and patterns of sleep. It has seven separate domains: (1) "subjective sleep quality"; (2) "sleep latency"; (3) "sleep duration"; (4) "habitual sleep efficiency"; (5) "sleep disturbances"; (6) "use of sleep medication"; and (7) "daytime dysfunction over the last month". Individuals score all items on a Likert scale, ranging from 0 to 3. As a result, the total scores on the PSQI are between 0 and 21, and the higher the total PSQI score is, the poorer the quality of sleep is. In daily clinical practice, often a cut-off score of 5[31] is used, meaning that participants who score below 5 have a good quality of sleep and participants who score above 5 have a poor quality of sleep. In the present study, the total PSQI scores were used for further analyses.

Sleep log

As part of the treatment adherence measurements for the schizophrenic and depressive patients,[32] the participants were asked to complete a sleep log for two weeks. It consisted of the following six sleep variables: (1) "total sleep time"; (2) "how many minutes awake during the night"; (3) "how many minutes awake before falling asleep"; (4) "how relaxing was your sleep"; (5) "did you feel exhausted"; (6) "how was your average performance level today". The sleep log was to be completed every morning just after awakening and every evening just before falling asleep.[33] Completing the sleep log took approximately 10 min a day, 140 min in total.

Procedure

Every participant in the group of patients with depression was asked to complete the BDI-II and the PSQI. The PANSS was completed by the patient's psychiatrist in the group of patients with schizophrenia, and these patients were also asked to complete the PSQI. All participants were asked to keep a sleep log and return it to their clinical psychologist at the end of the two weeks. At the end of the study, a debriefing was offered to all participants, in which they were individually informed of their test results. The study was approved by the local ethics committee (Ärztekammer Nordrhein, number: 2008331); moreover, the clinical trial has officially been registered under number NTR3132 at the Dutch Trial Register (see also http://www.trialregister.nl/trialreg/admin/rctview.asp? TC = 3132). Finally, the study was performed in accordance with the Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/).

Design and statistics

An experimental design was used in which the first categorical dependent variable was called "Absolute Treatment Adherence"; it was measured by using the returned sleep logs (i.e. if the patient returned the sleep log, the patient was considered as being treatment adherent *vs.* if the patient did not return the sleep log, the patient was considered as not being treatment adherent). The second continuous dependent variable was called "Degree of Treatment Adherence" because it consisted of the total number of days the participants had completed the sleep log, with 0 day meaning a very low degree of treatment adherence and 14 days meaning a very high degree of treatment adherence. In other words, "Absolute Treatment Adherence" showed whether a patient returned the sleep log or not while "Degree of Treatment Adherence" showed to what degree the patient was treatment adherent. Note that in the present study, a very narrow definition of the word "treatment" was used (e.g. completing and returning a sleep log) and did not include any pharmacological

Table 1: The number of adherent and non-adherent patients with depression and their mean scores and standard devitations on the BDI-II and the PSQII

Adherence	Instrument	Number	Mean	SD
Non-adherent	BDI-II	21	20.81	12.21
	PSQI	21	9.05	4.28
Adherent	BDI-II	37	19.24	11.28
	PSQI	37	9.51	4.17
Total	BDI-II	58	19.81	11.54
	PSQI	58	9.34	4.18

All $P > 0.05$; PSQI: pittsburgh sleep quality index; BDI-II: beck depression inventory-II

Table 2: The number of adherent and non-adherent patients with schizophrenia and their mean scores and standard deviations on the PANSS and the PSQI

Adherence	Instrument	Number	Mean	SD
Non-adherent	PANSS-total	16	67.81	19.56
	PANSS-negative	16	16.81*	6.76
	PANSS-positive	16	14.13	7.37
	PANSS-psychopathology	16	36.88	11.62
	PSQI	16	6.31	3.32
Adherent	PANSS-total	14	81.86	26.74
	PANSS-negative	14	23.43*	10.42
	PANSS-positive	14	20.71	26.44
	PANSS-psychopathology	14	44.86	14.36
	PSQI	14	8.57	4.50
Total	PANSS-total	30	74.37	23.86
	PANSS-negative	30	19.90*	9.14
	PANSS-positive	30	17.20	18.78
	PANSS-psychopathology	30	40.60	13.37
	PSQI	30	7.37	4.01

*$P < 0.05$; PANSS: positive and negative syndrome scale

interventions; neither did we use the sleep variables recorded in the sleep log to predict adherence. The scores on the BDI-II, the PANSS, and the PSQI served as "predictor" variables.

With SPSS version 22.0[34] a discriminant function analysis[35] was conducted with "Absolute Treatment Adherence" as a categorical dependent variable and the scores on the BDI-II and PSQI as predictor variables for the group of patients with depression. For the group of patients with schizophrenia, a discriminant function analysis was performed with "Absolute Treatment Adherence" as a categorical dependent variable and the scores on the PANSS and PSQI as predictor variables. Furthermore, two multiple regression analyses[36] were conducted with one analysis containing "Degree of Treatment Adherence" as the dependent variable and the score on the BDI-II, as well as the score on the PSQI, as independent variables for the group of patients with depression. In the second analysis, "Degree of Treatment Adherence" was used as the dependent variable, and the score on the PANSS, as well as the score on the PSQI, were used as independent variables for the group of patients with schizophrenia.

RESULTS

Absolute treatment adherence in the depression group

For the group of patients with depression, a discriminant function analysis was conducted in order to identify patients who were adherent to treatment *vs.* those who were not; this was done using the patient's scores on the BDI-II and the PSQI as predictors. The discriminant function analysis explained 100% of the variance, canonical $R^2 = 0.01$ (Note that this does not mean that the discriminant function accounts for 100% of the variance in the response variable; rather, this means that it is the only discriminant function extracted for the analysis).[37] The discriminant function analysis did not significantly differentiate the patients who were treatment-adherent from the ones who were not [$\Lambda = 0.99$, χ^2 (2) = 0.43, $P > 0.05$]. Table 1 summarizes the descriptive statistics of the group of patients with depression.

Table 3: Results of the multiple regression analysis for variables predicting degree of treatment adherence

Predictor	B	SEB	β
Constant	8.95	2.48	-
BDI-II	-0.03	0.07	-0.05
PSQI	0.13	0.21	0.08

$R^2 = 0.01$; B: unstandardized multiple regression coefficient; SEB: standard error of multiple regression coefficient; β: standardized multiple regression coefficient

Table 4: Results of the multiple regression analysis for variables predicting degree of treatment adherence

Predictor	B	SEB	β
Constant	-2.14	4.13	-
PANSS-positive	0.08	0.07	0.21
PANSS-negative	0.21	0.25	0 .29
PANSS-psychopathology	0.01	0.32	0.02
PANSS-total	-0.02	0.22	-0.06
PSQI	0.65	0.31	0.39*

$R^2 = 0.27$ *$P < 0.05$; B: unstandardized multiple regression coefficient; SEB: standard error of multiple regression coefficient; β: standardized multiple regression coefficient

Absolute treatment adherence in the schizophrenia group

In addition, a discriminant function analysis was conducted for the group of patients with schizophrenia. The discriminant model was used to identify patients who were adherent to treatment versus those who were not treatment-adherent by using the patient's scores on the PANSS (also divided into positive symptoms, negative symptoms, and general psychopathology) and on the PSQI as predictors. The discriminant function analysis explained 100% of the variance, canonical $R^2 = 0.26$. The discriminant function analysis did not significantly differentiate the patients who were treatment-adherent from the ones who were not [$\Lambda = 0.74$, χ^2 (5) = 7.67, $P > 0.05$]. The correlations between outcomes and the discriminant function revealed that the score on the PANSS-negative loaded highly onto the function ($r = 0.67$), followed by the score on the PSQI ($r = 0.50$). Table 2 summarizes the descriptive statistics of the group of patients

with schizophrenia.

Variables predicting degree of treatment adherence in the depression group

For the group of patients with depression, a multiple regression analysis with "Degree of Treatment Adherence" as the dependent variable and the scores on the BDI-II and the PSQI as predictors was conducted. Note that the adherent depression group had a mean of 13.44 days (SD = 2.02 days). Table 3 shows the results of the multiple regression analysis using the enter method for predicting "Degree of Treatment Adherence". The scores on the BDI-II and the PSQI were insignificant predictors.

Variables predicting degree of treatment adherence in the schizophrenia group

For the group of patients with schizophrenia, a multiple regression analysis was conducted with "Degree of Treatment Adherence" as the dependent variable and the scores on the PANSS-total (also split into PANSS-positive, PANSS-negative, and PANSS-general psychopathology) and on the PSQI as predictors. Note that the adherent schizophrenia group had a mean of 12.82 days (SD = 2.43 days). Table 4 shows the results of the multiple regression analysis using the enter method for predicting "Degree of Treatment Adherence". The scores on the PANSS-total, PANSS-positive, PANSS-negative, and PANSS-general psychopathology were insignificant predictors while the score on the PSQI was a significant predictor. Moreover, the score on the PANSS-negative and the score on the PSQI correlated significantly with "Degree of Treatment Adherence" (PANSS-negative: $r = 0.31$, $P < 0.05$; PSQI: $r = 0.39$, $P < 0.05$).

DISCUSSION

The general results of our study showed that for the group of patients with depression, in contrast to our hypotheses, symptom severity and quality of sleep did not significantly predict whether a patient would be adherent to treatment or not; the severity of depression and the quality of sleep did not differ significantly between treatment adherent and non-adherent patients. For the group of patients with schizophrenia, in line with our hypotheses, a relationship was found between symptom severity and treatment adherence, as well as between quality of sleep and treatment adherence; however, the directions of those relationships were contrary to our expectations. Experiencing more severe negative symptoms was found to be significantly related to better treatment adherence. Moreover, quality of sleep did serve as a significant predictor of treatment adherence; however, in general, the patients with schizophrenia who reported a worse quality of sleep were more treatment-adherent than those who reported a better quality of sleep and not vice versa.

The surprising findings that no relationships were found between quality of sleep and treatment adherence and between symptom severity and treatment adherence in the depression group are opposed to the findings by Ong *et al.*[2] who investigated the risk factors associated with dropping out of group cognitive-behavior therapy for insomnia and found that short sleep duration and elevated symptoms of depression at baseline could be especially associated with an increased risk of early therapy dropout. Also, in their study, Phillips *et al.*[23] reported that women with greater sleep disturbances had a higher level of depressive symptoms and poor adherence to their medication regimen. Noticeably, research that targets adherence to medication dominates research that targets adherence to treatment. Possibly, different risk factors may be related to these two forms of adherence, which might make a direct comparison between studies that target adherence to medication and studies that target adherence to treatment difficult. This suggestion remains to be verified. Overall, to date, only a few studies have been conducted on treatment non-adherence by patients with depression and its predictors,[38] so further research is warranted.

Nevertheless, in the group of patients with schizophrenia in the present study, symptom severity and quality of sleep may be related to better treatment adherence. Steger *et al.*[39] reported similar findings. The researchers assessed medication adherence in a sample of 216 patients with a first episode of psychosis; the assessments were done at program entry and three and six months later. They found an association between early resolution of negative symptoms and poor adherence. Patients whose positive symptoms had been resolved after three months of treatment did not show a change in adherence behavior compared to those whose symptoms persisted. On the contrary, early resolution of negative symptoms was significantly associated with less medication adherence compared to the patients whose negative symptoms persisted. Unfortunately, patients whose negative symptoms had been resolved but who were non-adherent experienced a worsening of both positive and negative symptoms at six month. Steger *et al.*[39] concluded that patients who experience a rapid reduction of negative symptoms must be closely followed, as they are at high risk for non-adherence. As a possible

explanation for these findings, the researchers stated that patients might associate reduction of negative symptoms with a return to normal functioning and, therefore, might decide that they no longer need medication. This assumption is supported by the finding of Quach *et al*.[40] whose study indicated that a high level of functioning after one year of treatment was associated with non-adherence at year two.

However, opposing findings have also been reported in the treatment adherence literature on patients with schizophrenia. A study by Tattan and Creed[41] regarding negative symptoms of schizophrenia and compliance with medication, for instance, found that patients with schizophrenia who had a poor medication compliance experienced a significantly greater severity of negative symptoms. Especially avolition, apathy, and alogia were related to poor compliance. The researchers presumed that patients who suffered from avolition and apathy would lack the motivation to regularly go to a satellite clinic. They also suggested that these patients might question the beneficial effects that the medication given at such clinics had had on their positive symptoms and might have instead focused on the limited effect that the medication had had on the negative symptoms from which they still suffer. Another point considered was that patients with alogia could possibly lack insight into their illness and, therefore, might not understand the importance of taking medication regularly. In sum, hitherto, as for depression, not much research has been conducted on the relationship between symptom severity and treatment adherence in patients with schizophrenia, and the results are conflicting.[39-41] Clearly, negative symptoms can be related to adherence, but further research is needed to gain a better understanding of this relationship.

An interesting new finding of our study is the fact that poor quality of sleep significantly predicted better treatment adherence in the group of patients with schizophrenia. This finding contradicts the finding in the study by Ong *et al*.[2] who reported that insomnia might be a risk factor for poor treatment adherence. To the authors' knowledge, this study was among the first to investigate a possible relationship between quality of sleep and treatment adherence in patients with schizophrenia; therefore, comparing this group of patients to another that has sleep disorders such as obstructive sleep apnoea and insomnia might be difficult. Possibly, the patients with a worse subjective quality of sleep were more treatment adherent because they could benefit from completing the sleep log and, hence, register their own sleeping patterns for a period of two weeks. Patients without sleeping problems might not understand the therapeutic use of registering their sleeping patterns and, therefore, might be more likely to not complete the sleep log.

In sum, treatment adherence appears to be a complex phenomenon and can only partly be associated with symptom severity and quality of sleep in patients with schizophrenia and patients with depression. Some studies indicate, for instance, that a weak therapeutic alliance and low insight might be related to poor adherence in patients with schizophrenia.[42] Another study by Spiekermann *et al*.[43] stated that patients with schizophrenia who had stronger cognitive impairments showed lower adherence behavior compared to those without cognitive impairments.

The present study has several limitations that should be discussed in order to correctly interpret its results. One limitation of our study is the fact that all data were derived from self-report inventories, which have their strengths and weaknesses.[44] However, the disadvantages, for instance, self-report measures being potentially biased by social desirability,[45] might have negatively affected the reliability of the results. Moreover, future studies might vary in their means to measure treatment adherence. For instance, attendance at a psycho-education group for patients with schizophrenia[46] could be additionally used to measure treatment adherence. Another limitation of our study is that a mixed sample of subjects, e.g., outpatients with schizophrenia and outpatients with depression, was used. Although we have presented our treatment-adherence results for the depression group and the schizophrenia group separately, having a larger sample of one patient group in order to investigate the possible relationships between symptom severity, quality of sleep, and treatment adherence would have been better because the main reasons related to non-adherence in patients with schizophrenia and those with depression may differ significantly. This is an important issue for future research. Finally, the exploratory nature of the analyses, as well as the cross-sectional study design,[47,48] used in the present study should be mentioned as further limitations, making it impossible to generalisze the results.

In conclusion, the current exploratory study revealed a significant positive relationship between symptom severity and treatment adherence, as well as a significant negative relationship between quality of sleep and treatment adherence in patients with schizophrenia, but no significant relationships between symptom

severity, quality of sleep, and treatment adherence were found in patients with depression. However, more research is needed to identify possible risk factors that can be related to treatment non-adherence to, in a second step, determine strategies to improve treatment adherence. Treatment non-adherence might be reduced, and at best prevented, if specific predictors can be related to the adherence behavior of psychiatric patients. Furthermore, identifying the characteristics of clinical patients who drop out of treatment could lead to improvements in the care these patients receive.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

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A case of Hashimoto's encephalopathy presenting with seizures and cognitive impairment

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ABSTRACT

Hashimoto's encephalopathy (HE) is a rare disease with unknown pathogenesis. An epileptic seizure is reported in association with HE. Here, the author reported an 18-year-old girl with a history of hyperthyroidism for one year. She was admitted to the hospital due to status epilepticus. Serum thyroid function test showed that the concentration of anti-thyroid peroxidase antibodies and thyroglobulin antibody were significantly elevated. Brain magnetic resonance imaging showed that multiple abnormalities varied from bilateral frontal, parietal, occipital-temporal lobe to cerebellum hemisphere. The patient's symptoms were significantly relieved after methylprednisolone therapy. At 3-month follow-up visits, she had been symptom free. HE is a diagnosis of exclusion and should be considered when evaluating a patient with cognitive dysfunction and high titers of anti-thyroid antibodies as it responds dramatically to steroids.

Key words: Hashimoto's encephalopathy; anti-thyroid antibodies; steroid responsive encephalopathy

INTRODUCTION

Hashimoto's encephalopathy (HE), also termed as "steroid-responsive encephalopathy associated with autoimmune thyroiditis", is a rare immune-mediated encephalopathic event which affects children and adolescents. It is characterized by altered mental status, seizures, and cognitive dysfunction. It was reported seizures happened in 66% of HE, among which status epilepticus in 12%.[1] We reported a young female patient with generalized, tonic-clonic seizures and cognitive impairment changes who responded well to steroid treatment.

CASE REPORT

An 18-year-old girl who suffered from status epilepticus was admitted to the hospital. She had a history of hyperthyroidism for one year, and was treated with propylthiouracil (50 mg/day). There was no family history of psychiatric diseases, seizures, or other problems. Her initial vital signs were stable. On physical examination, she was conscious, cooperative, and oriented to person and time. However, she had short-term memory and computing power loss [the mini-mental state examination (MMSE) score was 22, which was lower than normal] as well as bilateral limbs dystaxia. The white blood cell count was 7.55 × 10^9/L, hemoglobin 156 g/L, and C-reactive protein 0.09 mg/dL. Routine biochemical analyses of liver, renal, and blood glucose were all within normal limits. The serum sodium was 131 mmol/L, potassium 3.3 mmol/L, and chlorine 91 mmol/L. The cerebrospinal fluid (CSF) analysis displayed normal pressure (145 mmH_2O) and a normal cell count and protein and glucose levels. The serum and CSF of TORCH [toxoplasmosis, other (viruses), rubella cytomegalovirus, herpes (simplex viruses)] as well as the serum rapid plasma regain test, anti-human immunodeficiency virus antibody, epatitis B surface antigen and antineutrophil cytoplasmic antibody were negative. The plasma concentrations of sex hormone and cortisol were normal. Serum thyroid function test showed that triiodothyronine (T3) < 0.300 nmol/L(reference intervals 1.30-3.10 nmol/L), free triiodothyronine (FT3) < 0.400 pmol/L (reference intervals 3.10-6.80 pmol/L), upersensitive thyroidstimulating hormone (S-TSH) 0.01 mIU/L (reference intervals 0.27-4.20 mIU/L), anti-thyroid peroxidase antibodies (TPOAb) > 600.0 IU/mL (reference intervals 0.00-34.00 IU/L), thyroglobulin antibody (TGAb) 2,189.00 IU/mL (reference intervals 10.00-115.00 IU/mL). Electroencephalography showed a marked slowing of background rhythm as an indicator of encephalopathy but no activity corresponded with

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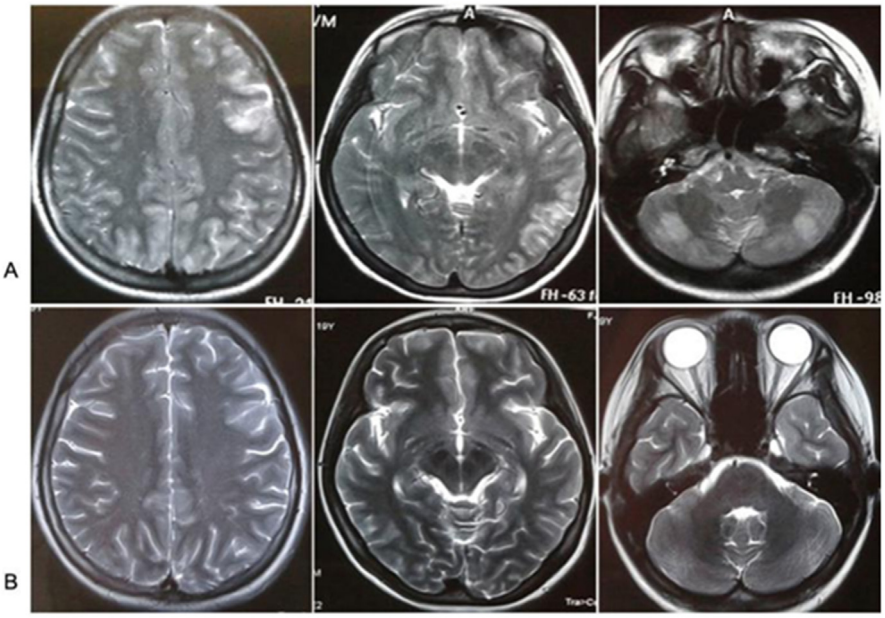

Figure 1: Brain magnetic resonance images (MRI) showed that multiple abnormalities varied from bilateral frontal, parietal, occipital-temporal lobe to cerebellum hemisphere on axial T2-weighted images (T2WI). Multiple abnormalities disappeared after steroids therapy. (A) MRI of the brain obtained before steroids therapy; (B) MRI of the brain obtained after steroids therapy

seizures. The color Doppler ultrasound examination of Thyroid and abdomen was normal. Results of CT scan of the head were unremarkable. Brain magnetic resonance images (MRI) showed that multiple abnormalities varied from bilateral frontal, parietal, occipital-temporal lobe to cerebellum hemisphere on axial T2-weighted images (T2WI) [Figure 1A]. The anticonvulsant, Tegretol (100 mg bid) was given to control the seizures. Given the indications for HE, the patient was firstly treated with intravenous injection of Dexamethasone 10 mg/day for 7 days, followed by 5 mg/day for 7 days. Finally, she was given with oral methylprednisolone 12 mg/day. Her symptoms improved siginificantly and neuropsychiatric symptoms fully resolved. Retest brain MRI showed that initial multiple abnormalities disappeared compared with initial brain MRI [Figure 1B]. Serum thyroid function test showed that T3 1.22 nmol/L, FT3 2.65 pmol/L, S-TSH 4.17 mIU/L, TPOAb 369.80 IU/mL, TGAb 1,102.00 IU/mL. On the 17th day, she was discharged on tapered doses of oral methylprednisolone. On 3-month follow-up visits as an outpatient, she had been symptom free without any seizure and MMSE score was 30.

DISCUSSION

As a rare steroid responsive neuropsychiatric syndrome, HE is associated with the serologic evidence of anti-thyroid antibodies when other causes of encephalopathy are excluded. The clinical manifestations of HE include cognitive impairment, various types of epileptic seizures, dystaxia and tremor, sleep disturbance and headache.[2] In this case, this female patient suffered from four generalized, tonic-clonic seizures and mild cognitive impairment as well as limbs dystaxia. Recently, HE has received extensive attention due to its treatability and unclear pathogenesis. In China, Hashimoto's encephalopathy is still not fully recognized because of its complex clinical manifestions and absence of specific biomarkers. The previous research demonstrated that cognitive impairment (84.6%) and psychiatric symptoms (38.5%) were the most frequent symptoms, however, seizures (30.8%) and myoclonus (7.7%) were relatively infrequent in thirteen consecutive patients with HE.[3] Therefore, presenting symptoms of HE may be quite variable.

The diagnosis of HE should be considered in patients presenting with the characteristic neuropsychiatric manifestations excluding other causes of encephalopathy. Generally, high levels of anti-thyroid antibodies in serum or CSF are important and helpful in the diagnosis of HE. They have no alteration in the CSF and/or imaging tests compatible with infectious, vascular, or neoplastic etiology, and response well to immunosuppressive therapy.[4] Non-specific electroencephalogram abnormalities are presented in the vast majority of patients, and brain MRI may display abnormalities in 49%, such as cerebral atrophy, focal cortical abnormality, diffuse subcortical abnormality and non-specific subcortical focal white matter abnormality.[5] In this female patient, the analysis of CSF as well as serum inflammation biomarkers was normal, indicating the exclusion of intracranial infection. Although the elevated CSF protein is common in HE, this change depends on the severity of the illness. Her brain MRI showed that multiple abnormalities varied from bilateral frontal, parietal, occipital-temporal lobe to cerebellum hemisphere. Neuroimaging results have no reliable diagnostic value in HE.

In the context of the typical clinical picture, high titres of antithyroid antibodies, in particular TPOAb, are diagnostic.[6] Recently, Blanchin *et al.*[7] reported that TPOAb from Hashimoto's encephalopathy patients

could bind cerebellar astrocytes in HE patients but not in Hashimoto thyroiditis patients. This may support the role of TPOAb in the pathogenesis of Hashimoto's encephalopathy. TPOAb is present in 95-100% and TGAb in 73% of patients with HE. Elevated serum level of TPOAb may be related with vasculitic type Hashimoto's encephalopathy and elevated serum levels of TPOAb and TGAb may be with diffuse progressive type of HE. However, the elevated titres of these antibodies can be tested in the healthy population. Therefore, the role of those antibodies and their pathophysiology are unknown. In addition, corticosteroid treatment is successful in most cases, which can further support the diagnosis of HE. Furthermore, other common causes of encephalopathy should be ruled out, such as intracranial infection, metabolic disease, electrolyte imbalance, poisoning or toxins, neoplasm, and the central nervous system involvement of vasculitic syndromes. With the aid of a detailed medical history and related auxiliary examination (such as CSF and MRI), it is easy to rule out these diseases.

Regarding treatment for HE, the patient's symptom improved significantly and rapidly after initiation of corticosteroid treatment, and eventually achieved a long-term stable remission. Clinical improvement with corticosteroid therapy is usually observed in the first 4-6 weeks. This positive response has been considered to be part of the definition of HE, but does not occur in all patients.[8] Other therapies such as plasmapheresis and immunosuppressant medications have been successfully used in patients non-responsive to corticosteroids. It was reported that only a few HE patients have been treated with Intravenous immunoglobulins.[9] Moreover, since the antithyroid antibodies could not been used as relapse markers of encephalopathys, the question of the continuation of the immunomodulatory of immunosuppressive drugs remains an open debate.[10]

In conclusion, HE is a rare disease associated with encephalopathy and autoimmune thyroiditis. Our research suggests that a high degree of suspicion is necessary to diagnose HE, especially in those patients with high levels of antithyroid antibodies and presented with unexplained encephalopathy, such as seizures and cognitive dysfunction, as in our case. Corticosteroid treatment is successful for HE in most cases, however, clinicians should be aware that relapses can occur early or even late after tapering of steroid use; therefore, a long follow-up period should be recommended.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

OAE Publishing Inc.

Good recovery of a patient with neurocysticercosis using two antihelminthic drugs combined with steroid

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ABSTRACT

Neurocysticercosis is the most common parasitic infection of the central nervous system. We present a case report of a neurocysticercosis patient with multiple cysts, who presented with new onset generalized tonic-clonic seizures. A 4-cycle treatment of 2 different antihelminthic drugs with dexamethasone and sodium valproate led to clinical improvement without any adverse reactions. The manifestations of neurocysticercosis are protean and the diagnosis should be considered whenever multiple cysts are seen on computed tomography or magnetic resonance imaging. The antihelminthic treatment of neurocysticercosis should be individualized, especially for patients with multiple cysts.

Key words: Neurocysticercosis; parenchymal neurocysticercosis; neurocysticercosis diagnosis; antihelminthic drug

INTRODUCTION

Neurocysticercosis has become a serious public health concern, especially in developing countries where the prevalence rate reaches 4%.[1] Neurocysticercosisis caused by the larval form of the tapeworm Taenia solium grow in cerebral parenchyma, ventricles and subarachnoid space, and is the leading cause of acquired epilepsy. Therapeutic measures include surgery, symptomatic therapy and antihelminthic drugs such as albendazole and praziquantel.[2] Evidence supporting existing treatment guidelines for neurocysticercosis is inadequate although there has been a comparison between the efficacy of albendazole and praziquantel.[2]

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CASE REPORT

A 45-year-old male was brought to a local hospital with a generalized tonic-clonic seizure which self-terminated after 2 min. Further such seizures recurred over the following 20 days. There was no headache, dizziness or vomiting. His weight was 80 kg. On examination of the central nervous system, higher mental functions were normal (30/30 mini-mental state examination). All cranial nerves were intact. All muscles tone and power, deep tendon and planter reflexes were all normal. The general examination was normal. The first brain magnetic resonance imaging (MRI) [Figure 1], undertaken at the local hospital, showed multiple large spherical viable cysts distributed evenly throughout the brain parenchyma, that were hyperintense (with respect to brain parenchyma) on the T2-weighted scan and

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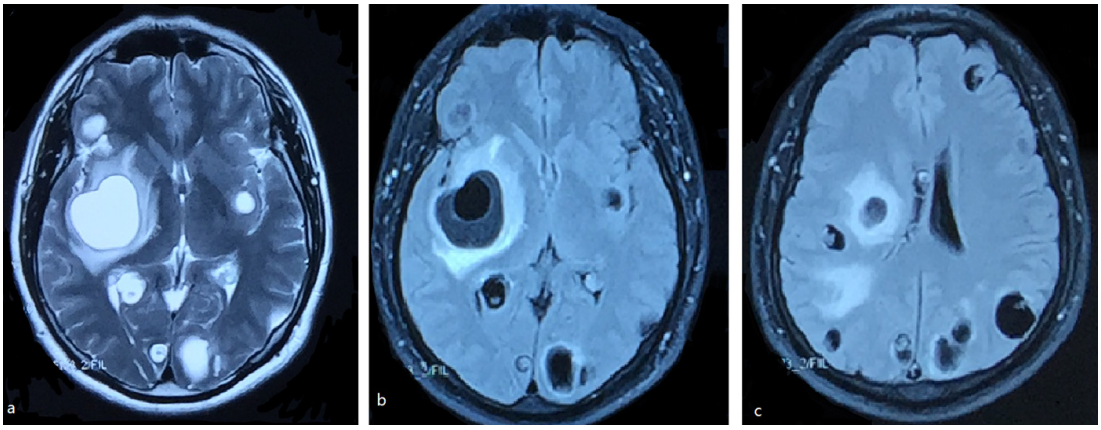

Figure 1: Brain magnetic resonance imaging from the local hospital showed multiple large cysts accompanied by perilesional oedema, in the presence of scolex (a: T2-weighted image; b and c: Flair image)

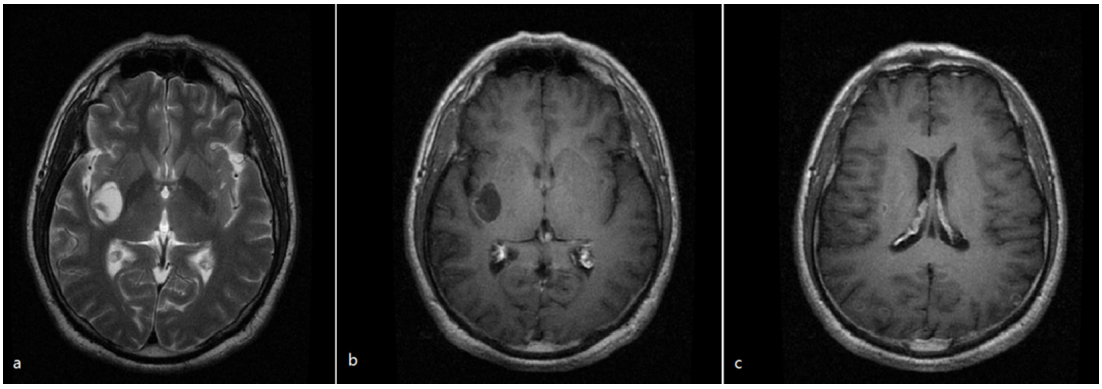

Figure 2: Magnetic resonance imaging (MRI) after 2 cycles of treatment with albendazole. (a) T2-MRI and gadolinium-enhanced MRI; (b) showed a single large cyst with cerebrospinal fluid-like signal in the right basal ganglia region; (c) gadolinium-enhanced MRI showed some ring-like or nodular contrast enhancements in occipital lobe

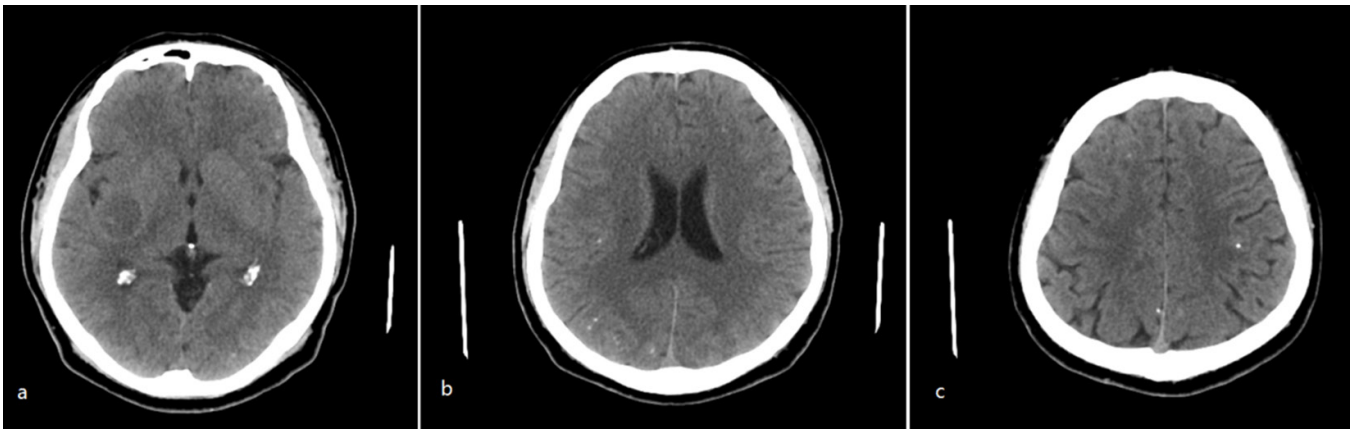

Figure 3: Computed tomography after 2 cycles of treatment with albendazole. There was a single large hypointense cyst in the right basal ganglion region (a) and the resolution of other cysts associated with calcification around the lateral ventricles (b) and in the cerebral cortex(c)

hypointense on the T1-weighted scan with ring enhancement. The patient was transferred to the Beijing Tiantan Hospital for diagnosis and treatment. Examination findings remained unremarkable. Blood cell counts and erythrocyte sedimentation rate were within the normal range. Cerebrospinal fluid analysis revealed an antibody of Taenia solium. On this basis, the patient was diagnosed with neurocysticercosis. Dexamethasone 20 mg/day was initially used and reduced to 10 mg/day after 6 days. Later, sodium valproate (500 mg bid) was added. Albendazole was given at a dose of 400-1,200 mg/day as the antihelminthic treatment for 17 days. After 1 month of treatment, the generalized seizures remitted and the patient's condition improved. He was discharged on day 23.

At follow-up 6 months later, the patient started to use albendazole again at a dose of 400 mg/day for 2 days, then added to 800 mg/day for 4 days, finally increased to 1,200 mg/day for 10 days. At 2 months, clinical recovery was accompanied by neuroimaging improvement. Most cysts have reduced in diameter, and 1 large cyst persisted in the right basal ganglia. These lesions showed ring-like or nodular contrast enhancement on brain MRI [Figure 2] and calcification on the brain computed tomography [Figure 3].

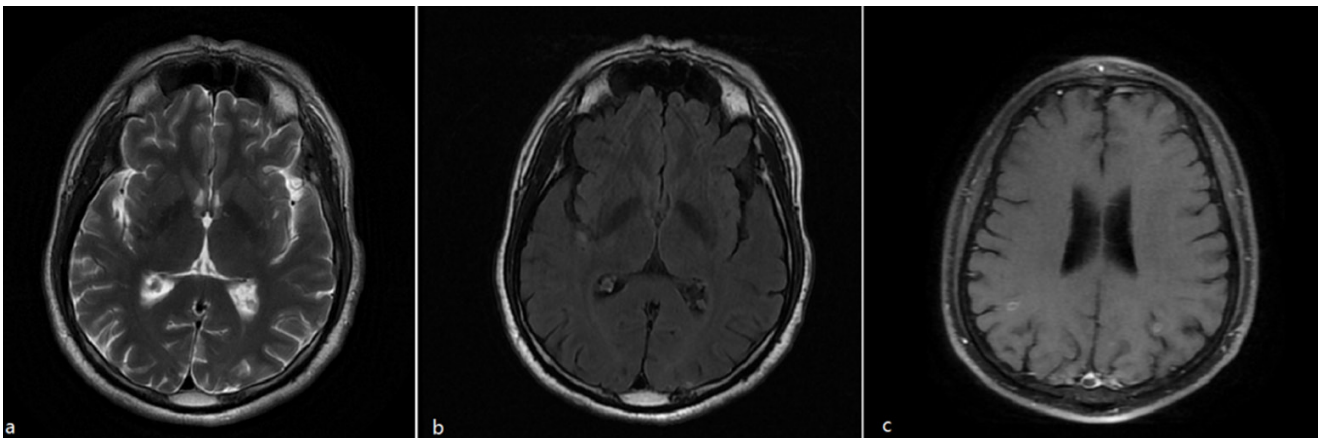

Figure 4: Magnetic resonance imaging at the end of treatment. All cysts were resolved on T2-weighted image (a) and Flair image(b). But a few ring-like or nodular contrast enhancements in the occipital lobe were found on gadolinium-enhanced MRI

Sixteen months after the original presentation, the patient was re-admitted to Beijing Tiantan hospital after a generalized tonic-clonic seizure and reporting formication with parasthesia on the left limbs for 2 months. Instead of albendazole, he was given praziquantel at an initial dose of 400 mg/day increasing to 1,800 mg/day (400 mg/day for 1 day, 600 mg/day for 4 days, 1,200 mg/day for 9 days and 1,800 mg/day for 2 days). He responded with no further seizures. 2 months after the therapy, the brain MRI revealed that the large right basal ganglia cyst had reduced in size.

He went back to our hospital after 4 months for further evaluation of his illness. We suggested another cycle praziquantel (600 mg/day for 3 days and 1,800 mg/day for 10 days). Then he was in a stable status and discharged. A brain MRI [Figure 4] showed complete resolution of the large cyst. He continued on the sodium valproate.

DISCUSSION

The clinical manifestations of neurocysticercosis are poorly specific without a "typical" syndrome owing to the variable factors including the number, type, size, localization of cysticerci, as well as the host immune response against the parasite. Our patient presented with a generalized tonic-clonic seizure, which in case series is the most frequent clinical manifestation of intraparenchymal neurocysticercosis.[3] Seizures secondary to neurocysticercosis are attributed to the local cortical inflammation arising from the death of the cysticercus, or gliosis associated with end-stage calcified lesions. A literature review states that 50% of patients with neurocysticercosis develop recurrent seizures.[4] According to the major diagnostic criteria, neurocysticercosisis diagnosed in the presence of suggestive lesions on neuroimaging studies (images of cyst and scolex) and positive serum anticysticercal antibodies detected by enzyme-linked immuno sorbent assay.[5] Our patient's earliest brain MRI was typical for neurocysticercosis, but it was unrecognised by the local hospital. Delayed diagnosis contributes to extensive greater burden of neurological and psychiatric morbidity, and so awareness of neurocysticercosis should be raised especially in regional primary hospitals. The detection of antibodies to Taenia solium glycoprotein antigens can support the diagnosis but, where negative, does not exclude neurocysticercosis.

Management of neurocysticercosis includes surgery, symptomatic therapy and antihelminthic drugs. The patient's outcome is evaluated according to the improvement of symptoms and lesion alleviation on neuroimaging studies such as the disappearance of perilesional oedema and reduction of number of viable cysts. There is no standard definition of what constitutes a resolved cyst. In accordance with a recent randomised controlled trial,[6] it is accepted that the absence of discernible hyperintense contents on T2 MRI could be the final marker of parasite degeneration. In the case presented, the MRI scans were undertaken approximately 2 months after the end of each cycle of treatment to evaluate the effect of the treatment. Surgery is thought primarily as an option for treating intraventricular cysts and so was not indicated in this case. Symptomatic therapy included antiepileptic drugs and corticosteroids. For our patient, we gave dexamethasone in order to attenuate the parenchymal inflammatory reaction, and sodium valproate to control the clinical seizures. Then we added an antihelminthic drug. Albendazole is a broad-spectrum antihelminthic drug. According to a recent meta-analysis of randomized trial, albendazole use is associated with resolution of active cysts and fewer generalized seizures in patients with parenchymal neurocysticercosis.[7] It is also reported that albendazole is particularly indicated for symptomatic patients presenting with multiple viable brain parenchymal cysticerci. Compared to patients with extraparenchymal lesions, albendazole is more effective in patients with parenchymal lesions.[8,9] The

usual recommended dose for albendazole is 15 mg/kg per day divided into two doses every 12 h for 1 week while the duration of antihelminthic therapy varies.[10] In this case, our patient started on albendazole at a low dose when he had multiple parenchymal cysts. After a 2-cycle treatment of albendazole, the perilesional oedema faded away, the number of large cysts reduced to one, and most of the cysts became calcified nodules. In contrast with most neurocysticercosis cases, where only one type of antihelminthic drugs is given, we utilized praziquantel to target a single unremitting large cyst. Praziquantel can destroy cysticercus cysts in parenchymal brain by changing the metabolism of intracellular calcium. It has been pointed out that praziquantel is favoured in patients with lower cyst burdens, while the multiple cysts barely changed after short course of praziquantel introduced by Pretell *et al*.[11] In contrast to albendazole, praziquantel is associated with more frequent adverse reactions (e.g. headache and vomiting). However, after another 2-cycle treatment of praziquantel in this case, the large cyst in the right basal ganglia region had dramatically resolved without adverse drug reactions. Recent studies about the relative efficacy of these two antihelminthic drugs seemed to demonstrate that albendazole might be the drug treatment of first choice in neurocysticercosis. While praziquantel has been shown to eliminate 60-70% of parenchymal brain cysts, albendazole has been found to destroy 80-90% of them. A meta-analysis revealed that albendazole was more effective than praziquantel regarding the clinical outcomes of patients with neurocysticercosis.[8] A randomised controlled trial also showed the increased antihelminthic efficacy of an albendazole plus praziquantel regimen, and that further seizures were less frequent in individuals with complete cyst resolution after antihelminthic treatment.[6] On the basis of those studies and this case report, we believe that the antihelminthic therapy should be rationally and individually based on different circumstances and that albendazole and praziquantel have equivalent efficacy at different stages of the disease. We suggest that the combination antihelminthic treatment of albendazole and praziquantel has potential benefits for patients with multiple cysticercus cysts in brain.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

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Status epilepticus in scleromyxedema

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ABSTRACT

Scleromyxedema is a rare dermatologic disorder, characterized by erythematous or yellowish lichenoid waxy papules. Neurological manifestations are rare but well-recognized. A 51-year-old woman, diagnosed with scleromyxedema, was admitted to the hospital with status epilepticus, caused by brain lesions, as disclosed in a brain magnetic resonance imaging (MRI). The patient was treated with anticonvulsants and corticosteroids and gradually recovered fully. A complete remission of the lesions was shown in a follow-up brain MRI. In cases with scleromyxedema and the presence of neurological manifestations, we need to pay attention to central nervous system involvement, especially when combined with brain MRI lesions, and treat the patient appropriately.

Key words: Scleromyxedema; epilepsy; status epilepsticus; dermatologic disorder

INTRODUCTION

Scleromyxedema is a rare dermatologic disorder, characterized by erythematous or yellowish lichenoid waxy papules. Neurological manifestations are rare but well-recognized in patients with scleromyxedema, among other extracutaneous complications.[1-4]

We report a patient with scleromyxedema presenting with refractory status epilepticus, caused by brain lesions, probably related to the disease.

CASE REPORT

A 51-year-old right handed Caucasian woman was admitted to the hospital with subacute onset of confusion, slurred speech and expressive aphasia that had developed in a period of 3 days. The patient had been diagnosed with scleromyxedema for 15 years and had been under regular follow up and monthly administration of immunoglobulin. Her general physical examination at admission showed changes in the cutaneous presentation of her scleromyxedema. This patient presented with diffuse confluent papulosquamous eruption and thickening of the skin on the face, body, arms and the legs with impaired joint mobility of her fingers, perhaps as a consequence of the skin thickening associated with her scleromyxedema. With regard to the neurological manifestations, aphasia and bilateral pyramidal weakness were found.

The patient became rapidly disorientated and developed generalized tonic-clonic seizures leading to convulsive status epilepticus. Intravenous diazepam and phenytoin (according to status epilepticus treatment protocol) was administered and status epilepticus was not resolved and general anesthesia and intubation in the intensive care unit was required to achieve seizures control.

Using magnetic resonance imaging (MRI), low signal intensity on T1-weighted images and high signal intensity on T2-weighted and fluid-attenuated inversion recovery images of the brain revealed bilateral fronto-parietal and left fronto-occipital cortical lesions [Figures 1 and 2]. Furthermore, "hyperintense vein sign" was observed in right frontal area, compatible with slow flow in isolated cortical veins [Figure 1]. After intravenous gadolinium administration, no abnormal enhancement was revealed [Figure 2].

Following a lumbar puncture, mildly elevated protein

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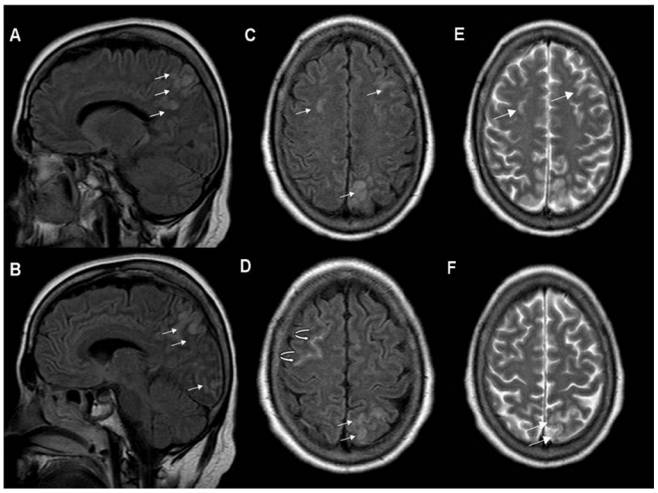

Figure 1: Sagittal fluid-attenuated inversion recovery (FLAIR) (A, B), axial FLAIR (C, D) and T2-weighted images (E, F) revealed multiple focal cortical areas of high signal intensity fronto-parietal and occipital (arrows) and "hyper-intense vein" compatible with slow flow in isolated cortical vein (curved arrow).

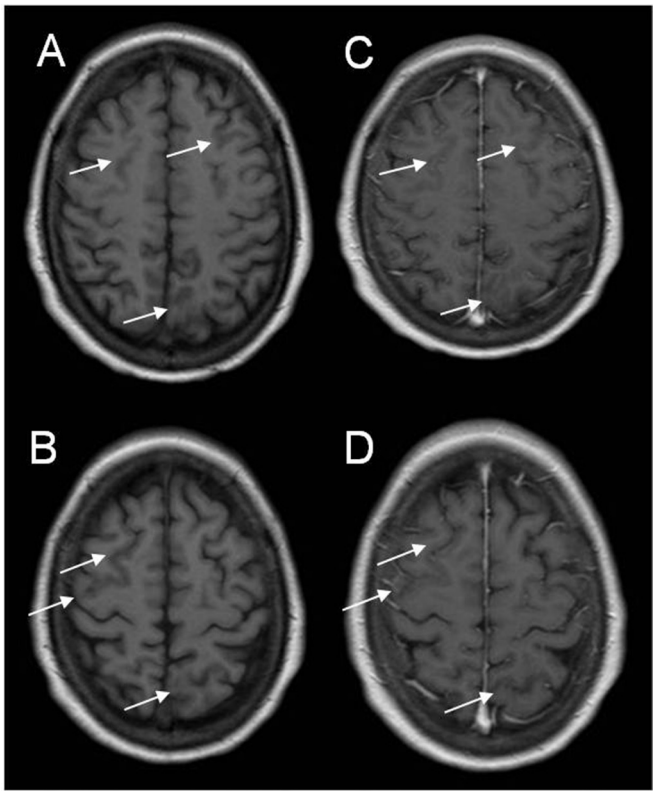

Figure 2: Axial T1-weighted images before (A, B) and after intravenous gadolinium administration (C, D) showed cortical areas with low signal intensity (arrows) without abnormal enhancement.

(TPR 77 mg/dL) was recorded and all other constituents of cerebrospinal fluid (CSF) were found within normal ranges. All CSF stains for microorganisms and polymerase chain reaction assays for herpes simplex virus type 1 (HSV-1), HSV-2, varicella-zoster virus, human cytomegalovirus and Epstein-Barr virus were also negative. Additional laboratory tests disclosed no other significant findings apart from monoclonal immunoglobulin G (IgG) gammapathy with lambda light chains and excessive proteinuria.

The patient remained under general anesthesia for 48 h.

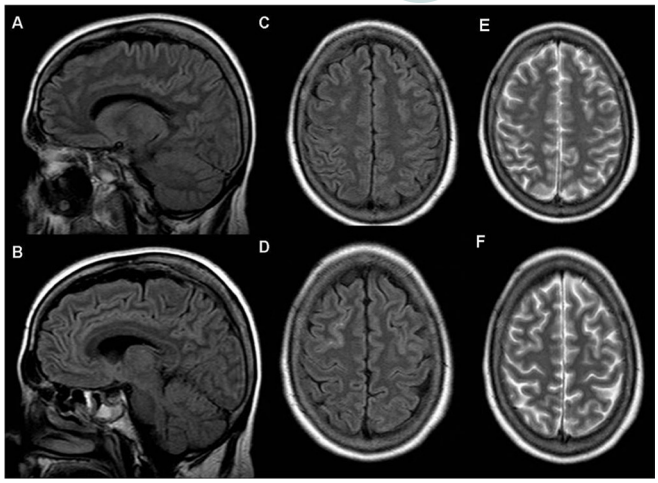

Figure 3: Sagittal fluid-attenuated inversion recovery (FLAIR) (A, B), axial FLAIR (C, D) and T2-weighted images (E, F) one month later, showed near total remission of the lesions.

Electroencephalogram after this 48 h-period showed that status epilepticus was resolved and demonstrated spikes followed by slow waves, over the left fronto-parietal region. The patient was transferred to the department of neurology under treatment with levetiracetam and corticosteroids (40 mg of dexamethasone). She demonstrated a gradual recovery and over the following week her language and motor function improved dramatically and the dosing of corticosteroids were reduced with decrements of 10 mg every 3 days.

The patient was discharged, after 25 days of hospitalization, without any neurological semiology. The follow up brain MRI one month later demonstrated almost complete remission of signal abnormalities [Figure 3]. The anticonvulsant treatment was tapered of (redcued) over a three month period without any further seizure occurrence.

DISCUSSION

Scleromyxedema also known as lichen myxedematosus or papular mucinosis is a rare skin disorder involving the face and the extremities that is characterized by erythematous or yellowish lichenoid waxy papules.[1,2] The pathogenesis of scleromyxedema remains unclear.[5] A proliferation of fibroblasts, deposition of acid mucopolysaccharides in the upper dermis and a monoclonal paraproteinemia-most often IgG monoclonal gammopathy are the predominant findings.[2,5] Extracutaneous manifestations are due to cardiovascular, rheumatologic, renal, hematologic and neurologic involvement.[1-4] Encephalopathy, transient focal neurological disturbances, progressive cognitive decline, seizures, peripheral neuropathy, carpal tunnel syndrome and myopathy are included in the neurological manifestations.[2,4] They could be partially explained by the paraproteinemia and the disruption of the blood-brain barrier with elevated CSF protein concentration in the central nervous

system (CNS) disorders or by the deposition of mucopolysaccharides within the nerve and muscle fibers in peripheral nervous system conditions.[2,3] The treatment of CNS involvement in scleromyxedema patients includes steroids, plasmapheresis, intravenous immunogloblin and chemotherapeutic drugs in various combinations.[4]

Brain imaging in patients with CNS involvement rarely shows lesions responsible for this condition and in the rare cases with lesions demonstrated, spontaneous remission remains unlikely.[2,4] In conclusion, in cases with scleromyxedema that present with neurological manifestations, we should consider central nervous system pathophysiology and evaluate possible bsrain abnormalities with detailed magnetic resonance images and treat the patient appropriately.

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Conflicts of interest

There are no conflicts of interest.

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Review

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Current and emerging therapies for neuromyelitis optica

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ABSTRACT

Neuromyelitis optica (NMO) is an autoimmune demyelinating disease that mainly affects the optic nerve and spinal cord, potentially resulting in blindness and paralysis. Once thought to be a clinical variant of multiple sclerosis, NMO is currently considered as a different disease with its own features due to the identification of a specific autoantibody against aquaporin 4. Given the high risk of disability, treatment should be launched once the diagnosis is established. Evidence from clinical practice showed that traditional immunosuppressive agents affecting the function of T and B cells could attenuate disease exacerbation. Recently, with better understanding pathogenesis of NMO, increasing bodies of novel therapies and therapeutic targets have been discovered. In this review, the authors discuss the current strategies of treating NMO in details and briefly introduce the potential therapies in future.

Key words: Neuromyelitis optica; aquaporin 4; therapies

INTRODUCTION

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease of the central nervous system (CNS) characterized by recurrent optic neuritis and transverse myelitis. A relapsing-remitting clinical course was observed in the most patients. Historically, NMO was thought to be a variant of multiple sclerosis (MS). Given its distinct clinical features, radiological changes and the identification of the autoantibody targeted to aquaporin 4 (AQP4),[1] it is now believed to be a different disease with its own diagnostic criteria, prognosis and treatment.

The epidemiology information of NMO is limited because of its rarity and commonly confusing with MS. Estimated incidence and prevalence of NMO ranges from 0.05-0.4 and 0.1-4.4 per 100,000 respectively.[2-5] It has a preference in non-Caucasian women.[6] The median age of disease onset is between 34-43 years, while children and elder people are also affected.[7] Unlike MS, patients with NMO are more possibly associated with other autoimmune diseases, like systemic lupus erythematosus, Sjogren's syndrome,[7] suggesting these patients may have a genetic tendency to autoimmune.

AQP4-IgG, a sero-biomarker of NMO, distinguishes NMO from other demyelinating disorders and was incorporated into the 2006 diagnostic criteria of NMO,[8] broadening the spectrum of NMO. In 2007, neuromyelitis optica spectrum disorder (NMOSD) was termed to encompass patients who have detectable serum AQP4-IgG but do not fully meet the diagnostic criteria (e.g. first-atack LETM, typical NMO with brain lesions, NMO with other autoimmune diseases).[9] In 2015, the international panel for NMO diagnosis unified the term of NMO and NMOSD, and developed diagnostic criteria for seropositive and seronegative NMOSD, respectively.[10]

The pathogenesis of NMO still remains ambiguous. It is currently considered that AQP4-IgG is of great importance in triggering NMO. AQP4 is a transmembrane protein expressed by astrocytes and controls the flow of water in cells.[11] Upon the penetration through the blood brain barrier (BBB) and binding with AQP4 on perivascular astrocyte end feet, AQP4-IgG activates the classical complement pathway

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which injured astrocytes, followed by marked NK cell, granulocyte and monocyte infiltration along with BBB breakdown, oligodendrocyte death and demyelination, microglia activation and even neuronal apoptosis.[12] Unfortunately, the mechanism of AQP4-IgG generation remains unclear; predictions include AQP4 molecular mimicry and infection.[13,14] Although T cells are rare in the CNS lesions, recent studies indicate that some T cell subsets may also involve in the pathological process. Th17 cells[13] and follicular helper T cells (Tfh)[15] significantly increase during the relapse period, which are critical for breaking down BBB and antibody production. Further investigation into the pathogenesis mechanism of NMO may facilitate the development of more therapeutic targets.

CURRENT THERAPIES OF NMO

Since NMO has a relapsing-remitting clinical course, the treatment of NMO is divided into two stages: management of acute attacks and prevention of future relapses. The main aims of acute therapies are to suppress the inflammatory response, minimize the irreversible neurological damages and accelerate recovery, whereas preventive therapies are designed to reduce relapse frequency. Treatment strategies for these two stages are discussed as follows.

Therapies of acute attacks

The most typical treatment for an acute attack of NMO is the administration of intravenous methylprednisolone (IVMP). Glucocorticoids exert profound anti-inflammatory and immunosuppressive effects, including down regulation of pro-inflammatory cytokines, reduction of adhesion molecules and matrix metalloproteinases, prevention of apoptosis of mononuclear cells and restoration of the BBB integrity.[16] Up to now, there have been no prospective clinical trials for acute treatment of NMO, meaning that the reliable dosages and duration have not been reviewed. Methylprednisolone is commonly prescribed for 1,000 mg per day for 3-5 days, followed by prednisone starting from 60-100 mg per day. If patients demonstrated no improvement after IVMP, plasma exchange (PE) should be considered. PE is recommended as a second-line therapy for refractory patients, accelerating the removal of antibodies, complements, pro-inflammatory cytokines and chemokines. Generally PE is performed daily or every other day for five cycles each of which remove 1.0-1.5x volumes of plasma.[17] Retrospective studies and case series have reported that the total remission rate of PE therapy was between 44-75%.[18] Male patients and early initiation therapy were associated with better improvement.[18] If patients demonstrated promising improvement after PE, it can be considered as the first-line therapy for the next acute attack. The treatment with intravenous immunoglobulin (IVIg), which is an ideal alternative for PE in other neurological disorders such as myasthenia gravis, multiple sclerosis and Guillain-Barré syndrome, in acute NMO has also been reported;[19,20] however, the improvement of the prognosis of steroid refractory individuals has not been clearly demonstrated, indicating IVIg therapy may need further investigation in acute NMO.

Prevention of acute attacks

Given its high recurrence rate and severe morbidity associated with one relapse, long-term immunosuppressive treatment should be instituted right after the recovery of the first attack. Due to its low incidence, data of prospective randomized controlled trials of preventive agents are still limited. For developing a treatment protocol, the effects of prior treatment, the short-term and long-term side effects, other system complications and even economic status of patients should be considered intensively.

Azathioprine

Azathioprine is shown to be the most popular oral immunosuppressive agent currently used in NMO treatment, which can effectively disrupt purine synthesis and inhibit the proliferation of T and B cells. A large retrospective study including 99 patients with NMO or NMOSD demonstrated that the annualized relapse rate (ARR) decreased from 2.20 to 0.52 during a median treatment interval of 22 months.[21] The usual initial dose is 50 mg/day, and subsequently increased to 2-3 mg/kg per day, which provides better improvement. As azathioprine may take full effect only after 3-6 months, oral steroids should be used simultaneously (1 mg/kg per day). A recent prospective trial has showed azathioprine plus low dose corticosteroid distinctly reduced the ARR (from 0.923 to 0) of the NMO patients in Southern China and 57.1% of patients were relapse-free during a median follow-up of 20 months.[22]

Mycophenolate mofetil

Mycophenolate mofetil is a potent immunosuppressive drug that inhibits the inosine monophosphate dehydrogenase and suppresses the proliferation of T and B cells. It has been demonstrated in a retrospective case series of 24 patients (median dose 2 g daily) that mycophenolate mofetil decreased the ARR from 1.28 to 0.09 over a median follow-up of 28 months, but the expanded disability scale score (EDSS) remained almost unchanged (6.0 pretreatment *vs.* 5.5. post-treatment).[23]

Mitoxantrone

Mitoxantrone, an inhibitor of topoisomerase II, is

firstly used in treatment acute leukemia and other malignancies. The potent ability of suppressing the development of some lymphocytes, especially B cells, guaranteed the therapeutic effect in treatment of some autoimmune diseases, such as aggressive relapsing MS. It was reported in 2006 among 5 patients with NMO.[24] Although 2 patients experienced relapse during a 2-year follow-up period, 4 of them benefited from the treatment (12 mg/m^2 monthly for 6 months and 3 cycles at 3-month interval for maintenance) and 3 gained long-term remission. In another case, Kim *et al.*[25] reported that 20 NMO patients infused with methotrexate (3-6 monthly cycles of 12 mg/m^2 followed by 6-12 mg/m^2 maintenance doses) showed a reduction of ARR (2.8 to 0.7) and EDSS (5.6 to 4.4).

Methotrexate

Methotrexate inhibits purine and thymidylate synthesis. A retrospective case of 14 NMO/NMOSD patients treated with methotrexate at a median dose of 17.5 mg weekly showed a significant decrease of ARR from 1.39 to 0.18. However, 13 out of 14 patients were co-treated with other immunosuppressive agents: oralprednisolone (n = 11), rituximab (n = 1), or tacrolimus (n = 1), and the impact of these remains unknown.[26]

Oral corticosteroids

In addition to intravenous administration of high dose for acute exacerbation, low dose of oral corticosteroids have been shown to be effective in maintaining long-term remission. A cohort of 25 Japanese patients, receiving low-dose prednisolone (2.5-20.0 mg daily) as monotherapy was retrospectively evaluated in a median period of 19.3 months. Results showed that ARR declined from 1.48 to 0.49. Meanwhile, this study indicated the therapeutic effect was dose-dependent. Patients receiving less than 10 mg/day were more susceptible to relapse.[27]

Rituximab

Rituximab (RTX) is a chimeric mouse/human anti-CD20 monoclonal antibody that specifically depletes peripheral CD20-positive B cells. This antibody binds to the surface antigen on B cells and activates complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity. It was firstly used in the treatment of lymphomas and leukemia. Recently, RTX has been reported to be effective in treating certain autoimmune disorders such as rheumatoid arthritis.[28] RTX potently suppresses autoimmune reaction by blocking the differentiation of B cells into plasma cells, antigen expression and release of cytokines simultaneously. Cree *et al.*[29] firstly reported that RTX is refractory to other immunosuppressive agents in an open labeled trial of treatment of NMO. In this study, RTX was infused weekly at 375 mg/m^2 for 4 weeks followed by 1,000 mg reinfusion if CD19+ B cells were detectable in the peripheral blood. Positive curative effect was observed in all cases with significant reduction of ARR (2.6 to 0) and EDSS (7.5 to 5.5). In another 5-year prospective study, Kim *et al.*[30] reported that 26 of 30 patients benefited from the treatment with a reduction of ARR and stabilization of EDSS 18 patients totally recovered in 24 months.

Dose-dependent strategies have been exploded in the treatment of NMO. Generally, there are two different regimens: one is discussed above in Cree's study while the other one is 1 g infusion at an interval of 2 weeks for twice. The dose of RTX was adopted from the treatment of B-cell malignancies which is considerably expensive and has a high probability of occurrence of side effects. A Chinese group reported that repeated infusion with reduced dosage of RTX to five patients with NMO/NMOSD was very effective in modulation of peripheral B cells and prevention of relapse.[31] The regimen was 100 mg per week for consecutive 3 weeks. One hundred milligram of RTX was reinfused when the percentage of the B cells circulation reached 1%. None of the 5 patients experienced relapse during the 1-year follow-up period. The median EDSS score slightly decreased from 4.5 to 4, while all the patients got a significant functional improvement after therapy. Despite the fact that this study indicated that responsiveness may achieve in a lower dose in Asian patients, further investigations with a sufficient number of patients are needed.

OTHERPROMISING THERAPIES

Complement-targeted therapy

As discussed above, complement activation pays an important role in the pathogenesis of NMO. Eculizumab, a humanized monoclonal anti-complement C5 antibody, inhibits its cleavage by C5 convertase and subsequently blocks the complement cascade. In an open label clinical trial of 14 patients, intravenously administration of 900 mg eculizumab every 2 weeks significantly reduced ARR and improved the patients' neurological deficits.[32] Twelve of 14 patients got no relapse after 12-month treatment, however one patient got meningococcal sepsis and sterile meningitis. In spite of this preliminary finding, the efficacy and safety of eculizumab should be further reviewed.

C5 is the key molecule of all the three complement pathways. The infectious adverse effect above was likely due to the inhibition of the alternative and lectin

pathways that are important in eliminating microbes. C1 is only involved in the classical pathway; inhibition C1 not only blocks the cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) effect, but also saves the anti-infection function of the alternative and lectin pathway. It has been discovered that C1-inhibiting monoclonal antibody is sufficient for preventing NMO in mouse models.[33] Another small-scale phase I clinical trial reported C1 esterase inhibitor significantly reduced the EDSS score of patients with NMO, and no obvious side effects showed up.[34] So the selective inhibition of the classical complement pathway is hypothesized as another more promising and safer complement-targeted therapy.

Inhibition of IL-6-IL-6 receptor signaling

A few studies have indicated that interleukin 6 (IL-6), increasing in NMO during relapse phase, is a potent driver of NMO relapsing to enhance the survival of plasma blast and secretion of AQP4-IgG.[15,35-37] Tocilizumab, an IL-6 receptor blocking antibody, reduced the survival of AQP4-IgG-secreting plasma blast and reduced NMO relapse. A few case reports showed that tocilizumabhasa positive impact on the patients' condition due to its ability of suppressing the CD20-plasma cells and decreasing the titer of AQP4-IgG,[38-40] suggesting tocilizumab is a promising alternative therapy for aggressive, immunosuppressive agents-refractory patients. Clinical trials in large scale are needed to examine the effectiveness and safety of tocilizumab in NMO treatment.

Restoring stability of blood brain barrier

Elevated level of albumin was detected in the CSF of patients with NMO during relapse, indicating the stability of BBB is damaged in the acute phase of NMO.[41] A few studies reported serum from AQP4-IgG positive patients increases the permeability of artificial BBB and reduces the expression of tight junction proteins.[42,43] Although the specific mechanism still remains unclear, majority of studies strongly suggested that the vascular endothelial growth factor (VEGF)[42] and matrix metalloproteinases[44] were involved. Currently the anti-VEGF monoclonal antibody, bevacizumab, is under clinical review of the clinical benefits in restoring the stability of BBB in NMO patients.[45]

Restraining the activity of neutrophils

Large number of neutrophils was detected in the perivascular lesions in both human cases of[46] and animal model[47] of NMO. Sivelestat, a neutrophil protease inhibitor, has been demonstrated with the inhibitory effect on migration of neutrophils and reductive effect on neutrophil-related tissue damages in a NMO model of mouse.[47] The authors further observed that neutrophils enter the CNS within 24 h after onset,[47] suggesting that Sivelestat may be effective in the treatment of acute NMO.

Eosinophils infiltration into the demyelinating lesions is another pathological hallmark of NMO.[48] Degranulation of mouse bone marrow-derived eosinophils significantly enhanced the AQP4-IgG-mediated ADCC and CDC effect in cultured spinal cord slices, indicating eosinophils granules are involved in the pathogenic process of NMO.[48] In the same study, cetirizine, the second generation of antihistamine, reduces tissue damage in a NMO model of mouse, which may be another option to control NMO.[48]

CONCLUSION

Corticosteroids, azathioprine, mycophenolate mofetil and rituximab are still the first-line therapy for NMO. With the dramatically increasing knowledge of NMO, a variety of novel therapies are under test and development, including complement-targeted therapy, IL-6 targeted therapy, restoring the stability of BBB and granulocytes inhibition, *etc.* The ultimate goal of NMO therapeutics is to develop a highly selective, low side effect therapy. This is challenging since clinical experience of treating NMO, a rare disease, is limited. So multiple-center, large-scale randomized controlled trials for treatment-naïve patients are needed to review the efficacy of these therapeutic approaches.

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Conflicts of interest

There are no conflicts of interest.

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Review

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Statins in acute neurologic disease: which one, which dose, when to start, and when not to stop

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ABSTRACT

Statins could have physiologic properties that may benefit patients that have been diagnosed with various acute neurological diseases. This review aims tosummarize the literature pertaining to stain use in acute neurological disease such as subarachnoid hemorrhage, intracerebral hemorrhage (ICH), cerebral ischemia (CI), traumatic brain injury, status epilepticus and meningitis. The authors reviewed published abstracts and manuscripts pertaining to experimental and clinical trials relevant to statins in acute neurological disease. Although acute statin therapy in the setting of subarachnoid hemorrhage might reduce delayed cerebral ischemia and mortality, it should not be considered standard care at this time. Acute statins therapy has not demonstrated anybenefit yet following an ICH or CI. Acute statin withdrawal may worsen outcome in acute CI. Observational and case-control studies suggest that pretreatment with statin at time of onset may be associated with better outcomes. Even though preclinical studies have shown statins to have beneficial effects, there has been no clinical evidence. In conclusion, current published studies have not shown that acute statin therapy has any benefical effects in acute neurologic diseases and therefore further large randomized clinical trials are needed.

Key words: Statin; dyslipidemia; stroke prevention; subarachnoid hemorrhage; intracerebral hemorrhage; cerebral infarction

INTRODUCTION

The drug class of potent inhibitors of cholesterol biosynthesis called the 3-hydroxy-methyglutaryl coenzyme A reductase inhibitors, are also commonly referred to as statins.[1] They are classified as a therapeutic class of lipid lowering agents and are established in the primary and secondary prevention of vascular diseases. Recent experimental and clinical evidence suggests that statins have cholesterol

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Table 1: Summary the published clinical studies for statin with subarachnoid hemorrhage

Study	Size (S:P)	Statin therapy	Initiation	Duration	Vasospasm	DCI	Poor outcome	Mortality
Randomized controlled trials								
Tseng *et al.*[15]	80 (40:40)	Pra 40 mg qd	72 h	14 days or discharged	43% *vs.* 63% ($P = 0.006$)	5% *vs.* 30% ($P < 0.001$)	43% *vs.* 53% ($P = 0.7$)	5% *vs.* 20% ($P = 0.04$)
Lynch *et al.*[16]	39 (19:20)	Sim 80 mg qd	48 h	14 days	26% *vs.* 60% ($P = 0.03$)	26% *vs.* 60% ($P = 0.03$)	N/A	N/A
Chou *et al.*[17]	29 (19:20)	Sim 80 mg qd	96 h	21 days or ICU discharged	68% *vs.* 50% ($P = 0.24$)	37% *vs.* 50% ($P = 0.41$)	63% *vs.* 50% ($P = 0.41$)	0% *vs.* 15% ($P = 0.23$)
Observational cohort study								
Kramer *et al.*[21]	150 (71:79)	Sim 80 mg qd (93%)	Previously used	14 days	42% *vs.* 41% ($P = 0.91$)	28% *vs.* 23% ($P = 0.46$)	28% in both group	N/A
McGirt *et al.*[22]	340 (170:170)	Sim 80 mg qd	N/A	14 days	25.3% *vs.* 30.5% ($P = 0.277$)	N/A	21.7% *vs.* 18.2% ($P = 0.416$)	18% *vs.* 15% ($P = 0.468$)
Kern *et al.*[23]	135 (72:58)	Pra 40 mg qd	N/A	14 days	52% *vs.* 50% ($P = 0.17$)	N/A	34.7% *vs.* 31.0% ($P = 0.95$)	20.8% *vs.* 13.7% ($P = 0.4$)
Kern *et al.*[23]	100 (49:51)	Sim 20, 40 mg qd	N/A	14 days	N/A	20% *vs.* 16% ($P = 0.74$)	N/A	14% *vs.* 27% ($P = 0.20$)

S:P: statin:placebo; Pra: pravastatin; Sim: simvastatin; qd: once a day; TCD: transcranial Doppler; DCI: delayed cerebral ischemia; N/A: not available; ICU: intensive care unit; H: hours.

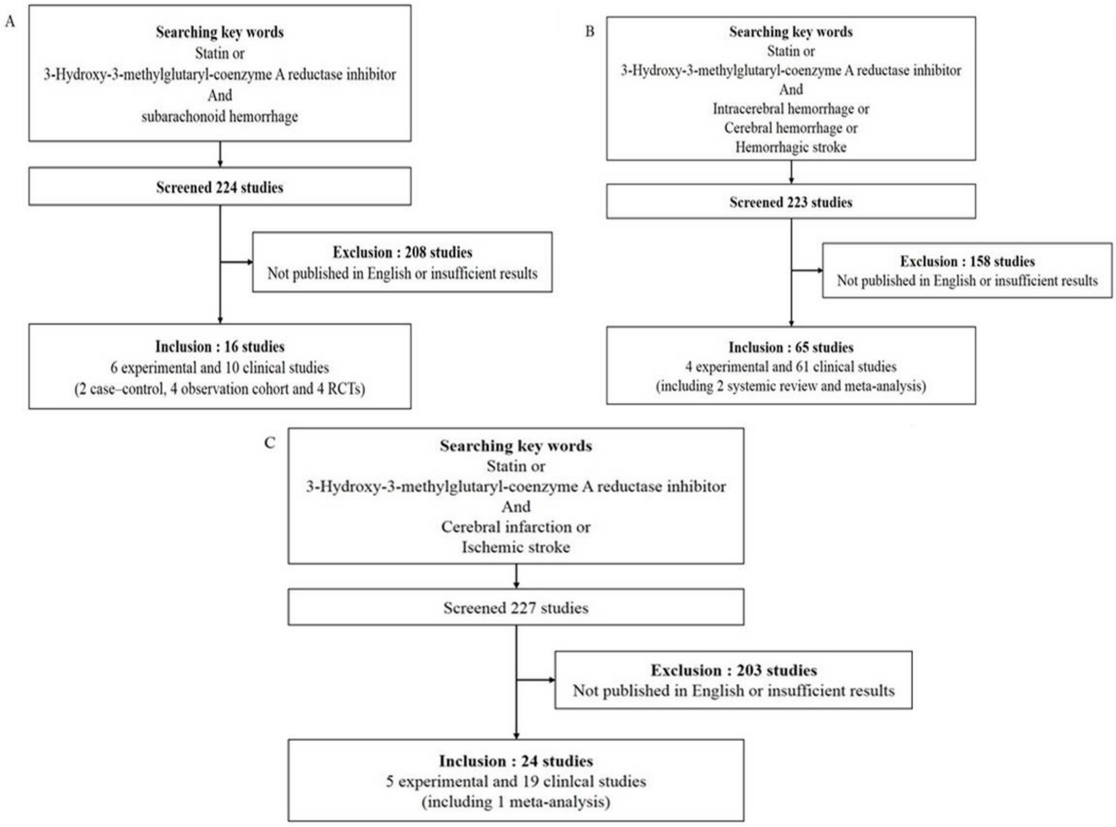

Figure 1: Flow chart of the literature selection for (a) subarachnoid hemorrhage, (b) intracerebral hemorrhage and (c) acute cerebral infarction. RCTs: randomized controlled trials

independent beneficial pleiotropic effects, including immunomodulation,[2] neuroprotection[3] and cellular senescence.[4] These properties could be beneficial in various acute neurologic diseases.

Statins were initially developed for secondary prevention of cardiovascular disease via secondary to dyslipidemia. Initial studies in neurological disease focused on patients with stroke or at high risk of stroke. Long-term statin therapy was associated with meaningful reductions in stroke, myocardial infarction and vascular death in both primary[5] and secondary stroke prevention.[6] Promising data has been published from studies of acute myocardial infarction[7] and casecontrolled studies of statin use prior to stroke[8] led to an interest in determining the role of statins in the acute setting, such asreducing early recurrence of ischemic event or beneficial for long-term functional outcome.

The aim of this review is to summarize the literature regarding the use of statins in common acute neurological diseases; subarachnoid hemorrhage (SAH), intracerebral

Table 2: Summary of the published clinical studies for statin with intracranial hemorrhage

Study	Study type	Size (s:n)	Statin therapy	Outcome		
				Timing	Good outcome (OR, 95% CI)	Mortality (OR, 95% CI)
Leker *et al.*[33]	Observation	312 (89:223)	Any	At discharge	2.97, 1.25-7.35 ($P = 0.015$)	0.25, 0.09-0.63 ($P = 0.004$)
Biffi *et al.*[28]	Observation	699 (238: 461)	Any	90 days	2.08, 1.37-3.17 ($P = 0.004$)	0.47, 0.32-0.70 ($P = 0.005$)
Biffi *et al.*[28]	Meta-analysis (6 obs + 1 RCT)	2521 (698: 1,823)	Any	90 days	1.91, 1.38-2.65 ($P < 0.0001$)	0.55, 0.42-0.72 ($P < 0.0001$)

Observed outcome with pretreatment of statin. s:n: statin: no statin; obs: observation; RCT: randomized controlled trial; ICH: intracerebral hemorrhage

Table 3: Risk of ICH with statin treatment

Study	Study type	Population	Size	Statin therapy	Risk of ICH
Goldstein *et al.*[34]	RCTs	Treatment after stroke (include TIA)	88 (s55: n33)	Atorvastatin 80 mg	OR 1.68 (95% CI 1.09-2.59, $P = 0.02$)
Hackam *et al.*[35]	Meta-analysis	Statin with various cause		Any	
	23 RCTs	526, 518 patients-years (median 3.9 years),497 ICH			RR 1.10 (95% CI 0.86-4.14)
	19 Obs	219,459 patients-years (median 3.0 years),14280 ICH			RR 0.94 (95% CI 0.81-1.10) for 12 observational cohort RR 0.60 (95% CI 0.41-0.88) for 6 case control study

RCTs: randomized controlled trials; TIA: transient ischemia attach; OR: odds ratio; CI: cerebral ischemia; ICH: intracerebral hemorrhage; RR: relative risk; Obs: observation

hemorrhage (ICH), cerebral infarction, traumatic brain injury (TBI), status epilepticus and meningitis. Studies were identified by performing a PubMed search using following key words: "statin" and each disease which was discussed in this review. Study selection was performed by two reviewers independently and the third reviewer would step in if there were any disagreement. The studies only published in English were selected. Discussion between reviewers was made to get a final consensus. The results were described in Figure 1. The main focus was the evidence for timing of statin initiation as well as specific agent and doses in the patient presenting to the emergency department and acute care unit.

SAH

Symptomatic cerebral vasospasm and delayed cerebral ischemia (DCI) is a major source of disability, unfavorable outcome and cause of death after aneurysmal SAH. Although the exact mechanism of SAH associated vasospasm and DCI are not well known, experimental studies suggest multifactorial pathogenesis involving inflammation, No depletion, endothelial injury, free radical and microvascular by autoregulation.[9-11] The pleiotropic effects of statin may be beneficial in attenuation of SAH associated vasospasm and DCI via inhibit the underlying mechanism of vasospasm and DCI. The results from 3 different animal models (mice, rabbits and dogs), have supported this hypothesis.[12-14]

Clinical evidence suggesting benefits in by reducing DCI and possibly vasospasm and early in-hospital mortality that has come from 6 randomized controlled trials (RCTs)[15-20] (two of which have only published as abstracts[19,20]), four observational cohort studies[21-24] [Table 1] and two case-control studies.[25,26]

All 6 RCT were phase II, single-center trials and none of which enrolled more than 100 patients.[15-20] One RCT[17] focused on patents with Fisher grade 3 and the other RCTs[15,16,18-20] included all grades. The statins used in these trials were either pravastain 40 mg[15,19] or simvastatin 80 mg[16-18,20] administrated within 96 h (range from 24-96 h) following aneurysmal SAH. Three RCTs administered statin for 2 weeks,[15,16,18] two used statin for 3 weeks,[17,20] and the other prescribed statins when patients were admitted to the intensive care unit.[19] Because the definition of vasospasm varied between trials, the effects of statins on vasospasm were inconsistent. Vasospasm-related DCIs was based on both neurological deterioration and neuroimaging findings in all trials. Statin therapy reduced the incidence of DCI in three trials,[15,16,18] but showed a non-significant trend to reduce the DCI in two trials[17,20] and was neutral in one trial.[18] None of these trials showed significant benefits of early statin therapy on functional outcomes by modified Rankin Scale or Glasgow Outcome Score.[15,17-20] Mortality was reduced by statin therapy in three RCTs,[15,17,20] but not in others.[18,19]

A meta-analysis with high quality four RCTs[15-18] showed that use of statin after SAH significantly reduced both DCI [odd ratios (OR) 0.41, 95% confidence interval (CI) 0.20-0.82, $P < 0.001$] and mortality (OR 0.29, 95% CI 0.09-0.93, $P = 0.04$).[27] When data from non-published

Table 4: Summary the published clinical studies for statin with acute cerebral infarct

Study	Study type	Size (s:n)	Outcome	
			Result	Definition
Aboa-Eboulé *et al.*[42]	Observation	953 (127:826)	OR 0.76 (95% CI 0.53-1.09, $P = 0.134$)	Good outcome
Marti-Favregas *et al.*[8]	Observation	167 (30:137)	OR 5.55 (95% CI 1.42-0.80, $P = 0.012$)	Good outcome at 3 months
Elkind *et al.*[43]	Observation	650 (57:593)	1.8% *vs.* 10.6% ($P = 0.04$)	Mortality at 3 months
Greisenegger *et al.*[44]	Observation	1,691 (152:1,539)	6% *vs.* 14%, OR 0.37 (95% CI 0.19-0.74, $P = 0.004$)	Severe stroke (mRS 5-6)
Flint *et al.*[45]	Observation Treatment pre- or during hospitalization	12,689 (6,294:6,395)	22.1% *vs.* 33.8%, HR 0.59 (95% CI 0.53-0.65, $P < 0.001$)	Mortality at 1 year

Pretreatment of statin and associated outcome. s:n: statin: no statin; OR: odds ratio; CI: cerebral ischemia; HR: heart rate

Table 5: Outcome after in-hospital cessation of statin therapy

Study	Study type	Size	Outcome	
			Result	Definition
Flint *et al.*[45]	Observation	468 of 3,749	46.2% *vs.* 22.1%, HR 2.5 (95% CI 2.1-2.9, $P < 0.001$)	Mortality at 1 year
Blanco *et al.*[46]	Randomized controlled	46 of 89	65.2% *vs.* 20.9%, OR 8.67 (95% CI 3.05-24.63, $P < 0.0001$)	Early neurologic deterioration
			60.0% *vs.* 39.0%, OR 4.66 (95% CI 1.46-14.91, $P = 0.043$)	Death or dependency

OR: odds ratio; CI: cerebral ischemia; HR: Heart rate

Table 6: Outcome after statin initiation in acute phase of ischemic stroke

Study	Study type	Size (s:n)	Outcome	
			Result	Definition
Flint *et al.*[45]	Observation	8,940 (2,545:6,395)	19.4 % *vs.* 33.8%, HR0.55 (95% CI 0.50-0.61, $P < 0.001$)	Mortality at 1 year
Kennedy *et al.*[47]	Randomized controlled	199:193	10.6% *vs.* 7.3%, RR 1.3 (95% CI 0.7-2.4, $P = 0.25$)	Stroke within 90 days
Squizzato *et al.*[48]	Meta-analysis of 7 RCTs	Total 431	OR 1.51 (95% CI 0.60-3.81)	Mortality

s:n: statin: no statin; OR: odds ratio; CI: cerebral ischemia; HR: heart rate; RR: relative risk; RCTs: randomized controlled trials

RCTs[19,20] was included in the analysis, statin therapy significantly reduced DCI (fixed model, OR 0.38, 95% CI 0.23-0.65, $P < 0.001$) and was associated with a trend toward reduced mortality (fixed model, OR 0.51, 95% CI 0.25-1.02, $P = 0.06$).[27]

Four single centers reported observation from cohorts that ranged from 49 to 170 patients of statin therapy following aneurysmal SAH.[21-24] These observational studies were considered to low quality because of relatively small sample sizes, heterogeneity in baseline, clinical management and definition of clinical outcome. A meta-analysis was performed using these 4-observation cohort studies, one case control study[25] and 6 RCTs which included 1,542 patients, whom 385 received statin.[27] Statin use after aneurysmal SAH was not significant associated with reduced DCI (OR 0.96, 95% CI 0.71-1.31, $P = 0.80$) or mortality (OR 1.16, 95% CI 0.78-1.73, $P = 0.47$). A more recent case-control study with atorvastatin suggested that the atorvastatin may have an anti-ischemic effect on imaging, but no clinical benefit after aneurysmal SAH.[26]

Consistent across all studied, there were no significant adverse effects associated with statin use after aneurysmal SAH. Asymptomatic elevation of liver enzyme within unexpected range was reported in 3 RCTs[15,17,18] with only 1 patient having to discontinue statin because of myalgia.[18]

ICH

Although case-control studies of statin use before ICH has demonstrated an association with favorable outcomes and reduced mortality after ICH,[28] there are no clinical studies of early initiation after ICH onset. Preclinical studies have shown beneficial effects on functional outcome in several animal models of ICH.[29-31] Pleiotropic effects of statin such as neuroprotection and stimulation of neurogenesis and synaptogenesis might be contributed to this benefit.[32] A multicenter observational cohort study in Israel, including 89 patients with statin from a total of 312 ICH patients, showed that the prior use of statins was associated with good neurologic outcome at discharge of the patients (OR 2.97, 95% CI 1.25-7.35, $P = 0.015$) and reduced mortality or discharge to a nursing facility (OR 0.25, 95% CI 0.09-0.63, $P = 0.004$) [Tables 2 and 3].[33] Another single center study compared 90-day functional outcome in 238 pre-ICH statin cases and 461 statin-free cases.[28] In this study, statin therapy was associated with improved functional outcome (OR 2.08, 95% CI 1.37-3.17, $P = 0.004$) and reduced mortality (OR 0.47, 95% CI 0.32-0.70, $P = 0.005$) without an effect on hematoma expansion. A meta-analysis was performed of 6 trials that usedstatins before ICH and the data showed a increased associationwith favorable outcomes (OR 1.19, 95% CI 1.38-2.65, $P < 0.0001$) and reduced mortality (OR 0.55, 95% CI

0.42-0.72, $P < 0.0001$).[28]

Although there have been concerned of statins increasing the risk of ICH,[34] recent evidence suggest that the statins did not increase the risk of ICH. The Stroke Prevention by Aggressive Reduction of Cholesterol Levels (SPARCL) study found increased risk of subsequent ICH (unadjusted hazard ratio 1.68, 95% CI 1.09-2.59) among subjects with prior stroke randomized to high-dose atorvastatin.[34] Mild antithrombotic properties and lipid lowering effect of statins might be explained mechanism of association between statins and risk of ICH. However, recent meta-analysis have been reported that low cholesterol concentrations with intensive statin therapy did not correlated with risk for ICH.[36] A recent large systematic review and meta-analysis of 23 randomized trials that provided a cumulative total of 526,518 patients years of follow-up with median 3.9 years found no evidence that statins were associated with developing ICH (risk ratio 1.10, 95% CI 0.86-4.14).[35] A second meta-analysis using 12 cohort studies that provide a total of 219,458 patient-years of follow-up or 6 case-control studies also did not show any risk of ICH with statin (each risk ratio 0.94, 95% CI 0.81-1.10 and risk ratio 0.60, 95% CI 0.41-0.88).

ACUTE CEREBRAL INFARCTION

There are numerous published work that demonstrates the beneficial effects of statins in animal models of ischemic stroke. These experimental models have evaluated effects of statin treatment prior to and after initiation of cerebral infarction.[37] Statins have been shown to improve endothelial function and increase cerebral perfusion in the ischemic penumbra by improving no production immediately after treatment initiation.[38,39] The anti-oxidative and anti-inflammatory properties of statins can affect secondary brain injury in the setting of ischemia.[40,41] A meta-analysis of 1,882 animals in 41 studies with ischemic occlusive stroke models showed that use of statin reduced infarction volume by 25% (95% CI 21-30%, $P < 0.001$) and improved neurologic outcome by 20.36% (95% CI 14-26%, $P < 0.001$).[37] Furthermore pretreatment with statin (median 14 days, range 5-14) was more effective than initiation after ischemia (median 4 h, range 1-12) in infarct size reduction (33.57%, 95% CI 28.47-38.53% *vs.* 16.02%, 95% CI 11.63-20.42%; $\chi^2 = 408$, $P < 0.001$) and improve neurologic outcome (26.52%, 95% CI 15.05-37.99% *vs.* 14.37%, 95% CI 7.26-21.48%; $\chi^2 = 17$, $P < 0.001$).

Most studies have shown that the use of statins at the time of ischemic stroke may confer a beneficial effect [Tables 4-6]. A population-based prospective study of 953 patients did not demonstrate early improvement in functional outcome after a first ischemic stroke event (OR 0.76, 95% CI 0.53-1.09, $P = 0.134$).[42] A small observational study noted more favorable outcomes in a 3-month period in which the patients were givenstatins for 3 months prior to the stroke statin group.[8] Pretreatment with statin was associated with decreased in-hospital mortality[43] and reduced stroke severity.[44] The largest observational study evaluated 12,689 cases with acute ischemic stroke and found that statin use before and during hospitalization was strongly associated with improved survival (hazard ratio 0.59, 95% CI 0.53-0.65, $P < 0.001$).[45]

The question of in-hospital cessation of statin therapy is an important as stroke patients may be dysphagic and NPO after admission. This important question was addressed in the same large observational cohort of 12,689 cases and statin discontinuation in the acute phase of stroke, even for a brief period, was associated with a substantially greater risk of death (hazard ratio 2.5, 95% CI 2.1-2.9; $P < 0.001$).[45] A small single-center randomized blinded study of statin withdrawal *vs.* continuation confirmed the need to continue treatment in this population. The acute statin withdrawal was associated with increase in early neurologic deterioration (OR 8.67, 3.05-24.63) and death/dependency (OR 4.66, 1.46-14.91).[46]

There is currently not enough evidence to confirm the beneficial effect of statin treatment in acute phase of ischemic stroke. A recent pilot clinical trial called "MISTICS" randomized 60 patients within 3 to 12 h after acute ischemic stroke to simvastatin or placebo for 90 days. This study showed that simvastatin therapy improved functional outcome (46.4% *vs.* 17.9%, $P = 0.02$).[49] However, there were safety concerns as statin therapy was associated with increased incidence of infection (OR 2.4, 95% CI 1.06-5.4) and a trend toincrease mortality (25.0% *vs.* 10.7%, $P = 0.16$). Other randomized trials of statin therapy in acute stroke were limited by insufficient recruitment and insufficient data for analysis.[47,48,50]

One potential strategy for translating the efficacy of statins in preclinical models may be to use very high doses or intravenous routes for statin initiation. The neuroprotection with Statin Therapy for Acute Recovery Trial[51] of 33 patients with acute ischemic stroke < 24 h of onset wastesting a short-term high-dose lovastatin at 1, 3, 6, 8, and 10 mg/kg per day for 3 days. Patients were followed for 30 days andclinical and laboratory outcome measured in this Phase IB trial and the maximum tolerated dose was estimated to be 8 mg/kg per day.

Despite the lack of evidence for treating acute stroke with statin, there is no doubt that in-hospital initiation should occur when statin therapy is indicated. The SPARCL study clearly defined the role of statins in secondary stroke prevention, yet did not address the best

time to initiate therapy.[52] Studies comparing in-hospital initiation with outpatient initiation of preventative therapies have consistently shown better compliance over the long-term.[53] For stroke patients, participation in a hospital-based prevention initiative with in-hospital initiation of antithrombotic, statin and antihypertensive was associated with very high rates of compliance at 3 months.[54] In-hospital initiation of statins is associated with high rates of continuation and achievement of National Cholesterol Education Program guideline goals.[55] In one study 92 statin-naïve patients with an indication for treatment, hospital initiation of statin therapy yielded a 93% rate of adherence, lowered mean low-density lipoprotein cholesterol levels from 120 to 78 mg/dL and increased the proportion of patients with low-density lipoprotein cholesterol levels lower than 100 mg from 36% to 88% at 3 months.

TBI AND SCI

Statins have demonstrated benefit in animal models of TBI and spinal cord injury (SCI). Statins treatment prior to experimental TBI was associated with reduced the cortical contusion volume[56] and cerebral edema.[57,58] Statin therapy after TBI in rat decreased post-traumatic apoptosis in hippocampus and peri-contusional cortex and increased neuronal proliferation leading to improvement of cognitive abilities.[59] The experimental studies in preclinical SCI models also suggest that statin treatment could significantly improve functional outcome via anti-inflammatory and anti-apoptotic effects.[60,61]

Clinical evidence for the effect of statins moderate to severe neurotrauma needs further extensive studying. Only one small prospective, randomized, double blind trail of statin treatment initiation within 24 h of moderate TBI was found.[62] The study included only 8 patients with statin and the 13 controls. The statin administration was associated with a reduced duration of amnesia (hazard ratio 53.76, 95% CI 1.58-1,824.64), but no difference in disability at 3 months.

STATUS EPILEPTICUS AND EPILEPSY

Animal models suggest that statin administration might be a therapeutic strategy for epilepsythrough neuroprotection in status epilepticus (SE) and prevention of epileptogenesis progression.[63-66] Lovastatin administration after pilocapine-induced SE suppressed mRNA expression of hippocampal cytokines (such as interleukin-1b, interleukin-6, tumor necrosis factor a, and kinin B1 recepter) and reduced SE-induced hypothermia.[63] Lovastatin also decreased cell loss in hippocampal CA1, CA3 and hilus of dentate gyrus after pilocapine-induced SE that is a critical step of epileptogenesis.[64] In the chronic temporal lobe epilepsy (TLE) model, 2 weeks administration of simvastatin after kainic acid-induced SE lead to not only attenuated microscopic morphological changes, but also reduced seizure activity in the brain at 4 to 6-month after SE.[65] On the other hand, 2 weeks treatment of atorvastatin did not affect the duration of SE or development of epilepsy in electrically induced rat TLE model.[66]

There is no evidence that the neuroprotective properties of statin will have a clinical benefit in acute epileptic syndromes.

CENTRAL NERVOUS SYSTEM INFECTION

Simvastatin can attenuate leukocyte invasion into the central nervous system (CNS) and systemic complication of pneumococcal meningitis in an experimental model of bacterial meningitis rodents.[67] Simvastatin treatment significantly reduced cerebrospinal fluid leukocyte counts with dose-dependent manner, but did not altered cerebellar bacterial titers. The marked hypothermia was dose-dependently reversed by statin treatment. This neuroprotective effects can be explained by anti-inflammatory pleiotropic property of the statin. Statin treatment did not result in an improvement of the clinical score or a reduction of increased intracranial pressure and blood-barrier breakdown. Clinical studies of the effects of statin treatment in acute CNS infection are lacking.

CONCLUSION

Some preclinical and clinical evidence has shown that statin therapy following SAH could be safe and beneficial in terms of reducing DCI and possibly cerebral vasospasm and early in-hospital mortality. However, methodology of clinical studies was varied and beneficial effects were inconsistent, statin therapy following SAH should not be considered standard care at this time. Statin use before ICH or before and during acute ischemic stroke is safe and can reduce in-hospital mortality and improve functional outcome, whereas statin withdrawal in the hospital after acute ischemic stroke, even for a brief period, can cause early neurologic deterioration and death. Despite the lack of clinical evidence for statin initiation after ICH or acute ischemic stroke, we can achieve better long-term compliance with in-hospital initiation when statin therapy is indication. Even though benefit in preclinical studies, there is no clinical evidence that the pleiotropic properties of statins will have a clinical benefit in neurotrauma, epilepsy and CNS infection.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

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Huge supratentorial cortical ependymoma in a young child: case report and literature review

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ABSTRACT

Supratentorial cortical ependymomas are uncommon in the pediatric population and extremely rare in very young children. Histologically, tumors of the anaplastic type are also less common in children. The authors report one case of anaplastic cortical ependymoma in a 16-month-old girl who presented with a 7-day history of left side weakness and rapid neurological deterioration. Brain imaging with computed tomography and magnetic resonance imaging scanning showed a huge right fronto-parietal cystic and solid lesion compressing the brain parenchyma. The young child was operated via a transparietal approach with gross total resection of the lesion. The tumor's histology was anaplastic ependymoma. Intensive chemotherapy was given post operatively and the patient remained well without recurrence after 20 months of follow-up.

Key words: Cortical ependymoma; young child; surgery; chemotherapy; prognosis

INTRODUCTION

Ependymomas are rare neuroectodermal tumors arising from ependymal cells of the ventricular system, choroid plexus, filum terminale or the central canal of the spinal cord. They account for 1.2-7.8% of all intracranial neoplasms.[1-3] Pure supratentorial cortical ependymomas (CE) are uncommon. To our knowledge, 49 cases of CE have been reported, of which 16 involved pediatric patients, with only 4 occurring in very young children (less than 3 years).[1-5] Here we report the 5th case of CE in very young child, treated surgically and with chemotherapy with a good outcome at 20 months of follow-up.

CASE REPORT

A 16-month-old female, with normal development milestones and without other past medical history, was admitted to our department of neurosurgery after presenting with 7 days of left-sided weakness. At initial neurological examination, the young child was conscious, had a left hemiparesis mainly affecting the upper limb, a normal head circumference for age and no papilledema. Brain computed tomography (CT) scanning revealed a huge right fronto-parietal cystic-solid lesion mesearing 76 mm × 70 mm × 70 mm. It was slightly hyperdense in its solid component, with thin calcifications and was well-demarcated from brain parenchyma [Figure 1]. During hospitalization, the patient had a focal seizure followed by rapid deterioration in consciousness and right pupillary dilatation. The patient was transferred immediately to the operating theatre and underwent in emergency cyst puncture with solid component biopsy. The outcome was good, with improvement in her clinical state and resolution of the hemiparesis.

The brain magnetic resonance imaging (MRI) performed after the first surgery revealed a large cortical fronto-parietal lesion, hypointense on T1, hyperintense on T2, markedly enhanced with contrast, with moderate surrounding edema [Figure 2]. The tumor was totally resected in an elective procedure using a right transparietal approach. The postoperative course was uneventful, without complications. Histological examination of the resected tumor showed perivascular pseudo rosettes of small round cells with mitosis (5 mitosis/10 fields) and necrosis. Immunohistochemical studies showed positivity to glial fibrillary acidic

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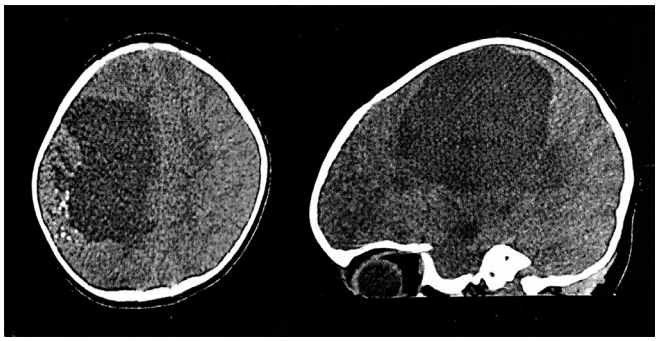

Figure 1: Preoperative brain computed tomography scan without contrast; axial view and sagittal reconstruction, showing a huge right fronto-parietal cyst and a slightly hyperdense cortical solid lesion with thin calcifications.

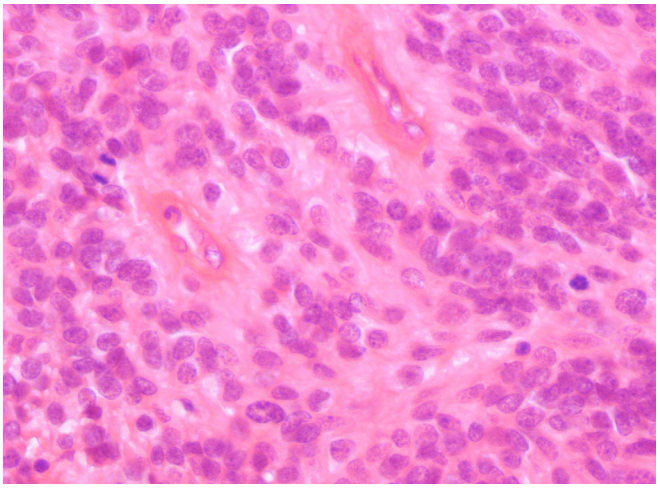

Figure 3: Photomicrograph of the tumor specimen showing marked hypercellularity, nuclear atypia and brisk mitotic activity. (HE, X40).

protein (GFAP), poly styrene 100, and keratin, but not to synaptophysin. Kalium iodidum (Ki)-67 was positive in 35%. These findings are in keeping with an anaplastic ependymoma, World Health Organization (WHO) classification grade III [Figure 3]. Cerebrospinal fluid studies were negative for malignant cells and no drop metastases were detected on neuroaxis MRI. Intensive chemotherapy with BBSFOPP protocol consisting of seven cycles of 3 courses alternating 2 drugs at each course (carboplatin/procarbazine, cisplatin/etoposide and vincristine/cyclophosphamide) was given post operatively for 16-month period. After 20 months of follow up, the patient remained neurologically normal without recurrence or metastasis on surveillance MRI [Figure 4].

DISCUSSION

Ependymomas account for 2-9% of all neuroepithelial tumors, and are graded as grade II (low grade), and grade III (anaplastic) according to 2007 WHO classification. They involve frequently the spinal cord and ventricular system, especially the fourth ventricle, and they commonly occur in children and young adults.[1]

Supratentorial CE is an uncommon ependymoma located in the superficial cortex and more often found

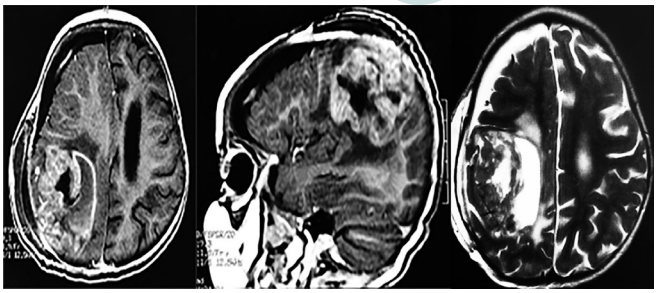

Figure 2: Post puncture magnetic resonance imaging (axial and sagittal T1 post gadolinium and axial T2 weighted images) showing a huge right parietal tumor, enhancing with contrast and with moderate peritumoral edema.

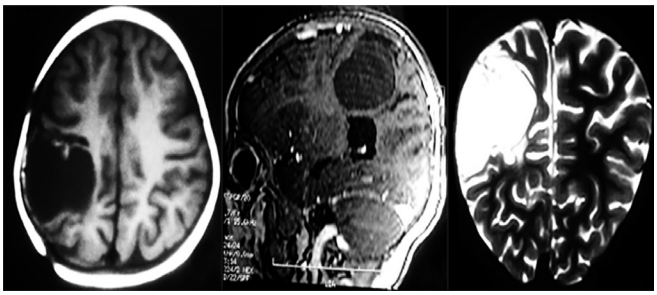

Figure 4: Follow-up magnetic resonance imaging at 20 months (axial T1, sagittal T1 with gadolinium and coronal T2 weighted images) showing complete removal of the lesion without recurrence.

in adults than children. There are only 49 cases reported in the literature.[1-5]

Ependymomas are known to arise from ependymal cells. The pathogenesis of the extraventricular tumor type remains uncertain. Pure cortical ependymomas may arise from embryonic remnants of ependymal tissue encapsulated in the developing cerebral hemispheres.[2]

CEs are rare in pediatric patients and exceedingly uncommon in very young children.

Among the 49 cases reported in the literature, 16 involved children and only 4 occurred in patients under the age of 3 years.[1,2,4,6]

Our patient is the 5th case reported of CE in those under 3 years of age.

The age of these 5 young children ranged from 12-24 months, with a mean age of 19.4 months. There was male preponderance with male to female ratio of 3:2 [Table 1].

The typical presentation of CE was with seizures and focal neurological deficits, and rarely with signs of raised intracranial pressure.[1,2,7]

Our patient presented with left hemiparesis, a seizure, and rapid deterioration with signs of intracranial hypertension, whereas the others four young children reported, presented either with seizures or with hemiparesis [Table 1].

Intracranial ependymomas appear on brain CT scans as

Table 1: The summary of previous reported young children supratentorial cortical ependymoma with our case.

References	Age (months) /gender	Location	Clinical presentation	Neuroimaging finding	WHO grade	Treatment	Follow-up
Lehman *et al*,[4]	12/F	Rt frontal	Seizures	Solid lesion not enhanced	III [clear cells]	GTR	No recurrence in 48 months
Lee *et al*,[2]	21/M	Rt fronto-parietal	Seizures	Diffuse enhancing mass, some focal calcifications	II	GTR	No recurrence in 12 months
Liu *et al*,[1]	24/M	Lt frontal	Right side weakness	Large mass, irregular peripheral enhancement no edema	III	GTR	Recur at 3 years after surgery alive at 4 years
Kambe *et al*,[6]	24/M	Rt parietal	Seizures	Solid mass, calcification homogeneous enhancement	II [tanycytic]	GTR	No recurrence in 20 months
Our case	16/F	Rt fronto-parietal	Left side weakness, seizures, ICH signs	Large solid cystic lesion heterogeneous enhancement	III	GTR	No recurrence in 20 months

F: female; M: male; Rt: right; Lt: left; WHO: world health organization; GTR: gross total resection; ICH: intracranial hypertension

isodense or mildly hyperdense soft tissue lesions with frequent large cyst, calcifications in 50%, hemorrhage in approximately 10% and, often, heterogeneous enhancement.[8]

MRI is the brain imaging modality of choice for ependymoma. CE generally appears as large, well-demarcated lesions, T1 hypointense and T2 hyperintense, showing cyst formation. After gadolinium administration, T1 weighted images usually show heterogeneous enhancement of the solid component.[8] Frontal and parietal region are the most common locations.[8]

These radiologic findings are, however, non-specific and glioblastomas, pleomorphic xanthoastrocytomas, oligodendrogliomas, primitive neuroectodermal tumors, astroblastomas and angiocentric gliomas should be considered in the differential diagnosis of a large supratentorial cortical lesion.[1,8-11]

Histologically, the classic cellular features of ependymomas include round to oval nuclei with evenly dispersed stippled chromatin, perivascular pseudo rosettes or true ependymal rosettes. Unusual morphological features like clear cells, spindle cells and giant cells can also be seen.[1,4]

Anaplastic ependymomas are characterized by increased cellularity, cytological atypia, increased mitotic activity, microvascular proliferation and/or pseudopalissading tumor necrosis. There is normally a clear interface between tumor and adjacent brain tissue and relative uniformity of tumor cell nuclei.[1,4] However, some authors have described ependymomas, that infiltrate at their peripheries.[4]

Immunohistochemical studies revealed frequent immunoreactivity for GFAP, S100, a perinuclear dot-like or ring-like positivity for epithelial membrane antigen and/or membranous with or without perinuclear dot-like staining pattern for cluster of differentiation 99.[1,4]

Immunohistochemical cell proliferation markers, that have some specificity for ependymomas are available. MIB-1 L1 and Ki-67 are associated with high-grade ependymomas, as in our case. Recently, further markers have been found, notably topo-II-α and p53 and murine double min 2 protein expression which are correlated with high-grade tumors and a poor prognosis.[12]

Although nearly 70% of all ependymomas diagnosed in the pediatric population are histologically benign, CEs are more frequently anaplastic in young children (3/5) [Table 1].

Given the superficial location, radical resection is the treatment of choice for CE.

There has been much debate about adjuvant radiotherapy and chemotherapy.

Radiotherapy was thought to increase the length of survival and reduce or postpone tumor recurrence. It is used in patients with anaplastic ependymomas in cases of partial resection of either benign or malignant tumor.[13,14] Whole brain irradiation with additional local fractions is recommended by many authors in malignant ependymoma.[14]

In very young children, delayed radiotherapy and a period of close observation after gross total resection may be chosen for some cases to defer the side-effects of radiotherapy.[4,6,13,15]

Chemotherapy has mainly been studied in children with incomplete tumor resections and multiple agents have been given in an effort to delay or avoid irradiation. These studies suggest that chemotherapy offers limited

benefits.[15]

The four patients previously reported had gross total resection without adjuvant therapy, whereas our patient had intensive postoperative chemotherapy.

Recurrences of CE are less frequently seen than for the intraventricular type. Among the 5 young children reported, recurrence only occurred in one and at 3 years [Table 1].

CE appear to have a relatively favorable prognosis compared with other supratentorial ependymomas.[7,13] All the young children reported, including our case, are alive after a mean follow-up of 29.6 months.

We believe that successful gross total resection of CE is the best prognostic factor for long-term survival with both WHO grade II and grade III lesions.

In conclusion, supratentorial CE is a very rare tumor type that should be considered in the differential diagnosis of a large cortical lesion in very young children.

Gross total resection is the treatment of choice, which can afford good prognosis with long-term survival.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Commentary

Meningeal inflammation and multiple sclerosis

Li-Ping Liu

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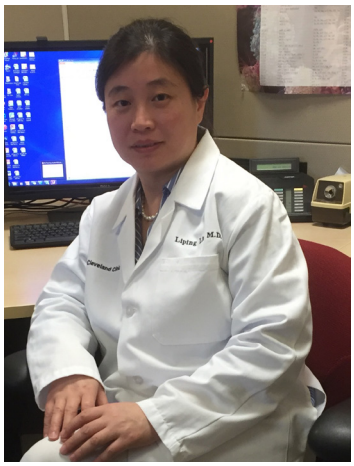

Dr. Liu obtained her Ph.D. from Fudan University, Shanghai, China. She currently is a Project Scientist at the Cleveland Clinic, Cleveland, Ohio, USA. Dr. Liu has a record of remarkable publications in top impact journals such as *Nature Neuroscience*. She is an internationally recognized scientist with the expertise in the neuroinflammation and neurodegenerative diseases.

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Inflammation in MS is characterized by infiltration of peripheral immune cells into the CNS, especially in the meninges.[1-4] The infiltration into meninges, which has been referred to as tertiary lymphoid tissues (TLTs), is a likely first step preceding infiltration into the CNS parenchyma. These invading autoreactive immune cells destroy myelin, the insulation surrounding neuronal axons, and cause demyelination in subpial and cortical areas, promoting disease pathogenesis. Experimental autoimmune encephalomyelitis (EAE), a widely used murine model of MS, also shares this characteristic.[5,6] However, what cellular components and molecular pathways support infiltrating lymphocyte retention and production within the meninges as well as further invading into the CNS parenchyma are still not clear. Pikor *et al*.[7] first reported that stromal cells in the inflamed CNS meninges (TLTs) play an important role in the neuroinflammatory process of MS. They also demonstrated that collaboration between the encephalitogenic T helper 17 (Th17) cell and lymphotoxin pathways contributes to the formation of TLTs.

First, they made use of the SJL/J EAE model, which mimics progressive MS, to investigate the location and cellular composition of TLTs.[7] They found that T cells were the initial population in meningeal TLTs, while B cells invaded the meninges later. Based on a previous report showing the importance of Th17 cells in promoting TLTs,[6,7] they established an adoptively transferred encephalitogenic Th17 in the SJL/J EAE model. They found that Th17 cells could rapidly populate, proliferate, and secrete cytokines in the meninges. The TLTs formed by Th17 cells and B cells were also associated with subpial and parenchyma demyelination, as shown by histological staining.[7] Thus the authors have established a good model (Th17 cells A/T model) for further investigation of the cellular and molecular mechanisms of TLTs.

What are the roles of infiltrating Th17 cells in the remodeling of stromal cells in the meninges? They found that 1 out of the 4 populations of stromal cells in meninges, meningeal FRC-like cells [a subpopulation

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of gp38(+)CD31(-) which are PDGFα$^-$PDGFβ$^+$ and PDGFα$^+$PDGFβ$^+$], which closely resembled lymph node fibroblastic reticular cells, were dramatically increased in EAE meninges regardless of the genetic background of mice.[7] From *in vivo* and *in vitro* studies, they found that Th17 cells and their soluble mediators IL-17 and IL-22 could remodel fibroblasts and up-regulate extracellular matix proteins and metal matrix proteinase 9, chemokines (murine macrophage inflammatory protein-3α, murine exodus-2, human GRO/melanoma growth stimulatory activity), and cytokines in the meninges. Inhibition of both IL-17 and IL-22 resulted in reductions in the fibronectin network and clinical severity of disease.[7] These results indicate that infiltrating Th17 cells contribute to building a suitable environment in the meninges to support their survival.

How do stromal cells in TLTs support infiltrating Th17 cell retention and proliferation in the meninges? Lymphotoxin beta receptor (LTBR) is a member of the tumor necrosis factor superfamily[8] and is expressed on stromal cells, dendritic cells, and macrophages, whereas its ligand LTab is expressed on embryonic Lymphoid tissue inducer cells, as well as innate lymphoid cells, B cells, natural killer cells, and activated T cells. Stromal cell chemokine secretion and stromal cell maturation are depended on the lymphotoxin pathway. The author applied LTBR-Ig treatment or used LTBR-deficient mice in a Th17 cells A/T model and found that stromal cell remodeling was comparable between wild-type mice and LTBR-deficient mice. TLT formation was also undisturbed in LTBR-Ig-treated mice.[7] All of this evidence suggests that the early steps of TLT formation are not dependenton the leukotriene pathway. Interestingly, they also found that LTBR signaling in radio-resistant stromal cells was required for the maturation of stromal cells and accumulation of B cells in TLTs as well as for T cell cytokine (IL-17 and interferon-γ) production in the CNS by using bone marrow chimeric mice which had LTBR deficient in radial-sensitive cells in Th17 cell A/T EAE model.[7] Meninges stromal cells activated by Th17 cells and their cytokines IL-22 and IL-17 also secreted IL-6 and IL-23, which were involved in Th17 cell polarization and maintenance.[7] So the specific stromal cells in TLTs, which share similarities with lymphoid tissue stroma, interact with Th17 cells and support Th17 cell retention and proliferation in meninges.

Furthermore, expression of lymphotoxin αβ (Ltαβ) on Th17 cells was also found to be required to propagate inflammation and disease progress in Th17 cell A/T EAE model.[7] Moreover, increased levels of Ltαβ (LTBR ligand) on activated CD4+ T cells were found in MS patients, but not in healthy controls.[7]

Collectively, these results suggest that infiltrating Th17 cells remodel the meningeal stromal cells and initiate the formation of TLTs during EAE. The remodeled stromal cells retain and promote the production of Th17 and the accumulation of B cells. The collaboration between LTB on Th17 cells and LTBR on meningeal radio-resistant cells is very crucial for the induction and progression of MS. This highlights the importance of the interaction between immune cells and non-immune cells (stromal cells) in the pathogenesis of MS.

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Conflicts of interest

There are no conflicts of interest.

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Autoimmune encephalopathies in children: diagnostic clues and therapeutic challenges

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ABSTRACT

Neuronal surface antibody syndromes (NSAS) encompass a variety of disorders associated with "neuronal surface antibodies". These share clinical and neuroradiological features that pose challenges related to their recognition and treatment. Recent epidemiological studies show a clear predominance for the glutamate-N-methyl-D-aspartate receptor encephalitis in both adults and pediatric population. Despite this, the overall NSAS's incidence remains underestimated, and diagnosis persists to be not always easy to achieve. Based on current literature data, in this paper the authors propose a diagnostic pathway to approach and treat pediatric NSAS. An autoimmune etiology can be suggested through the integration of clinical, immunological, electrophysiological and neuroradiological data. On that basis, a target treatment can be started, consisting of corticosteroids and intravenous immunoglobulin or plasma exchange as a first-line immunotherapy, followed by second-line drugs including rituximab, cyclophosphamide or mycophenolate mophetil, if the case. In children a prompt diagnosis and a targeted treatment may lead to a better clinical outcome. Nevertheless further studies are required to assess the need of more tailored treatments according to long-term outcome findings and prognostic factors in different NSAS.

Key words: Autoimmune encephalitis; children; diagnosis

INTRODUCTION

Over the last decade there has been an increase in the identification of forms of encephalitis associated with "neuronal surface antibodies" (NSAbs). These have been labelled as "neuronal surface antibody syndromes" (NSAS).[1]

NSAS differ from encephalitis due to antibodies directed against intracellular neuronal antigens for a different etiopathogenetic mechanism, a weaker association with paraneoplastic syndromes, a better response to immunotherapy and a higher incidence in the pediatric population.[2-6] Pathogenesis predominantly involves humoral immune response, while cellular immune response activation may coexist in a variable proportion, according to the different forms. Target antigens include proteins with various roles in neuronal function, ranging from synaptic transmission and plasticity to ions channels' clustering and modulation, and including also glutamic acid decarboxylase (GAD) enzyme when exposed on cellular surface during exocitosis.[1,7]

A number of studies reporting NSAS in infancy suggest that, so far, their incidence has been probably underestimated, due to the fact that they are still often unrecognized or identified at a later stage.[8,9]

In pediatric forms, as in adults one, females can be over-represented, and a history of other antibody-mediated condition is easily detectable.[10] Conversely, in children rather than adults, a paraneoplastic cause is less probable and the role of fever or intercurrent infections in supporting the autoimmune process is less clear.[1,10]

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Table 1: Details of NSAS encephalitis in adults and children

Target	Antigen	Adult forms: Patients	Clinical signs	Neuroimaging	EEG pattern	Tumor association (%)	Outcome	Pediatric forms: Incidence	Peculiar symptoms
Glutamate-N-methyl-D-aspartate receptor (NMDA-R)									
Glutamate-N-methyl-D-aspartate receptor subunits	GluNR1/ GluNR2/	F 80% Age:1-80 y (median 20 y)	Psychosis or epileptic seizures followed by insomnia, amnesia, MD, catatonia, AI, coma[11-13]	Normal (50%) Non specific pattern or DMN involvement (50%)	-GS and FIED -Extreme delta brush (25%)	Age dependent -OT (10-50% > 18 y)[11] - ADC (rare)	Monophasic (75%): excellent if early treated (good response to IM/tumor removal). Relapsing-remitting (25%): normal in between	40% of the total[14]	Seizures > psychosis occurrence (age dependent) EEG: Rarely extreme delta brush.[11-13]
Voltage-gated potassium channel (VGK)									
VGKC-complex	VGKC undefined targets (intracellular epitope? interacting proteins?)	M 60% Age:1-86 (median 55)	Peripheral nerve hyperexcitability, Morvan's syndrome, encephalopathy, behavioural changes, seizures, short term memory loss. Possible coexistence with LEMS.[1,17]	LE, possible atrophy at MRI follow-up	GS/FS (temporal) and bilateral (temporal) FIED	Age dependant Thymoma, SCLC/ADC/ HM (rare)	Variable response to IM	F = M Age: 1-16 y (< 50 cases)	Broad clinical spectrum: fever followed by SE, encephalopathy, GDR, MD (Cho, My, Tr, MT), CA, AI, RS,TLE; RE.[18-20]
Leucine-rich glioma inactivated-1 component	LGi1	M 65% Age: 30-80 y (median 60 y)	Prodromal faciobrachial seizures, short term memory loss, psycosis, hyponatremia (60%), tonic seizures/ myoclonus.[21]	LE generally progressing to hippocampal/whole brain atrophy	-GS/FS (temporal) and bilateral (temporal) FIED	-Thymoma (< 10%) -SCLC/ADC/HM (rare)	Monophasic with complete recovery (rare) or relapsing desease course evolving to memory impairment, TLE, psychiatric disorders.	Not reported	Not reported
contactin-associated protein-like 2	CASPR2	M 85% Age: 45-80 y (median 60 y)	Morvan's syndrome; psychosis, seizures, memory impairment hyponatremia (60%).[22]	-Normal (especially in Morvan's syndrome) -aspecific T2/FLAIR abnormalities -LE (25%) No progression to hippocampal atrophy/sclerosis	-GS/FS and GED/FIED	Thymoma (0-40%) SCLC/ADC/HM (rare)	Generally monophasic with good outcome (good response to IM). Relapses may occur.	M* Age: 2 y,6 y (2 cases)	GBS without encephalitis.[23] Complete recover
Alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid- glutamate receptor (AMPA-R)									
	GluA1/2	F 90% Age: 40-80 y (median 60 y)	Seizures, short term memory loss, disorientation, psychosis. Frequent coexisting autoimmunities.[24]	LE	GS or FS and GED/FIED	Lung (70%), breast, thymoma (rare)	Monophasic (50%) good response to IM/tumor removal Relapsing remitting disease course (50%).	Not reported	Not described (GluA3). RE (occasional reports) doubt pathogenicity[25]
Metabotropic gutamate receptors									
Type 1	mGluR1	F 100%* Age: 20-50 y*	Cerebellitis.[26]	-Normal (generally) -cerebellar T2/FLAIR abnormalities evolving in atrophy -LE (rare)	Normal	HL (70%)	Monophasic or chronic disease course (correlation with titer). Good response to IM	Not reported	Not reported
Type 5	mGluR5	F 50%* Age: 15-45 y*	Ophelia syndrome	LE	FS (bi-temporal) or FIED; FED or GED	HL (70%)	Monophasic or chronic disease course (correlation with titer). Generally good response to IM	M 15 y (1 case)	Confusion, anxiety, fear, extreme agitation, auditory/ visual hallucination, GS. Complete spontaneous recovery.[27]
Gamma-aminobutyric acid receptors ($GABA_A$-R and $GABA_B$-R)									
$GABA_A$-R associated protein (clustering and anchoring receptors)	GABARAP, α1/β3	M 60% Age: 2-74 y (median 22 y)*	SE/RES, encephalopathy, RS, SPS, CA	LE	FS or GS + FED and FIED	None	Variable	Age: 2-16 y (7 cases)	SE/RES, encephalopathy, Refractory seizures, SPS (rare).[28] Association with HL (1 case)
$GABA_B$-R subunit 1/2	GABAB1 GABAB2	F 50% Age: 25-75 y (median 60 y)	Prominent seizures (FS, GS), short term memory loss, psychosis	LE (often asymmetric)	FS or GS + FED and FIED	SCLC/thymus/NET (50%)	Monophasic or chronic disease course: good response to IM. Occasional relapses.	Age: 3 y, 16 y (2 cases)	Lethargy, MD, opsoclonus, ataxia, seizures. Variable outcome MRI: diffuse changes in cortical and subcortical structures.[29-31]
Dopamine receptor									
Type 2	D2-R	M 50% Age: 6-31 y (median 20 y)	SydCho, PANDAS, TS, encephalopathy, psychosis, sleep disorders	T2/FLAIR Basal ganglia abnormalities	Generally GS	Exceptional	Monophasic or relapsing remitting; good response to IM.	F = M Age: 2-17 y (median 7 y)	Comparable to adult forms Relapsing remitting course.[32]
Glycine receptor (Gly-R)									
Gly-R subunit α1	Gly-Rα1	M 80% Age: 30-60 y (median 50 y)	Prominent PERM and SPS; hyperekplexia, spinal My, CA, encephalitis, FS, brainstem disfunction (rare)	Normal (ca. 70%) LE or unspecific (rare)	Normal	Typically none; thymoma/lung (exceptional)	Monophasic or chronic disease course: good response to IM. Occasional relapses.	F 80% Age:14 M-5 y, (3 cases)	-PERM, hyperekplexia rigidity, My, no consciousness impairment; -explosive-onset epileptic encephalopathy; RFS, speech and behavioural disturbance. Variable outcome.[33,34]
Dipeptidyl-peptidase-like protein 6									
Cell surface auxiliary subunit of the Kv4.2 potassium channel	DPPX	M 80% Age: 13-75 y (median 53 y)	Prodromal severe gastrointestinal dysfunction, encephalopathy, agitation, hallucinations, tremor, PERM, My, startle with muscle rigidity, sleep disturbances, AI seizures (rare)	-Normal -Aspecific T2/FLAIR abnormalities (rare)	Normal	None	Good response to IM but generally relapsing	One case reported (13 y)	Comparable to adult forms.[35]
Neuronal cell adhesion molecule IgLON5									
Neuronal cell adhesion protein IgLON5	IgLON5	M 50% Age: 52-76 y (median 59 y)*	Prominent sleep dysfunction, abnormal sleep movements, OSAS, Cho, CA, progressive memory loss, mild gait imbalance.[36]	Normal	Normal	None	Chronic disease course refractory to IM	Not reported	Not reported
Glutamic-acid-decarboxylase									
Glutamic-acid-decarboxylase isoenzime65	GAD65	F 80% Age:15-80 y (median 60 y)	Neurocognitive disorders, LE, TLE with RE, SPS, PERM, CA; DM1 (if titer < 20 U/ml)-Frequent coexisting autoimmunities;[37] EMG abnormalities	-Normal -LE or mesio-temporal, brain stem, cerebellum and spinal cord T2/FLAIR abnormalities evolving into atrophy.	-Normal -GS or FS, FED (SE) and/or GED	Thymoma, lung, colon, pancreas, breast, thyroid, renal cell carcinoma (rare)	Chronic disease course: usually poor response to IM (more resistant than those with cell surface antibodies)	M = F Age: 5-16 y (median10 y)	Comparable to adult forms Variable response.[38]

F: female; M: male; y: years; m: months; *: less than 20 cases reported; IM: immunotherapy. NMD: default mode network; LE: limbic encephalitis; GS: generalized slowing; FS: focal slowing; FIED: focal interictal epileptic discharges; FED, GED: generalized epileptic discharges; OT: ovarian teratoma; SCLC: small cell lung carcinoma; ADC: adenocarcinomas; HM: hematological malignancies; HL: Hodgkin Lymphoma; NET: neuroendocrine tumor; LEMS: Lambert Eaton myastenic syndrome; RE: Rasmussen's; RSE: refractory status epilepticus; SE: status epilepticus; RS: refractory seizures; RFS: refractory focal seizures; TLE: temporal lobe epilepsy, SydCho: Sydenham chorea, TS: Tourette's syndrome. GDR: global developmental regression, AI: autonomic instability; MD: movement disorders; Cho: chorea; My: myoclonus; Tr: tremor; MT: motor tics; CA: cerebellar ataxia; SPS: Stiff-person syndrome; PERM: progressive encephalomyelitis with rigidity and myoclonus; DM1: diabetes mellitus type 1; OSAS: obstruction sleep apnea syndrome.

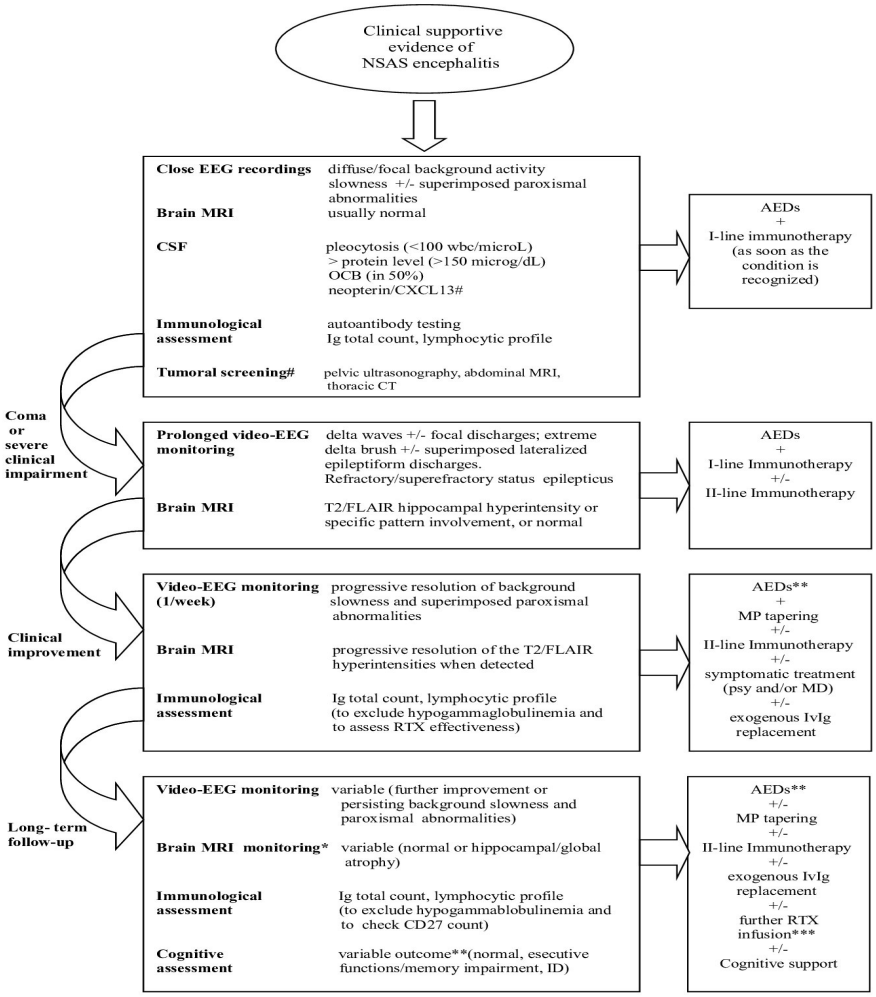

Figure 1: Diagnostic investigations and treatment of encephalitis related to neuronal surface antibody syndrome (NSAS) in children according to clinical steps. IvMP + IvIg +/-PE: intravenous methylprednisolone + intravenous immunoglobulin + plasma exchange (I-line immunotherapy). EGG: electroencephalogram; MRI: magnetic resonance imaging; CSF: cerebrospinal fluid; OCB: oligoclonal band; CXCL13: chemokine (C-X-C motif) ligand 13; CT: computed tomography; II-line immunotherapy: RTX (rituximab), Cyc (cyclophosphamide) or MMF(mycophenolate mophetil); AEDs: anti epileptic drugs; #: in anti NMDA-R encephalitis; *: timing according to patient an specific disorder; **: withdrawing according to patients/specific disorder and EEG findings; ***: according to immunological assessment at follow-up; psy: psychosis; MD: movement disorders; ID: intellectual disability; MP: methylprednisolone

Glutamate-N-methyl-D-aspartate receptor encephalitis is the most frequent form of NSAS in children.[11-14] According to up-to-date researches it is also the most common pediatric form of encephalitis, with the only exception of acute demyelinating encephalomyelitis.[15,16] With reference to other pediatric NSAS, reports are mostly anecdotal, with the only exception of the forms associated with voltage-gated potassium channel complex (VGKC) antibodies.[17-20] [Table 1]

In this paper the authors propose a diagnostic pathway based both on literature and the experience that may help to obtain accurate identification of pediatric NSAS, with the aim to start an adequate and early treatment, and achieve a better clinical outcome.

DIAGNOSTIC CLUES

When a healthy child presents with unexpected symptoms such as seizures, sudden behavioral changes and movement disorders, causes like infections and traumas must be ruled out, together with toxic, metabolic and neoplastic factors. Another issue to be excluded is a previous central nervous system disease history. Once left aside all this, an autoimmune etiology should be always taken into account.

Longitudinal clinical, neurophysiological and neuroradiological findings facilitate the diagnostic pathway, and often provide information suggestive of specific NSAS variants [Figure 1].

Moreover, abnormalities at electroencephalogram (EEG) and magnetic resonance imaging (MRI) may be

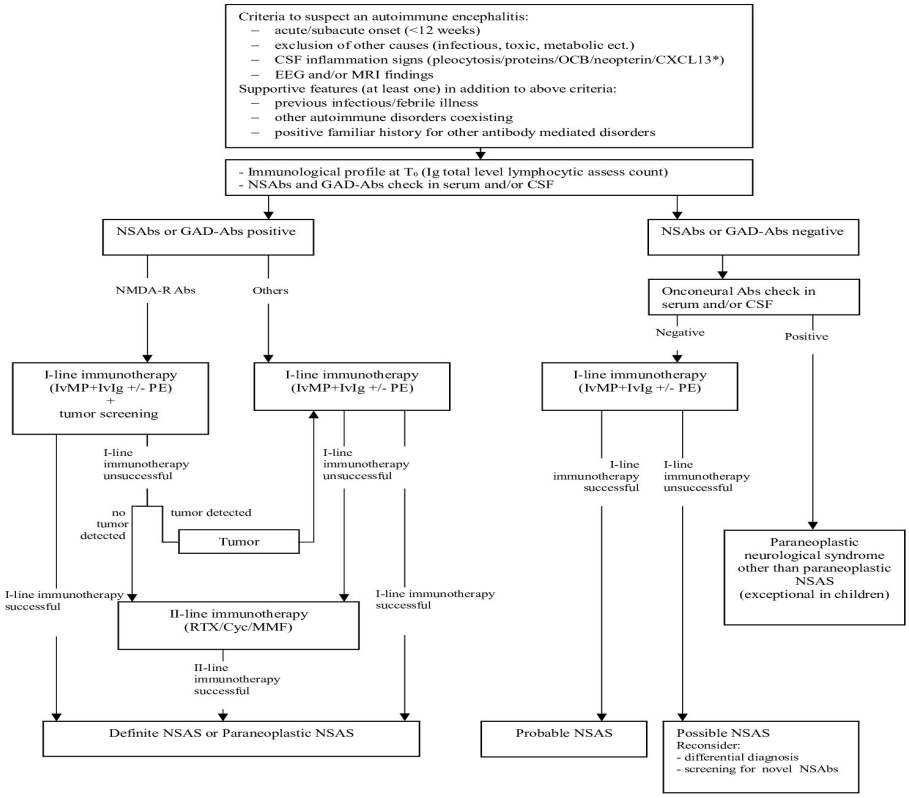

Figure 2. Flowchart for approaching the recognition of encephalitis related to neuronal surface antibody syndrome (NSAS) in children [modified from Zuliani *et al.*[1] 2012 and Suleiman *et al.*[8] 2013]. NSAbs: neuronal surface antibodies; CSF: cerebrospinal fluid; CXCL13: chemokine (C-X-C motif) ligand 13; MRI: magnetic resonance imaging; *: in NMDA-R encephalitis; GAD-Abs: antibodies to glutamic acid decarboxylase; *: GAD positivity is defined as > 1,000 u/mL.[8] OCB: oligoclonal bands; IvMP: intravenous methylprednisolone; IvIg: intravenous immunoglobulin; PE: plasma exchange; RTX: Rituximab; Cyc: cyclophosphamide; MMF: mycophenolate mophetil.

roughly specific for the different phases of the disease, allowing monitoring its progression.

As reported in adults, the different forms of pediatric NSAS share an overlap of clinical signs and symptoms. Typically, onset is acute/subacute (< 12 weeks) and occurs simultaneously or after a stressful event, such as infections, fever or cephalalgia. Behavioral and sleep disorders, confusion and short term memory impairment are often present, but epileptic seizures are generally the first obvious sign in children, consisting in focal seizures arising from bi-temporal lobes or, less frequently, generalized ones.[8-10] In this phase EEG recordings usually shows a diffuse background activity slowness, sometimes associated with superimposed paroxysmal abnormalities,[9,39] and MRI findings are usually normal or non specific.

Usually, a more acute stage follows, characterized by reduced consciousness, refractory/super-refractory epilepticus status and a progressive sinking into a coma lasting days to weeks.[40,41] Ventilator and/or other vital parameters support are often required,[13] as well as a heavy sedation that might easily hide eventual associated symptoms.[28]

In this phase, interictal EEG recordings may show a diffuse high delta waves activity with focal discharges, refractory multifocal status epilepticus[38,39] or less frequently, extreme delta-brush with superimposed periodic lateralized epileptiform discharges.[39] A close EEG monitoring is mandatory, inclusive of at least one 24 h video-EEG recording (duration to increase according to the clinical picture). On MRI, in this stage common findings are a T2/FLAIR hyperintensity in the medial temporal lobes involving the hippocampus, and/or the prefrontal areas and the cingulate gyrus. Only few exceptions are detectable, for example a frequent persistent negative findings in dipeptidyl-peptidase-like protein-6 encephalitis, an extensive multifocal or widespread diffuse abnormalities in NMDAR and GABAA-R encephalitis, and a basal ganglia involvement in D2-R encephalitis.[42,43]

A slowly progressive improvement of the interictal activity usually follows, sometimes preceded by a previous sleep background activity reorganization (personal observation), which generally gives way to a drug-resistant epilepsy although, in a minority of cases, a complete recovery is achieved.[44]

According to Suleiman classification, in order to help in the diagnosis 5 different autoimmune epilepsy categories are identifiable, on the basis both of the data obtained and of the immunotherapy response. Treatment may lead to remission, but the response depends on the specific form and possible association with malignancy.[7,45] In some forms, such as NMDA-R encephalitis, a complete recovery occurs in about 80% with a very low mortality rate.[13,14,46-48] When a complete remission does not occur, sequelae ranging from drug-resistant epilepsy associated with cognitive decline, to milder cognitive impairment are easily detectable.[1,12,13,47]

Following the treatment phase, EEG interictal activities slowly improve and longitudinal MRI studies can reveal a trend toward complete resolution, especially in some specific NSAS and when a rapid immunotherapy is administered. A more severe course towards a global atrophy predominantly affecting hippocampus, frontal and parietal regions usually characterizes the remaining cases.[43] An EEG monitoring including awakeness and sleep recordings (e.g. 40 min cad) as well as a neuroradiological surveillance are thereby suggested.

Ideally, CSF and autoimmune responses should be investigated as soon as there is suspicion of autoimmune encephalitis.

CSF FINDINGS

As in adult, CSF usually shows a mild to moderate lymphocytic pleocytosis (< 100 white blood cells/μL), increased protein concentration (< 150 mg/dL), normal glucose level and frequently elevated IgG index; oligoclonal bands result detectable in about 50% of cases.

In children, elevated CSF neopterin can be used as an additional marker of CNS inflammation.[10,49]

A recent study underlies the role of the CXCL13 chemokine as a potential CSF biomarker of clinical outcome in anti-NMDAR encephalitis, its prolonged or secondary elevation suggesting a limited response to immunotherapy, an higher risk of relapses, and thereby the need to a more aggressive therapeutic approach.[50]

IMMUNOLOGICAL FINDINGS

As soon as an autoimmune condition is suspected (at prodromal or early acute stage) and before immunotherapy is started, a serum and/or CSF sample must be taken for autoantibody testing, and a Ig total level as well as a study of lymphocytic profile should be performed, in order to get a value to compare later on with [Figure 2].

If NSAbs are detected, the diagnosis is usually easily achieved. On a practical level, the expanding NSAbs spectrum could make it difficult to choose which antibody to check first. In order to direct the diagnostic pathway, indirect immunohistochemistry on rat brain tissue or immunocytochemistry in primary rat neuronal culture can highlight staining patterns evocative for surface (e.g. neuropil) or intracellular antigens.

This preliminary screening must be confirmed by more specific tecniques, such as cell-based assay, ELISA or radioimmunoassay.[51] A blended approach reduces the false positive rate,[52] providing an efficient diagnostic tool for pediatric NSAS.

A paraneoplastic cause is much less probable in children, so that testing for onconeural antibodies (Hu, Ma2, CV2/CRMP5, Ri, amphiphysin) can be not strictly necessary, at first instance at least. Nevertheless, if a paraneoplastic clinical picture shows-up, a commercial immunoblotting assay specifically designed can be properly used to manage a differential diagnosis.

TUMOR SURVEILLANCE

Because of the low prevalence of malignancy in pediatric NSAS, many authors consider tumor surveillance not strictly necessary in the first instance, with the only exception related to NMDA-R encephalitis. Nevertheless it may become increasingly relevant in patients who are older at the time of clinical onset.[13]

TREATMENT

Due to antibodies pathogenicity, treatment is focused on reducing the serum antibodies titer. There is no consensus on the immunotherapy approach to carry out, but it has become increasingly clear that starting treatment as early as possible is crucial to achieve a better clinical outcome.[12,48]

At seizure onset, antiepileptic therapy, with few exceptions, usually results ineffective.[9]

During the acute phase, as the diagnostic work out may take time, starting immunotherapy empirically is highly recommended. Although some patients

undergo a complete recovery spontaneously, this is not frequent and it is not possible to identify the patients with a favorable outcome. Taking time before treatment waiting for immunological results or tumoral screening in anti-NMDAR cases is not recommended, not only because of the severity of the clinical findings but also because patients not promptly treated may be at higher risk for relapse.[12,53]

To date no consensus has been achieved on the treatment scheme to be used, and the available protocols are heterogeneous.

The first-line therapy usually includes a short course of high-dose steroids (methylprednisolone MP; 30 mg/kg/day i.v. per 3-5 days) followed by or combined with intravenous immunoglobulin (IvIg) administration (0.4 g/kg/day per 5 days).

Steroids are then tapered using 1-2 mg/kg/day orally, on average for another 12 weeks, adjusting the dose according to patient tolerability or possible side effects. If no benefit is noticed during steroid treatment, plasma exchange (PE), 3-5 cycles, should be considered.

In case the first-line treatment is unsatisfactory, a second-line immunotherapy should be started. It usually consists of rituximab 375 mg/mq per week every other week for 4 weeks,[54,55] cyclophosphamide (Cyc) 750 mg/m^2, 3 times or mycophenolate mophetil 600 mg/m^2, alone or in combination.[1,13]

The immunotherapy's effectiveness can be checked with HIC on frozen rat brain tissue to assess the lack of immunostaining.

In the meanwhile, antiepileptic treatment is usually continued, even though its real impact in modifying the epileptic course remains uncertain as long as the immune mechanism starts to decrease itself. The decision whether to withdraw antiepileptic drugs or not should be made according to the patient, the specific disorder and EEG findings in the follow-up.

Psychiatric symptoms and involuntary movements, when present, can be treated symptomatically, and medications with a broad effect on multiple symptoms are usually recommended. Long acting benzodiazepines, sedatives such as clonidine, and anticonvulsivant drugs may be helpful in improving abnormal movements and mood instability. The management of psychiatric symptoms is more challenging: sedative and sleep medications other than benzodiazepines seem to be the most effective, while antipsychotic drugs are less efficacious and often associated with adverse events.[56]

Finally, during the remission and stabilization phases a gammaglobuline total check should be repeated in order to detect rituximab induced hypogammaglobinemia, that can eventually be treated with a replacement of an extra dose of exogenous IvIg.[10,57]

Regarding relapse risk prevention, no data are available so far on the preventive value of chronic long term IvIg administration, but encouraging results come from the chance of monitoring the CD19+ and CD27+ lymphocytes value every 2 months, re-administering rituximab in case of their further increase.[58]

DISCUSSION

The early recognition of an immune mechanism underlying a neurologic disorder provides a chance to start early treatment and to achieve a better outcome.

Guidelines for NSAS in children have been recently developed,[10] extrapolated from a previous study by Zuliani[1] referred to adults, and mainly differing from it since focused on the higher epilepsy occurrence among pediatric symptoms.[10,59] As in Zuliani's, the role given to immunotherapy response becomes a retrospective feature that helps with the classification itself. This points out that, whenever a specific antibody is detected, the diagnosis of NSAS is easily achieved; conversely, the hypothesis lies in a shady area. In this paper, based on a review of the literature and the experience, the authors provide a simplified pathway that may facilitate the identification and the early treatment of these forms. Concerning the diagnostic algorithm many questions remain unanswered.

A field that requires further work is the differential diagnosis among the individual forms of NSAS but this was beyond the aim of the paper. The spectrum of signs and symptoms is wide and it is often difficult to achieve a specific diagnosis on clinical ground only because of the overlapping of clinical signs. The recognition of some highly characteristic clinical features is sometimes possible and further work using an integrated approach combining EEG, neuroimaging and early identification of the underlying immunological mechanism is highly recommendable, as it can lead to an early appropriate treatment and to the possibility of a perceivable clinical improvement.

Few reports are available to date about the predictive value of electrophysiological findings in NSAS.[60] Identifying specific EEG pathological patterns may help not only in distinguishing the different forms, but also in recognizing them at an initial stage, with the aim of administering an early treatment. Moreover MRI-patterns, despite providing a supportive feature in the diagnostic flow-chart orientation, are often quite a specific,[43] and thereby usually insufficient, if alone, to get specific NSAS distinguishable from each other, especially at clinical onset.

Correlations between electrophysiological and neuroradiological data are then mandatory, but remain partly unexplored. Modern neuroradiological techniques are now increasingly available and could be useful to better understand pathophysiological mechanism and disclose predictive outcome data in different NSAS.[43]

Moreover, no consensus is obtained to date about the sensibility and specificity of serum *vs.* CSF testing. Although a study performed by Gresa-Arribas *et al.*[61] in 2014 demonstrated the higher CSF testing reliability in NMDA-R encephalitis, consensual data lack on the others NSAS. Moreover, the same work stressed out a positive correlation between the antibody titer and the risk of relapse, but no predictive threshold value has been established to help in deciding to resort to a retreatment or a chronic immunotherapy.

Finally, concerning the therapeutic approach, strategies tailored to the individual syndrome should be outlined, considering a less aggressive approach for those with a usually better outcome, such as NMDA-R encephalitis.[46,47] This must be done by taking into account the possible side effects of immunotherapic drugs and a "risks *vs.* benefits" assessment per single patient.[62]

CONCLUSION

In children NSAS clinical picture is heterogeneous, often overlapping and still poorly outlined. Anyhow, the recognition of some characteristic clinical features are sometimes possible, and can help the diagnostic approach with the aim to start a proper and early treatment. Nevertheless, further studies on larger prospective pediatric cohorts and randomized treatment trials are required in order to assess the need to tailor more or less aggressive treatments according to long term outcome findings and prognostic factors in different NSAS.

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Conflicts of interest

There are no conflicts of interest.

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Review

Necroptosis: a new link between cell death and inflammation

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ABSTRACT

Necroptosis is a type of newly identified cell death induced by apoptotic stimuli under conditions where apoptotic execution is prevented. Studies over the past 10 years have revealed the molecular mechanism of necroptosis and challenged the old conception that necrosis is un-programmed. Recently, more and more data have emerged suggesting a close association between necroptosis and inflammation. In this review, the authors summarized the current knowledge of the mechanism of necroptosis, focusing on tumour necrosis factor α induced necroptosis and the roles of necroptosis in regulating inflammation. In particular, we discussed the occurrence of necroptosis and its relation with inflammation in neurological diseases hoping to provide new insight for the research and treatment of neuroinflammatory disorders.

Key words: Necroptosis; inflammation; neurodegenerative diseases

INTRODUCTION

Death is the most common ultimate fate of cells when challenged by death signals. By ultrastructural morphology, cell death is mainly classified as apoptosis, autophagy, or necrosis.[1] During past decades, extensive studies have been performed on apoptosis and autophagy, and have pictured very elegant molecular mechanisms for apoptosis and autophagy. By utilizing these mechanisms, apoptosis and autophagy can be finely regulated. Therefore, these 2 types of cell death are regarded as "programmed cell

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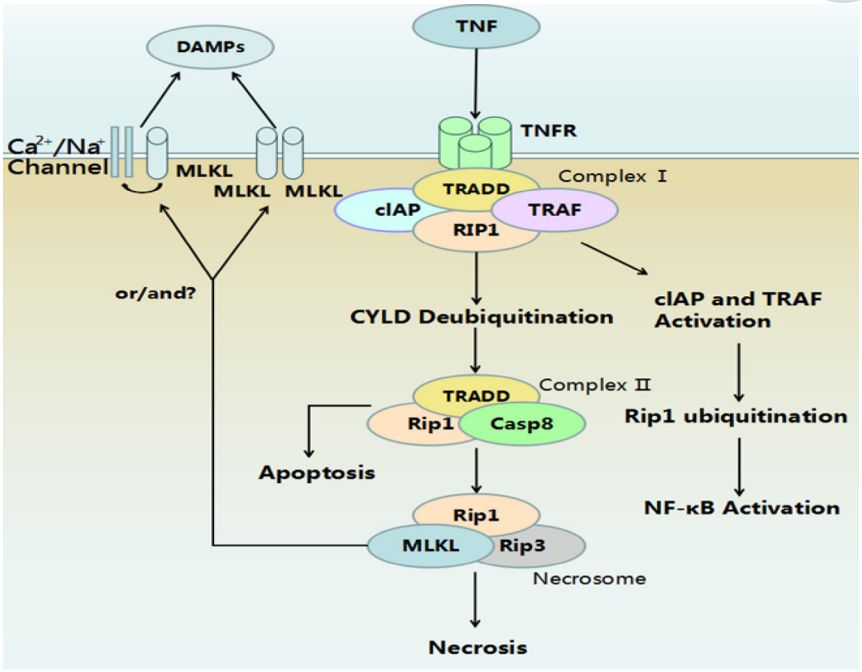

Figure 1: Mechanism of tumour necrosis factor-α (TNF-α) induced necroptosis. Binding of TNF-α to its receptor results in formation of Complex I. Activation of cIAP and tartrate resistant acid phosphatase activates downstream NF-κB signaling and subsequently promote cell survival. Complex II acts as a switch between apoptosis and necroptosis. Activation of caspase-8 guides the cells to apoptosis. Inhibition of caspase-8 leads to formation of a necrosome. Membrane translocation of phosphrylated MLKL disrupts cell membrane. The mechanisms underlying the lysis of cytoplasmic contents during necrosis are still unclear. DAMPs: damage associated molecular patterns; MLKL: mixed lineage kinase domain-like protein; TNF: tumour necrosis factor; TNFR: tumour necrosis factor receptor; cIAP: calf intestinal alkaline phosphatise; TRADD: tumor necrosis factor receptor associated death domain; TRAF: TNFR-associated factors; RIP1: receptor-interacting protein 1; CYLD: cylindromatosis; Casp8: caspase-8; RIP3: receptor-interacting protein 3; NF-kB: nuclear factor kappa.

death". Necrosis, however, has long been thought as acute and uncontrollable, largely owning to its elusive molecular mechanism, and thus been thought as "un-programmed".

In the year of 2005, Dr. Jun-Ying Yuan[2] at Harvard University reported a novel type of necrosis, which occurred in cells when the apoptosis machinery is inhibited, but extracellular apoptotic stimulation persisted. This type of necrosis can be inhibited by a chemical named Necrostatin-1 (Nec-1), which suppresses the activity of receptor-interacting protein 1 (RIP1), suggesting that the cell death is molecularly regulated. Because the dying cells the researchers originally identified showed a mixture of ultrastructural features of both apoptosis and necrosis, for example, condensation of chromatin, disruption of the cell membrane and lysis of cytoplasmic contents, it was termed as necroptosis (necrosis + apoptosis). Later on, it was found to show mostly the morphological features of unregulated necrotic death.[3]

MOLECULAR MECHANISM OF NECROPTOSIS

Since Dr. Yuan's publication, many studies have been performed on the occurrence and molecular mechanism of necroptosis. Most of the current knowledge about necroptosis comes from tumour necrosis factor α (TNF-α) induced necroptosis. Necroptosis is initiated when death signals such as TNF-α and Fas bind to their membrane receptors. This ligation leads to the formation of a membrane associated protein complex, named complex I.[4] Complex I is composed by: (1) proteins which have a death domain such as tumor necrosis factor receptor (TNFR)-associated death domain (TRADD), Fas-associated death domain (FADD); (2) RIP1; (3) TNFR-associated factors (TRAF), such as TRAF2 or TRAF5, and (4) cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2.[5] TRADD acts as an adaptor for recruiting RIP1 to TNFR1. Subsequently, TRAF2/3/5 and cIAPs are added into the protein complex.[6] If E3 ubiquitin ligase is activated, TRAF2/5 and cIAP1/2 can ubiquitinate RIP1, which results in stabilization of the RIP1-containing plasma membrane associated complex that activates nuclear factor kappa and mitogen-activated protein kinases, and thus promoting cell survival.[7] Therefore, protein complex I determines the fate of cells to either survival or death.[8]

Activation of necroptosis signalling starts with deubiquitination of RIP1 and other components by deubiquitinating enzyme cylindromatosis, which removes ubiquitin chains from RIP1, thus, destabilizing Complex I.[9] Deubiquitinated RIP1 is released from Complex I and combines with FADD, TRADD,

receptor-interacting protein 3 (RIP3) and caspase-8 to form Complex II.[10] Active caspase-8 can cleave and inactivate RIP1 and RIP3, thereby promoting the exogenous apoptosis pathway.[11]

If caspase-8 is inhibited, RIP1 and RIP3 will remain active and combine together by the common RHM domain to take part in forming a necrosome, which initiates a downstream signal cascade resulting in necroptosis.[12] Although RIP1 and RIP3 are both essential in the process, over-expressed RIP3 can induce necroptosis without enough RIP1, but not vice versa.[13] It has been demonstrated that RIP3 activates downstream signalling pathways, in particular, the phosphorylated mixed lineage kinase domain-like protein (MLKL),[14] which plays a central role in the execution of necroptosis. Two models have been proposed for its function: (1) acts as a platform at the plasma membrane for the recruitment of Ca^{2+} or Na^{+} ion channels,[15] (2) as a direct pore-forming complex on cell membranes through binding of the amino-terminus of the 4-helical bundle domain to negatively charged phosphatidylinositol phosphates.[16] Previous studies have suggested that phosphoglycerate mutase 5 involved mitochondrial fragmentation might be the key downstream molecule of MLKL for necroptosis execution.[17] However, recent evidences have challenged this idea.[18]

The mechanism of necroptosis discussed above is outlined in Figure 1. Besides the TNF-α induced extrinsic necroptosis, an intrinsic necroptosis signalling initiated by intracellular reactive oxygen species has been proposed recently. Translocation of p53 has been suggested to play a role in ischemia induced intrinsic necroptosis.[19] More detailed mechanisms of intrinsic necroptosis remain to be elucidated.

NECROPTOSIS AND INFLAMMATION

It is known that apoptosis triggers minor or no inflammation, while necrosis induces inflammation via releasing damage associated molecular patterns (DAMPs), such as nuclear high mobility group box-1 proteins, mitochondrial DNA, and IL-1 family cytokines.[20] The insertion of MLKL into cell membranes immediately suggested a possible role of MLKL in the release of DAMPs. Because DAMPs stimulate pattern-recognition receptors such as toll-like receptors, necroptosis is thought to be beneficial in innate immune responses. For example, vaccinia virus encodes an inhibitor of caspase-1 and 8. In cases of vaccinia virus infection, the cells exhibit RIP3-dependent necroptosis, which mobilize immune cells against viruses.[21] Therefore, in certain virus-infected diseases, necroptosis seems to be an evolutionarily cellular anti-virus strategy.

In terms of bacterial infection, it has long been known that TNF is an important driver of bacterial sepsis,[22] suggesting that necroptosis may also be a pro-inflammatory factor in the bacterial infection-induced inflammation. In consistent with above mentioned role of RIP/MLKL-dependent necroptosis in the destructive inflammation after virus infection, RIP3 deficient mice are more resistant to TNF induced systematic inflammation.[23] However, some studies showed that $RIP3^{-/-}$ macrophages respond almost normally to liters per second stimulation, indicating that RIP3 may not be crucial for acute inflammation after bacterial infection.[24] In line with this idea, RIP3-dependent necroptosis and TNF expression was observed in tuberculosis infected tissue,[25] suggesting a role of necroptosis in the bacterial induced chronic inflammation.

In addition to its roles in infectious diseases, necroptosis has also been demonstrated to be involved in chronic sterile inflammation. For example, up-regulation of RIP3 and phosphorylated MLKL were detected in alcoholic and drug-induced liver injury. RIP3 depletion, or necrostatin-1 (Nec-1) administration can significantly protect liver cells from these injuries.[26] In ischemia-reperfusion conditions, necroptosis was reported in multiple tissues, including brain, heart, kidney and retina.[27-29] In other chronic inflammatory diseases such as atherosclerosis, receptor-interacting serine/threonine-protein kinase 3-dependent macrophage necroptosis has been thought of as a direct driver of atherosclerotic plaque formation.[30] Although it has been clearly demonstrated that necroptotic cells release DAMPs, how DAMPs mediate this necroptosis-triggered sterile inflammation remains to be experimentally validated.

It should be pointed out that many studies used Nec-1 to inhibit necroptosis. However, recent studies reported that Nec-1 has off-target effects. Besides inhibiting the kinase activity of RIP1, it inhibits the activity of endoleamine 2,3-oxygenase, which by itself, modulates inflammation.[31] Therefore, one should be sure to explain the results obtained solely by Nec-1 treatment.

NECROPTOSIS AND NEUROLOGICAL DISEASES

Necroptosis was initially identified in ischemic brain.

As the molecular mechanisms of necroptosis have been gradually discovered, necroptosis has been reported in more and more neurological diseases.

Spinal cord injury (SCI) is well known for its devastating effects on patients. One pathological feature of SCI is secondary injury characterized by chronic inflammation, astrogliosis and cavity formation.[32] Previous studies have demonstrated that application of Nec-1 can be protective for SCI, but which cells undergo necroptosis is unknown.[33,34] Our recent studies demonstrated that RIP3 and phosphorylated MLKL are up-regulated in reactive astrocytes and microglia after SCI.[35,36] Reactive astrocytes, which line the spinal cavity, die by M1 microglia/macrophage induced necroptosis partially through toll like receptor/myeloid differentiation 88 signalling.[35] Microglia, the major player of chronic inflammation post-SCI, die through endoplasmic reticulum stress involved necroptosis.[36] These researches raised the straightforward question of how necroptosis regulateschronic inflammation after SCI.

Multiple sclerosis is another neurodegenerative diseases characterized by demyelization and chronic inflammation. The link between inflammation and demyelination has long been recognized. A recent study from Prof. Jun-Ying Yuan's group reported that TNF-α induces the death of oligodendrocytes in a RIP1/3 dependent manner.[37] In the mouse model of Gaucher's disease, systemic TNF-α and IL-1β are elevated, and RIP3 is up-regulated in microglia and neurons. RIP3 knockout can significantly ameliorate the development of disease and prolong the survival of animals.[38] Amyotrophic lateral sclerosis (ALS) is the most adult onset motor neuron degenerative disease, in which inflammation is the most striking hallmark of pathological changes. Recently, it has been demonstrated that in the spinal cord of the ALS model, motor neurons also undergo necroptosis.[39] These studies suggested that necroptosis in different neurodegenerative diseases is cell type specific. The underlying mechanisms remain to be further investigated.

CONCLUSION

In summary, this progress brings us the new concept that necrosis can be chronic and controllable, although the mechanism of necroptosis remains far from fully revealed. The current evidences suggest that pro-inflammatory factors, e.g., TNF-α, can induce necroptosis, which in turn triggers inflammation. As we know, inflammation is complex and dynamic. The outcome of inflammation depends on the coordination of different types of immune cells. Even sub-populations of immune cells change their phenotypes in the course of inflammation, such as the M1-M2 switch of microglia/macrophages.[40] How necroptotic cells affect the different immune cell populations during the different time-phase of inflammation, or how necroptotic cells influence the phenotype of immune cells remain to be further investigated.

Recently, several studies showed that RIP1 and RIP3 might also be involved in inflammation independent of necroptosis.[41] For example, Inflammasome activation and release of IL-1 in smac-mimetic treated macrophages or in caspase-8 deficient dendrite cells are dependent on RIP3,[42] suggesting a cell-death independent role of RIP3 in inflammasome activation. This should be considered when evaluating results. Combined results from MLKL deficient cells or mice may be helpful for clarifying the point. In view of the importance of necroptosis and its roles in inflammation, better understanding of the interaction between necroptosis and inflammation will be helpful for treatment of inflammatory diseases.

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Conflicts of interest

There are no conflicts of interest.

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Case Report

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Differentiation of radiation necrosis from glioblastoma recurrence after radiotherapy

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ABSTRACT

The standard treatment of glioblastoma, the most common type of primary-brain-tumor, involves radiotherapy with concomitant temozolomide chemotherapy. A patient with glioblastoma, post radiotherapy developed magnatic resonance imaging (MRI) changes consistent with either radiation-induced tumor necrosis or tumor recurrence. Perfusion MRI was suggestive of radiation necrosis, but magnetic resonance spectroscopy and ^{99m}Tc-Tetrofosmin single photon emission computed tomography was indicative of tumor recurrence. Positron emission tomography scan was not available. Tumor recurrence was documented by biopsy. Several advanced imaging methods are available to differentiate tumor recurrence from radiation necrosis in glioblastoma patients. However, in inconclusive cases, brain biopsy should be performed for definite diagnosis.

Key words: Glioblastoma multiforme; single photon emission computed tomography; magnatic resonance imaging; spectroscopy; radiotherapy

INTRODUCTION

Glioblastoma multiforme (GBM) is the most frequent primary-brain-tumor in adults. Its profound cellular heterogeneity, presence of stem cells and highly invasive characteristics[1] renders this tumor difficult to treat, exhibiting high recurrence rates and poor survival.[2] The initial therapeutic intervention involves maximal surgical resection, if the tumor is surgically accessible, otherwise a needle biopsy, to document the diagnosis and determine the histologic grade, suffices. The mainstay of post-surgical therapy involves conventional radiotherapy, with an approximate

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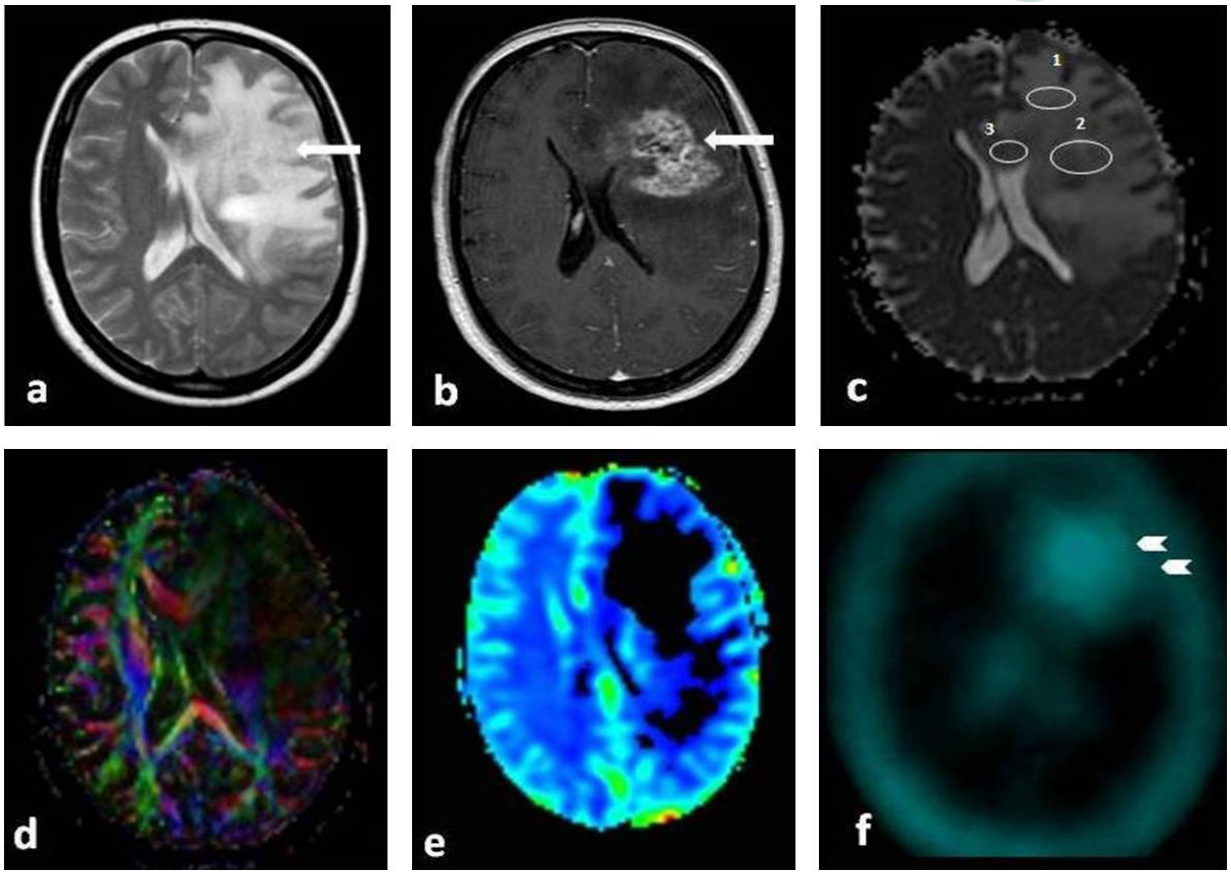

Figure 1: T2-W axial images (a), T1-W axial images with intravenous contrast administration (b), demonstrated an extensive edematous lesion with mass effect and heterogeneous enhancement (arrows), characteristic of both tumor recurrence and radiation necrosis. The diffusion weighted imaging (DWI) demonstrated decreased values of the apparent diffusion coefficient (ADC) maps (c) ($1.2\text{-}1.8 \times 10^{-3} mm^2$) consisted with malignant tumor infiltration. However, the fractional anisotropy (FA) (d) maps showed increased white matter tracts destruction (low FA) and the perfusion maps (CBV) (e) depicted very low perfusion, both consisted with radiation-induced necrosis. SPECT (f) revealed increased metabolic activity (arrowheads) consisted with tumor recurrence

total radiation dose of 5,500 to 6,000 rads over 6 weeks period, and with concomitant temozolomide chemotherapy.[3] Further chemotherapy with temozolomide may be given for approximately 1 year thereafter. In any step during post radiotherapy follow-up, change of any imaging characteristic requires differentiation between tumor progression, requiring therapy change, and radiation-induced necrosis, requiring mostly symptomatic therapy.[4] In the present case the above question was raised in a young patient requiring a large number of diagnostic tests to be performed, which produced conflicted results requiring partial tumor resection for the correct diagnosis.

CASE REPORT

A 35 years old woman was diagnosed with a left-frontal astrocytoma, grade II, 5 years prior to admission to our hospital, during which time it was partially resected without further treatment. Two years prior to admission, she had a recurrence, and a new resection revealed progression to glioblastoma multiforme (grade IV). She underwent standard radiotherapy (6,000 rads) with concomitant temozolomide therapy, followed by 1 year of 5 days per month temozolomide chemotherapy. Subsequently, she remained stable on no further therapy until 1 year later when she developed progressive right hemiparesis, expressive aphasia and seizures. At that time, an MRI was performed demonstrating, in the T2-W axial images [Figure 1a] and T1-W axial images with intravenous contrast administration [Figure 1b], a mass lesion consistent with either tumor recurrence or radiation necrosis. Diffusion weighted imaging (DWI) [Figure 1c] showed restricted diffusion consistentwith malignant tumor infiltration. However, the fractional anisotropy (FA) [Figure 1d] maps and the perfusion maps (CBV) [Figure 1e] were consistent with radiation-induced necrosis. Subsequently, a ^{99m}Tc-Tetrofosmin single photon emission computed tomography (SPECT) was performed [Figure 1f], which revealed increased metabolic activity, indicative of tumor recurrence. Magnetic resonance (MR) spectroscopy demonstrated decreased N-acetylaspartate (NAA) and increased

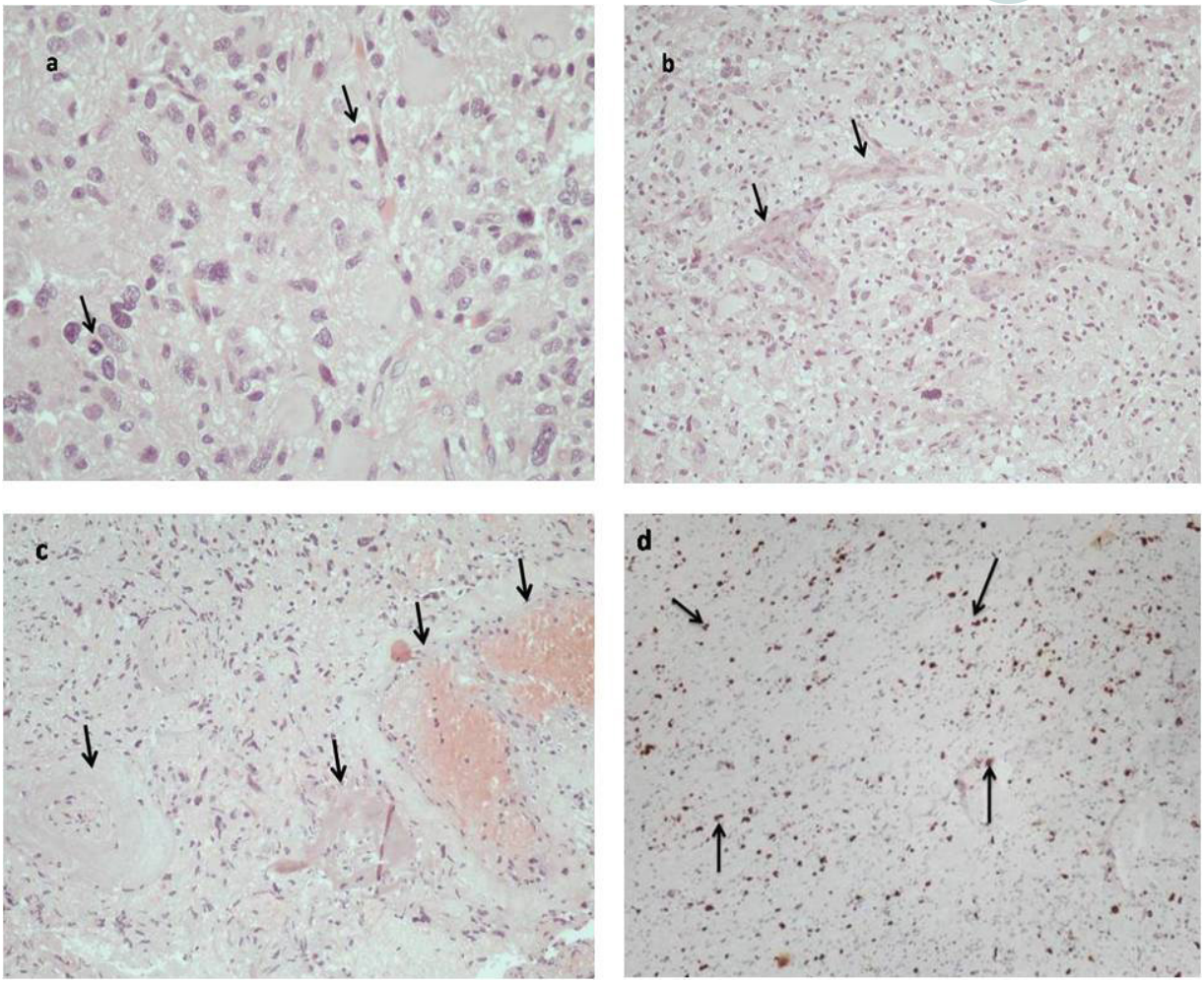

Figure 2: (a) Histological examination revealed a neoplasm with highly pleomorphic cells exhibiting several mitoses (arrows); (b) microvascular proliferation was also evident, a common finding in high grade gliomas (arrows); (c) some areas of the biopsied tumor showed evidence of radiation-induced changes, such as necrosis (arrows) and vascular changes such as telangiectasia and hyalinization of vessel walls adjacent to areas of hypercellular tumor tissue (arrows); (d) the proliferation marker MIB1 (Ki67) was expressed in a high proportion of tumor cells (arrows).

choline (Cho) with ratio Cho/NAA > 3.63, compatible with infiltration of malignant cells with high metabolic activity (not shown). Positron emission tomography (PET) scan was not available atour institution.

Due to discrepancy between the imaging tests, a partial surgical resection was performed for accurate diagnosis. Histological examination showed evidence of tumor recurrence in most of the resected tissue [Figure 2a and 2b]. There were some small parts of the tumor exhibiting radiation-induced changes as well [Figure 2c]. The high proliferation rate of the tumor cells denoted an aggressive tumor [Figure 2d]. The patient was started on daily low dose temozolomide (50 mg/m^2 of body surface per os) administration and oral dexamethasone at 6 mg/day, and she remained alive and stable 6 months after the operation.

DISCUSSION

After the standard initial therapy, consisting of a combination of radiotherapy with temozolomide chemotherapy[3] followed by plain temozolomide therapy, glioblastoma multiforme usually recurs within the first year from therapy.[5] However, at that point it may be difficult to differentiate between radiation necrosis of the tumor *vs.* tumor recurrence, since both conditions behave similarly on traditional imaging modalities (Standard CT or MRI with and without IV contrast administration).[6] In these cases, more advanced imaging modalities can be employed to differentiate between tumor recurrence from radiation necrosis, such as PET[7] and perfusion/diffusion MRI.[8] However, in many countries, PET is not widely available, necessitating the use of other nuclear medicine modalities such as SPECT with various tracers.[9]

MR spectroscopy is another alternative imaging method to improve our diagnostic capabilities in brain tumor evaluation, however, this modality is still not widely available.[10] NAA is an acetylated

amino acidconcentrated in neurons that functions as a neuronal marker. Any destruction of neurons, such as noted by high grade malignant tumors or radiation-induced necrosis, decreases the amount of NAA and thus reduces its peak in MR spectroscopy. Choline (Cho) is an essential nutrient that, in addition to being a precursor in acetylcholine synthesis, is a basic component of sphingomyelin and phosphatidylcholine, both constituents of cell membranes. During MR spectroscopy, Increased Cho indicates higher cellularity, as seen in tumors, and decreased Cho indicates radiation-induced necrosis. In general, an increased ratio of Cho/NAA is indicative of brain tumor growth. In our patients, a Cho/NAA > 3.63 was considered a strong suggestion for tumor recurrence, rather than radiation necrosis. Partial resection of the tumor mass and histologic examination demonstrated the presence of recurrent tumor. However, in some areas of the mass, the occurrence of radiation-induced changes may at least partially explain the discrepancy between the various imaging modalities.

In conclusion, the differentiation of treatment induced necrosis from glioblastoma recurrence or progression is imperative in order to employ the appropriate treatment. Although it may be impossible to differentiate such a condition with conventional imaging, new technology, such as combination of diffusion/perfusion MRI, MR spectroscopy with either PET or SPECT, should be able to accurately diagnose the patient's condition. In rare cases, such as in ours, biopsy may be needed if the imaging methods are contradictory.

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Conflicts of interest
There are no conflicts of interest.

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Commentary

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Sex differences in Alzheimer's disease

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Dr. Yi Xing, Department of Neurology, Xuan Wu Hospital, Capital Medical University, Beijing 100050, China. Research interest: the effects of hormones and metabolism on the pathogenesis of neurodegenerative disorders, especially Alzheimer's disease. Publications: eight SCI articles as the first author, including progress in Neurobiology, *Journal of Alzheimer's Disease*, *et al.*

Recently, Koran *et al.*[1] published an article, named "Sex differences in the association between Alzheimer's disease (AD) biomarkers and cognitive decline" in *Brain Imaging and Behavior*. The result proved that there were sex-specific associations between biomarkers of AD. This article added evidence to the theory of sex differences in AD.

Sex difference is a common phenomenon in AD and manifests in many ways. Females are disproportionately affected by AD. It is well known that females have higher prevalence of AD than males. In China, for urban population, the AD prevalence in male is 1.27%, while in female, it is 3.54%; for rural population, this difference is even more dramatic: 1.95% in men *vs.* 6.30% in women.[2] However, sex differences in the incidence of AD are still inconsistent. Studies tend to show that there are no sex differences in incidence of AD until very late of age when females have the higher incidence.[3] However, females seem to have higher risk of progression from mild cognitive impairment (MCI) to AD. A meta-analysis showed that the risk of progression from MCI to AD is 1.33 fold higher in women than in men.[4]

The pathological changes of AD are characterized by the deposition of extracellular amyloid-β (Aβ) and the presence of neurofibrillary tangles. Pathological studies showed that females had more global AD pathology than males, especially for neurofibrillary tangles. More interestingly, females were more susceptible for AD pathological changes. It was reported that 1 additional unit of AD pathology was associated with a nearly 3-fold increase in the risk of clinical AD in men; while for women, this odd increased to 20-fold.[5]

Nowadays, researchers seek to observe brain pathological changes *in vivo*, using magnetic resonance imaging (MRI), positron emission tomography or cerebrospinal fluid (CSF). More and more studies on biomarkers of AD are underway. MRI is a common and convenient method to help

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us observe brain atrophy. After 1,368 MRI scans, a study demonstrated that brain atrophic rates were about 1-1.5% faster in females than males.[6] The levels of $A\beta_{42}$ and tau protein in CSF are promising biomarkers of AD. Although by now, there is no remarkable evidence suggesting sex differences in $A\beta_{42}$ and tau levels in CSF, Koran's study showed that associations between $A\beta_{42}$ and tau levels in CSF and brain atrophy in MRI were sex-specific. A total of 348 normal control, 565 patients with MCI and 185 patients with AD from Alzheimer's disease neuroimaging initiative were included in this study. Participants were followed for an average of 2.5 years. A significant interaction between sex and CSF $A\beta_{42}$ levels was found on longitudinal hippocampal atrophy and longitudinal decline in memory and executive function. A similar interaction between sex and CSF tau levels was also observed. With the decrease of CSF $A\beta_{42}$ levels or increase of tau levels, females showed faster hippocampal atrophy and cognitive decline compared with males. This result was not modified by different diagnosis.

The authors pointed out in discussion that the result could be explained by age-related changes in estrogen levels. Actually, the decline of estrogen levels after menopause is only one of the reasons that are related to sex differences in AD. The interaction between genes, hormones and social environments may be the key to unraveling broad sex differences in AD.

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Conflicts of interest

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Editorial

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Astrocyte, reactive astrocytes and self-regulative apoptosis in the neuroinflammation

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Astrocyte, one of the most abundant glial cell types, actively functions in stabilizing neural circuits and synaptic transmission in the central nervous system (CNS). Astrocytes not only provide metabolic and trophic supports to various CNS neurons and but also actively work in assisting synaptic transmission and plasticity. A line of growing evidences have documented that astrocytes present as an essential coordinatorin neural circuit function.[1] Firstly, calcium signaling or calcium wave calcium (Ca^{2+}) between neighboring astrocytes contribute to establishment of a huge astrocytic glial network by gap-junctions, which has updated the understanding of astrocyte function in CNS, and led to an idea that astrocytes are powerful regulators of neuronal spiking, synaptic plasticity and brain blood flow as well.[2] The Ca^{2+} wave in astrocyte processes may also precede onset of hyperemia and function as regulators of neurovascular coupling.[3] Secondly, astrocytes can also fast respond to sensory stimulation and involve in generation of neuronal rhythmic activity, and blockade with a Ca^{2+} chelator can sufficiently prevent neurons from a rhythmic bursting, indicating that astrocytes partially and critically contribute a fundamental neuronal firingpattern or generation of rhythmic activity.[4] Thirdly, distinct astrocytic transporters like well-known glutamate transporter 1 (GLT-1) and dynamic diffusion play a physiological modulating role in shaping synaptic transmission between neurons. Glutamate action time in synaptic transmission is controlled by the astrocytic GLT-1, i.e. while impairing GLT-1 diffusion could slow kinetics of excitatory currents or prolonged time course of synaptic glutamate transmission.[5]

Reactive astrocytes, a most common pathological hallmark, contribute to pathogenesis or progression of neurological disorders like trauma, ischemia, Alzheimer's and Parkinson's disease (PD). Astrocytes can *in vitro* and *in vivo* respond to various stimuli in trauma, ischemia and diseased conditions, fast change morphology and functional properties, and many appear asactivated or become reactive astrocytes. Reactive astrocytes undergo phenotypic changes and contribute to pathogenesis or progression of neurological disorders. For instance, those reactive astrocytes have been proposed to be incompetent bystanders in epileptogenesis as a result of cellular changes rendering them unable to perform housekeeping functions in diseased CNS.[6] Astrocytes modulate excitatory and inhibitory balance by regulating astrocytic uptake of gamma amino acid butyric acid and glutamate efficiency.[7] The reactive astrocytes or astrogliosis resulting from neuronal hyperexcitability further render inhibitory activity in epilepsy. New findings have thus challenged us to consider an important contribution of activated astroglial cells in epileptogenesis in the acquired epilepsy, although epilepsy has long been considered as a disease caused byabnormal increasing bursts of excitatory neurons, exclusively.[6,7] In addition, the reactive astrocytes are characterized with high level of G protein-coupled receptors such as adenosine receptor, which were

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evidently implicated in regulation of learning and memory activity in CNS. Conditional removal or down-regulation of these specific receptors enhanced memory in aging or Alzheimer's disease (AD) animals, indicating abnormal increase of adenosine receptor A2a in reactive astrocytes might contribute to AD-linked memory loss.[8]

Reactive astrocytes may display a self-regulativeapoptosis for modulation of over-activated astrocytes or functional balance in the neuroinflmmatory event or diseased CNS. Cell apoptosis was earlier identified in the reactive astrocytes, but its real significance remains unclear in the neuroinflammation and diseased conditions. Astrocytic apoptosis *in vitro* and *in vivo* might attribute to Ca^{2+} overload, mitochondrial dysfunction, oxidative stress and NF-κB signaling activation.[9] It is known that obvious inflammatory injury of CNS neurons occur in pathogenesis of neurodegenerative diseases such as AD and PD. While the reactive astrocytes and microglial cells dominate in the inflammatory reaction, major histocompatibility complex II (MHC II) levels were significantly up-regulated in midbrain of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD and MHC II was mainly localized in astrocytes and microglia, in which MHC II mediated T cell activation in initiating immune reaction and in participating disease progression.[10] Besides, astrocyte activation and apoptosis was observed in lipopolysaccharide (LPS)-triggered inflammation.[11] Hyper- or over-activated astrocytes appear as hypertrophied morphology and glutamate transporter 1 up-regulation or functional changes that afterwards affect microglia-involving cytokine generation and inflammation. The reactive astrocytes underwent necroptosis in the animal model with spinal cord injury through M1 microglia/macrohpage-mediating way.[12] By *in vivo* and *in vitro* studies with LPS and MPTP models, we demonstrated that LPS plus cytokines or MPTP insult could result in obvious activation of astrocytes from the ventral midbrain and cerebral cortex, followed by appearance of apoptosis in a proportion of those reactive astrocytes. By mechanical analysis, apoptosis of reactive astrocytes was resulted from bax and cleaved caspase 3 up-regulation, and might be possibly related to significant up-regulation or activation of inducible gas-1 signaling.[13] In addition, N-Myc Downstream-regulated gene 2 (NDRG2) was also found to involve in the astrocytic apoptosis and inhibition of NDRG2 expression reduced astrocytic apoptosis in ischemia.[14] The ischemia-induced autophagy also influenced apoptosis of reactive astrocytes and down-regulation of autophagy caused time-dependent changes in extrinsic and intrinsic apoptotic pathways, indicating that autophagy in astrocytes might also act as an early adaptive response before initiation of apoptosis and necrosis.[15]

Taken together, we hypothesize that apoptosis of reactive astrocytes may also possibly present a self-regulator for those over-activated astrocytes or functional balance between neurotrophic and inflammatory properties in neuroinflammation, while reactive astrocytes work actively as aparticipatorinastrocyte-microglial communication and microglia-dominating inflammatory response. Nevertheless, one critical question regarding this self-regulatory apoptosis of reactive astrocytes in ameliorating the neuroinflammatory injury should still merit further extensive investigations to elucidate its exact roles in pathogenesis and disease progression of various neurological disorders.

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Conflicts of interest

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Case Report

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Progressive muscle cramps with pain as atypical initial presentations of amyotrophic lateral sclerosis: a case report

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease and is a progressive and devastating neurodegenerative disease that affects both lower and upper motor neurons. Muscle cramps, which are characterized by a sudden, painful, involuntary contraction of muscles, are not rare in ALS patients. However, muscle cramps do not normally present early in ALS and therefore not used for the initial diagnosis of ALS. In this paper the authors present a case of ALS with initial manifestation of progressive painful muscle cramps in the absence of muscle weakness. This case might help people to recognize atypical foremost presentations of ALS and therefore formulate effective therapies.

Key words: Muscular cramps; amyotrophic lateral sclerosis; motor neuron disease

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive, fatal neurodegenerative disorder.[1,2] The worldwide incidence of ALS is around 1.7-2.3% per 100,000 population per year and the average age of onset is 61.8 and 49.8 for Western countries and China individually. The occurrence rate of men is higher than women.[3-6] The pathogenesis of motor neuron disease (MND) remains largely unclear, genetic factors and various exogenous risk factors are suggested to be associated with MND, including rural residency, alcohol consumption, smoking, toxic substance, glutamate metabolism disorder and aberrant autoimmunity.[4-6]

Typical clinical presentations of ALS include asymmetric weakness of limbs (60-80%), bulbar symptoms (20%), respiratory muscle weakness (1-3%), generalized weakness in limbs and bulbar muscles (1-9%), axial onset with muscle weakness, muscle atrophy and fasciculations.[7,8] Muscle cramping with pain presents in 7-12% of ALS patients.[9] Muscle cramps, are not rare in ALS patients, but rarely act as initial symptom without muscle weakness of the ALS patients. Some studies reported that muscle cramps could appear during the early phase or prodromal phase of ALS, and muscle cramps could help in the early diagnosis of ALS.[10,11] In this paper we present a rare case with initial manifestation of progressive painful muscle cramps in the absence of obvious muscle weakness.

CASE REPORT

A 56-year-old Chinese woman presented with progressive limb muscle cramps with pain and walk difficulty for the last 7 months. She had no special medical history before. At the beginning, the muscle cramps started in her left lower limb, 4 months after the onset, muscle cramps were aggravated, and slowly

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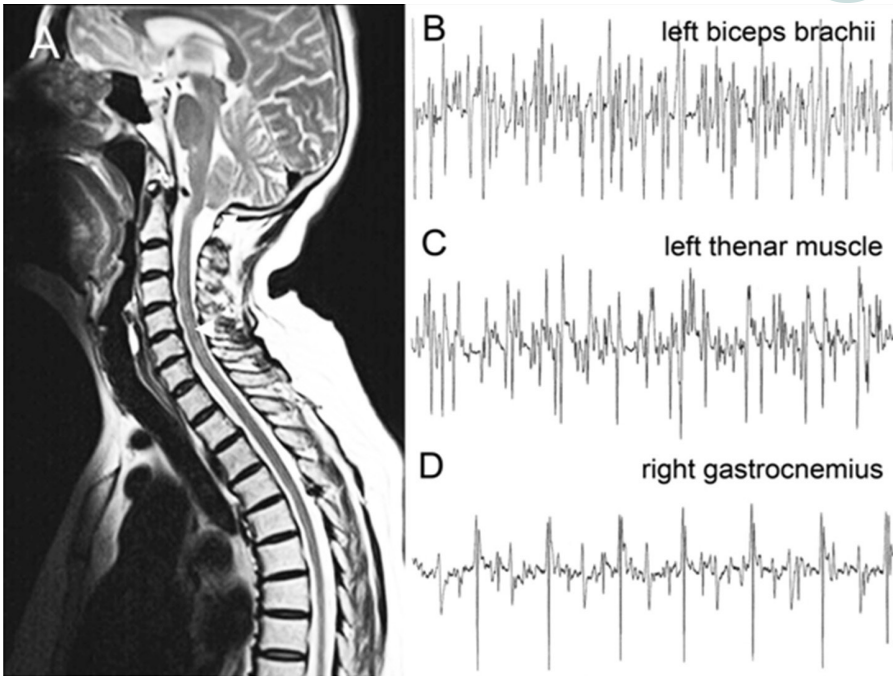

Figure 1: Brain and spine image and electromyography of the ALS case. (A) Brain MRI suggests Arnold-Chiari malformation type I (arrow). Spine MRI reveals slight prominence of intervertebral disks at C4/5, C6/7 and L4/5-L5/S1, and no abnormality was observed in the whole spinal cord. (B-D) Electromyography showed denervation potentials in the left biceps brachii (B), the left thenar muscle (C) and right gastrocnemius (D). ALS: amyotrophic lateral sclerosis; MRI: magnetic resonance imaging

progressed to her right lower limb and left upper limb in turn. Muscle cramps could be induced by touching her skin of limbs and chest. Muscle cramps frequently broke out during sleeping, and could be alleviated slightly by standing. Stiffness and weakness developed in the lower limbs for 4 months after symptom onset. Fasciculations, combined with tight feeling of the hips, was seen in both lower extremities and left upper limb. Then she had difficulty in walking, button-up her clothes and bradykinesia. There was no numbness and muscle atrophy of her tongue, face, hands and limbs. She had no complaints of dysphagia, dysarthria or dyspnea and history of alcohol drinking or cigarette smoking, no family history of neurological diseases.

Physical examination revealed a normal body figure, well-oriented in place, time and person, with no unconsciousness. Cranial nerve examination revealed no abnormalities. Examination of limbs revealed spastic rigidity in both lower limbs, with 4/5 muscle power in both lower limbs, 4/5 muscle power in left upper limb, and 4/5 to 5/5 muscle power in right upper limb. There was no atrophy of the limbs, trunk and lingual muscles. Sensations and cerebellar examinations were unremarkable. Deep tendon reflexes in the 4 limbs were asymmetrical that left limbs were hyperactive and right limbs brisk. Hoffman's reflexes and patellar clonus were negative. Left ankle clonus was positive. Babinski's signs were presented in both sides. For laboratory examinations, blood cell count, urine analyses, liver function, renal function, muscle enzymes (creatine kinase, creatine kinase-MB, aspartate aminotransferase, lactate dehydrogenase, hydroxybutyrate dehydrogenase), serum vitamin B12, autoantibodies and tumor biomarkers were normal. Intracranial pressure and cerebral spinal fluid analysis were normal. Brain and spine magnetic resonance imaging (MRI) suggest Arnold-Chiari malformation type I [Figure 1A], which was not explainable for the symptoms and abnormalities of physical examinations. Spine MRI revealed slight prominence of intervertebral disks and no abnormality was observed in the whole spinal cord [Figure 1A]. Electromyography (EMG) showed neurogenic changes in the left biceps brachii, the left thenar muscles and bilateral gastrocnemius [Figure 1B-D]. Evoked potentials examination showed prolonged latency period in the left side of the visual evoked potential and event-related potential-P300. Motor evoked potential, somatosensoroy evoked potential and brainstem auditory evoked potential, nerve conduction, ambulatory electroencephalogram and electrocardiogram were normal.

According to the revised 2,000 criteria,[12] the patient was diagnosed with clinically probable ALS, which was confirmed by other hospitals in Beijing. The oral administration of diazepam and baclofen was initiated; muscle cramps were relieved a bit. The patient did periodic follow-ups in our outpatient

clinics, but her symptoms did not improve with the use of the muscle-relaxants prescribed.

DISCUSSION

ALS has many varied manifestations as first symptoms, which are vital to help for early diagnosis, and understand the natural course the disease progression.[13] Atypical presentations include cramps and fasciculations in the absence of muscle weakness,[14] frontal-temporal dementia,[15] weight loss[16] and cardiovascular consequences.[17] Clinical characteristics of our case include the progression of muscular painful cramps with upper neurons signs, limb weakness with lower motor neuron signs and electrodiagnostic (EMG/nerve conduction velocity) evidence of denervation. Besides MNDs, other etiology of muscle cramps includes metabolic disorders, electrolyte disturbances, medications, *etc*.[14] Our patient had no special medical history and laboratory examinations which support the other disease. As to pathophysiology, the muscle cramps may arise from spontaneous discharges of the motor nerves, this may origin at neuronal level and then transferred through the nerve trunks to the muscular fibers or it may begin at nerve trunks level by activation of abnormal cholinergic receptors that function as trigger points.[18] The neurodegeration in ALS might be caused by a complicated interaction of glutamate excitotoxicity, generation of free radicals, superoxide dismutase 1 enzymes, resulting in impaired axonal structure or transport defects.[2,14] A "dying-forward" process is proposed to explain these clinical characteristics.[19]

Hitherto, there is no disease-modifying therapeutics for ALS. The main managements for patients with ALS are the symptomatic treatments, including muscle relaxants, anticonvulsants for cramps, fasciculations and spasticity, physiotherapy for weakness or disability, and ventilator support for dyspnea,[2,14] These treatments can alleviate symptoms, improve the quality of life and increase the life expectancy.

In conclusion, our case presents a rare initial manifestation of progressive painful muscle cramps in the absence of muscle weakness. It helps to improve our early recognition of the atypical initial presentations of ALS, and formulate effective symptomatic therapies to improve the life quality and survival of the patients.

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Conflicts of interest
There are no conflicts of interest.

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Review **Open Access**

Population of inflammatory cells in intracranial aneurysm with the special insight to the development of novel diagnostic and therapeutic approaches

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With the background of neurosurgeon, molecular biologist and pharmacologist, Dr. Tomohiro Aoki's research aims to develop novel therapeutic drugs to treat intracranial aneurysms or novel diagnostic modalities. He desires to collaborate with both clinicians and basic researchers to proceed researches.

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ABSTRACT

Intracranial aneurysms (IAs) can cause a lethal subarachnoid hemorrhage after rupture. The prevalence of IA is high in the general public; however, the annual risk for the rupture of an incidentally found lesion is relatively low. Therefore, it is crucial to selectively diagnose rupture-prone IAs among many diagnosed IAs, and properly treat such IAs before rupture. Recent studies using human IA specimens or experimentally-induced IAs in animals have revealed some important findings regarding the role of inflammatory cells infiltrating IA lesions. Currently, IA is considered an inflammatory disease of the intracranial arterial walls. Macrophages are presumably a major type of inflammatory cells regulating the pathogenesis of IAs through the production of a wide range of pro-inflammatory factors. Based on a series of studies, macrophages could be a diagnostic target for rupture-prone IAs. Currently, the potential diagnostic method to detect iron-engulfing macrophages in IA lesions by magnetic resonance imaging is reported. In this review, the authors will summarize the findings regarding the inflammatory cell types present in IA lesions and discuss future prospects for the development of a novel diagnostic method identifying rupture-prone IAs.

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INTRODUCTION

Despite the existence of intensive treatments and modern technical advancements in medical care, subarachnoid hemorrhage caused by the rupture of an intracranial aneurysm (IA) has a poor prognosis with a mortality rate of up to 50%.[1] In addition, subarachnoid hemorrhage can cause sudden death, even in the productive population, making this disease socially important. Given such a devastating outcome and difficulty in treatment once the subarachnoid hemorrhage develops, rupture-preventing treatment of IAs is essential. Currently, many IAs are incidentally found before rupture through a medical checkup of the brain, particularly in developed countries. Indeed, in a Japanese cohort, the majority of unruptured IAs were found incidentally.[2] The detection of unruptured IAs enables prophylactic interventions for the prevention of rupture and the subsequent onset of subarachnoid hemorrhage. Currently, IAs with a high probability of rupture are holistically selected using morphological aspects such as size and shape, anatomical aspects such as location, and other confounding factors which, according to some guidelines and previous cohort studies, increase the likelihood of rupture, such as a previous or family history of subarachnoid hemorrhage, race (Japanese or Finnish), current smoking status, or the presence of hypertension. IAs with a high risk for rupture are surgically treated using microsurgical or endovascular procedures.[3-5] In order to predict the risk of IA rupture more objectively and accurately, a scoring system has been established based on a meta-analysis of 6 prospective cohort studies on the annual rupture risk of IAs.[3,6] However, the lack of a diagnostic method to qualitatively estimate the rupture risk for each IA is currently a major concern in IA treatment. The natural consequence is that IAs with a lower probability of rupture are sometimes surgically treated with a considerable risk for complications, or lesions on the verge of rupture are simply observed, resulting in a devastating outcome. Therefore, a novel qualitative diagnostic method should be established in order to reduce inappropriate decisions regarding surgical intervention.

Another important concern regarding current IA treatment for rupture prevention is the lack of medical treatment (expect for medical care targeting risk factors such as hypertension) for patients with IAs ill-suited for surgery, including patients with small IAs or elderly patients with significant comorbidity.[6] Considering the poor outcome associated with subarachnoid hemorrhage after onset, the intrinsic risk of complications related to surgical manipulations, and the nature of unruptured IAs as asymptomatic lesions, medical IA treatment should be established for patients without surgical indications, or as an alternative to surgical procedures. Currently, statin is considered to be a candidate therapeutic drug for IAs as our previous case-control study demonstrated that statin usage reduced the incidence of subarachnoid hemorrhage due to the rupture of IAs.[7] In addition, a prospective randomized trial examining the inhibitory effect of statins on the progression and rupture of human IAs, known as the Small Unruptured Aneurysm Verification Prevention Effect against Growth of cerebral Aneurysm Study Using Statin study (Japan), is in progress. However, the mechanisms underlying the pathogenesis of IAs need to be further examined in order to develop effective and safe medical treatments. Thus, knowledge regarding the cell types regulating the pathogenesis of IAs is essential to the identification of diagnostic or therapeutic targets. Although the histopathological examination of human IAs demonstrated the presence of hyaline deposits, sub-intimal fibrin deposition, and laminar thrombosis in lesions, particularly in ruptured IAs,[4,8,9] thereby implicating endothelial dysfunction as a potential target for medical therapy, we focus on the inflammatory infiltrates found in IA walls in this short review given previous findings that the inflammatory response is crucial in the pathogenesis of IAs.[10-13]

INFLAMMATORY CELLS IN IA LESIONS

Histopathological analysis of surgically dissected or autopsy-harvested IA specimens has revealed the presence of inflammatory cells in IA lesions. Kataoka *et al*.[9] demonstrated an increased presence of inflammatory infiltrating immune cells in ruptured human IAs compared to that in unruptured IAs with a positive correlation between inflammatory infiltrates and degenerative changes in the arterial walls, suggesting a role for inflammatory cells in the rupture of IAs. Inflammatory cells found in human IA lesions include macrophages,[14-16] neutrophils,[17] T lymphocytes[14,15] and mast cells.[16,18,19] Among these types of inflammatory cells, the contribution of macrophages, neutrophils, and mast cells to the pathogenesis of IAs has been supported by experimental studies using animal IA models. Below, we review the evidence for each cell type.

T cells

T cells are a major cell type participating in acquired immunity. T cells are differentiated in the thymus from their precursors mainly into CD4-positive and CD8-positive T cells. These differentiated T cell subsets are then distributed throughout the body and are further differentiated into effective subtypes according to the microenvironment *in situ*, including CD8-positive T

cells as well as T helper 1, 2, and 17 cells (Th1, Th2, and Th17). Some T cells function as a node of acquired immunity and regulate inflammatory responses in a coordinated manner with other cell types in the microenvironment. As expected, T cells play a crucial role in various diseases, including inflammatory and autoimmune diseases.[20] The accumulation of T cells in human IA lesions has been pathologically demonstrated, suggesting the contribution of this cell type to the rupture of IAs.[14,15] Although a recent study has demonstrated the predominance of Th1 and Th17 subsets over Th2 in ruptured IAs,[21] the detailed role of T cells in the pathogenesis of IAs, including which subset/subtype of T cells regulates IA formation and rupture, remains to be elucidated.

Mast cells

Mast cells are characterized by a large number of cytoplasmic granules and are well recognized as mediators of certain types of inflammation including allergic inflammation.[22,23] Mast cells play a role in inflammation through degranulation of cytoplasmic granules, which contain a variety of cytokines and pro-inflammatory factors such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-3, IL-4, IL-6, IL-8, IL-13, and transforming growth factor-beta (TGF-β).[24-29] Once mast cells are activated, they release large amounts of pro-inflammatory factors from the granules into the extracellular space, resulting in the induction of inflammatory responses within the microenvironment. Mast cells significantly participate in the pathogenesis of various inflammation-related diseases, including vascular diseases such as atherosclerosis,[24,30-33] via the release of pro-inflammatory factors from granules.

In the case of IA, the presence of mast cells in human IA lesions has been demonstrated.[16,19] Hasan *et al*.[16] further demonstrated that the number of mast cells located in IA lesions is larger for ruptured compared to unruptured IAs, suggesting a role for mast cells in the rupture of IAs. In addition, Ollikainen *et al*.[19] demonstrated a positive correlation between the number of mast cells present in IA lesions and neovascularization and iron deposition (presumably due to microhemorrhage), suggesting the contribution of mast cells to degenerative changes in the media. However, the exact contribution of mast cells to the pathogenesis of IAs is difficult to discern from studies using human specimens; thus, the pivotal role of mast cells to the pathogenesis of IA has been clarified using animal IA models.[34,35] These studies have demonstrated an increase in the number of mast cells in IA lesions during progression of the disease. In order to demonstrate a causal relationship between the presence of mast cells and IA progression, the researchers treated rats with emedastine difumarate (1-(2-ethoxyethyl)-2-(hexahydro-4-methyl-1H-1,4-diazepin-1-yl)-benzimidazole difumarate) and tranilast (N-(3,4-dimethoxycinnamoyl) anthranilic acid), two widely-used inhibitors for limiting mast cell degranulation. Inhibition of mast cell degranulation effectively reduced lesion size and degenerative changes of the media through the inhibition of inflammatory responses, including reduced NF-κB activation, monocyte chemoattractant protein-1 (MCP-1) expression, macrophage infiltration, matrix metalloproteinases (MMPs) and IL-1β expression, which facilitate pathogenesis.[10,13,36-39] Moreover, *in vitro* experiments using a primary culture in which vascular smooth muscle cells and mast cells prone to degranulation are co-cultured, confirmed the contribution of mast cell degranulation to the activation of medial smooth muscle cells.[35] Given that factors suppressed by the administration of degranulation inhibitors are known to facilitate IA formation and progression,[10,13,36-39] mast cells or mast cell degranulation inhibitors may be a therapeutic target for IA treatment. It is encouraging that mast cell degranulation-inhibitors are already widely used as anti-allergic drugs; thus, medical therapy targeting mast cells may be possible in near future.

Neutrophils

Neutrophils, defined using surface markers such as CD11b, Ly-6G and Ly-6C, are antigen-presenting cells. This cell type is recruited by chemoattractant factors such as chemokine (C-X-C motif) ligand 1 (CXCL1)[40] and is present in the inflammatory microenvironment, migrating to inflammation sites and exacerbating inflammatory responses by secreting various pro-inflammatory factors in response to cytokines present *in situ*. Factors released from neutrophils include proteases and digestive enzymes such as myeloperoxidase, collagenase, elastase, and cathepsin.[20] Neutrophil turnover during inflammation is usually rapid; therefore, this cell type is traditionally believed to be a mediator of the acute inflammatory response. However, recent studies have revealed the contribution of neutrophils to the pathogenesis of diseases with long-lasting inflammation, referred to as chronic inflammatory diseases. For example, neutrophils are the most abundant cell type in colitis-associated colon cancer induced in mice and have been shown to be as a major source of cytokines regulating inflammatory responses, including TNF-α and IL-6, which contribute to the transformation/proliferation of cancer cells.[41,42] The crucial contribution of neutrophils to the pathogenesis of cancer has been clarified in a study demonstrating that the genetic depletion of

C-X-C chemokine receptor 2, the receptor for CXCL1, resulted in a significant reduction of tumorigenesis.[41]

In case of IAs, the presence of neutrophils in IA lesions has been demonstrated both in human specimens[17] and in animal models.[17,37] In human IAs, myeloperoxidase-positive cells, including neutrophils, are abundantly present in IA lesions.[17] Genetic loss of myeloperoxidase in mice significantly reduces not only the incidence of IAs, but also delays the onset of subarachnoid hemorrhage. This suppressive action due to the loss of myeloperoxidase is accompanied with the suppression of CXCL1 and other pro-inflammatory factors. These results suggest a role for myeloperoxidase, presumably produced by neutrophils, in the incidence and rupture of IAs. Intriguingly, as the loss of myeloperoxidase suppresses CXCL1 expression, myeloperoxidase may contribute to the formation of a positive feedback loop among neutrophils, which amplifies and exacerbates inflammation. Thus, myeloperoxidase may function to form a vicious cycle leading to the progression of IAs. Therefore, myeloperoxidase, or another factor regulating the activity of neutrophils, could be an ideal therapeutic target in the treatment of IAs.

Macrophages

Macrophages, defined by the expression of surface markers including CD11b, CD68, CD163, are also antigen-presenting cells.[43] It is, of course, the major cell type evoking inflammatory responses. The accumulation of macrophages in human IA lesions has been demonstrated. Chyatte *et al*.[15] demonstrated that number of macrophages was larger in IA lesions than in control arterial walls, and thus proposed a role for macrophages in the formation and rupture of IAs. Frösen *et al*.[14] analyzed the pathology of surgically-dissected IA walls and found that macrophage infiltration, apoptosis, the loss of endothelial cells, thrombosis in the lumen, and the proliferation of medial smooth muscle cells were more frequently and robustly observed in ruptured IAs than in unruptured IAs, suggesting a role for macrophages in the rupture of IAs. Furthermore, as macrophage infiltration was correlated with the proliferation of medial smooth muscle cells and both were increased in ruptured IAs collected within 12 h after rupture, the authors proposed an additional role of macrophages in the repair of the arterial walls of IA lesions.[14] Hasan *et al*.[16] examined macrophage subsets (M1 and M2) in IA walls. M1 and M2 are defined according to the expression of several markers with inducible nitric oxide synthase (iNOS) expressed in M1 and CD163 expressed in M2 macrophages.[44] Traditionally, M1 and M2 macrophages are believed to function oppositely in inflammatory settings with M1 exacerbating and M2 ameliorating inflammation. Hasan *et al*.[16] found that, in unruptured IAs, M1 and M2 populations were similar, but in ruptured IAs, the balance shifted to M1-predominance over M2, indicating a role for M1 in the rupture of IAs. However, careful attention is needed regarding the role of specific macrophage subsets in the rupture of IAs because subarachnoid hemorrhage as the consequence of rupture robustly induces acute inflammation, and thereby presumably shifts the balance among macrophage subsets.

Recent experimental studies using animal IA models have clarified the crucial role of macrophages in IA formation and progression. Using a rat model, Aoki *et al*.[37] demonstrated that macrophages are the major inflammatory cell type in IA walls with the remaining cell types consisting of T lymphocytes and neutrophils. The authors also demonstrated the presence of mast cells in IA walls in an experimental model; however, macrophages occupied the majority of inflammatory cells.[35] In order to examine the contribution of macrophages to IA formation and progression, mice deficient in monocyte chemoattractant protein-1 (MCP-1), a major chemoattractant for macrophages, were subjected to an IA model.[36] The genetic loss of MCP-1 almost completely inhibited the infiltration of macrophages in lesions and significantly suppressed IA formation to the level seen in un-treated wild type mice.[36] The effect of a genetic loss of MCP-1 on IA formation has also been demonstrated in another study by Kanematsu *et al*.[45] In addition, Aoki *et al*.[36] administered the dominant-negative form of MCP-1, known as 7-ND, in a rat IA model by injecting expression plasmid in the femoral muscle, and demonstrate a suppression of IA formation and progression, further confirming the crucial role of MCP-1-mediated macrophage infiltration in IA formation and progression. In the IA walls of MCP-1-deficient mice, the inflammatory response, including NF-κB activation as well as iNOS and MMP-9 induction, was remarkably ameliorated compared with that of wild type mice,[36] supporting the role of macrophages as an inducer of inflammation in lesions. The importance of macrophages in the pathogenesis of IAs is also supported by Kanematsu *et al*.[45] in which the pharmacological depletion of macrophages using clodronate liposome remarkably suppressed IA formation in mice. MCP-1 expression overlaps with NF-κB in endothelial cells during the early stage of IA formation,[36] indicating that NF-κB-dependent MCP-1 expression and trans-endothelial migration of macrophages occurs via MCP-1. Indeed, mice deficient in the NF-κB p50 subunit show significantly less induction of MCP-1 in lesions, supporting the dependency of NF-κB on MCP-1 expression in

lesions.[13] Given that the high wall shear stress loaded on bifurcation sites of intracranial arteries where IA forms is considered to be a trigger of IA formation through extensive studies conducted in mainly 3D-computational fluid dynamics analyses of human IA lesions,[10,46-48] one potential factor triggering NF-κB-dependent MCP-1 expression in endothelial cells is presumably this hemodynamic force. However, further studies are needed in order to corroborate whether macrophages indeed migrate across endothelial cells during IA formation and progression. Given these findings, IA is now considered to be a macrophage-mediated inflammatory disease of the intracranial arteries. Therefore, macrophages could be a target in the development of a novel diagnostic method and in the development of a therapeutic drug.

MACROPHAGE IMAGING

Given the devastating consequences of an aneurysmal subarachnoid hemorrhage as well as the relatively low annual risk of rupture,[2] it is crucial to appropriately select rupture-prone IAs among many unruptured IAs while avoiding unnecessary surgical intervention through the proper estimation of the rupture risk for each lesion. For example, a qualitative evaluation using non- or minimally-invasive magnetic resonance imaging (MRI) of IA lesions could be a candidate diagnostic method. Given the crucial contribution of macrophage-mediated inflammatory responses to the pathogenesis of IAs,[10,36,45,49] macrophages as well as factors secreted from or factors recruiting macrophages can be considered potential targets for imaging. Macrophages are an antigen-presenting cell; thus, macrophages actively search for and engulf foreign bodies by nature. Through their phagocytic activity, contrast agent-engulfing macrophages can be theoretically visualized using MRI. Indeed, many studies have demonstrated the application of this type of imaging technique for macrophage-mediated pathological conditions or diseases, including infection and atherosclerosis.[50,51] In these studies, ferumoxytol, which is an ultra-small superparamagnetic particle of iron oxide approved by the Food and Drug Administration (FDA) for the treatment of Iron Deficiency Anemia due to chronic renal failure, was applied as a contrast agent. Recently, the use of macrophage imaging for IAs has been reported.[52-55] In a series of reports, Ferumoxytol was intravenously administered to patients with pre-diagnosed unruptured IAs and ferumoxytol-engulfing macrophages were visualized using a T2 star weighted image. In addition, in order to validate the MRI findings, the presence of iron particles was histologically confirmed using Prussian blue staining of $CD68^+$ macrophages in the IA walls identified as macrophage-rich lesions from ferumoxytol-MRI.[52] Intriguingly, anti-inflammatory drugs (non-steroidal anti-inflammatory drugs) decreased signals in this imaging method, which was accompanied with a decrease in the macrophage count within lesions,[53] suggesting that macrophage-imaging using ferumoxytol-MRI can positively reflect macrophage-rich or poor lesions. Importantly, these findings suggest the potential of this imaging method as a surrogate marker to non-invasively monitor disease activity. Unfortunately, ferumoxytol has a risk of a fatal allergic reaction and the FDA has strengthened the warning concerning usage of this drug as a contrast agent for diagnostic purposes. Therefore, a novel contrast agent for macrophage imaging with excellent biocompatibility and adequate safety is needed. In addition, because red blood cells contain a large amount of iron in the hemoglobin, macrophage-imaging using iron-containing particles need to be evaluated after the subtraction of signals derived from red blood cells in the vasculature. Unfortunately, these subtraction procedures are often time-consuming and make interpretation quite difficult. Hence, to overcome the technical limitations related to iron-particle-based macrophage-imaging methods, positive contrast agents such as nano-particles containing gadolinium may be beneficial. Furthermore, with the use of a nano-particle based technique, a novel drug delivery system targeting macrophages may be possible. If so, IAs with abundant macrophage infiltration can be selectively targeted and treated by a cytotoxic agent in order to reduce macrophage-mediated inflammation in IA lesions.

As described above, macrophages have a wide range of subpopulations with different characteristics.[44] In human IA lesions, the M1 and M2 subpopulations are detected using immunostaining.[16] Further, as indicated in the previous report demonstrating a marked predominance of M1 over M2 in ruptured IAs compared to that in unruptured lesions,[16] a particular subpopulation of macrophages may alone contribute to the rupture of IAs. If so, the detection of this specific subpopulation would be helpful in discriminating rupture-prone IAs from stable lesions. Thus, next-generation imaging modalities for subpopulation-specific visualization of macrophages are desired. In addition, MRI contrast agents that target enzymes produced by inflammatory cells may be useful in diagnosing rupture-prone 'dangerous' lesions as suggested in a recent review article.[55] For example, myeloperoxidase is an enzyme produced by myeloid-linage cells such as neutrophils. Enzymatic activity of this protein can be monitored by MRI using gadolinium-chelating bis (5-hydroxytrytamide) derivatives of diethylenetetraamine pentaacetic acid as a contrast

agent.[56] Hence, if such a kind of contrast agent can be developed for clinical usage, rupture-prone IAs could presumably be differentiated from stable lesions.

CONCLUSION

The accumulation of histopathological and experimental evidence from human IA specimens and animal IA models has significantly promoted the conceptual understanding of the pathogenesis of IA and has defined IA as a macrophage-mediated chronic inflammatory disease of the arterial walls. This recent advancement in the understanding of IA pathogenesis greatly encourages the development of not only novel diagnostic methods for the detection of rupture-prone IAs, but also the development of medical therapy targeting macrophages. In the near future, the diagnosis and treatment of unruptured IAs will enter a phase of major change. We hope that many patients with this disease will be more properly diagnosed and more safely treated.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

No patient involved.

Ethics approval

This article does not contain any studies with human participants or animals.

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Inhibition and reversal of growth cone collapse in adult sensory neurons by enteric glia-induced neurotrophic factors

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ABSTRACT

Aim: Previous studies show enteric glia (EG)-conditioned medium promotes neurite outgrowth in adult dorsal root ganglia (DRG) derived sensory neurons. This EG-conditioned medium contains various neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotropic factor (GDNF), and neurotrophin-3 (NT-3). This study attempts to determine the importance of these neurotrophic factors in enabling DRG-derived sensory neuron axons to overcome the inhibitory guidance cues released from the glial scar. **Methods:** A Semaphorin 3A (SEMA3A) growth cone collapse model was used on cultured rat DRG. Neutralizing antibodies to each neurotrophic growth factor in question (NGF, BDNF, GDNF and NT-3) were applied to the EG-conditioned medium to evaluate the factor's individual importance in preventing growth cone collapse. **Results:** EG-conditioned medium inhibits and reverses growth cone collapse in adult DRG neurons when added either 1 h before or concurrently with SEMA3A. When administered 40 min after the initial SEMA3A-induced collapse, EG-conditioned medium was able to reverse the growth cone collapse. Individual inhibition of all the neurotrophic factors, except for BDNF in the co-treatment setting, resulted in increased growth cone collapse. **Conclusion:** NGF, BDNF, GDNF, and NT-3 are all variably involved in preventing or reversing SEMA3A-induced growth cone collapse in pre-, co-, and post-treatment time settings. However, no individual neurotrophic factors appear to be essential to promoting neurite outgrowth.

INTRODUCTION

Spinal cord injury (SCI) can result in a complete or partial loss of sensation and paralysis at and below the site of injury and represents a large burden of disease.[1] SCI damage occurs in 2 phases: the primary and secondary phase. The primary phase is the membrane shearing and axonal tearing caused by mechanical stress.[1] The secondary phase is the ischemia, apoptosis, and necrosis that occurs post-trauma.[2,3] Glial scarring at

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the injury site is a major mechanical barrier to axonal regeneration. Studies have found that the glial scar contains many molecules that inhibit axonal growth.[4-8] Semaphorin 3A (SEMA3A) is one of these molecules and is highly expressed in the glial scar.[9-11] SEMA3A is part of the Semaphorin family of inhibitory guidance cues specific to vertebrae, and it is normally present during the development of the central and peripheral nervous systems.[12-14] Semaphorins signal through complex neuronal receptors that contain neuropilin-1 and plexins, which are significantly expressed in axotomized dorsal root ganglion (DRG).[13] This indicates that SEMA3A is a key mediator of axon retraction and growth cone collapse.[13,15] To date, multiple studies have observed the effects of SEMA3A on DRG-derived neurons in animal models.[16-21] Two of them employed growth cone collapse assays adapted from studies on embryonic neurons.[16,18]

Enteric glia (EG) are the support cells of the enteric nervous system (the nervous system of the gut) and share a number of characteristics with central nervous system (CNS) astrocytes. It has been previously reported that *in vivo* locally transplanted EG facilitate ingrowth of transected dorsal root axons toward their targets through the spinal cord and across the non-permissive peripheral/CNS boundary.[22] These EG induce the regeneration of neurofilament-positive dorsal root axons into the injury site of rats given spinal cord crushes (using the clip-compression model).[23] Additionally, there is published data that suggests EG secrete nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT-3) in culture and that these growth factors at least partially mediate neurite outgrowth in DRG neurons in response to EG.[24] Studies have shown that all of these growth factors imbue some resistance to SEMA3A-induced growth cone collapse in cultured embryonic DRG neurons,[25,26] although collapse sensitization varies with developmental stage.[26-28] A study by Wanigasekara *et al*.[18] is of particular importance to our study, as it indicated that NGF, GDNF, and NT-3 inhibit SEM3A-induced collapse in adult DRG-derived sensory neurons when administered 1 h before collapse.

We hypothesized that incubation with EG-conditioned medium would inhibit and perhaps reverse SEMA3A-mediated growth cone collapse in cultured DRG neurons, and theorized that inhibiting any one of the neurotrophic factors in the EG-conditioned medium would decrease its overall efficacy at preventing SEMA3A-mediated collapse, regardless of whether the conditioned medium was applied before, during, or after the addition of SEMA3A. The objective of this study was to provide evidence that neurotrophic factors are involved in mediating axonal regeneration and neurite outgrowth as well as shed light on the roles of specific neurotrophic factors in mediating neurite outgrowth.

METHODS

Enteric glia extraction and culture

All experiments were performed in accordance with the requirements of the Animals for Research Act of Ontario, Canada and the Guidelines of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board at our institution. Both EG extraction from the small intestines of adult female Wistar rats and EG identification confirmation by staining for glial fibrillary acidic protein and myelin protein zero (MPZ) were performed using previously described methods[29] as adapted in Hansebout *et al*.[24] The presence of glial fibrillary acidic protein, in conjunction with the absence of MPZ, is considered to be indicative of EG.[21,24] EG were cultured as per[16] in Dulbecco's modified eagle medium (DMEM/F) 12 1:1 (Invitrogen D8437) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The medium was changed every 2-3 days, and the cells were then subcultured using 0.25% trypsin-ethylene diamine tetraacetic acid (Invitrogen 25200-056).

DRG extraction and culture

DRG were extracted from six- to sixteen-week old adult male Wistar rats and cultured as per Hall[30] and as described by Hansebout *et al*.,[24] plating 3 × 10^4 cells/well in a 6-well tissue culture plate. Non-neuronal cells were reduced with a 4 h incubation in dispase/collagenase. They were then plated on a standard petri dish. Neurons generally have difficulty adhering to the petri dish surface, and the neurons mixed in solution were subsequently removed and replated. As per Reza *et al*.,[16] each well was coated with 3 mL of 0.01% poly-L-lysine 48 h before extraction. The poly-L-lysine was replaced with 2 ng/mL laminin in HBSS 24 h before extraction to facilitate DRG attachment. The neurons were acclimatized to culture conditions for 4 days before each experiment. Half of the 3 mL of supplemented neurobasal medium was replaced with fresh medium every other day.

Generation of enteric glial-conditioned medium

The EG medium was conditioned as per Hansebout *et al*.[24] EG derived from adult male Wistar rats were seeded at 4 × 10^4 cells/well into a six-well plate coated with rat tail collagen, with tissue culture inserts covered in 3T3 mouse embryonic fibroblasts.

The EG were bathed in DMEM/F12 medium (Invitrogen 11330-032) supplemented with 20% FBS and 1% P/S. EG from passages 6-10 were acclimated to DMEM/F12 containing 2% FBS and 1% P/S. Twenty-four hours later, cells were rinsed in phosphate buffered saline (PBS, pH 7.35) and subsequently bathed in supplemented neurobasal medium. After another 24 h, the medium was conditioned and centrifuged for 5 min at 4,000 *g* to remove any particulates.

Collapse assay

A collapse assay was adapted from Reza *et al*.[16] and experimental conditions were modified from Hansebout *et al*.[24] EG-conditioned medium was administered before, during or after SEMA3A (R&D System 1250-S3) mediated collapse (pre-treatment, co-treatment, post-treatment). Each time setting was assessed using serial assays. To test individual growth factor involvement, antibodies to one of NGF, BDNF, GDNF or NT-3 were added to select wells. Each neurotrophic factor was investigated with or without conditioning media (+/-CDN) and with or without antibody (+/-aB). The negative (model; -CDN/-aB) and positive (+CDN/-aB) control groups show the effect of enteric glia on SEMA3A-mediated collapse before the addition of the antibodies. Cells were incubated at 37°C and PBS was used as a vector control for antibodies in each experiment arm. The final working concentration of SEMA3A in all of the wells was 100 ng/mL. The final antibody concentration in each well was 2.5 µg/mL. All cells were later fixed in 4% perfluoroalkoxy alkanes (PFA) containing 10% sucrose and subsequently stained. The detailed procedures are as follows:

Pre-treatment

Half of the medium in each well was replaced with EG-conditioned medium or normal supplemented neurobasal medium for positive and negative controls respectively. Each EG-conditioned and control neurobasal group also received either anti-NGF (RD System AMK0208091), anti-BDNF (Millipore AB15130P), anti-GDNF (RD System AFW0408071), or anti-NT-3 (Chemicon AB1780SP) dissolved in PBS to reach a working concentration of 2.5 µg/mL while controls were given PBS in equal volumes. After a 1-h incubation, all cultures were then treated with 100 µg/mL SEMA3A in PBS (R&D Systems 1250-S3-025) to obtain a bath concentration of 100 ng/mL as per Wanigasekara *et al*.[18] and Reza *et al*.[16] One hour later, cells were fixed and stained.

Co-treatment

This experiment was almost identical to the previous one with the exception of the timing of the treatment. Half of the medium in each well was replaced with EG-conditioned medium or normal supplemented neurobasal medium for the comparison groups containing 200 ng/mL SEMA3A to obtain a bath concentration of 100 ng/mL. The medium in the treatment groups also contained either anti-NGF, anti-BDNF, anti-GDNF, anti-NT-3 added in PBS to achieve a well concentration of 2.5 µg/mL. Equal volumes of PBS were added to control wells. The cultures were incubated for 1 h and then fixed and stained.

Post-treatment

Similar to the previous experiments, half of the medium in each well was replaced with fresh neurobasal medium containing 200 ng/mL SEMA3A to obtain a bath concentration of 100 ng/mL. After a 40-min incubation, 2.5 mL of the bathing medium was replaced with either EG-conditioned medium with SEMA3A or supplemented neurobasal medium with SEMA3A. Pilot studies revealed that 1.5 mL of EG medium was inadequate to prevent collapse after SEMA3A administration in our model. Also added was either anti-NGF, anti-BDNF, anti-GDNF, anti-NT-3 in PBS for a final concentration of 2.5 ng/mL or equal volumes of PBS for controls. These cells were incubated for another 20 min for a total 1-h incubation period before fixing and staining.

Staining

Phalloidin staining, which specifically binds to F-actin, was used to visualize the actin-dense cytoskeleton of the growth cones.[31,32] Specific methods were modified from Hansebout *et al*.[24] After a 10-min fixation in a 4% PFA and 10% sucrose solution, the cells were washed in PBS, permeabilized with 0.05% Triton X-100 for 5 min, and then treated with 1% bovine serum albumin for 30 min to reduce background staining. The cells were then stained for 1 h with Alexa-488 phalloidin (Invitrogen A12379) to visualize the growth cones and cell morphology and then counter-stained with Propidium iodide (Sigma) to visualize the nuclei. After a double wash in PBS and a final wash in double-distilled water, coverslips were mounted onto glass slides using VectaShield mounting medium (Vector Laboratories H-1,000), and edges of the coverslips were sealed with clear nail polish.

Data gathering

The ratio of collapsed to uncollapsed growth cones was measured as per Kapfhammer *et al*.[33] and Brown *et al*.[34] noting the approximate cell body size of each neurite, so as to exclude large-diameter DRG neurons that have been shown to be unresponsive to SEMA3A.[16] Since the study used isolated DRG from adult rats rather than explanted embryonic DRG as used in the study by Kapfhammer *et al*.[33] growth

cone morphology was not as obvious; therefore, we examined the stained neurons under the 40× objective of a Leica fluorescent microscope. A minimum of 20 neurite-containing photographs were taken per slide and the first 50 different neurons were counted per slide in a horizontal strip manner. Growth cones were scored as "uncollapsed" (any flattened lamellipodia and 2 or more filopodia) or "collapsed" (bullet-shaped neurite tip sometimes with a single filopodium originating at the neurite tip and no lamellipodium), as previously described.[16] Only axons that were longer than the majority of other axons were scored; axons that were in contact with another cell surface were ignored. The results are represented as the percentage of collapsed growth cones out of the total number of growth cones counted.

Statistical analysis

Analyses were performed on combined data from at least 3 separate experiments (n = 150) using Graphpad 6.0, with 95% confidence intervals. Data were plotted as the mean ± the standard error of the mean and were compared by a two-way ANOVA followed by a Dunnett's *post hoc* test, comparing the means to the positive control (+CDN/-aB). Differences were significant if $P < 0.05$.

RESULTS

Pre-treatment

Will inhibition of NGF, BDNF, GDNF, and NT-3 significantly decrease the effect of the EG-conditioned medium on growth cones in a SEMA3A-mediated collapse model when the conditioned medium is administered to DRG neurons prior to SEMA3A application?

In this collapse assay, DRG were incubated in either EG-conditioned medium or supplemented neurobasal medium for 1 h. Antibodies or PBS (for controls) were also added at this time. One hour later, SEMA3A was applied. This is the first study to test the hypothesis that pre-treatment with EG-conditioned medium can prevent SEMA3A-induced growth cone collapse, as shown in Figure 1. The red bar represents the SEMA3A model (-CDN/-aB) and demonstrates that normal ranges of collapse were observed. The orange bar represents the percentage of collapse when DRG were treated with EG-conditioned medium only (+CDN/-aB). Comparing the SEMA3A model with the cultured DRG in the EG-conditioned medium demonstrates that the EG-conditioned medium (orange bar) had a positive effect on growth cones, significantly preventing their collapse in a pre-treatment setting ($P < 0.0001$). The supplemental neurobasal medium with antibody comparison [Figure 1, grey bars] was included to ensure that antibodies alone would not have an effect on growth cone collapse.

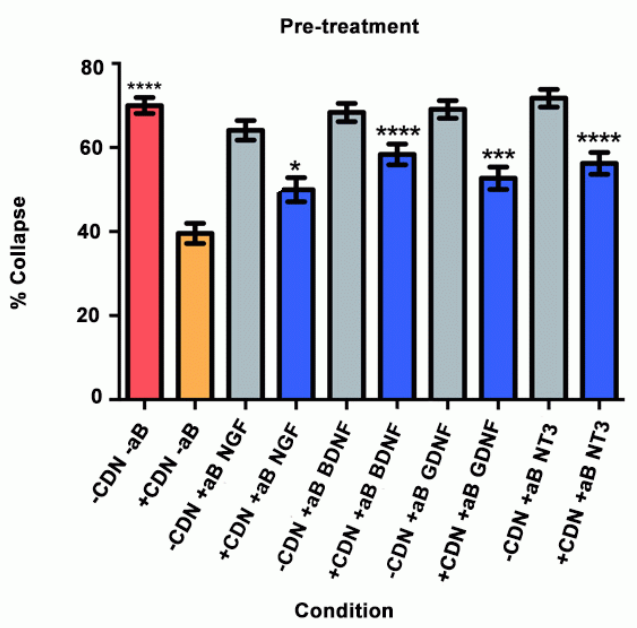

Figure 1: Pre-treatment experiment results. *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$

This experiment also tested the hypothesis that by inhibiting a specific neurotrophic factor, the ability of EG-conditioned medium to prevent SEMA3A-induced collapse would be decreased. In all of the groups treated with EG-conditioned medium and an inhibitory antibody (blue bars), the percentage of growth cone collapse was significantly higher when compared with the EG-conditioned medium alone (orange bar) (anti-NGF, $P < 0.05$; anti-BDNF, $P < 0.0001$; anti-GDNF, $P < 0.001$; anti-NT3, $P < 0.0001$) [Figure 1]. Representative DRG neuron images from each sub-group in the pre-treatment group appear in Figure 2.

Co-treatment

Will inhibition of NGF, BDNF, GDNF, and NT-3 significantly decrease the effect of the EG-conditioned medium on growth cones in a SEMA3A-mediated collapse model when the conditioned medium is administered to DRG neurons concurrently with SEMA3A?

In this collapse assay, SEMA3A and EG-conditioned medium were added concurrently to the DRG cultures. Antibodies or PBS (for controls) were also added at this time. The supplemental neurobasal medium with antibody comparison [Figure 3, grey bars] was included to ensure that antibodies alone would not have an effect on growth cone collapse.

The red bar represents the SEMA3A model (-CDN/-aB) and demonstrates that normal ranges of collapse were observed [Figure 3]. The orange bar represents the percentage of collapse when DRG were treated with EG-conditioned medium only (+CDN/-aB). Comparing the SEMA3A model with the cultured DRG in the EG-conditioned medium demonstrates that the EG-conditioned medium (orange bar) had a positive effect on growth cones, significantly preventing their collapse in a co-treatment setting ($P < 0.0001$).

In the groups treated with both EG-conditioned medium and an inhibitory antibody against NGF, GDNF, and NT-3 (blue bars), the percentage of growth cone collapse was significantly greater when compared

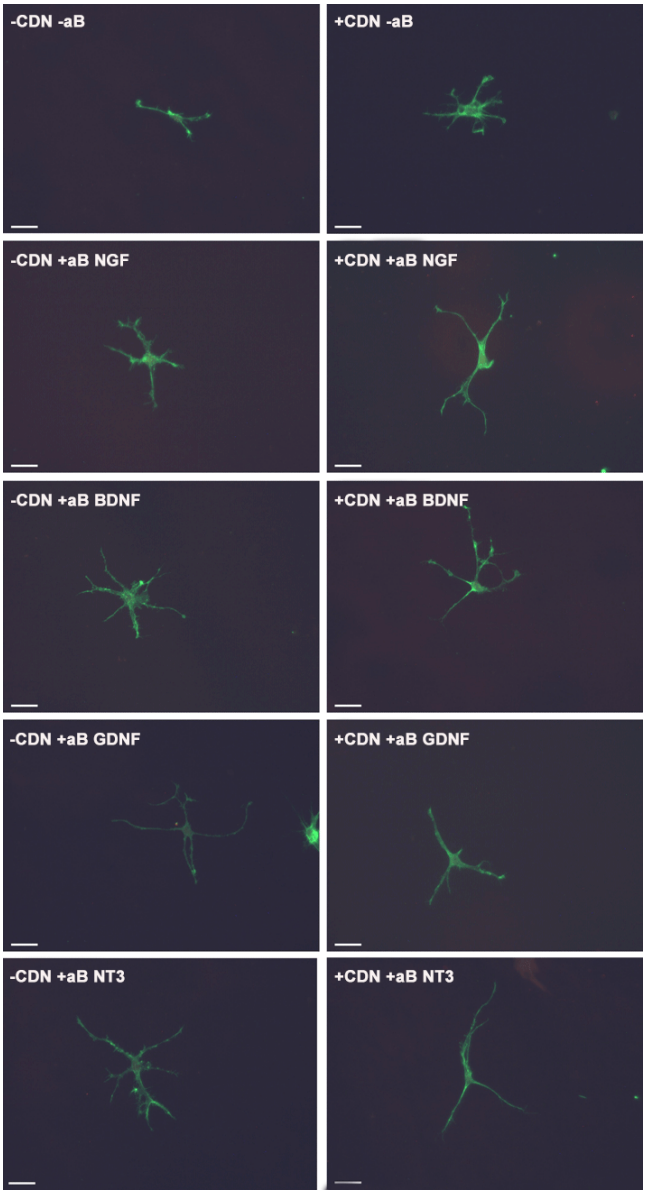

Figure 2: Images of DRG neurons from each sub-group in the pre-treatment experiment. Each white bar represents 25 μm. DRG: dorsal root ganglion

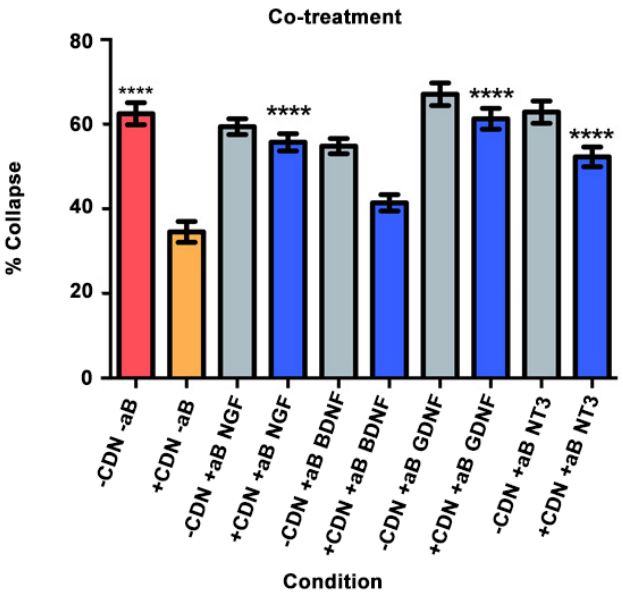

Figure 3: Co-treatment experiment results. ****$P < 0.0001$

with the EG-conditioned medium alone (orange bar) (anti-NGF, $P < 0.0001$; anti-GDNF, $P < 0.0001$; anti-NT3, $P < 0.0001$) [Figure 3]. There was no significant difference between the levels of collapse with pure EG-conditioned medium (orange bar) and EG-conditioned medium with anti-BDNF antibody (blue bar; $P > 0.05$). Representative DRG neuron images from each sub-group in the co-treatment group are shown in Figure 4.

Post-treatment

Will inhibition of NGF, BDNF, GDNF, and NT-3 significantly decrease the effect of the EG-conditioned medium on growth cones in a SEMA3A-mediated collapse model when the EG-conditioned medium is applied to DRG neurons after SEMA3A?

We used a collapse assay similar to the one in the pre- and co-treatment experiments. In this setting, cultured DRG neurons were bathed in 100 ng/mL of SEMA3A in supplemental neurobasal medium for 40 min. At that time, 2.5 mL of the medium was exchanged with either EG-conditioned medium with SEMA3A or supplemental neurobasal medium with SEMA3A. Also added were inhibitory antibodies or PBS (for controls). The DRG were then incubated for a final 20 min.

As illustrated in Figure 5, the red bar represents the SEMA3A model (-CDN/-aB) and indicates that normal levels of collapse were observed in the post-treatment experiment. The orange bar illustrates that post-treatment with EG-conditioned medium (+CDN/-aB) significantly prevented and/or reversed the SEMA3A-induced growth cone collapse when compared with the SEMA3A model (-CDN/-aB; red bar, $P < 0.0001$).

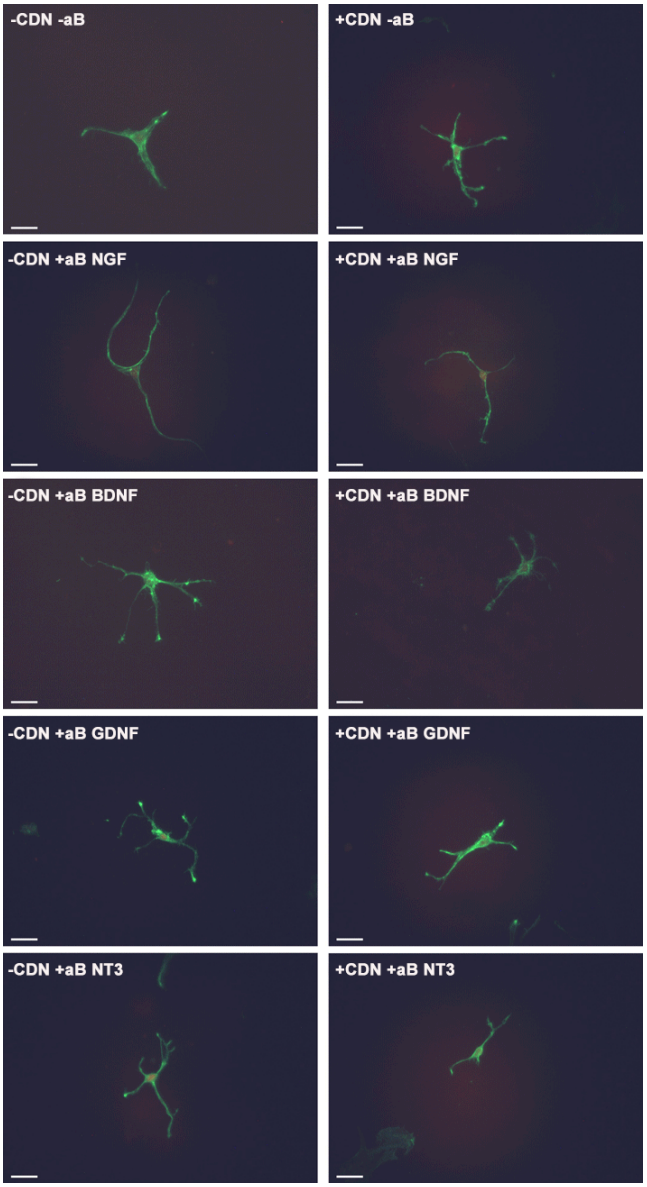

Figure 4: Images of DRG neurons from each sub-group in the co-treatment experiment. Each white bar represents 25 μm. DRG: dorsal root ganglion

In all groups treated with EG-conditioned medium and an inhibitory antibody (blue bars), the percentage of growth cone collapse was significantly higher when compared to EG-conditioned medium alone (orange bar) (anti-NGF, $P < 0.0001$; anti-BDNF, $P < 0.0001$; anti-GDNF, $P < 0.0001$; anti-NT3, $P < 0.0001$) [Figure 5]. Representative DRG neurons images from each sub-group in the post-treatment group are shown in Figure 6.

DISCUSSION

The experiments demonstrate the novel finding that EG-conditioned medium may protect DRG growth cones against SEMA3A-mediated collapse when applied before, concurrently, and after SEMA3A. The results show that the EG-conditioned medium, which contains NGF, BDNF, GDNF, and NT-3, is neuroprotective when applied prior to or concurrently with SEMA3A-induced collapse, and regenerative when applied 40 min after said collapse. In previous studies, it has been shown that when NGF, GDNF or neuritin is applied to DRG overnight, it is protective against SEMA3A-induced collapse.[18] In addition, Hansebout *et al*.[24] found that EG-conditioned medium expressed NGF, BDNF, GDNF, and NT-3 and could induce neurite growth in cultured DRGs.

This phenomenon may be due to either neurotrophic factor inhibition or reversal of SEMA3A-induced DRG apoptosis.[35] Transplanted adipose derived stem cells into nerve conduits of rat DRG showed differentiation of the adipose derived stem cells and the subsequent release of NGF, BDNF, GDNF and NT-4. This was linked with a reduction in DRG mRNA expression of apoptotic factors Bax and caspase-3 and an increase in expression of the anti-apoptotic factor Bcl-2.[35]

These experiments also suggest the novel finding that NGF, BDNF, GDNF, and NT-3 are all involved in the process of preventing or reversing SEMA3A-induced collapse, but no individual neurotrophic factor is essential to this process. In almost all cases, the application of treatment medium with inhibition of individual neurotrophic factors appeared to result in significantly increased levels of collapse compared to the pure EG-conditioned control medium. However, individual inhibition did not cause full collapse, as determined by the SEMA3A control group. This is most apparent in the pre-treatment and co-treatment

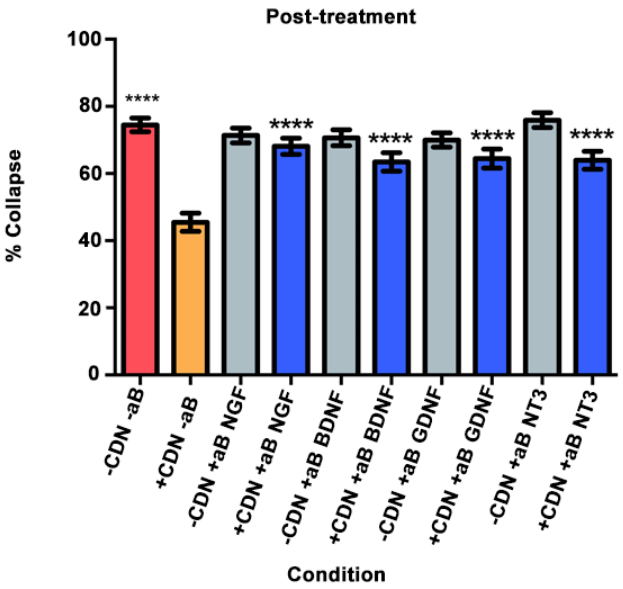

Figure 5: Post-treatment experiment results. ****$P < 0.0001$

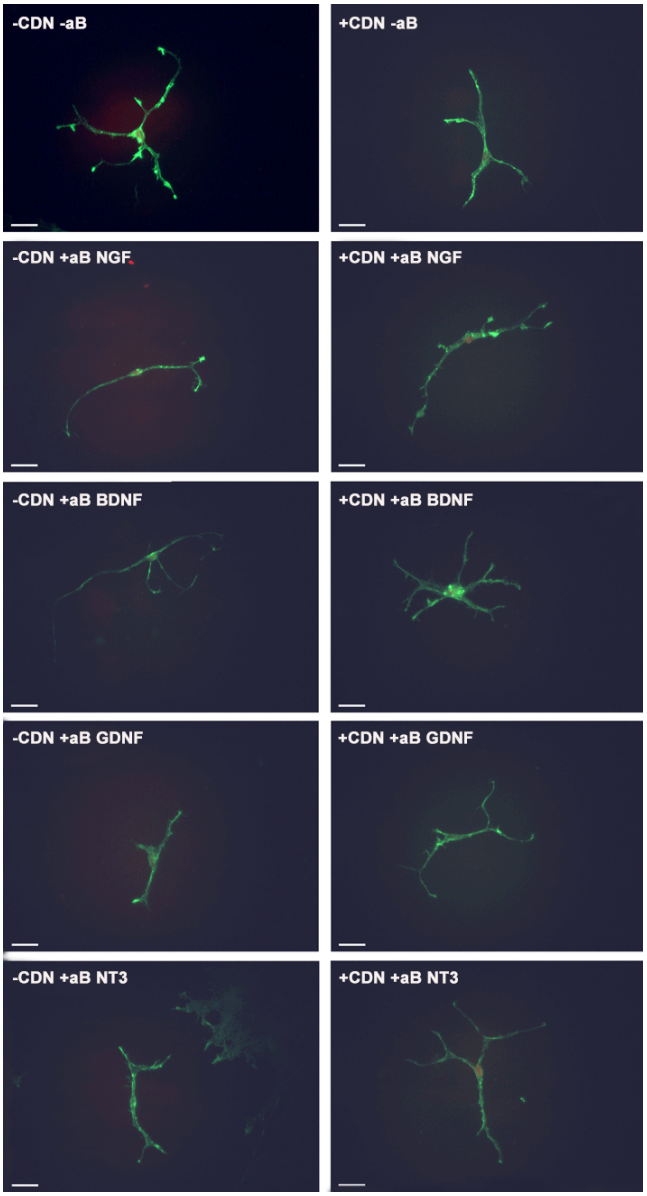

Figure 6: Images of DRG neurons from each sub-group in the post-treatment experiment. Each white bar represents 25 μm. DRG: dorsal root ganglion

settings. In fact, inhibition of BDNF in the co-treatment setting did not cause a significant increase in collapse, suggesting a negligible role for BDNF in that time setting. Combined, these results suggest that while these neutrophic factors play large roles, none are individually essential to the neuroprotective process.

The impact of each neurotrophic factor appears to be variable. In the pre-treatment setting, inhibition of BDNF or NT-3 resulted in the greatest increases in the percentage of growth cone collapse when compared with the pure EG-conditioned medium. This suggests that BDNF and NT-3 play the most important roles in preventing SEMA3A-induced collapse in the pre-treatment setting. In the co-treatment setting, inhibition of NGF, GDNF, and NT-3 caused the greatest increases in growth cone collapse; conversely, when compared with the pure EG-conditioned medium, inhibition of BDNF did not appear to result in a significant increase in growth cone collapse. This suggests that NGF, GDNF, and NT-3 are the most important neurotrophic factors in preventing or reversing SEMA3A-induced collapse in the co-treatment setting.

In the post-treatment setting, inhibition of all neurotrophic factors resulted in significant increases in growth cone collapse when compared with the pure EG-conditioned medium. This suggests that all the neurotrophic factors in this study play an important role in reversing SEMA3A-induced growth cone collapse in a post-treatment setting. This particular setting is unique in that it tests both inhibition and reversal of growth cone collapse because it is the only time frame where SEMA3A-induced collapse had already begun before the EG-conditioned medium was introduced. This may suggest why all 4 neurotrophic factors were important in the post-treatment setting.

Previous studies have analyzed the combined effect of neurotrophic factors on neuronal development and regeneration in different settings. Madduri *et al.*[36] observed that NGF and GDNF work synergistically in axon development; GDNF plays a greater role in axon elongation while NGF plays a greater role in axon branching. In another study that analyzed the length of neurite outgrowth, the results demonstrated that individual inhibition of BDNF and GDNF resulted in decreased neurite length, but inhibition of both neurotrophic factors resulted in the greatest reduction in length.[37] Furthermore, Hansebout *et al.*[24] showed that NGF, BDNF, GDNF, and NT-3 all play individual roles in DRG neurite growth. In other SEMA3A models, Wanigasekara *et al.*[18] found that SEMA3A-sensitive neurons were heterogeneous in their expression of NGF, GDNF, and neuritin receptors. Their study suggests that all of these factors have a role in axonal and growth cone regeneration. Finally, Ben-Zvi *et al.*[27] demonstrated that SEMA3A, NGF, BDNF, and NT-3 all play roles in determining whether DRG survive in mouse embryo models.

The post-treatment model attempts to mimic the clinical setting of post-trauma treatment of spinal cord injury. Our study is novel in that it suggests the entire complement of neurotrophins is necessary to maximally reverse and prevent further insult to the damaged area. However, no individual factor appears to be essential to the process. Since inhibition of each neurotrophic factor (NGF, BDNF, GDNF, NT-3) resulted in an increase in growth cone collapse, it suggests EG

secrete each of these factors in biologically relevant quantities *in vitro*. Therefore, this secretion might also be a mechanism that can mediate their beneficial effects *in vivo* post SCI.

Weakness of our study include that it does not verify if all neurotrophic factors were neutralized by the antibodies. While as per the manufacturer guidelines, this should have been achieved, a future study could include a secondary enzyme-linked immunosorbent assay to confirm lack of free floating neurotrophic factors. In addition, this study does not indicate if these four neurotrophic factors work together in a synergistic manner or through individual mechanisms. Similar to previous studies, further experiments where multiple neurotrophic factors are co-inhibited could shed light on this question. Furthermore, as an animal model, it has limited human application. A future study repeated in a human DRG cell line, using human EG, may provide better translational knowledge.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

No patient involved.

Ethics approval

All experiments were performed in accordance with the requirements of the Animals for Research Act of Ontario, Canada and the Guidelines of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board at our institution.

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Case Report **Open Access**

D-cycloserin, a NMDA-agonist may be a treatment option for anti-NMDAR encephalitis

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ABSTRACT

Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is caused by reversible neuron dysfunction associated an autoantibody-mediated decrease of NMDAR in the entire brain. A N-methyl-D-aspartate (NMDA) -agonist treatment for anti-NMDAR encephalitis might have a role considering its specific mechanism. The authors used D-cycloserine, a partial NMDA-agonist in a refractory case with prolonged intensive care unit duration. A 13-year-old female presented with headache, cognitive deterioration, generalized seizures, coma and hypoventilation with required mechanical ventilation. Anti-NMDAR antibodies were identified in cerebrospinal fluid and serum confirming anti-NMDAR encephalitis. The patient was refractory to first-line and second-line immunotherapy and removal of ovary teratoma. D-cycloserine was then administered and her symptoms improved gradually and significantly. This is the first reported case in which D-cycloserine was applied to this disease. D-cycloserine might be a potential option as specific treatment in anti-NMDAR encephalitis.

INTRODUCTION

Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is caused by reversible neuron dysfunction associated autoantibodies against NMDAR.[1,2] Most cases with anti-NMDAR encephalitis respond to immunotherapy. However, some cases are refractory to immunotherapy and have prolonged intensive care unit duration.[1] Specific symptomatic treatment for anti-NMDAR encephalitis might have a role considering the mechanism of NMDAR dysfunction in the disorder. However, the current symptomatic medication, such as anti-epileptics and neuroleptic are usually less effective in anti-NMDAR encephalitis. Therefore, we think a N-methyl-D-aspartate (NMDA)-agonist might be used to reverse its neuron dysfunction. We reviewed the literatures on clinical application of NMDA-agonists and found that D-cycloserine, a

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partial NMDA-agonist, may be a reasonable option. We then applied the medicine to a patient with anti-NMDAR encephalitis refractory to immunotherapy. The patient's parents signed informed consent for publication of this report.

CASE REPORT

A previously healthy 13-year-old female presented with headache and nausea without fever. One week later, she progressively developed agitation, cognitive deterioration, generalized seizures, coma and hypoventilation with required mechanical ventilation. Brain magnetic resonance imaging was normal. Cerebrospinal fluid (CSF) analysis demonstrated pleiocytosis (180 white cells/μL) with a normal protein and glucose concentration. Anti-NMDAR antibodies was identified in CSF and serum confirming anti-NMDAR encephalitis. The patient was initially treated with intravenous immumoglobulin (IVIg) and methylprednisolone with no improvement. Four weeks after the onset, ovary teratoma was identified on ultrasound and laparoscopic resection was performed with pathological diagnosis of teratoma. After the operation another 2 cycles of IVIg, cyclophosphamide (IV 500 mg/m^2 monthly) and mycophenolate mofetil（1,000 mg/d）was added because of insufficient clinical response. Five months after initial presentation, no remarkable clinical improvement was observed. The patients were still unconscious with recurrent seizure, chorea, tackycardia and episodes of hypoventilation requiring mechanical ventilation. D-cycloserine was then administered 125 mg/d for 1 week followed 250 mg/d. Two weeks after the first administration of D-cycloserine, her symptoms improved gradually. Seizure and choreic movements were reduced and hypoventilation disappeared, leading to discharge from the intensive care unit. Two weeks later, a further improvement was observed. She was able to understand simple orders. Seizure and chorea had disappeared. D-cycloserine was reduced to 125 mg/d and then stopped. The patient is functionally normal now with modified Rankin Score 0 at her last follow-up 1 year from disease onset.

DISCUSSION

We report a case of severe anti-NMDAR encephalitis refractory to immunotherapy and response to D-cycloserine. This is the first reported case in which this NMDA-agonist was applied to this disease. Our observation indicates that D-cycloserine may be a therapeutic option for anti-NMDAR encephalitis.

The cellular mechanisms of anti-NMDAR encephalitis are a specific and reversible decrease of NMDAR surface density due to immunoglobulin G-mediated internalization. The internalization is caused by crosslinking of the receptors by the autoantibodies.[2] As we know, antibody-mediated receptor internalization had been demonstrated for myasthenia gravis.[3] Symptomatic treatment with pyridostigmine in myasthenia gravis is relatively specific considering its mechanism. It is reasonable for neurologist to find and apply a NMDA-agonist to treatment of anti-NMDAR encephalitis.

D-cycloserine, known as anti-tuberculotic medicine, has been widely introduced to neuropsychiatric studies, since its central activation mechanism as a partial NMDA-agonist has been found.[4,5] Increasing evidence suggests that D-cycloserine may be effective in various psychiatric diseases, including schizophrenia, anxiety disorders, addiction, major depression and autism as well as in neurological diseases, including dementia, Alzheimer's disease and spinocerebellar degeneration.[5,6] D-cycloserine acts at the glycine-binding site of the NMDA receptor, which is located at its NR1 subunit. As a partial agonist, D-cycloserine acts like an agonist at low doses but has antagonistic features with high doses. The doses of D-cycloserine used for modulation of neuroplasticity are lower than for antituberculotic indication. The typical application in antituberculosis therapy is 250 to 500 mg twice daily. In the neuropsychiatric studies, D-cycloserine was administrated at a dose of 50 mg/d to 250 mg/d.[5,6] The pharmacological properties of D-cycloserine indicate its potential as central nervous system modulator. The maximum concentration in blood is reached 2 h after oral application. About 54-79% of oral intake reaches the CSF.[7,8]

There are some interesting observations in which NMDA antagonist including ketamine may improve symptoms of anti-NMDAR encephalitis.[9] However, the administration of NMDA antagonist to anti-NMDAR encephalitis is not consistent with the mechanism of NMDAR hypofunction in the disorder and in our practice there were not obvious improvement after administration of ketamine or memantine to our patients with anti-NMDAR encephalitis. Recently, Heresco-levy *et al*.[10] reported the clinical and electrophysiological effects of D-serine in a schizophrenia patient positive for anti-NMDAR antibodies. D-serine was administrated in 6 weeks to their patient in which dose were increased gradually from 1.5 to 4 g/day.

This preliminary study is limited by the fact that spontaneous remission of symptoms even after severe and long disease duration is still expected in

anti-NMDAR encephalitis while the treatments with cyclophosphamide and mycophenolate may take 3-4 months to have responses. We cannot draw conclusions that the clinical improvement was a consequence of the D-cycloserine treatment.

In conclusion, D-cycloserine seems to be a promising symptomatic treatment for anti-NMDAR encephalitis. Further studies are warranted to evaluate the effectiveness of D-cycloserine. Understanding the mechanisms underlying the disorder will lead to discover novel therapy including other NMDA-agonists.

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Conflicts of interest
There are no conflicts of interest.

Patient consent
Obtained.

Ethics approval
The patient was treated within the standards of our institute and the report was approved.

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Case Report **Open Access**

Takayasu's arteritis - aphasia as an initial presentation

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ABSTRACT

Takayasu arteritis (TA) is an uncommon disease of young women, characterized by granulomatous vasculitis of medium and large arteries. Neurological involvement is reported in only a minority of patients and occurrence of neurological syndromes as the first manifestation of disease has been rarely reported. We present clinical, laboratory and imaging findings of a 40 years old lady with TA, who initially presented with clinical manifestations of stroke in form of aphasia. The rarity of the disease and especially such a presentation can cause considerable delay in the diagnosis and treatment.

INTRODUCTION

Atherosclerotic and embolic disease is common causes of ischemic stroke in both young and old patients. In the young however, a wider array of systemic and vascular diseases must be given consideration. Systemic inflammatory or autoimmune diseases, hypercoagulable states and vascular diseases such as dissection are responsible for about 20% of cases, while no certain cause is found in about one-third of young stroke victims.[1]

Takayasu arteritis (TA) is a chronic inflammatory disease of unknown etiology, characterized by granulomatous vasculitis of large and medium sized arteries, especially aorta and its branches.[2] During the course of the disease, neurological involvement (such as transient ischemic attack, stroke, and cranial nerve palsies) is seen in 10-20% of cases.[3-5] However, occurrence of neurological syndromes as the first manifestation of the disease has been rarely reported.[6-9]

CASE REPORT

A 40-year-old Indian female with no significant past medical history presented with sudden onset aphasia. There was no history of limb weakness or facial asymmetry. There was no known family history of cardiac, cerebrovascular or autoimmune disease. On

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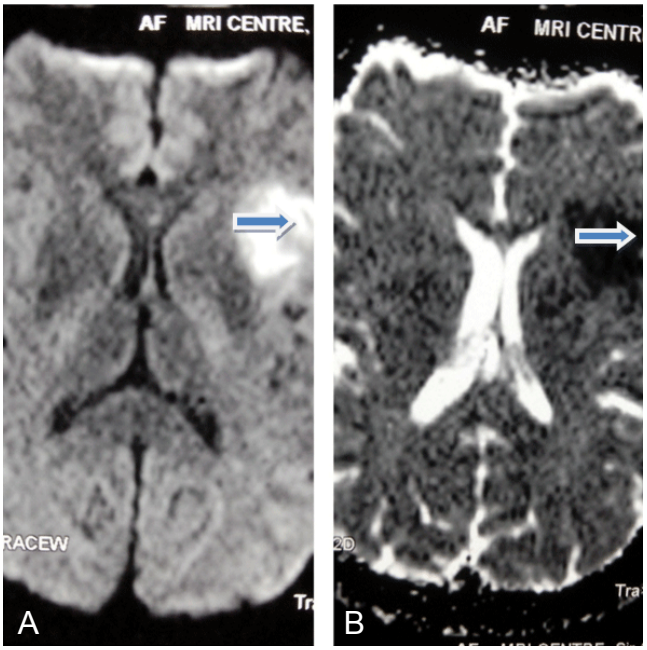

Figure 1: (A and B) Magnetic resonance imaging brain diffusion weighted imaging/apparent diffusion coefficient showing acute left perisylvian infarct

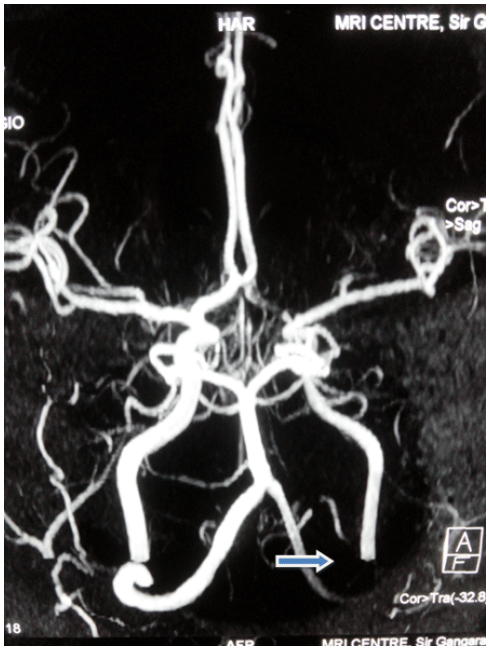

Figure 2: Magnetic resonance angiography brain showing arteritic disease involving the left internal carotid and vertebral artery

examination, the patient was moderately built and afebrile. Her left and right radial and brachial pulses were absent. Blood pressure in right upper limb was unrecordable and left upper limb was 120/70 mmHg. Blood pressure recorded in the lower limbs was 130/80 mmHg on both the sides. There was a thrill associated with a bruit over the right carotid artery. All the lower limb pulses were felt. She had wernicke's aphasia, no facial asymmetry, limb power was normal. Cardiovascular examination was normal. There were no renal vascular bruits and optic fundi did not reveal any abnormality.

Her erythrocyte segmentation rate was 50 mm/h, total counts 5,200 cells/μL, C-reactive protein was positive, and chest X-ray was normal. Electrocardiogram and holter study was within normal limit. Echo cardiography revealed normal study. Serum lipoprotein(a), homocysteine, Vit B12 level, lipid profile, Factor v leiden gene mutation, anticardiolipin antibodies, lupus anticoagulant, protein C, protein S, antithrombin-3 levels were found to be normal.

Magnetic resonance imaging brain revealed left perisylvian acute infarct [Figure 1]. Magnetic resonance (MR) angiography brain revealed arteritic disease involving the left internal carotid and vertebral artery [Figure 2]. MR angiography thorax and abdomen with contrast revealed diffuse concentric mural wall thickening involving ascending arch and the descending aorta causing luminal compromise [Figure 3a]. Right brachiocephalic, left common carotid and the left subclavian artery showed diffuse wall thickening with significant luminal compromise in right brachiocephalic artery. MR angiography neck vessels revealed significant Aorto-arteritic changes affecting all the major vessels [Figure 3b]. Given the clinical examination, laboratory studies and the findings on MR angiogram, diagnosis of Takayasu's arteritis was made. She was started on steroids and methotrexate was added subsequently. She is doing well and is also in follow up with rheumatologist. The patient is consented and agrees with this publication.

DISCUSSION

Takayasu's arteritis, also known as pulseless disease, is a chronic inflammatory disease of unknown etiology that affects the aorta and its main branches and is characterised by chronic vessel inflammation leading to wall thickening, fibrosis, stenosis, and thrombosis. It is a rare disease and was first reported in 1905 by Mikito Takayasu,[10] an ophthalmologist, in a case with peculiar changes in the central retinal vessels. This disorder is most common in Japan and to date more than 5,000 patients have been registered by the Japanese government.[11] Women are affected in 80% to 90% of cases, with an age of onset that is usually between 10 and 40 years.[12] It has a worldwide distribution, with the greatest prevalence in Asians.[13] Panja's series of 650 cases of TA, the largest series in India reported an incidence of stroke of 22%.[14]

The etiology of Takayasu's arteritis is unknown, but evidence suggests an autoimmune process, given

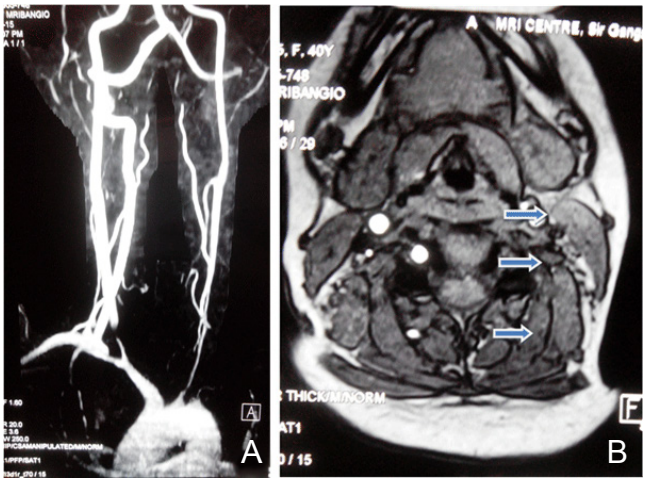

Figure 3: Magnetic resonance angiography. (A) Image of neck and thorax showing significant aorto-arteritic changes affecting all the major vessels; (B) image of neck vessels axial section showing significant aorto-arteritic changes affecting all the major vessels

the association with certain human leukocyte antigen (alleles and other autoimmune processes such as sarcoidosis and inflammatory bowel disease. It is also suggested that tuberculosis may have an association, given a high prevalence of active and past infection in patients with Takayasu's arteritis.[15]

The pattern of Takayasu's arteritis is typically triphasic, consisting of a systemic nonvascular phase, a vascular inflammatory phase, and a quiescent "burnt out" phase.[16] The symptoms of Takayasu's arteritis vary depending on the site and degree of arterial lesions. Most patients initially present with non-specific symptoms, such as fever, night sweats, malaise and arthralgia. As the disease progresses, symptoms of end organ disease due to the ischemia, including renovascular hypertension or coronary artery disease, may develop. As inflammation progresses, stenotic lesions develop and patient develop associated symptoms. Diminished or absent pulses, vascular bruits, hypertension, retinopathy, aortic regurgitation, congestive cardiac failure, neurological manifestation and pulmonary artery involvement are some of common manifestations of these patients.

Neurological complication occurring in the chronic phase of the disease, range from asymptomatic disease to catastrophic neurological impairment and most commonly include headache, dizziness, visual disturbances, convulsive crisis, transient ischemic attack, stroke and posterior reversible encephalopathy syndrome.[17]

The American College of Rheumatology established classification criteria for the diagnosis of Takayasu's arteritis: age at disease onset ≤ 40 years; claudication of the extremities; decreased pulsation of one or both brachial arteries; difference of at least 10 mmHg in systolic blood pressure between the arms; bruit over one or both subclavian arteries or the abdominal aorta; arteriographic narrowing or occlusion of the entire aorta, its primary branches, or large arteries in the proximal upper or lower extremities, not due to arteriosclerosis, fibromuscular dysplasia, or other causes.

The presence of three out of six criteria is required for diagnosis and demonstrates a sensitivity of 90.5% and a specificity of 97.8%.[16]

Ultrasound, computer tomography and magnetic resonance angiography (MRA) have shown promise in the diagnosis of TA. MRA provide high resolution detail of vessel wall thickness and lumen calcification; also allow the vessel wall thickness and lumen configuration.

Steroids are the mainstay of treatment for Takayasu's arteritis. Steroid unresponsive patients can be treated with cytotoxic drugs including cyclophosphamide, azathioprine, and methotrexate. Treatment should aim to control disease activity, preserve vascular competence with minimal long term side effects. Surgical treatment is offered to those with severe stenosis of renal artery, extremity claudication, stenosis of three or more cerebral vessels, or evidence of coronary artery involvement.

Our patient had no past history of systemic manifestations like fever, joint pains, and weight loss. Neurological deficit heralded the onset of disease. She had five out of six of the criteria set forth by the American College of Rheumatology and was thus diagnosed with Takayasu's arteritis.

In conclusion, when confronted with patients with neurological problems, we should be aware of rare but possible causes, which may be treatable or at least positively modifiable with correct and timely diagnosis. Although neurological manifestations are common in patients with Takayasu's arteritis in the chronic phase, acute stroke as an initial presentation has rarely been reported. Our patient shows that Takayasu's arteritis should be considered in the differential diagnosis of young stroke. This case again emphasises the importance of looking for peripheral pulses and recording blood pressure in all four limbs at least in young stroke patients.

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Conflicts of interest
There are no conflicts of interest.

Patient consent
Obtained.

Ethics approval
The patient was treated within the standards of our institute and the report was approved.

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Commentary **Open Access**

Auto-reactive B cells in MuSK myasthenia gravis

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On [*Guptill JT, Yi JS, Sanders DB, Guidon AC, Juel VC, Massey JM, Howard JF Jr, Scuderi F, Bartoccioni E, Evoli A, Weinhold KJ. Characterization of B cells in muscle-specific kinase antibody myasthenia gravis. Neurol Neuroimmunol Neuroinflamm 2015;2:e77*]

Acquired myasthenia gravis (MG) is a protoypical autoimmune disease caused by a dysfunction of neuromuscular transmission at the postsynaptic part. Patients experience fluctuating muscle weakness that increases with exertion. It is typically classified into clinical subtypes depending on distribution of involved muscles, onset age, thymic pathology, and auto-antibodies. While the most common auto-antibodies are targeted towards the skeletal muscle acetylcholine receptor (AChR), the list of target molecules of pathogenic auto-antibodies has been expanding to include the muscle specific tyrosine kinase (MuSK), low-density lipoprotein receptor-related protein 4 and agrin.[1-5]

MuSK MG in particular has been of great interest. It is clinically characterized by bulbar predominant manifestation, marked atrophy of the involved facial muscle, frequent myathenic crisis, poor outcome with conventional immunosuppressants, intolerance to acetylcholinesterase inhibitors, and fewer thymic pathologic changes.[6] The disease is also known for its unique immunological features. The pathogenic autoantibodies are mainly immunoglobulin G4 (IgG4),[7] which unlike the IgG subtypes in AChR MG (IgG1 and IgG3) does not activate the complements and effector cells. Little is known about the precise cellular components and molecular mechanisms in MuSK MG.

Guptill *et al*.[8] have recently reported the characteristics of B lymphocytes in MuSK MG patients. They performed polychromatic flow cytometry and enzyme-linked immuno sorbent assays in peripheral blood samples from MuSK MG patients, and compared immunological features of the patients to those of healthy controls. They found no differences in the frequencies of total B cells and B cell subsets (naive, memory, class-switched, plasmablasts and transitional cells) between the healthy controls and MuSK MG patients who had not been treated with rituximab (anti-CD20 monoclonal antibody). There

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was no difference between immunosuppressed and non-immunosuppressed patients either. However, the plasma B-cell activating factor (BAFF) levels were significantly increased in MuSK MG patients; another main finding of this study is that *in vitro* stimulation of peripheral blood mononuclear cells resulted in lower percentages of B10 cells in MuSK MG patients compared to controls.

Despite the limitations of small number of patients and heterogeneous treatments, this study provides novel insights and understanding of the immunopathology of MuSK MG. First, this study supports the emerging pathogenic role of BAFF which is a cytokine essential for the survival and differentiation of B cells. A clinical trial of belimumab, the monoclonal antibody that targets BAFF, is currently in progress for MG patients. It will be interesting to see if the drug is effective for MuSK MG which is often refractory to conventional immunosuppressive treatment. Second, B10 cells are recently characterized regulatory subset of B cells producing IL-10. With no change in Treg frequency and function, the reduced B10 cells observed in this study suggests a potential mechanism of breakdown in the immune tolerance in MuSK MG.[9] Future studies of larger number of patients would help further elucidate the precise immunobiology of this rare autoimmune disease.

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Conflicts of interest
There are no conflicts of interest.

Patient consent
No patient involved.

Ethics approval
This article does not contain any studies with human participants or animals.

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Editorial **Open Access**

Serum immuno-biomarkers in gliomas

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Gliomas comprise the majority of malignant primary brain tumors in adults and remain a devastating diagnosis.[1] Despite therapeutic advances, median survival in the radiation and temozolamide era for the most common and most aggressive tumor type, glioblastoma (GBM), is 14-20 months and tumor recurrence is universal.[2] A promising new direction to combat GBM is immunotherapy, which emerged from intensive research revealing that although highly immunogenic, GBM actively suppresses the host anti-tumoral immune response throughout a variety of mechanisms (reviewed in[3] and[4]). Current therapies to abrogate tumor immunosuppression and unleash T-cell mediated killing of GBM include: inhibitors of immune-checkpoints that are exploited by GBM to enhance tumor survival (therapeutic antibodies against CTLA-4 and PD-1, which are overexpressed in tumor-infiltrating lymphocytes, and against PD-L1, which is overexpressed on GBM cells and tumor-infiltrating immune cells); vaccination strategies against single or multiple tumor-associated peptides; infusion of autologous adoptive CAR T-cells primed against GBM-specific antigens; and inhibition of the immunomodulatory indoleamine 2,3-dioxygenase (IDO) pathway, which is also overexpressed in GBM.[3,5] Recent work by Alexiou *et al*.[6,7] published in this journal contributes to the use of immune mediators as prognostic surrogates of GBM aggressiveness and survival and may be clinical indicators of which patients are likely to benefit in current and future immunotherapeutic trials.

In the first article, a prospective, single-institution, observational study of GBM patients treated with tumor resection followed by chemotherapy and radiation (under the Stupp regimen[8]) for up to 1 year, Alexiou *et al*.[6] demonstrate a lower neutrophil-to-lymphocyte ratio (NLR) to be an independent prognostic predictor of survival. Using a cutoff value of NLR < 4.7, both overall survival (OS) and progression-free survival (PFS) were significantly longer. This study supports other work[9] which also revealed NLR as an independent prognostic predictor using a cut-off value of 4, and is consistent with other studies demonstrating the prognostic value of NLR in other tumor types. Importantly, both studies examined the NLR prior to administration of corticosteroids as corticosteroid use is itself an independent negative prognostic indicator, regardless of other chemo- or radiotherapy given.[10,11]

Extensive work has explored the role of tumor-infiltrating lymphocytes (TILs) in glioma, both as markers of tumor aggressiveness and with patient survival, namely that infiltration of cytotoxic CD8+ T lymphocytes are decreased and CD4+ and CD4+/CD25+/FoxP3+ (Treg) populations of T lymphocytes

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increased in higher grade tumors, with increased Treg infiltration indicating a worse prognosis.[12] Furthermore, it has been shown than TILs are enriched in the mesenchymal molecular GBM subclass,[13] which has also been correlated with improved response to immunotherapy.[14] Han *et al*.[15] describe a prospective, single-center series of GBM patients comparing intratumoral infiltration of macrophages and T cells and corroborating pre-treatment NLR (using a cut-off value of 4) with survival, establishing that serum neutrophil and lymphocytes levels (and thus the NLR) reflect the presence of TILs within the tumor milieu. In another study by Berghoff *et al*.[16] a single-center retrospective analysis ("Vienna cohort"), improved survival was not associated with TIL density (or PD-L1 overexpression), in contrast the other studies. Routine analysis of TILs is difficult in clinical settings, so it is crucial that the study of Alexiou *et al*.[6,7] mirrors the use of serum kynurenine and tryptophan levels as another biomarker of the immunosuppressive effect of IDO catabolism in gliomas, which demonstrated prognostic significance for OS in a prospective, multi-center observational trial of GBM patients.[17] Taken together, the work of Alexiou *et al*.,[6] Bambury *et al*.,[9] and Han *et al*.[15] elaborate upon the role of TILs in GBM via an easily obtainable, non-invasive, serum biomarker proxy. These papers reinforce and expand upon the paradigm that GBM is a lymphocyte-suppressing tumor as well as that lymphocyte down-regulation is itself a marker of an aggressive tumor pathology.

The second article by Alexiou *et al*.,[7] also a prospective, single-institution observational study of patients undergoing surgery for intracranial tumors (not limited to GBM), revealed that lower serum IgE levels in the GBM patients were a prognostic marker associated with poorer survival and higher-grade in gliomas, although the result was of marginal significance statistically. IgE levels in gliomas and meningiomas were also significantly lower than in metastatic tumors. This supports prior work on IgE and allergies in the development of malignancies. For example, in a large, multi-center, case-controlled study of self-reported medically-diagnosed allergies, there was an inverse relationship between allergies and gliomas. Furthermore, this association was incidence responsive, the greater number of medically-diagnosed allergies the lower the glioma risk.[18] A number of previous studies also suggest that a general atopy phenotype rather than a specific allergy type/allergen is correlated with decreased glioma risk,[18,19] which is consistent with the total serum IgE results discussed here. Thus, it is not a limitation of Alexiou's study that allergen-specific IgE antibodies were not elucidated.

GBM continues to be a catastrophic tumor, with eventual recurrence and poor survival, in no small part due to tumor-based immunosuppression. Immunotherapy is a promising addition to the current standard of care. The studies highlighted here by Alexiou *et al*.[6,7] emphasize the growing role of immune cells and antibodies in the understanding of both gliomagenesis, glioma progression, and the development of aggressive and treatment-resistant tumor types. They do not identify a causal role for certain inflammatory mediators either as protective or tumor-suppressive, but rather begin to pinpoint immune phenotypes of GBM. Furthermore, they provide easily measurable biomarkers for immunosuppressive GBM phenotypes, which are prognostic for patient survival and are clearly applicable to clinical trials for novel immunotherapies. Subsequent work is needed to correlate these findings to other prognostic GBM markers or subtypes (e.g. MGMT promoter methylation, presence of IDH1 mutations, *etc*.) and imaging characteristics (e.g. pseudoprogression following both standard treatment and immunotherapy is thought to be an immune-mediated response[20]). In fact, immunotherapy-induced changes on neuroimaging have been incorporated in to the newest response assessment guidelines ("iRANO"[21]), but whether these serum biomarkers correlate remains unclear. It is equally important to identify any mechanistic role of IgE, and also CD4+ Th2 cells, CD4+/CD25+/FoxP3+ Treg cells, or the IgE-driving cytokines IL-4 and IL-13, in the glioma milieu and the promulgation of tumor immunosuppression. Of further value would also be to expand upon the prognostic findings described here to determine if the serum NLR or IgE levels are useful to assess treatment response.

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Editorial **Open Access**

Interleukin-1beta: a common thread between inflammation, pain and opioid tolerance

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Chronic pain is a major health issue in our society that clearly impacts quality of life. Thirty to forty percent of the population in the United States suffer from chronic pain and its total cost have been estimated at 560-635 billion dollars annually.[1] Even if research progresses to develop novel analgesics, opioids remain the gold standard to treat pain. However, opioid treatment is associated with several adverse side effects including analgesic tolerance and opioid-induced hyperalgesia (OIH). OIH is of major importance and the use of morphine continues to increase. Analgesia tolerance corresponds to a progressive decrease of analgesia produced by a given dose of opioid upon chronic administration, resulting in the need to increase the dosage in order to maintain the initial analgesic effect. OIH usually clinically presents itself as the development of hypersensitivity to painful stimuli. OIH is well established in humans in different types of pain such as post-surgical pain, cancer pain and musculoskeletal pain.[2-4] Hence, clinicians face a dilemma to decided to either treat or not treat chronic pain with opioids which the knowledge of the patient developing pain hypersensitivity that may develop into opioid dependence. OIH is not yet completely understood and different mechanisms have been identified for this adaptive process to occur following opioid administration. These included the sensitization of primary afferent neurons and enhanced release of glutamate, hyperexcitability of second order neurons to excitatory neurotransmitters. However, more recently glial cells have been shown to play an important role in OIH. Receptors expressed in both microglia and

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astrocytes become activated in OIH.[5]

Long-term potentiation (LTP) is a sensitization of synapse (homo- and heterosynaptic) that enhances the strength of the synapse and its signal transduction. Increase LTP can cause hypersensitivity and may lead to hyperalgesia and has been shown to be involved in OIH.[6] In addition to glutamate-NMDA receptor mediated LTP in OIH, glial cells and released pro-inflammatory mediator have also been implicated in LTP in OIH. For example, cytokines interleukin-1beta (IL-1β) and tumor necrosis factor-α (TNF-α) can enhance post-synaptic potentials leading to neuronal excitation in the spinal cord. Cytokines in the central nervous system act mostly through the activation of glial cells to induce the release of other mediators that trigger LTP and hyperexcitability or neurons that leads to OIH.[7] The pro-inflammatory cytokine, IL-1β plays a major role in host defense and inflammation and is associated with inflammatory pain and opioid analgesia. In rodent models, peripheral administration of IL-1β produced hyperalgesia and reduced morphine analgesia while contributing to morphine tolerance.[8,9] At the molecular level, the interaction of IL-1β and the opioid system is shown by the finding that IL-1β increased the levels of mu opioid receptor (MOR) mRNA in primary astrocytes, neurons and in neural microvascular endothelial cells.[10-12] Other cytokines, including IFNα, TNFα, IL-4 and IL-6, also increase the expression of MOR in neuroblastoma cells and peripheral immune cells.[13-15] These results and others show that cytokines interact with endogenous opioid systems but explicit molecular mechanisms remain elusive. Interleukin-1beta mediates its effects through the interleukin-1 receptor type 1 (IL1R1) protein, which is a member of the Toll-like/IL-1R1 (TIR) domain family of membrane receptors.[16] Like the Toll-like receptors, the IL1R1 receptor signals through a complex of accessory proteins and downstream signaling events including activation of the JAK-STAT, MAPK, and NF-κB pathways.[17] Functional studies in cell lines show that transcription factors from the JAK-STAT, MAPK and NF-κB signaling pathways alter MOR gene transcription after cytokine stimulation.[10,18,19]

The NOD-like receptor protein 3 (NLRP3) inflammasome and downstream release of IL-1β are involved in pain conditions such as post-operative pain, post-herpetic neuralgia, diabetic peripheral neuropathy and spinal cord injury and if not controlled can lead to neuropathic pain.[20] In these and other forms of pain conditions, opioid such as morphine remains the gold standard analgesic and opioid use for pain management has dramatically increased, with little assessment of the pathological consequences on the primary pain condition. Recent data has shown that prolonged treatment with morphine doubled the duration of pain associate with nerve injury independent of opioid-receptor selectivity.[21] Morphine-mediated persistence of pain was attenuated following co-administration with the IL-1 receptor antagonist (IL-1ra).[21] Prolonged morphine use can activate glial toll-like receptors such as the toll-like receptor 4 (TLR4) which following priming ensures neurotoxicity, immune mediated amplification of nociceptive signaling in the spinal cord.[5,21-23] Evidence has also shown that morphine can directly compromise opioid-induced analgesia by promoting proinflammation via a TLR4 dependent mechanism and can potentiate mechanical allodynia.[24,25]

Reactive microglia has been implicated in playing a key role in morphine-mediated persistent pain conditions as demonstrated with the use of glial cell blockers.[26-29] It is noteworthy that while there are many reports that have described the importance of neuroinflammation in analgesic tolerance, since 2002 only a dozen few have focused on immune mechanism for OIH with four of the studies showing that the blockade of IL-1β reduced OIH.[30-33] In astrocytes, morphine exposure has shown to trigger astrocytes activation and lead to the upregulation of IL-1β.[34] Also, more recently, ultra-low dose morphine induced OIH was found to selectively activate astrocytes.[35] Together, this indicates that concurrent activation of microglia and astrocytes are involved in OIH.

In conclusion, my hypothesis is that opioid tolerance is a consequence of OIH. The increase in pain sensitivity caused by OIH masks opioid analgesia and if this continues would lead to opioid tolerance. At the molecular level, increased, chronic use of opioids would cause a decrease in MOR expression contributing to a loss of any analgesia mediated by opioids. Therefore, in the future it would be key to determine the cellular chronological order involved in increasing synaptic activity (i.e. LTP), which is normally mediated by increased levels of glutamate in the synaptic cleft and is removed by astrocytes. Current research shows that the common thread that may lead to OIH is the pro-inflammatory cytokine, IL-1β. Morphine alone can increase the expression and release of IL-1β from activated microglia and this increase may disrupt glutamate homeostasis. Recent evidence has shown that IL-1β can down-regulate the expression of GLT-1 and directly elevate the levels of glutamate and trigger the release of ATP from glia.[21] Increased glutamate, ATP and reactive oxygen species may contribute to excitotoxicity and chronic inflammatory and therefore the cycle may continue until morphine is discontinued. Current and previous data supports the

rationale to further examine whether the management of pain with opioids such as morphine contributes to a neuroinflammatory challenge that then leads to opioid tolerance and other pain comorbidities.

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Editorial **Open Access**

Emerging roles of microglia cells in the regulation of adult neural stem cells

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MICROGLIA CELLS

Microglia cells are antigen presenting cells with myeloid origin that constituted approximately 10% of all glial cells of the adult mouse brain.[1] In the early embryo, the microglial precursors are located in the yolk sac and progressively migrate throughout the primitive brain.[2] In the adult brain, some of microglial cells derive from the bone marrow, but this process only takes place when the brain is lesioned.[1] Microglial cells can display 4 morphological and functional stages: (1) resting microglia; (2) active microglia; (3) phagocytic microglia; and (4) senescent microglia.[3] One of the functions of microglia is to provide the cell-mediated immunity response against pathogens by releasing chemokines or cytokines.[1] However, the immune response and debris removing are not the only functions of microglial cells. Recently, it has been identified that microglia cells regulate neuronal apoptosis during the early brain development and modulate synaptic function. Strikingly, recent evidence indicates that microglia cells can regulate neurogenesis in the adult brain.

THE VENTRICULAR-SUBVENTRICULAR ZONE

The adult mammalian brain possesses specialized regions, also referred to as niches that host stem cells with neurogenic potential. One of these niches is the ventricular-subventricular zone (V-SVZ), an

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epithelial layer located in the lateral walls of the lateral ventricles.[4] In the adult V-SVZ, three cell populations have been identified: type-B cells, type-C cells and type-A cells.[5] The putative neural stem cell (NSC) is the type-B cell, an astroglial cell that can be identified by the expression of the glial fibrillary acidic protein, glutamate aspartate transporter, brain lipid binding protein, platelet-derived growth factor receptor α, CD133, Id1, Tailless, vascular cell adhesion molecule 1, epidermal growth factor receptor (EGFR), and others.[4,5] The activation of B1 cells depends on signaling pathways including sonic hedgehog, wingless-related integration site, Notch, bone morphogenetic proteins, ephrins, retinoic acid, betacellulin, stromal derived factor-1, pigment epithelium-derived factor and some intrinsic signals (Peroxiredoxin 1, sulfur oxide 2, arsenic-resistance 2, scute homolog 1, neuron-glia 2, Oligodendrocyte lineage transcription factor 2).[4,5] After activation, type-B1 cells produce transit-amplifying progenitors (type-C cells) that express the EGFR and the transcription factors Dlx2 and Mash1.[5,6] Type-C cells divide and give rise to neuroblasts (type-A cells) that migrate to the olfactory bulb through the rostral migratory stream and become mature interneurons.

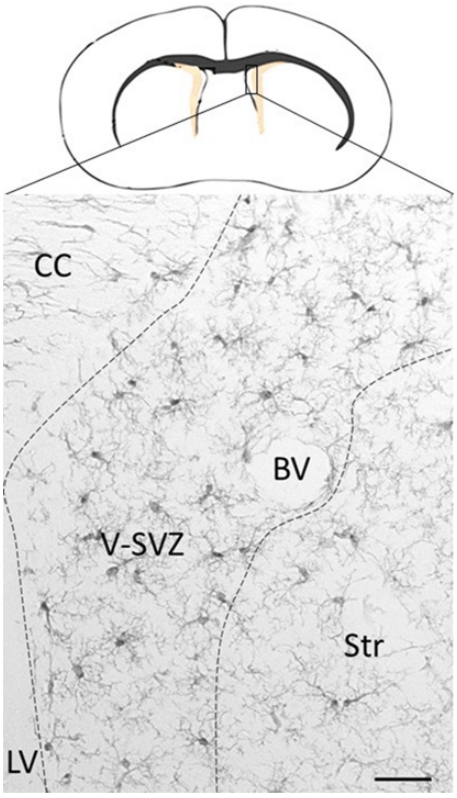

Figure 1: Microglia cells in the adult V-SVZ of mouse brain. Schematic bran section: The adult V-SVZ is the neurogenic niche lining the lateral walls of the lateral ventricles (LV). Photomicrography: Microglia cells labeled with anti-Iba1 antibodies and revealed with 3,3'-Diaminobenzidine (DAB) technique. At resting stage, these cells exhibit ramified cell morphology and numerous thin processes. Note that microglia cells are more abundant in the V-SVZ as compared to adjacent brain regions: corpus callosum (CC) and striatum (Str). These cells are also in close contact with blood vessels (BV). Bar = 20 μm

THE RELATIONSHIP BETWEEN MICROGLIA CELLS AND THE V-SVZ

In the adult brain microglia cells are present all along the V-SVZ [Figure 1] and remain in an intimate contact with niche cells.[7] The first interaction between the V-SVZ and microglia begins when microglia cells begin to populate the embryonic brain. At the early stages of brain development an excessive number of neurons are produced. This surplus of neurons needs to be eliminated and microglia cells are the responsible effectors of that function. Thus microglial cells are crucial to maintain the balance of neurons and normal postnatal brain development.

In the adult V-SVZ microglia cells stimulate neurogenesis by releasing soluble factors within the niche.[5] This is a very complex process that requires the molecular feedback between NSCs and microglia cells, which release and express a myriad of molecules, such as: CD200, vascular endothelial growth factor, transforming growth factor β in NSCs and CD200R, reactive nitrogen species/reactive oxygen species, insulin-like growth factor 1, tumour necrosis factor-α, Toll-like receptor-9, chemokine fractalkine, chemokine fractalkine receptor (CX3CR1), adenosine triphosphate-sensitive potassium channel IL-1β, leukemia inhibitory factor, interferon-ɣ.[7,8,9] In the V-SVZ, microglia cells presents certain degree of activation level and constantly release cytokines and neurotrophic factors with respect to other brain areas. This phenomenon suggests that NSCs are regulated by microglia cells. All of these events occur under physiological conditions, but under pathological circumstances these signals can be magnified. After activation, microglia cells enter into a phagocytic state to remove cell debris and damaged. Phagocytic microglia releases neurotrophic factors and cytokines that activate NSCs, thus trigger cell survival and neural regeneration after lesion. Microglial phagocytosis is one of the principal mechanisms to regulate and preserve the homeostasis in the production of neural progenitors in the postnatal brain. Microglia also eliminates aberrant cells that might later give rise to malignant cells or brain tumors.

THE V-SVZ/ROSTRAL MIGRATORY STREAM MICROGLIA AND THEIR ROLE IN THE V-SVZ

In a recent report, Xavier *et al.*[10] demonstrate the presence of a microglial subpopulation in the V-SVZ

and the rostral migratory stream (RMS) of CX3CR1-enhanced green fluorescent protein mice, which have a locus of the fractalkine receptor CX3CR1 replaced by the gene encoding green fluorescent protein. They found a subpopulation of microglia (V-SVZ/RMS microglia) that displayed distinct morphologies depending on the zone where they reside. The authors observed that some cells of this V-SVZ/RMS microglia presented an alternatively activated macrophage phenotype and released IL-4 and IL-10 cytokines via signal transducer and activator of transcription 6 phosphorylation.

The authors suggested that the differences in morphologies respect other brain areas may be due to a lower expression of purinergic receptors P2RY12, P2RY6, and P2RY1. Purinergic receptors use ATP for their activation and appear to regulate the processes and phagocytosis in microglial cells at non-neurogenic regions. In their study, Xavier *et al*.[10] found that P2YR12 expression was virtually absent in the V-SVZ/RMS compared with the surrounding areas. Interestingly, the P2RY6 purinergic receptor, involved in phagocytosis process, is rarely expressed in the V-SVZ/RMS, which suggests that phagocytic events are not frequent in the V-SVZ and RMS. Conversely, P2RY1 expression is highly expressed in the V-SVZ/RMS region in migrating neuroblasts, reaffirming the importance of P2RY1 in the migratory process of new neurons. Ablation of microglia increases the number of cells Ki67+ and BrdU+ in the V-SVZ/RMS and disrupts neuroblast migration. This evidence suggests that microglia play critical roles in neuroblast survival and migration in the V-SVZ.

In summary, microglial cells are the most important immune-cell mediator in the adult brain that preserve tissue homeostasis, regulate synaptic pruning, and sculpt neural circuitry. These cells are important components of the V-SVZ and RMS that play key roles in neurogenesis, neural survival and cell migration. Studying the relationship between NSCs and microglia cells is important for understanding the homeostatic mechanism that control the V-SVZ neurogenenic niche and surrounding areas. Nonetheless, this neural-immune interaction is still not well understood. Thus, further studies are needed for clarifying the molecular events implied in the neurogenic process and determining its clinical relevance.

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No patient involved.

Ethics approval

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Review **Open Access**

Immune-to-brain signaling and substrates of altered behavior during inflammation

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ABSTRACT

During the systemic inflammatory response to acute infection, and when in a safe environment, endothermic mammals typically display reduced activity and food intake, increased sleep, and the adoption of a curled-up position. These changes in behavior, in concert with fever, are adaptive in that they contribute to host survival. The present review addresses the immune-to-brain signaling pathways as well as possible neural substrates mediating reduced exploration and food intake during acute systemic inflammation. These involve rapid activation of peripheral nerves and glutamatergic brainstem circuits as well as slower IL-1β action in the brain activating limbic and possibly ventral hypothalamic structures. Although mostly adaptive acutely, behavioral changes during inflammation may also reflect brain dysfunction in severe sepsis-associated delirium or become maladaptive and result in depression due to medical conditions that involve long-term inflammatory episodes with pain or discomfort. The mechanisms underlying these conditions are presently ill-understood even though neuroinflammation and neurodegeneration occur during and subsequent to sepsis-associated brain dysfunction, respectively.

INTRODUCTION

Fever and reduced activity and food intake as adaptive host responses to infection

The finding in the 1970s that peripheral administration of non-steroid anti-inflammatory drug-type antipyretics lowered survival of different species of animals after their inoculation with bacteria provided a conclusive piece evidence in favor of the idea that fever was beneficial for survival of infected organisms.[1-3] But fever is an energetically costly response often requiring an increase in metabolism of 30-50%.[4] Text books of human and veterinary medicine often have mentioned reduced activity and appetite along with the occurrence of fever during infectious disease.[4] From an energy balance point of view on endotherms, it makes sense to reduce energy expenditure in the form of physical activity, such as exploration of one's environment, during fever. But, given the adaptive value as well as the high energy costs of increasing body temperature in

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response to infection, one may wonder how come then that the infected organism refrains from taking in more energy? In fact, reducing food intake upon infection may be an adaptive response as well since force-feeding mice during acute bacterial infection, it was indeed found to increase mortality.[5]

Sickness behavior as motivated behavior

Benjamin Hart[4] stated that in a "sickness behavior" perspective "the sleepy or depressed or inactive animal is less motivated to move about using energy that could fuel metabolic increases associated with fever". To consider sickness a motivation, like fear, hunger, thirst and other motivational states, implies that its expression is flexible depending on other motivations. Thus, it is important to show that its occurrence does indeed depend on environmental conditions. Interestingly, rats that depend entirely on hoarded food for their consumption, when rendered sick by bacterial lipopolysaccharide (LPS) endotoxins, continued to hoard more food, even though they did not consume it, than did animals injected with LPS that had the possibility to hoard, but also received food in their cage.[6] Thus, the expression of sickness behavior depends on the external conditions and, in this case, likely on the motivation to hoard food. Based on these and other observations, sickness behavior is now considered as the expression of a motivational system that reorganizes the organism's perception and action.

Sepsis-associated encephalopathy and delirium

Septic encephalopathy or brain dysfunction occurs in up to 70% of sepsis patients.[7] Encephalopathy was replaced by delirium due to a general medical condition in Diagnostic and Statistical Manual of Mental Disorders-IV and described as a disturbance in consciousness or perception or change in cognition characterized by reduced ability to focus or sustain attention and fluctuating changes in mental status, ranging from confusion to coma. However, functioning of the entire neuraxis and peripheral nerves can be disturbed during sepsis. Indeed, abnormal or slowed postural or protective reflexes have often been reported to occur during sepsis.[8-10] Therefore, and notwithstanding the fact that sickness behavior can clearly be adaptive in response to an acute infection, it should also be clear that during severe sepsis important cerebral dysfunction can occur.

Why immune-to-brain signaling?

Fever can be defined as "a state of elevated core temperature" that is "due to an elevation of the set-point of body temperature, according to which the higher temperature is actively established by the operation of thermo-effectors".[11] Since the set-point of body temperature is regulated by the preoptic hypothalamus, this gave rise to the question how the immune system signals the brain to bring about fever when animals are infected with bacteria.

The view of sickness behavior as being due to a motivation also implies immune-to-brain signaling. Indeed, even though the brain circuits underlying every single postulated motivational system are not known in full detail, motivations are mediated by brain circuits comprising the hypothalamus and limbic system. Thus, the occurrence of sickness behavior in response to exposure of animals to bacteria also begged the question as to how such events are signaled to the brain. In what follows the actions of the pro-inflammatory cytokine interleukin-1 (IL-1) on peripheral nerves, brain circumventricular organs and the blood-brain barrier (BBB), IL-1 transport across the BBB and IL-1 synthesis in the brain will be discussed as immune-to-brain signaling pathways.

FROM IMMUNE-TO-BRAIN SIGNALING TO NEUROINFLAMMATION?

Intereukin-1 as a mediator of immune-to-brain signaling that cannot passively cross the BBB

Once bacteria or their components have entered host tissues, they activate innate immune cells, including monocytes-macrophages and neutrophils, to generate an inflammatory response mediated by cytokines, such an interleukin-1β (IL-1β).[12,13] Peripheral injection of IL-1β mimics the symptoms of sickness and the signs of disease normally seen after infection.[14] Conversely, systemic administration of the naturally occurring IL-1 receptor antagonist (IL-1ra) alleviates or blocks systemic bacterial LPS-induced fever in rats.[15,16] In addition, peripheral IL-1ra also attenuates the reduction in locomotor activity and social interactions after systemic LPS injection.[17] Thus, IL-1 mediates, at least in part, fever and sickness behavior when these occur in response to the administration of bacterial LPS. However, the fact that IL-1 is a hydrophilic large peptide of 17 kDa means that it cannot passively cross the BBB separating the brain parenchyma from blood. Consequently, proposing and testing IL-1-mediated immune-to-brain signaling pathways became a topic of intense research activity from the 1990s onwards.

Circulating IL-1 acting in brain circumventricular organs lacking a BBB

In the early 1980s, antipyretics were already known to inhibit the synthesis of prostaglandins, a family of small lipophilic mediators. IL-1 was subsequently found to induce the formation of prostaglandin E2 (PGE2) by stimulating the synthesis of the rate-limiting enzyme

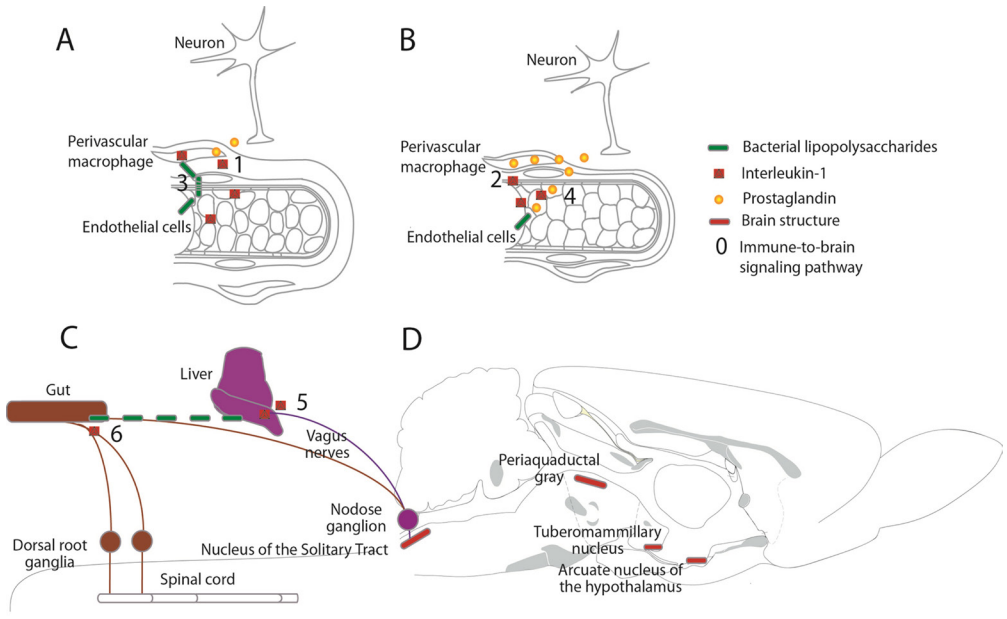

Figure 1: Immune-to-brain signaling pathways involving interleukin-1 in brain circumventricular organs (A), at the blood-brain barrier (B) and peripheral nerves (C) and substrates of altered behavior (D) during peripheral inflammation secondary to infection

cyclooxygenase in monocytes, fibroblasts, muscular and nervous tissue.[18] Around the same time, lesions of the anteroventral wall of the third brain ventricle, which contains the organum vasculosum of the lamina terminalis (OVLT), a brain cirumventriular organ where the blood-brain barrier is absent, were shown to suppress the fever response to peripheral administration of bacterial LPS or IL-1.[19,20] In addition, local injection of PGE2 into the OVLT resulted in higher fever than its administration in the preoptic area.[21] This led to the first hypothesis of IL-1-mediated immune-to-brain signaling pathway according to which circulating IL-1 acts in the OVLT to induce the production of PGE, which, in turn, modulates thermosensitive neurons in the preoptic area [Figure 1A-1].[22]

Transport of circulating IL-1 across the BBB

A more general alternative hypothesis of immune-to-brain signaling was put forward after it was shown that intravenously administered radioactive recombinant IL-1α or β entered the brain by a saturable transport mechanism [Figure 1B-2].[23,24] Subsequent studies provided evidence indicating that transport over the brain endothelium making up the BBB contributed more to the presence of intravenously injected IL-1 in the brain than the contained leakage from a circumventricular organ.[25] At the same time evidence for the presence of IL-1 receptors in the brain accumulated.[26-34] Moreover, administration of IL-1ra into the lateral brain ventricle was found to attenuate the reduction in social exploration and food-motivated behavior, but not the fever response, after systemic IL-1β injection.[35,36] These findings thus clearly indicate that IL-1 can act in the brain to bring about changes in behavior after its peripheral injection.

Brain production of IL-1

As early as 1984, bioactive IL-1 had been detected in the brains of mice that were given systemic bacterial LPS endotoxin and showing signs of sickness behavior.[37] In 1992, the presence of IL-1β immunoreactive mononuclear cells around blood vessels of the central nervous system (CNS) was observed several hours after an intravenous injection of a high dose of bacterial LPS in rats.[38] When using lower doses of bacterial LPS injected either intravenously or intraperitoneally, cells in the circumventricular organs and choroid plexus were found to synthesize IL-1β [Figure 1A-3].[39-43] Soon after this, mRNA for the LPS-recognizing receptor Toll-like Receptor 4 was found to be expressed in these organs.[44] Interestingly, IL-1β was also found to be synthesized by cells with microglial morphology in brain parenchyma adjacent to circumventricular organs, including in the arcuate nucleus of the hypothalamus and in the nucleus of the solitary tract, beyond 4 h after peripheral LPS injection.[43] Bioactive IL-1 is found in plasma but not in the brain 2 h after systemic LPS administration, whereas at 6 h IL-1 is bioactive in the brain but not in plasma.[45] When IL-1ra was given into the lateral brain ventricle at the time that brain IL-1 was bioactive, it was found to attenuate reduced social exploration without affecting the fever response after peripheral LPS injection.[46] The subsequent finding that peripheral administration of a neutrophil-neutralizing antiserum attenuates brain IL-1β expression as well as the reduction in locomotor activity

24 h after systemic injection of LPS suggests that neutrophil infiltration into brain provides an important source of IL-1β at later time points.[47] Hence, brain IL-1 production and action sustains sickness behavior after systemic LPS administration, but not necessarily fever.

Circulating IL-1 inducing prostaglandin synthesis at the BBB

In the early 1990s, a second form of the rate-limiting prostaglandin synthesizing enzyme cyclooxygenase (COX) was identified and found to be induced along brain blood vessels after peripheral administration of bacterial LPS or IL-1β.[48-50] Concurrently, it was shown that most IL-1 receptors in the rodent brain were expressed along blood vessels making up the BBB [Figure 1B-4].[31-34] This led to the hypothesis according to which circulating IL-1 acts on its signaling receptor expressed by brain endothelial cells to induce COX-2-mediated prostaglandin production, which, given their lipophilic profile, can diffuse across the BBB and activate prostaglandin receptors on neurons to bring about sickness symptoms.[48,49] Testing of this hypothesis using mice in which endothelial cells were deficient in COX-2 or PGE synthase showed that although the fever response to an intraperitoneal injection of IL-1β was abolished in these animals, the reduction in locomotor activity was not affected.[51,52] So, in accordance with a hypothesis put forward in 2002,[53] BBB prostaglandin synthesis underlies IL-1β-induced fever, but not necessarily sickness behavior.

IL-1 action on peripheral nerves

At least two of the classical symptoms of local inflammation, heat and pain, correspond to sensory modalities and thus involve neural activation. Interestingly, local IL-1β application under the skin of a rat paw was shown in 1994 to increase the sensitivity to mechanical and heat stimuli and to augment electric activity of sensory nerve fibers.[54] Based on these considerations, IL-1 was proposed to act on neural sensory afferents to signal the brain and bring about symptoms of sickness [Figure 1C-5]. In accordance with this hypothesis, subdiaphragmatic vagotomy was shown to attenuate the reduction in social exploration and food-motivated behavior, conditioned taste aversion, increased sleep and hyperalgesia as early as 2 h after intraperitoneal administration of IL-1β or bacterial LPS.[55-59] Reversible inactivation of the dorsal vagal complex, which contains the central terminals of vagal sensory fibers, by local anesthesia also restored social exploration after intraperitoneal LPS administration.[60] Moreover, the febrile responses to systemic administration of low doses of IL-1β or LPS were also attenuated by prior subdiaphragmatic vagotomy, whereas fevers after higher doses were unaffected by this procedure.[61-68] Furthermore, selective chemical lesions of C-fiber afferents after intraperitoneal injection of capsaicin in adult rodents were found to also attenuate the first phase of the fever response in response to systemic administration of LPS.[69] This suggests that LPS-induced rapid fever responses may involve vagally-mediated immune-to-brain signaling with later fever peaks or prolonged fever depending on prostaglandin synthesis at blood-brain interfaces.

Soon after the first vagotomy studies, intravenous IL-1β administration was found to increase afferent discharge activity of the hepatic and gastric branches of the vagus nerve in a prostaglandin-dependent way.[70-72] Subsequently, vagal paraganglia and the nodose ganglion containing the neuronal cell bodies of the vagus nerve were observed to bind IL-1ra and to express the signaling IL-1 receptor.[72,73] In addition, spinal sensory afferent cell bodies in dorsal root ganglia also express mRNA coding the signaling IL-1 receptor and their peripheral fibers respond to local administration of IL-1β by increasing their activity as well as their sensitivity to heat *in vitro*.[74,75] Interestingly, ganglia of both vagal and spinal sensory nerves express TLRs and some bacteria have been shown to directly activate sensory neurons.[76-78] Taken together, these findings suggest that low doses of IL-1β or bacterial fragments may act on sensory nerve fibers to signal the central nervous system to give rise to early fever, hyperalgesia and sickness behavior [Figure 1C-6].

ACTIVATION OF NEURAL SUBSTRATES OR INITIATION OF NEURODEGENERATION DURING SYSTEMIC INFLAMMATION?

Neural substrates of acute sickness behavior

Possible neural substrates of bacterial LPS-induced hypophagia

The basomedial hypothalamus plays an important role in the long-term regulation of food intake. Interestingly, lesions of the arcuate nucleus of the hypothalamus [Figure 1D] exacerbated the anorectic effect of peripheral IL-1β administration.[79] However; antagonizing the action of α-melanocyte stimulating hormone, which is produced by neurons of the arcuate nucleus of the hypothalamus, on central melanocortin receptors has been found to alleviate hypophagia after the peripheral administration of either IL-1β or LPS from 8 h onwards.[80,81] These findings indicate that the overall role of the arcuate hypothalamus is to counter reduced food intake, even though activation of some of its composing neurons does seem to play a role in sustained inflammation-associated hypophagia.

The brainstem mediates short-term regulation of food

intake and glutamatergic projections from the nucleus of the solitary tract [Figure 1D] to the parabrachial nuclei reduce food intake.[82] Interestingly, brainstem metabotropic glutamate receptor antagonism was found to attenuate hypophagia and to increase food intake during the first 6 h after peripheral LPS to a greater extent than in vehicle-treated animal.[83] In parallel, intra fourth ventricle administration of this metabotropic glutamate receptor antagonist also reduced expression of the cellular transcription activation marker c-Fos in the nucleus of the solitary tract and lateral parabrachial nuclei.[83] These findings suggest that brainstem glutamatergic circuits are part of the neuronal substrates that rapidly reduce food intake under inflammatory conditions.

Potential neural substrates of bacterial LPS-induced reduced exploration

Interestingly, all intervention strategies restoring social exploration after intraperitoneal LPS injection also reduce induction of the cellular transcription activation marker c-Fos in the central nucleus of the amygdala (CEA) and the oval bed nucleus of the stria terminalis (ovBNST).[46,60,66] The amygdala and the bed nucleus of the stria terminalis project to the ventrolateral periaqueductal gray (vlPAG) [Figure 1D] in the pons,[84] the stimulation of which induces immobility and reduced social interactions.[85] Thus, c-Fos expressing neurons in the CEA and ovBNST may inhibit GABAergic neurons projection to the vlPAG resulting in immobility and reduced social interactions.[53] In addition, reduced exploration of different environments and devices has been shown to be associated with c-Fos expression in the ventral tuberomammillary nucleus [Figure 1D] after peripheral bacterial LPS injection.[86,87] Reduced activation of the ventral tuberomammillary nucleus may therefore be part of the neural substrates underlying reduced environmental exploration during sickness.

The realization that endogenous IL-1β can act in the brain to bring about sickness behavior raised the question as to where in the brain it binds to the signaling IL-1 receptor to reduce social and environmental exploration. Although the hippocampus is one of the most prominent sites of neuronal IL-1 receptor expression (see transport of circulating IL-1 across the BBB), no published study to date seems to have critically addressed the involvement of hippocampal IL-1 receptors in mediating sickness behavior. It is important to point out that this is not because such approaches are not available. Indeed, several groups have employed hippocampal overexpression of the IL-1ra. These studies addressed the role of hippocampal IL-1 in mediating responses to psychological stressors, such as electrical shocks and chronic isolation, and not those occurring upon exposure to infectious microorganisms or their components.[88,89] However, the findings of Chaskiel *et al*.[83] show that selective lesioning of IL-1 receptor-expressing cells in the hippocampus does not alter the reduction in social exploration after intracerebroventricular administration of IL-1β in mice. Thus, IL-1 receptors in the hippocampus do not seem to mediate the component of sickness behavior that involves reduced exploration.

Severe sepsis may lead to neurodegeneration

Magnetic resonance imaging of septic patients with brain dysfunction has indicated the presence of vasospasms in the medial cerebral arteries and ischemic strokes in brain gray matter as well as white matter edema.[90-92] (see for review[93]) Post mortem examination of brains of patients who died from sepsis revealed intracerebral hemorrhage, necrotic vessels with infiltrating leukocytes, increased perivascular spaces, microglial activation, cerebral IL-1β and TNF-α expression, neuronal apoptosis as well as perivascular dissociation of myelinated fibers and demyelination.[91,94,95] Clinical research thus clearly indicates the occurrence of neuroinflammation that may, in turn, lead to neurodegeneration.

Recently, several groups have employed cecal ligature and puncture (CLP) in rodents to study CNS dysfunction associated with sepsis. In these models, food intake and social interactions were found to be reduced during the first days, while activity and body temperature were altered and some conditioning learning tasks impaired for several weeks after sepsis induction.[96-99] Increased cerebral pro-inflammatory cytokine expression, impaired BBB function, cortical perivascular edema, glial cell activation, brain leukocyte adhesion and infiltration as well as neuronal death and degeneration in cortical and subcortical areas have all been observed from the first day of CLP onwards.[96-105] Thus, relevant animal models of sepsis have been shown to result both in transient sickness behavior and in long-term learning deficits that are accompanied by neuroinflammation and neurodegeneration.

CONCLUSION

During the systemic inflammatory response to acute infection, and when in a safe environment, endothermic mammals typically display reduced activity and food intake, increased sleep, and the adoption of a curled-up position. These changes in behavior, in concert with fever, are adaptive in that they contribute to host survival. Although the precise neurobiological substrates still need to be worked out, they are brought about by immune-to-brain signaling pathways that involve rapid activation of peripheral nerves and glutamatergic brainstem circuits as well as slower IL-

1β action in the brain activating limbic and possibly ventral hypothalamic structures. Notwithstanding the fact that they are mostly adaptive acutely, behavioral changes during inflammation may also reflect brain dysfunction in severe sepsis-associated delirium or become maladaptive and result in depression due to medical conditions that involve long-term inflammatory episodes with pain or discomfort. The mechanisms underlying these conditions are presently ill-understood even though neuroinflammation and neurodegeneration occur during and subsequent to sepsis-associated brain dysfunction, respectively.

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Case Report **Open Access**

A case report of acute pediatric bacterial meningitis due to the rare isolate, *Pseudomonas putida*

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ABSTRACT

Acute bacterial meningitis (ABM) is the medical emergency which warrants an early diagnosis and an aggressive therapy. Despite the availability of the potent newer antibiotics, the mortality caused by ABM and its complications remain high in India, ranging from 16% to 32%. The aim of this case report is to present the rare isolation of *Pseudomonas putida* from cerebrospinal fluid sample. Besides this, the author also emphasizes the importance of correctly identifying the organism and thus the selection of the most accurate antibiotic from the susceptibility profile to allow for early recovery and to improve the patient outcome and survival.

INTRODUCTION

Bacterial meningitis can cause death if not treated early and aggressively both in the developed and developing countries.[1] Untreated, the mortality approaches 100%, and even with the current antibiotics and advanced pediatric intensive care, the mortality rate of disease is approximately 5% to 10%.[2] Worldwide, the neurological

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aftereffects of the meningitis in the survivors following the hospital discharge approaches 20%.[2,3] Risks of long-term disabling secondary results were highest in low-income countries, where the burden of bacterial meningitis is the greatest. Most of these reported results could have been averted by vaccination with Hib, pneumococcal, and meningococcal vaccines.[3] Hence early diagnosis and appropriate management of children with meningitis is critical as it can be difficult to diagnose as the symptoms and signs are often nonspecific especially in young children.[2]

CASE REPORT

A 5-year-old girl, a known case of opsomyclonus syndrome and is therefore being treated for this autoimmune condition with steroids for the past 2 years, she was referred Lotus hospital on March 3th, 2015, with the symptoms of fever, vomitings (7-8 episodes) and reduced appetite for the last 48 h and altered sensorium for the last 24 h. She had the past history of ataxia. On assessment, the growth and the development were appropriate for her age. Her heart rate was 168/min, respiratory rate was 61/min, blood pressure (BP) was 77/38 mmHg. On physical examination, she had sunken eyes and wound over the knee. Her tongue appeared to be dry. Respiratory exam showed that she had tachypnea. Central nervous system examination showcased that she was drowsy and Glasgow coma score (GCS) was E3V3M4 and hypotonia was present in the lower limbs. Pupils were bilaterally equal and reacting to the light. On abdominal exam, abdomen was distended. In view of the poor GCS, she was intubated and mechanical ventilator support was continued. Her blood gases were monitored regularly.

Her complete blood picture was normocytic and normochromic. The cell counts and erythrocyte sedimentation rate were within normal limits except neutrpohils (neutrophilia) and platelets (thrombocytosis). Blood urea nitrogen, serum calcium, serum creatinine (1.2 mg/dL) and serum electrolytes were out of range. Her serum glutamic oxaloacetic transaminase was 152 IU/L and her test results for malarial antigen were negative. Her blood ammonia was within normal range. Complete urine examination showcased 10 pus cells/high power field whereas the culture results showed that she was sterile. Routine examination of her stool was negative. Blood cultures were negative for bacterial growth. Oxygen saturation was 55%. Ultrasonography of the abdomen showed mild ascitis and her fundus examination was normal.

In view of the history and clinical features, she was diagnosed as meningitis with status epileptics with the lower respiratory tract infection. Cerebrospinal fluid (CSF) analysis showed a normal white blood cell count (0.4 cells/cumm and lymphocytes 100%), normal proteins (26.6 mg/dL) and elevated sugar levels (112.7 mg/dL).

Gram stain did not show any organism and pus cells. However, CSF culture grew Gram-negative organism which on further biochemical evaluation was identical to *Alcaligenes fecalis*. The organism was later on identified as *Pseudomonas putida* with automated identification system, VITEK®2 (BIOMERIEUX, USA).

The patient was treated initially with injection of piperacillin with tazobactum, vancomycin, meropenem, acyclovir and maintenance IV fluids. In addition to this, she received injection phenytoin followed by phenobarbitone and anticerebral edema measures; computed tomography scan of the brain was normal. As per the clinical findings, a possibility of severe sepsis with septic shock was considered. Her 2D ECHO was done and showed normal heart with mild, bilateral pleural effusion, inferior vena cava was non-collapsed and dilated. Her fluid bolus was optimized and she was commenced on the vasoactive agents in view of refractory shock. C-reactive protein was elevated (46 mg/L).

Pseudomonas putida displayed *in vitro* sensitivity to amikacin, ciprofloxacin, levofloxacin and minocycline and moderately sensitivity to gentamicin and cefepime. It was totally resistant to piperacillin and tazobactum, cefoperazone and sulbactum, cotrimoxazole, doripenem and tigeycycline. Hence the antibiotics were change to amikacin. Gradually her hemodynamics improved with the reversal of shock state.

Her chest X-ray showed the right lower lobe consolidation. She was extubated after 6 days and received chest physiotherapy. On the day of discharge, March 26th, 2015, her BP was 110/54 mmHg, and oxygen saturation was 98% and all organ systems examinations were normal.

DISCUSSION

Acute bacterial meningitis (ABM) is the dangerous disease if found in young children and has a high rate of fatality and risk of neurological handicaps.[4] In the developed countries, *N.meningitidis* and *S.pneumoniae* are the most prevalent cause of the acute bacterial meningitis[2] whereas *H.influenzae*, *N.meningitidis* and *S.pneumoniae* are responsible for ABM in the developing countries.[4,5]

In a Spanish prospective observational study, 69.4% and 30.5% cases of meningitis are community and nosocomially acquired respectively.[6] The etiologic agents of community acquired meningitis are *H.influenzae*, *N.meningitidis* and *S.pneumoniae* whereas nosocomial meningitis is caused by Gram-negative bacilli (GNB) and *Staphylococcus* sp.[6] Another Spanish neonatal meningitis study revealed 55.6% and 44.37% of meningitis cases were vertically and nosocomially transmitted respectively. *S.agalactiae* was reported in 48.5% confirmed cases of meningitis and in other cases *E.coli* and *S.epidermidis* were isolated from 26.5% and 24.5% of the cases respectively.[7]

In an 8-year study from the Northern region of India, the majority of the patients (83.8%) were younger than 12 years and majority of them were infants (36.7%). Majority of the meningitis cases (69.2%) were community acquired and 30.8% were hospital acquired. Overall, *S.aureus* predominated during the 8 years study period accounting for the total of 38% of all isolates followed by *Pseudomonas* sp (12%) and *E.coli* (11%).[8]

In the present study, *Pseudomonas putida* was isolated from a 5-year-old girl. Bareja *et al*.[9] in a study on the trends in bacteriology of meningitis reported, *P.aeruginosa* to be responsible for 9.23% of pediatric meningitis cases, out of which majority of them (29.4%) were seen in 1 to 3 years old children, less frequently were observed between 3 to 12 months of age group children (17.64%) and in 3 to 5 years old children (17.64%). Archibald *et al*.[10] reported 2 cases of *Pseudomonas aeruginosa* childhood meningitis. Yang *et al*.[11] isolated *P.putida* in CSF causing meningitis in 2 of their 55 patients (5%) with *P.putida* infections.

In the present study, Gram stain did not show any organism and pus cells. In addition to this, blood and urine culture were sterile. CSF culture grew GNB resembling *Alcaligenes fecalis* (identified with limited number of conventional biochemical tests) and later on identified as *Pseudomonas putida* with automated VITEK® 2 (BIOMERIEUX, USA) system. Modi *et al*.[12] in a study on 252 CSF samples in patients with acute childhood bacterial meningitis; 162 (64.3%) were smear positive and 200 (79.4%) were and culture positive. Bareja *et al*.[9] reported only 58% Gram stain positive samples and 23.5% culture positive. Almost similar results of positive Gram stain (67%) and cultures (50%) were reported by Chinchankar *et al*.[4]

In the present study, CSF analysis showed normal cell count, normal protein and elevated sugar. Bareja *et al*.[9] found increase in the cell count in more than 90% of their culture positive specimen. On the contrary, Modi *et al*.[12] reported the cell count of CSF sample to vary from no cells to sheets of cells; they also reported high mean level of protein (90.2 ± 11.5 mg/dL) and a mean sugar level of 32.2 ± 3.4 mg/dL.

We did not perform CSF C-reactive protein, latex agglutination test (LAT) and polymerase chain reaction (PCR). Chinchankar *et al*.[4] reported CSF C-reactive protein and LAT positive in 41% and 78% of the cases respectively while culture was positive in only 50% of the cases. Finlay *et al*.[13] in their study, LAT confirmed the etiology of meningitis in 60% cases of *S.pneumoniae*, 93% of *H.influenzae* type B and 39% of *N.meningitidis*. It also explains that though Gram stain and LAT were positive in 50% of the cases after receiving the antibiotics, LAT is beneficial to identify the causative agent and to start the early treatment and vaccination of the patient, specially in case of meningococcal types A and C. On the contrary, low sensitivity of LAT (13.5%) was reported by Tarafdar *et al*.[14] in a brief report on culture negative meningitis.

Broad range PCR for the early detection of bacterial meningitis showed 100% sensitivity, 98.2% specificity, 94% positive predictive value and 100% negative predictive value.[15] Similarly, the other analytical study displayed 54.5% sensitivity of the multiplex PCR in comparison with Gram stain (29.2%) and culture (34.5%).[16]

Intially, in this case patient was treated with piperacillin and tazobactum, meropenem and vancomycin. *Pseudomonas putida* displayed *in vitro* sensitivity to amikacin, ciprofloxacin, levofloxacin and minocycline and moderate sensitivity to gentamicin and cefepime. It was totally resistant to piperacillin and tazobactum, cefoperazone and sulbactum, cotrimoxazole, doripenem and tigeycycline. Later on the antibiotics were upgraded and patient gradually recovered. Results of the *in vitro* susceptibility test suggested that imipenem and ceftazidime were more effective than the other antimicrobials against *P.putida*.[11] Similarly, as per other CSF antibiogram analysis all strains of *Pseudomonas* sp were sensitive to imipenem.[12]

In conclusion, ABM is the medical emergency with high mortality rates. Rapid diagnosis and treatment are critical. We report a rare case of *P. putida* meningitis which was successfully treated. An infection with rare organisms is possible and a high index of suspicion can lead to accurate diagnosis and treatment in these cases.

Traditional lab methods such as Gram stain and culture are used for identification of organism. PCR is the rapid,

accurate, sensitive and specific method for diagnosis of meningitis as this assay detects 10 to 100 CFU/mL of bacteria in CSF.[16]

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Conflicts of interest
There are no conflicts of interest.

Patient consent
Obtained.

Ethics approval
The patient was treated within the standards of Lotus Hospitals (where the work was done) and the report was approved.

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Case Report **Open Access**

The expanding spectrum of pediatric anti-glutamic acid decarboxylase antibody mediated CNS disease - a chance association?

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ABSTRACT

Central nervous system autoimmunity in the pediatric age group represents an evolving constellation of various syndromes distinct from the adult age group. One of the rarely described pathogenic auto-antibodies (ab) is the one directed against glutamic acid decarboxylase (GAD). While its pathogenic role is controversial, literature concerning adult patients abounds with heterogeneous presentations with epilepsy often as part of limbic encephalitis or chronic temporal lobe epilepsy and cerebellar ataxia accompanying endocrinopathies or paraneoplastic disorders. Diagnosis is often delayed until late adulthood. The authors report hitherto under-reported syndromes in the pediatric age group. The first case was a 3-year-old boy with sub-acute myoclonus-ataxia following a flu-like illness akin to para-infectious cerebellitis. The second case was a 7-year-old girl with long-standing chronic extratemporal partial epilepsy and electrical status epilepticus in sleep (ESES) with right hemiparesis and developmental delay. Investigations revealed two-four fold elevations in titres of GAD-65-ab. The absence of systemic diseases like diabetes and the dramatic clinical response to steroids as well as intravenous immunoglobulin in both the cases argued for GAD-ab mediated neuronal injury rather than a chance association. The concern exists regarding other potentially co-existent auto-ab to gamma-amino butyric acid and glycine receptors, and demonstration of intrathecal synthesis of GAD-ab would be ideal. This entity should be contemplated in children presenting with acute/sub-acute onset episodic or progressive ataxia or refractory cryptogenic focal epilepsy syndromes, epileptic encephalopathy such as ESES and worsening neurological deficits. These children ought to be maintained on regular follow-up for monitoring evolution of other autoimmune disorders in adult life.

Quick Response Code:

INTRODUCTION

The spectrum of autoimmune encephalitis is ever expanding, with presentations outside the distinctively symptomatic groups being recognized every day. The array of intraneuronal and cell surface antibodies are fairly well elucidated with the former associated with paraneoplastic etiology and poor response to immunosuppressive agents.[1] While the clinical spectrum of non-paraneoplastic encephalitis, like anti-*N*-methyl-D-aspartate receptor (NMDAR) encephalitis and voltage-gated potassium channel (VGKC) encephalitis, has been well described,[1] the presentations of anti-glutamic acid decarboxylase antibody (GAD-ab) positive encephalitis are unclear. GAD-ab is directed to an intracellular enzyme and therefore considered to be the unlikely pathogenic moiety in itself. However, epilepsy and cerebellar ataxia represent the 2 most common neurological syndromes described in adults with this antibody.[2] *In vitro*, GAD-ab from patients with neurological syndromes induce a suppression of gamma amino-butyric acid (GABA) release.[3] The diagnostic value of low titres of GAD-ab in a patient with a neurological syndrome is unknown, as opposed to high titres as was seen in recently described case series in patients with autoimmune endocrinopathies, like type 1 diabetes mellitus (DM1) or central nervous system (CNS) autoimmunity, such as limbic encephalitis.[2,4]

The largest reported cohort of 9 adult patients with GAD-ab mediated limbic encephalitis was notable for a poor response to treatment in comparison with VGKC antibody positive encephalitis, however, only a minority had been given the benefit of immunomodulation with immuoglobulin.[4] Currently, anti-GAD-ab disorders of the CNS in the pediatric age-group are rarely reported. Here we report a case series in the pediatric age group with variable clinical presentations, course and treatment response with the 2 patients demonstrating definite elevations in anti-GAD 65 antibody titres, adding to the evolving clinical conundrum of CNS autoimmunity in childhood.

CASE REPORT

Case 1

A 3-year-old boy, product of a non-consanguineous parentage with normal birth and development presented to us with subacute onset incoordination of upper and lower limbs along with scanning dysarthria. A flu-like prodrome was noted nearly 20 days prior to onset. Around 10 days into illness parents had also noted sudden jerky movement of extremities. His examination was notable for pancerebellar involvement with multifocal stimulus sensitive myoclonus. There was no evidence of any behavioural or cognitive decline, limb weakness, seizures, opsoclonus or any extrapyramidal involvement with no history of drug/toxin exposure. Blood biochemistry and serology were normal. Based on the possibility of a post-infective or a paraneoplastic immune mediated myoclonic ataxia syndrome, magnetic resonance imaging (MRI), cerebro-spinal fluid (CSF) study including lactate levels, electroencephalogram (EEG) and somato-sensory evoked potentials were ordered and were normal. A search for a neoplastic focus with ultrasonography abdomen and chest X-ray was negative. Twenty-four hours urine vallinly mandelic acid and metanephrine tests were conducted to exclude occult neuroblastoma and urine aminoacid estimation to exclude alkaptonuria was also normal. He was empirically started on a course of intravenous (IV) methylprednisolone after excluding any active infection. Over the period of the next 2 weeks, he had resolution of all his symptoms and the steroids were tapered off over 6 weeks. However, he presented to us 6 months later with recurrence of the same complaints with much more severe symptoms along with irritability, hyperactivity and temper tantrums. Considering his recurrent course and apparent steroid responsiveness, a further search was done with repeat MRI, including MRI abdomen, CSF oligoclonal bands and autoimmune panel of antibodies including NMDA, VGKC, and GAD 65 as well as anti-aquaporin antibodies. His antibody panel revealed an elevated GAD 65-abtitre of 10.7 IU/mL (0.0-5.0 IU/mL) by enzyme-linked immunosorbent assay (ELISA) and the rest of the investigations were negative including blood tandem mass spectroscopy. Considering the severity of symptoms he was started on a simultaneous course of intravenous methylprednisolone (20 mg/kg for 5 days) and immunoglobulin (400 mg/kg for 5 days) with which all his symptoms completed remitted in 1 week. He was maintained on oral steroids with plans for a longer duration of maintenance and slow taper. After nearly 24 months of follow-up he is symptom free with preserved motor and cognitive abilities and is presently off steroids.

Case 2

A 7-year-old girl, product of a non-consanguineous parentage with normal birth and development history, presented with habitual seizures since 2 years of age without any initial precipitating event. Her seizure semiology was suggestive of frontal lobe epilepsy with more than 95% events being nocturnal events occurring out of sleep and characterized by head and eye deviation to the right side with right upper limb abduction, clonic jerks and right facial jerks. Since the onset of seizures parents noted delay in subsequent development as well as a shift of handedness from

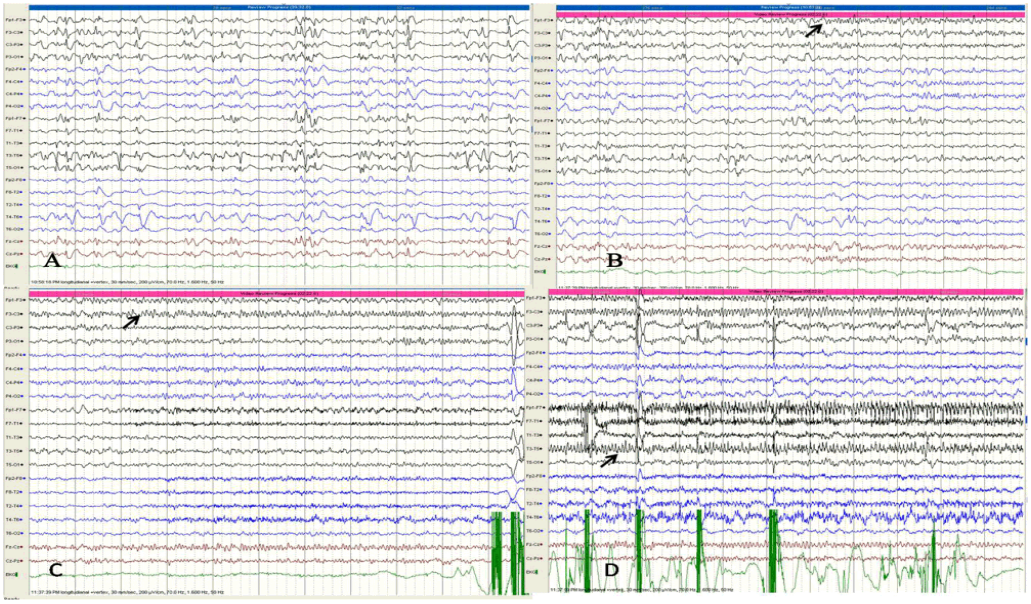

Figure 1: (A) Interictal activity in Case 2 during the first video EEG, consistent with an atypical left hemispheric dominant electrical status epilepticus in sleep; (B) ictal onset (arrow) in the form of low voltage fast activity over the left fronto-centro-parietal regions during hemiclonic seizure; (C) propogation of ictal activity over the left centro-parietal region (arrow); and (D) ictal activity during the established hemiclonic phase (arrow) demonstrating involvement of the left hemisphere with spread to the left posterior cortex. EEG: electroencephalogram

right to left. She presented to us nearly 4 years into her illness by which time she had frequent clustering of right hemiclonic seizures, which were drug refractory and in addition to serially prolonged episodes of Todd's paresis. Her examination revealed a developmental age of 3.5 years with right pyramidal signs. There were no epilepsia partialis continua. The initial MRI taken 1 year into the illness revealed non-specific volume loss over the left posterior cortex. A 12-h video EEG recording [Figure 1B-D] detected 6 complex partial seizure of left hemspheric semiology, five of left frontocentral ictal onset and one of left posterior head region onset. The interictal data showed frequent left frontocentral, left posterior temporal and occipital interracial epileptiform discharges with intrahemispheric and secondary bilateral synchrony, with sleep records showing electrical status epilepticus in sleep (ESES) [Figure 1A]. As she was on a combination of carbamazepine, phenobarbitone and levetiracetam, a possibility of sodium-channel blocker mediated worsening with ESES was considered and carbamazepine was gradually withdrawn and replaced by valproic acid. One month later she presented with increased nocturnal seizures, and she was commenced on lamotrigine which was subsequently withdrawn due to drug allergy. After another month, she developed simple partial and complex partial status epilepticus with worsening right hemiparesis. She was treated with a fosphenytoin-midazolam infusion along with continued polytherapy in view of seizure clusters and a 5-day pulse of IV methylprednisolone was administered, considering the possibility of left hemispheric focal encephalitis. The repeat MRI showed diffuse bilateral generalised parenchymal atrophy with asymmetric dilatation of ventricles (left more than right) with subtle asymmetric loss of grey-white differentiation over the left posterior quadrant [Figure 2]. Her CSF evaluation including immunoglobulin G (IgG) index at that time was normal. Her serum autoimmune panel comprising of blood and CSF: NMDAR antibody, VGKC antibody, anti-thyroid antibodies and anti-GAD antibody, which revealed elevated GAD 65-ab titre of 21 IU/mL by ELISA (0.0-5.0 IU/mL). Following discharge, she developed a phenytoin allergy. She was withdrawn off oral steroids over 2 months with only infrequent brief nocturnal seizures and near complete recovery of hemiparesis. Three months later she was re-admitted with seizure clustering and right hemiparesis. Following the initiation of IV steroids, she developed complex partial status epilepticus. She required ventilation with midazolam anesthesia and intravenous lacosamide, following which she recovered over 1 week. She was administered IV immunoglobulin 2 g/kg over 5 days with which her hemiparesis also recovered. She was maintained on cyclical immunoglobulin 1 g/kg 6-8 weekly for 3 cycles along with a tapering schedule of oral steroids. Presently 12 months into follow-up she experiences brief nocturnal simple partial seizures, her right hemiparesis has recovered by more than 80% and schooling has resumed. She is maintained on a regular schedule of valproic acid, lacosamide, clobazam and levetiracetam. Her repeat GAD 65-ab titre remains elevated (11 IU/mL).

DISCUSSION

Our case series demonstrates the heterogeneity of

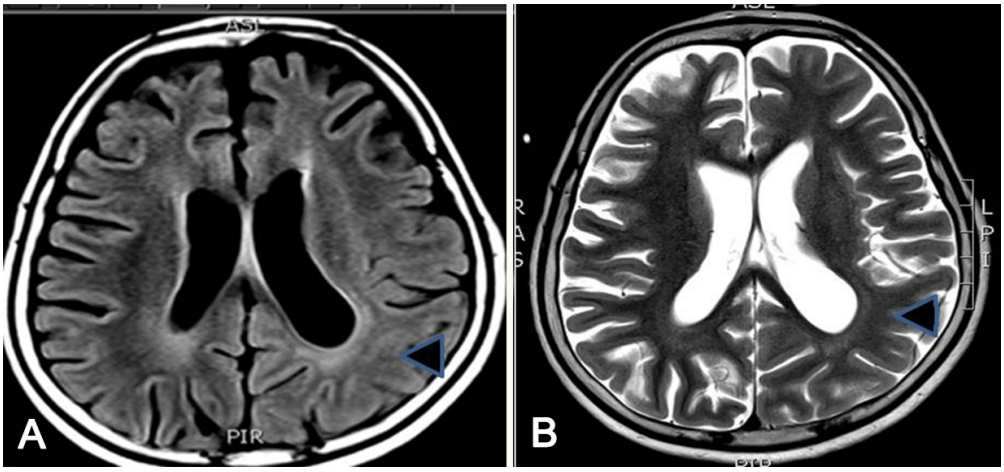

Figure 2: (A) FLAIR axial image; (B) T2-Weighted axial image. FLAIR and T2W axial MRI sequences in case 2 demonstrating global atrophy with loss of grey white matter differentiation over the left posterior quadrant. This was done 1 month after the first episode of complex partial status epilepticus. FLAIR: fluid attenuated inversion recovery; T2W: T2-weighted; MRI: magnetic resonance imaging

clinical presentations of presumed GAD mediated autoimmunity in the pediatric age-group. Despite the inability to ascertain intrathecal synthesis of GAD-ab, treatment was directed by clinical suspicion of the same and subsequent responses. Sero-prevalence of GAD-ab raises controversies on its role in CNS autoimmunity. GAD is the rate limiting enzymein the formation of inhibitory neurotransmitter GABA. The enzyme has 2 isoforms-GAD 65 and GAD 67, which differ substantially in their amino terminal, but function in synergy to maintain a physiological GABA level, with the former more active during stress and the latter assuming more of a housekeeping function.[5] GAD 65 is an intracellular protein, but it has been suggested that it could be exposed on the cell surface during exocytosis from GABA-ergic neurons, allowing a pathogenic ab-antigen interaction to occur. GAD 65-specific autoantibodies are also seen in some patients with other neurologic diseases, such as myoclonus, stiff person syndrome, pure cerebellar ataxia.[2,5] High GAD-ab levels, usually more than 100-fold higher that those found in DM1, are present in up to 80% of patients with stiff person syndrome (SPS), a subgroup of patients with late onset isolated cerebellar ataxia, epilepsy or brain stem dysfunction and are usually associated with type 1 diabetes or poly-endocrine autoimmunity, both of which were not seen in our patients.[6]

The causative immunological mechanisms remain speculative at best, and the clinical spectrum of GAD-ab associated disease may depend on the specific immunological response that is elicited. While it is believed that GAD-ab are unlikely to be pathogenic, *in vitro* studies suggest that IgG from patients can mediate effects on cerebellar neurons.[3,7] One explanation could be the intracellular uptake of these antibodies with subsequent inhibition of GABA synthesis, as has been demonstrated with ampiphysin antibodies associated with paraneoplastic SPS.[8] A more plausible explanation is that other unknown antibodies in sera positive for GAD-ab are the pathogenic moiety; the possible co-existence of antibodies for other membrane antigens, i.e. GABA-B receptor and glycine receptor requires further evaluation.[9,10] Furthermore, GAD-ab titers are significantly higher in the sera of adult patients with CNS involvement, some of whom also have evidence of intrathecal synthesis.[2] Due to lack of availability we could not confirm the antibody status using cell-based assays. There is enough evidence in literature that the epitopes of classical intracellular or onconeuronal antigens (Hu, Yo, Ri, CRMP5, Ma2, amphiphysin) are resistant to protein denaturation, and hence detectable by immunoblot or ELISA, as well as immunohistochemistry using mammalian brain, most commonly rat or mouse.[11] This is contrasted to antibodies to cell surface or synaptic protein ssuch as those against NMDA and VGKC wherein the reactivity is usually lost when the antigen is denatured so that these antibodies cannot be detected by standard immunoblot or ELISA. Detection of these antibodies requires either an immunohistochemistry protocol adapted to cell surface antigens, the use of cultures of live neurons, or cell-based assays in which recombinant antigens are expressed in mammalian cells. It is evident that the specificity and sensitivity of these assays vary among laboratories even when the same techniques are used. Because the reading of the tests is done by visual assessment, the interpretation of low serum titers can be misleading, and some sera produce non-specific background reactivity that may be interpreted as a positive result, although this rarely occurs when CSF is used. Another study however demonstrated absence of serum cross reactivity to NMDA and VGKC antibody in patients with suspected anti GAD mediated

Table 1: Presentations with CNS manifestations in non-neoplastic GAD positive patients (bold font indicative of patients with paediatric GAD-ab mediated diseases)

Author	No. of subjects (age at diagnosis)	Presentation	Serum Ab titre Mean (SD)	MRI	CSF Ab	Treatment	Outcome
Honnorat *et al*.[6]	14 (40-70 years)	Cerebellar ataxia	37,300 (30,460) U/mL	Cerebellar atrophy/ normal	Intra-thecal synthesis in 6/9	NA	Variable
McKnight *et al*.[16]	5 (3-36 years)	Chronic Drug resistant epilepsy	> 1,000 U/mL in 3; > 10 U/mL in 2	Normal in all	NA	NA	Chronic epilepsy
Mata *et al*.[15]	2 (20, 47 years)	Memory decline, seizures	72-87.5 U/mL	Temporal lobe HI	46-54.1 U/mL	Steroids, IVIG, PLEX	Partial benefit
Saiz *et al*.[2] (largest series)	50 (13-79 years)	Variable (predominant ataxia, SPS, drug resistant epilepsy)	> 2,000 U/mL	Temporal lobe HI, variable	High IgG index	Variable	Variable
Ozkan *et al*.[17]	2 (9 months, 6 years)	Acute ataxia, status epilepticus with involuntary movements	1.48-1.79 U/mL (ref < 1)	Normal	2.16 U/mL	Steroids, IVIG	Improved
Malter *et al*.[16]	9 (17-66 years)	Cognitive decline, seizure (presentation as limbic encephalitis)	1,798-12,030 U/mL	Medial temporal hyperintensity, PET hypometabolism	29-235 U/mL	Steroids, IVIG	None seizure free
Present series	2 (3, 7 years)	Chronic extratemporal partial epilepsy with ESES, subacute myoclonus ataxia	10.7-21 U/mL (ref 0-5.0 U/mL)	Grey matter loss with subcortical HI	NA	Steroids, IVIG	Improved

CNS: central nervous system; GAD: glutamic acid decarboxylase; Ab: antibody; SPS: stiff person syndrome; ESES: electrical status epilepticus in sleep; SD: standard deviation; ref: reference value; MRI: magnetic resonance imaging; HI: hyperintenisty; PET: positron emission tomography; IgG: Immunoglobulin G; CSF: cerebrospinal fluid; NA: not available; IVIG: intravenous immunoglobulin; PLEX: Plasma exchange

epilepsy with titres ranging between 6 to > 200,000 IU/mL with only 7 out of 15 subjects demonstrating high titres > 1,000 IU/mL.[12] This variability in serum titres is also demonstrated in Table 1. This indicates that low titres, as also demonstrated in another case series, need not be neglected in a clinically relevant scenario.[13] However, clinical and serological follow-up are likely to ascertain the significance of these mildly elevated titres in pediatric patients reported here.

Table 1 reflects the rarity of pediatric GAD-ab mediated CNS autoimmunity. In a large series, the spectrum of GAD-ab positive spectrum of diseases was associated with a variety of neurological and non-neurological entities.[2] The mean age was between 50-60 years in this series. In the adult population a female gender predilection with predominant presentation as limbic encephalitis is noted. In contrast, presentations of both our patients were unique. The first child presented with a subacute ataxia-myoclonus syndrome with good response to steroids that was previously undescribed in adult series. This constellation has been previously noted in anti-NR1 receptor NMDA-ab mediated encephalitis in addition to the well described paraneoplastic opsoclonus-myoclonus syndrome.[14] The other child had refractory focal epilepsy with lateralizing neurological deficits and a prolonged course of 4 years resembling a left hemispheric focal encephalitis versus a large malformation of cortical development. The latter's MRI features were also more in favour of focal encephalitis in the absence of discrete cortical pathology with the development of progressive grey matter volume loss, which may also be attributed to refractory seizures and the effect of anti-epileptic drugs. However, the clinical scenario was distinct from the well-described limbic encephalitis. In most adult series, GAD-ab were requested at the time of diagnosis of type 1 diabetes more than a decade or two after the onset of the epilepsy. As evident in Table 1, patients in series No. 2 had drug-resistant temporallobe epilepsy associated with hippocampal sclerosis, with 1 patient diagnosed to have celiac disease.[15] Patients with epilepsy reported in the largest series have ranged from chronic epilepsy with hippocampal sclerosis or co-existent heterotopias and one patient was diagnosed to harbour GAD-ab many years after the diagnosis of epilepsy following development of oscillopsia with nystagmus and subsequent detection of CSF oligoclonal IgG bands and intrathecal synthesis of GAD-ab.[2] Although the frequency of high GAD-ab levels in patients with epilepsy is low and ranges from 0% to 4%, GAD-ab-positive patients are more likely to have chronic drug-resistant epilepsy.[16,17] Rasmussen's encephalitis-like presentation has also been reported in a 6.5-year-old male with type I DM who presented with epilepsia partialis continua for days with detectable GAD-ab.[18] The presentation of Case 2 as ESES, refractory partial epilepsy and focal deficits with GAD-ab is previously undescribed in the literature, although a report of onco-neural antibody mediated ESES in a child with neuroblastoma and opsoclonus-myoclonus syndrome exists.[19] While the response of GAD-ab mediated epilepsies has been shown to be far from satisfactory in terms of seizure outcomes following immune-modulation or surgery, especially in temporal lobe epilepsies,[20] our experience has demonstrated significant improvement with immunomodulation during sub-acute worsening of encephalopathy, seizures, focal deficits in Case 2 with chronic extratemporal partial epilepsy. Though GAD-ab may just reflect the presence or later risk of

concomitant DM1 and other endocrine autoimmune disorders, longitudinal follow-up in our patients is likely to be provide more insight. The antibody titres were not extremely elevated as in reference cases in Table 1, however, the possibility of an alternative diagnosis has been reliably excluded by investigations. Despite the inability to demonstrate intrethecal synthesis, the dramatic response to immunomodulation demonstrated in both patients highlights the significance of evaluating for these antibodies in children with chronic refractory partial epilepsy with epileptic encephalopathy of uncertain etiology and acute-subacute myoclonus-ataxia syndromes.

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Conflicts of interest
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Patient consent
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Ethics approval
Ethics approval was obtained from Institute Review Board for case reports.

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Editorial **Open Access**

Schizophrenia and comorbid sleep disorders

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Prof. Maurits van den Noort is a regular professor (Research Group of Pain and Neuroscience) at Kyung Hee University in Seoul, South Korea. Moreover, he is a visiting professor at the Free University of Brussels in Belgium. He is especially interested in schizophrenia, depression, sleep disorders, fMRI, and TMS.

Schizophrenia is a severe psychiatric disorder that has a worldwide prevalence of 0.5%[1] and poses a high cost to society.[2] The disorder is characterized by positive symptoms, such as hallucinations and delusions,[3] negative symptoms, such as impaired emotional functioning and behavioral disruptions (e.g. flat affect, difficulty in starting activities and completing them, *etc.*),[4] and cognitive symptoms, such as deficits in executive functioning, impaired working memory, and attention problems.[5] Less known to the general public is the fact that a large number of the patients with schizophrenia suffer from sleep disturbances, such as reduced sleep efficiency, reduced total sleep time, and increased sleep latency.[6] Surprisingly, those sleep problems in patients with schizophrenia are also often under-estimated in daily clinical practice.[7]

In the Diagnostic and Statistical Manual of Mental Disorders 5th Edition (DSM-V),[8] sleep-wake disorders are classified into 10 disorders or disorder groups (e.g. insomnia disorder, restless legs syndrome, circadian rhythm sleep-wake disorders, *etc.*). Patients suffering from sleep-wake disorders have problems with respect to the quality, the timing, and the total amount of sleep,[8] leading to distress and impairment in their social and cognitive functioning.[9]

Several treatments have been used in patients with

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schizophrenia, as well as patients with sleep-wake disorders. Pharmacological treatment with first- and second-generation antipsychotics (e.g. amisulpride, clozapine, olanzapine, risperidone, *etc.*) is still the most frequently used treatment in patients with first-episode and long-term schizophrenia.[10] However, mainly because of the adverse effects of the pharmacological treatment, non-pharmacological add-on treatments, such as cognitive behavioral therapy,[11] are being increasingly used. Sleep-wake disorders are mostly treated with pharmacological interventions, such as benzodiazepines, zolpidem, zaleplon, *etc.*, but unfortunately, side-effects are common here, as well.[12] In addition, non-pharmacological interventions, such as cognitive psychotherapy, sleep hygiene, relaxation therapy, acupuncture, *etc.*,[13,14] are used.

Previous research involving patients with schizophrenia and comorbid sleep disorders has shown that a relation exists between sleep problems and cognitive functioning.[15] For instance, in a recent study, a significant negative relationship was found between the number of sleep problems and the working memory performance; i.e. the more severe the patient's sleep problems was, the lower the patient's working memory performance was.[15] However, more research is warranted, and to date, many questions remain unanswered: Firstly, how are poor sleep and decreased social and cognitive functioning in patients with schizophrenia related? Secondly, what role does the pharmacological treatment of patients with schizophrenia play in their impaired sleep and social and cognitive functioning? For instance, benzodiazepines are known to suppress rapid-eye-movement (REM) sleep,[12] and when patients stop such medications, episodes of increased REM sleep are more numerous.[12] Because REM sleep plays a role in the learning process, as well as in memory consolidation,[12] future research should clarify whether benzodiazepines might have a negative influence on cognitive functioning, e.g. working memory, in patients with schizophrenia and comorbid sleep disorders. Thirdly, how does the pharmacological treatment of comorbid sleep disorders in patients with schizophrenia interfere with the pharmacological treatment of the positive and the negative symptoms of those patients? These questions need to be investigated and answered in future studies so as to improve further the treatment and the quality of life of patients with schizophrenia and comorbid sleep disorders.

Here, an important finding is that in previous research, patients with schizophrenia tended to underestimate their problems on self-report inventories.[16] Therefore, in future research, both "objective" (e.g. electroencephalography, actiwatches, *etc.*) and "subjective" (e.g. self-report inventories, such as the Pittsburgh Sleep Quality Index,[17] Munich Parasomnia Screening,[18] *etc.*) measurements must be used if the efficiencies of various pharmacological and non-pharmacological treatments of patients with schizophrenia and comorbid sleep disorders are to be determined with accuracy.

To conclude, many patients with schizophrenia suffer from comorbid sleep-wake disorders. Therefore, in daily clinical practice, sleep needs more attention in the treatment of patients with schizophrenia so that such patients receive optimal treatment and their qualities of life are increased. Finally, evidence for adding "disturbed sleep" as one of the characteristic symptoms of schizophrenia in the DSM system seems to be mounting.[8,15]

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Review **Open Access**

Neurological manifestations in Fabry disease

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ABSTRACT

Fabry disease (FD) is a rare, progressive, multisystem and highly debilitating disease. FD is an X-linked lysosome storage disorder that results in α-galactosidase A deficiency. The subsequent accumulation of glycosphingolipids is more evident in vascular endothelium and smooth-muscle cells. The resulting effect of the deposition is generalized inflammation and vasculopathy, which can also affect the central and peripheral nervous system. FD progresses with kidney dysfunction, angiokeratoma of the skin, cardiomyopathy, cerebrovascular events and neurological disorders. In the present review, the neurological manifestations of FD are summarized with emphasis on cerebral vasculopathy, cochlear nerve dysfunction, psychiatric and cognitive symptoms, autonomic dysfunction and peripheral neuropathy. Enzyme replacement therapy is also discussed in the light of its more prominent effects when administered early in life, which make it essential to diagnose FD as soon as possible.

INTRODUCTION

Fabry disease (FD; Online mendelian inheritance in man #301500) is a rare, progressive, multisystem and highly debilitating lysosome storage disorder, resulting in α-galactosidase A (α-Gal A) (*300644) deficiency. FD birth prevalence is approximately 1:40,000 and more than 600 mutations in the α-Gal have been described. The disease is X-linked inherited,[1,2] and X-inactivation in women may render them vulnerable to severe manifestations of FD.[3,4] Even with the same gene mutation there is an intrafamilial variability of phenotypical presentation of FD, leading to variable signs, symptoms and severity of the disease.[5]

α-Gal A deficiency leads to progressive accumulation of glycosphingolipids such as globotriaosylceramide (GL-3) in various tissues and organs. The accumulation is predominantly in vascular endothelial and smooth-muscle cells. In the 19th century, William Anderson and Johannes Fabry described angiokeratoma of the skin as the first clinical sign of the disease. Subsequently, identification of kidney dysfunction,[6] cardiomyopathy,[7] gastrointestinal disorders,[8] cerebrovascular events[9] and other neurological disabilities was reported. These conditions are the most severe clinical manifestation of

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FD and may lead to increased morbidity and mortality, with concomitant reduction in life expectancy.[10] While the literature on the skin, kidney and cardiovascular manifestations of FD has been consistent, there have been very few reports on the neurological aspects of the disease.

The aim of this article was to describe the neurological manifestations in FD. They may occur at any stage of the disease, including its onset. Therefore, although relatively rare, FD is a differential diagnosis for young individuals with unexplained neurological manifestations.

CEREBRAL VASCULOPATHY

The prevalence of cerebrovascular diseases such as stroke events in FD patients is 4-6%. These events may be the first clinical manifestation of the disease and are more often observed between the ages of 18 and 55 years, affecting both genders equally.[11]

Cerebral endothelial vasculopathy in FD is not fully understood, but it is accepted that GL-3 accumulation and polymorphisms of pro-thrombotic genes can modify Virchow's triad and create a pro-thrombotic state.[12,13] These alterations include changes to interleukin-6-G-174C, G894T of endothelial nitric oxide synthase, factor V G1691A mutation and protein Z A-13G or G79A.[13] Few studies have concentraded on intravenous thrombolytic therapy for acute ischemic stroke in FD patients and the outcomes are not fully understood.[14]

Although ischemic stroke and transient ischemic attacks are the main types of cerebrovascular events in FD, cerebral hemorrhage, microbleeding, cerebral venous thrombosis and cervical carotid dissection have also been reported. The main etiology of stroke comes from the effect of the disease on the small arteries. The posterior circulation (vertebrobasilar system) is often more involved than the carotid system.[15-18]

White matter lesions are a reflection of secondary microangiopathy involvement of the central nervous system. As many as 80% of these patients present these abnormalities on magnetic resonance imaging (MRI), even without clinical symptoms of focal neurological involvement. Increased cerebral blood flow, vascular hyper-reactivity and GL-3 deposition ultimately induce cell dysfunction and increase interstitial pressure, thus generating vulnerability of elongated perforating arteries and leading to reduction of cerebral blood flow.[19]

Brain microangiopathy in FD can be misdiagnosed as multiple sclerosis due to intermittent disseminated sensory deficits and white matter lesions fulfilling the McDonald criteria.[20-22] However, T2-FLAIR MRI of the white matter usually produces asymmetric and confluent images, with little involvement of the corpus callosum and no enhancement of the lesion through gadolinium. There are no lesions in the spinal cord. These characteristics help differentiating FD images from multiple sclerosis.[23] Vertebrobasilar system ectasia, proteinuria, cardiac hypertrophy and histories of death among young relatives (renal, cardiac or cerebrovascular causes) are other frequently found elements in these patients' medical history.[24,25]

Calcification of cerebrovascular dolichoectasia in cerebral white matter and thalamus (pulvinar region) is due to dysfunction of the cerebrovascular circulation and to GL-3 accumulation. Cerebrovascular hyperperfusion reflects the increased vascular reactivity and the effect on the nitric oxide pathway, while increased oxidative stress and formation of peroxynitrite can create persistent vasodilation and increased risk of atherosclerosis.[26]

COCHLEAR NERVE DYSFUNCTION

The data in the literature on the pathogenesis of cochlear dysfunction in FD are limited. It has been hypothesized that GL-3 accumulation in the cochlear nerve can progress to hearing loss, especially at 2-3 kHz.[27]

PSYCHIATRIC AND COGNITIVE SYMPTOMS

High prevalence of neuropsychiatric symptoms, such as depression and neuropsychological deficits, reduces quality of life among FD patients. Although the pathophysiological mechanisms have not been fully elucidated, cerebral vasculopathy is involved. Furthermore, FD patients may be chronically distressed by pain and psychosocial impairment.[28-30]

AUTONOMIC DYSFUNCTION

Hypohidrosis, reduced saliva flow and impaired tear production may be present and have mechanisms that are not fully understood. GL-3 accumulation in autonomic ganglia and dysfunction of eccrine glands are found in patients with FD. Gastrointestinal symptoms (which may be associated with autonomic dysfunction) are the second most common clinical manifestation among children and young adults with FD.[8] During unexplained attacks of abdominal pain, the patients may also suffer from postprandial flatulence and bouts of diarrhea.[31,32]

PERIPHERAL NEUROPATHY

Peripheral neuropathy has an important negative impact on quality of life among FD patients. It has been described as being present since the beginning of GL-3 deposition, i.e. from these patients' first years of life. It affects both genders equally, and is often associated with fever and pain during exercise. The pain may last for periods ranging from minutes up to several days, and may be incapacitating.[31-35]

As mentioned above regarding other neurological manifestations of FD, the pathophysiological mechanisms of neuropathy are not fully understood either. It has been proposed that inhibition of central nociceptors would occur as a result of constant activation of nociceptive afferents, in association with neuronal dysfunction, Wallerian degeneration, activation of the inflammatory cascade, vasa nervorum ischemia and molecular changes in the peripheral nociceptor, similar to dying-back neuropathies.[36-40] In addition, disproportion and dysfunction of axonal sodium channels would increase the frequency of nociceptive discharge. This last topic has practical importance, since pain treatment in these patients must be carried out using sodium channel-blocking drugs such as carbamazepine. Involvement of distal, small Aδ- myelinated fibers and C-unmyelinated fibers is prevalent among patients with symptoms relating to temperature. It is important to establish a uniform quantitative assessment battery for sensitive symptoms.[41-45]

ENZYME REPLACEMENT TREATMENT

Enzyme replacement therapy with humanized recombinant α-Gal A (agalsidade beta, or more recently, agalsidase alpha) reduces the secondary clinical events relating to FD by 60% to 80%. The effect of this enzyme replacement is seen in prevention of cerebral, renal and cardiological life-threatening events, which, in untreated patients, are responsible for more than 90% of deaths.[46,47] There is evidence that early treatment with enzyme replacement therapy can stabilize vascular disease progression and decrease the risk of stroke.[48] Patients may have different response to treatment with agalsidade alpha or beta.[49-51]

CONCLUSION

Neurological manifestations of FD are often related to significant morbidity and mortality. Early detection and specific treatment of neurological involvement in cases of α-Gal A deficiency may result in improved quality of life for patients with FD.

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Patient consent

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Ethics approval

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Possible role of microparticles in neuroimmune signaling of microglial cells

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ABSTRACT

Aim: Submicron fragments termed microparticles (MPs), derived from all major central nervous system cell types including neurons and glia (microglia, astrocytes, oligodendrocytes), have emerged as novel intercellular signaling agents. This study tested the hypothesis that MPs derived from activated microglia, which represent the mononuclear phagocyte system in the brain, could induce pro-inflammatory and cytotoxic responses of microglia in an autocrine or paracrine manner. **Methods:** Human THP-1 monocytic cells were used to model human microglia. MPs derived from these cells were reapplied to THP-1 cells and their secretion of neurotoxins and cytokines was measured. **Results:** When exposed to lipopolysaccharide (LPS) or mitochondrial transcription factor A in combination with interferon (IFN)-γ, THP-1 cells released MPs. When reapplied to THP-1 cells, MPs induced the release of secretions that were toxic to human SH-SY5Y neuroblastoma cells, as well as monocyte chemoattractant protein-1. The cytotoxicity of THP-1 cells induced by MPs derived from IFN-γ plus LPS-treated THP-1 donor cells was enhanced in the presence of IFN-γ. The MPs released by THP-1 cells were not directly toxic towards SH-SY5Y cells. **Conclusion:** Our data support the hypothesis that activated microglia-derived MPs could act as signaling agents that are recognized by microglia to cause pro-inflammatory and cytotoxic responses.

INTRODUCTION

Microglia are a distinct population of mononuclear phagocytes that represent the innate immune system in the brain.[1] Under physiological conditions, the phagocytic responses of microglia ensure proper functioning of neuronal cells as they remove harmful material and repair injured tissue.[2] However, chronic activation of microglia, due to recognition of pathological formations associated with central nervous system (CNS) disorders including the amyloid-beta (Aβ) and α-synuclein aggregates observed in Alzheimer's and Parkinson's disease respectively can contribute to disease progression.[3,4] The state of prolonged over-activation of microglia is characterized by increased secretion of pro-inflammatory cytokines as well as reactive oxygen and nitrogen species.[5-9] Similar adverse activation of microglia occurs in response

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to other endogenous molecules such as damage-associated molecular patterns (DAMPs) released by damaged or dying cells.[10] DAMPs identified in the CNS include the DNA-binding proteins high-mobility group box 1 (HMGB1) and mitochondrial transcription factor A (TFAM).[11,12]

Recently, microparticles (MPs) have emerged as novel intercellular signaling agents, which can be released by all brain cell types. MP release from microglia is upregulated following their activation and such microlia-derived MPs appear to possess immunomodulatory properties similar to HMGB1.[13-16] Originally considered as inert platelet by-products, MPs are now known to be a heterogeneous population of membrane-derived vesicles ranging in diameter from 0.1 to 1 μm, which participate in intercellular signaling.[17,18] The structural composition and content of MPs differs based on a variety of factors, including the cell type of origin and the nature of the inducing stimulus.[18]

As MPs can mediate intercellular communication, they have been implicated in the regulation of various physiological processes, including, cell proliferation, coagulation, and inflammation.[19] Recent studies have also identified MP involvement in disease processes and the contribution of MPs to the progression of some neurodegenerative disorders has become increasingly evident.[20-24] Elevated levels of MPs have been detected in the plasma and cerebrospinal fluid of individuals suffering from Alzheimer's disease, multiple sclerosis, and cerebral malaria.[20,25,26]

Upon activation by adenosine triphosphate, microglia and astrocytes have been found to release MPs carrying the pro-inflammatory cytokine interleukin (IL)-1β.[15,27] Moreover, microglia-derived MPs have been shown to transfer inflammatory stimuli to other microglial cells, which then express pro-inflammatory genes such as IL-1β and IL-6.[28] These findings suggest that inflammatory mediators may be liberated from glia-derived MPs and subsequently interact with surrounding cells, thus contributing to the neurotoxic environment observed in neuroinflammatory diseases. Therefore, elucidating MP involvement in glial cell-mediated neuroinflammation may identify additional targets for the development of therapeutic strategies aimed at neuropathologies with neuroinflammatory components.

Although MP release by all major CNS cell types has been demonstrated, the role of MPs as mediators in glia-neuron communication remains to be fully characterized.[15,16,27] Since MP release is upregulated upon activation and during apoptosis or necrosis; we hypothesized that MPs may act as endogenous DAMPs with immunomodulatory properties similar to HMGB1 or TFAM.[15,29,30] We demonstrated that human mononuclear phagocytes release MPs when activated by pro-inflammatory molecules. We also showed that THP-1 monocytic cell-derived MPs act in an autocrine or paracrine manner to induce secretions of pro-inflammatory cytokines and cytotoxins by these monocytic cells.

METHODS

Reagents

Sodium dodecyl sulphate (SDS), N,N-dimethylformamide (DMF), 0.05% trypsin with ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), Dulbecco's modified Eagle medium: nutrient mixture F-12 Ham (DMEM/F12), penicillin/streptomycin stock solutions, bovine serum albumin (BSA), diethanolamine, and the Pierce bicinchoninic acid (BCA) Protein Assay Kit were purchased from ThermoFisher Scientific (Ottawa, ON, Canada). Dimethylsulfoxide (DMSO), lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Human interferon (IFN)-γ and enzyme-linked immunosorbent assay (ELISA) development kits for IL-6, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein-1 (MCP-1) were purchased from Peprotech (Embrun, ON, Canada). The FITC Annexin-V Apoptosis Detection Kit was purchased from BD Biosciences (Mississauga, ON, Canada). Nanobead NIST traceable particle size standards were purchased from Polysciences Inc. (Warrington, PA, USA). Recombinant human TFAM was a gift from Dr. K. Wolthers (University of British Columbia Okanagan Campus, Kelowna, BC, Canada).

Cell culture models

The human monocytic THP-1 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The human neuroblastoma SH-SY5Y cell line was donated by Dr. R. Ross (Department of Biological Sciences, Fordham University, Bronx, NY, USA). Cell cultures were grown in DMEM/F12 media containing 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL), and incubated at 37°C in humidified 5% CO_2 and 95% air atmosphere.

MP isolation

MPs were isolated using previously published protocols, which were modified as follows.[15,27,31] Human monocytic THP-1 cells were counted using a hemocytometer, centrifuged for 7 min at 450 *g*, and seeded into 12-well plates at 0.5 million cells/mL in

DMEM/F12 with 5% FBS. Following 30-min incubation, cells were stimulated by exposing them in duplicate wells to TFAM (2.5 µg/mL), IFN-γ (150 U/mL), IFN-γ plus TFAM (0.5 µg/mL), IFN-γ plus LPS (0.5 µg/mL), or vehicle solution (phosphate buffered saline, PBS). Following a 24-h incubation period, cultured supernatants from each well were collected into individual 50 mL centrifuge tubes and total cell numbers from each treatment were counted using a hemocytometer. A differential centrifugation procedure consisting of three steps was then performed at 4°C: (1) 5 min at 300 *g*; (2) 20 min at 1,200 *g*; (3) 30 min at 10,000 *g*. After the first two centrifugation steps, the supernatants were collected and transferred into new 50 mL tubes. After the third step, however, the supernatants were discarded. The pellets were re-suspended in 1 mL of sterile PBS and the MP samples were washed by centrifugation for 30 min at 10,000 *g*. The MP pellet was re-suspended in 1X Annexin-V binding buffer from the FITC Annexin-V Apoptosis Detection Kit. The MP samples were stored at -20°C.

MP detection and quantification

Flow cytometry

To detect and quantify MPs released by THP-1 cells, the MP samples were analyzed using flow cytometry according to previously described methods.[32] Briefly, 0.1 mL from each MP sample was combined with 5 µL of Annexin-V-FITC stain and incubated at room temperature in the dark for 15 min. Annexin-V was used as it binds to the externalized phosphatidylserine on the surface of MPs.[33] Following incubation, samples were centrifuged for 30 min at 10,000 *g* to remove any unbound Annexin-V-FITC. The MP pellet was resuspended in 0.1 mL of 1X Annexin-V binding buffer and analyzed using the MACSQuant Analyzer 10 with MACSQuantify software (Miltenyl Biotec). The MP size gate was defined using 0.5 µm and 1.0 µm calibration beads and events within the MP gate were further discriminated by Annexin-V label. A logarithmic scale was used for side scatter, forward scatter, and fluorescence channels. MPs were identified as Annexin-V positive events within the MP size gate with fluorescence intensity above a control sample not stained with Annexin-V-FITC.

BCA protein assay

Protein concentration in the MP samples was measured using the BCA protein assay as previously described.[34] Briefly, 10 µL of each MP sample were analyzed according to the instructions provided for the Pierce BCA Protein Assay Kit. BSA standards (0.0125-2 mg/mL) prepared by diluting the BSA stock solution (2 mg/mL) in distilled water were used to construct a standard curve from which the protein concentration in the MP samples was calculated. MPs were used in experiments at a protein concentration of 10 µg/mL.

Toxicity of MP-stimulated monocytic THP-1 cells towards neuronal SH-SY5Y cells

To study the cytotoxicity of monocytic THP-1 cells induced by MPs, supernatant transfer experiments were performed as previously described.[35] Briefly, THP-1 cells were seeded into 96-well plates as 250 µL aliquots at a concentration of 0.5 million cells/mL in DMEM/F12 with 5% FBS. Following 30 min incubation, THP-1 cells were stimulated with IFN-γ (150 U/mL), THP-1-derived MPs (10 µg protein/mL), IFN-γ plus THP-1-derived MPs, or left unstimulated by adding MP vehicle solution (1X Annexin-V binding buffer). After 48 h incubation, THP-1 cell supernatants were transferred onto SH-SY5Y cells seeded 24 h earlier into 96-well plates at 0.2 million cells/mL in 200 µL DMEM/F12 with 5% FBS. In addition, THP-1 cell supernatants were collected for IL-6, TNF-α, and MCP-1 ELISA measurements. THP-1 cell viability was assessed using the MTT assay. SH-SY5Y cells were incubated for additional 72 h at which point an MTT assay was performed to assess their viability.

Direct toxicity of MPs derived from stimulated THP-1 cells towards neuronal SH-SY5Y cells

To determine whether the MPs isolated from stimulated human monocytic THP-1 cells were directly toxic to human neuronal SH-SY5Y cells, the following experiment was performed. Human neuronal SH-SY5Y cells were seeded into 24-well plates as 400 µL aliquots per well at a concentration of 0.2 million cells/mL in DMEM/F12 with 5% FBS. Following 24-h incubation, SH-SY5Y cells were treated with MP vehicle solution (1X Annexin-V binding buffer) or MPs (10 µg protein/mL) isolated from unstimulated THP-1 cells or THP-1 cells that had been stimulated with IFN-γ in combination with LPS. Following 72-h incubation of SH-SY5Y cells with the MPs, the MTT assay was performed to assess neuronal cell viability.

MTT cell viability assay

Viability of the cells used during experiments was assessed using the MTT assay as previously described.[36,37] This assay is based on the ability of viable cells to reduce the water-soluble tetrazolium dye MTT to an insoluble purple formazan product, which can be measured spectrophotometrically. MTT (0.5 mg/mL) was added to the wells containing cultured cells and the plates were incubated for 1 h at 37°C. A 20% SDS/50% DMF solution was then added at a 1:1 ratio to each well to solubilize the formazan crystals. After an additional incubation period of 3-4 h at 37°C, 0.1 mL aliquots from each well were transferred

onto 96-well plates for optical density measurement at 570 nm using a microplate reader. The cell viability was expressed as a percent of the value obtained from cells treated with medium only.

ELISA

Concentrations of IL-6, TNF-α, and MCP-1 in cell-free supernatants from THP-1 cells stimulated for 48 h with THP-1-derived MPs were measured using Peprotech ELISA development kits according to the manufacturer's instructions. The detection limits for the IL-6, TNF-α, and MCP-1 ELISAs were experimentally determined to be 0.007 ng/mL, 0.09 ng/mL, and 1.02 ng/mL, respectively.

Data analyses

Statistical analyses of the data were conducted using SPSS software (version 22.0, IBM SPSS, Chicago IL, USA) and GraphPad PRISM software (version 6.0, GraphPad Software Inc, La Jolla CA, USA). Randomized blocks design analysis of variance (ANOVA) followed by Fisher's least-significant difference (LSD) *post hoc* test was used to determine significance of findings. Data are presented as means ± standard error of the mean (S.E.M.). Statistical significance was considered at a *P*-value less than 0.05. Data from 3-11 independent experiments for each figure are presented.

RESULTS

MP release by stimulated human monocytic THP-1 cells

The ability of various stimuli to trigger MP release by monocytic THP-1 cells was investigated. THP-1 cells were stimulated for 24 h with IFN-γ or a combination of IFN-γ plus LPS, which has previously been shown to induce maximal stimulation of these cells.[38] THP-1 cell supernatants were collected and MPs isolated using differential centrifugation. Representative scatter plots shown on Figure 1A-C illustrate that MPs were released by unstimulated and stimulated THP-1 cells as shown by the Annexin-V positive events within the MP size gate. The number of MPs released per million THP-1 cells was calculated. Figure 1D shows that significantly more MPs were released by THP-1 cells stimulated with a combination of IFN-γ plus LPS compared to cells stimulated with IFN-γ alone ($P = 0.001$) or unstimulated cells ($P = 0.001$).

Effects of THP-1 cell-derived MPs on THP-1 monocytic cell viability and their toxic secretions

Next, we studied whether MPs derived from unstimulated and stimulated THP-1 cells induce cytotoxicity of THP-1 cells in a paracrine fashion. MPs derived from unstimulated THP-1 cells (control) or THP-1 cells that had been stimulated with IFN-γ alone or IFN-γ plus LPS were added to THP-1 cell cultures. At the concentration tested, none of the isolated MPs significantly affected THP-1 cell viability [Figure 2A]. Cell-free supernatants from the THP-1 cells treated with different types of MPs were transferred onto SH-SY5Y neuroblastoma cells to determine secretion of cytotoxins by MP-stimulated THP-1 cells [Figure 2B]. The MPs isolated from unstimulated THP-1 cells as well as those isolated from IFN-γ-stimulated THP-1 cells, did not induce THP-1 cell toxicity towards SH-SY5Y cells. However, the MPs isolated from THP-1 cells stimulated with IFN-γ plus LPS induced cytotoxicity of THP-1 cells, resulting in a statistically significant decrease in viability of the neuronal cells compared to SH-SY5Y cells exposed to supernatants from unstimulated THP-1 cells ($P = 0.013$). The THP-1 cell toxicity induced by the MP population from IFN-γ plus LPS-stimulated THP-1 donor cells was also significantly different from the effect of the supernatants from THP-1 cells stimulated with MPs isolated from unstimulated THP-1 donor cells ($P = 0.001$) and THP-1 cells stimulated with IFN-γ alone ($P = 0.048$).

IFN-γ enhances THP-1 cell cytotoxicity induced by stimulated THP-1 cell-derived MPs

The synergistic effect of IFN-γ and THP-1 cell-derived MPs on THP-1 cell cytotoxic secretions was also studied [Figure 3A]. The combination of IFN-γ and IFN-γ plus LPS-stimulated THP-1 cell-derived MPs was significantly toxic towards THP-1 cells compared to THP-1 cells treated with vehicle solution alone ($P = 0.0001$), IFN-γ alone ($P = 0.002$), and MPs isolated from IFN-γ plus LPS-stimulated THP-1 cells alone ($P = 0.0001$). Supernatants from the differentially treated THP-1 cells were transferred onto SH-SY5Y cells and their viability was assessed after 72 h incubation. Figure 3B illustrates that the cytotoxicity of MPs derived from IFN-γ plus LPS-stimulated THP-1 donor cells was enhanced by IFN-γ being present during the incubation of THP-1 cells with these MPs.

MPs derived from TFAM plus IFN-γ-stimulated THP-1 cells induce cytotoxicity of THP-1 cells

The ability of MPs derived from TFAM-stimulated monocytic THP-1 cells to induce cytotoxic secretions from THP-1 cells was also investigated. MTT assay showed no toxicity of the isolated MPs towards THP-1 cells at the concentration tested [Figure 4A]. Treatment of THP-1 cells with MPs isolated from TFAM-stimulated THP-1 donor cells did not cause secretion of cytotoxins by THP-1 cells. However, MPs from THP-1 cells stimulated with TFAM in combination with IFN-γ induced THP-1 cell cytotoxicity resulting in a statistically significant decrease in SH-SY5Y cell viability compared

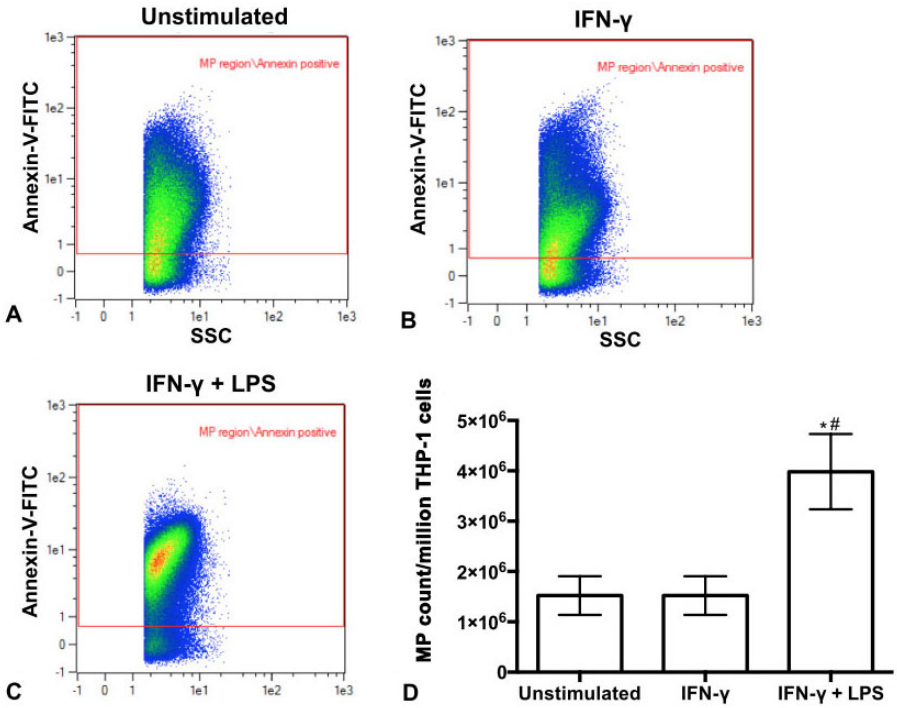

Figure 1: Flow cytometry scatter plots identify MPs released by human THP-1 monocytic cells that were unstimulated (A) or stimulated with IFN-γ (B) or IFN-γ plus LPS (C). Different colors reflect the density of MPs present, going from highest density (red) to lowest density (blue). The MP count per million THP-1 cells (D). $^{*}P = 0.001$ *vs.* unstimulated; $^{\#}P = 0.001$ *vs.* IFN-γ. MPs: microparticles; LPS: lipopolysaccharide

to cells exposed to supernatants from unstimulated THP-1 cells ($P = 0.01$) as well as to SH-SY5Y cells exposed to supernatants from THP-1 cells treated with MPs from unstimulated THP-1 donor cells ($P = 0.004$) and THP-1 cells stimulated with either IFN-γ ($P = 0.011$) or TFAM alone ($P = 0.0001$, Figure 4B).

MPs derived from stimulated THP-1 cells are not directly toxic towards SH-SY5Y neuronal cells

To rule out the possibility that the effects on SH-SY5Y cell viability observed following their exposure to supernatants from MP-treated THP-1 cells might have been due to MPs that were transferred to SH-SY5Y cells with the supernatant, we performed a control experiment where SH-SY5Y cells were treated directly with MPs. MPs (10 µg protein/mL) isolated from THP-1 donor cells that had been stimulated with IFN-γ plus LPS were incubated with SH-SY5Y cells for 72 h. The MTT assay showed no direct toxicity of the isolated MPs towards SH-SY5Y cells at the concentration tested [Figure 5].

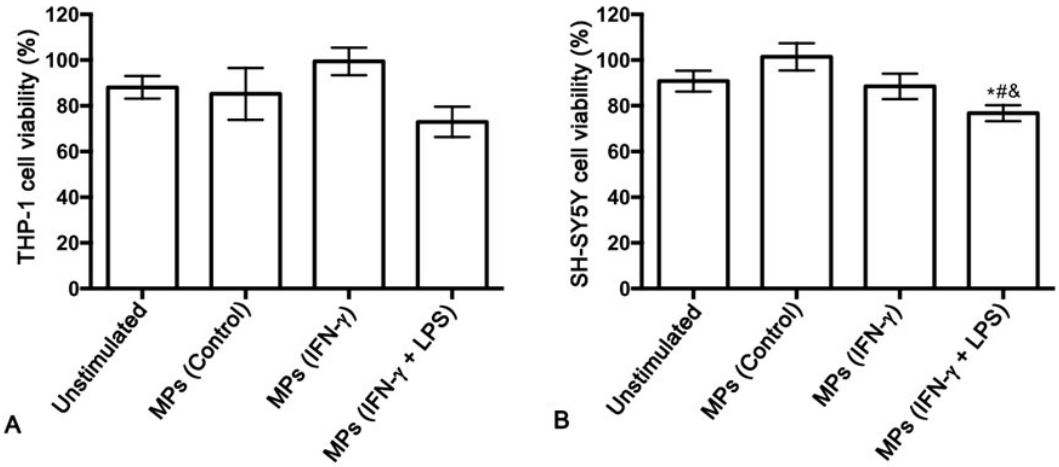

Figure 2: MPs isolated from IFN-γ plus LPS-stimulated human monocytic THP-1 donor cells induce toxicity of THP-1 cells towards human neuronal SH-SY5Y cells. $^{*}P = 0.013$ *vs.* unstimulated; $^{\#}P = 0.001$ *vs.* MPs (Control); $^{\&}P = 0.048$ *vs.* MPs (IFN-γ). LPS: lipopolysaccharide

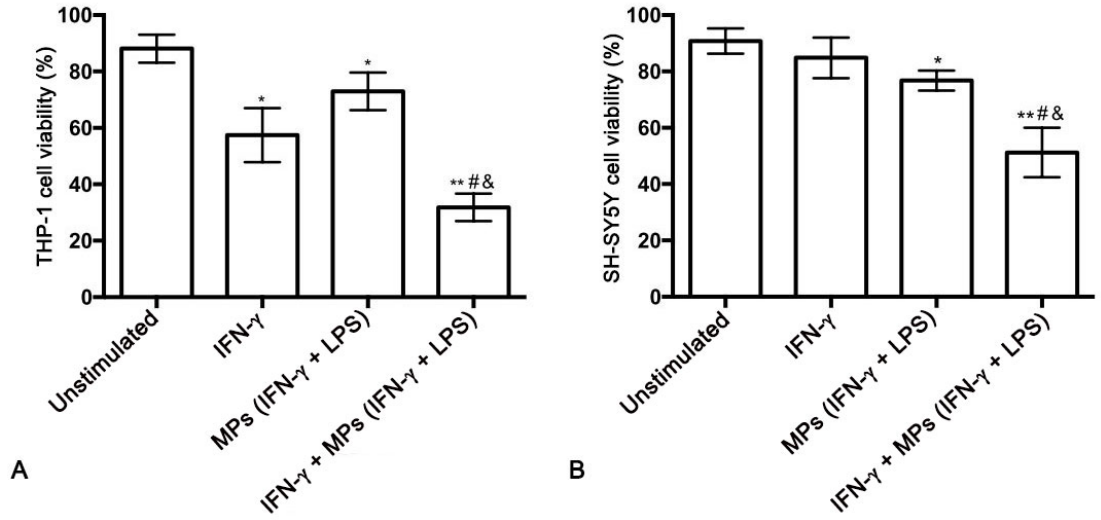

Figure 3: IFN-γ enhances cytotoxicity of monocytic THP-1 cells induced by stimulated THP-1 cell-derived MPs. $^{*}P = 0.034$, 0.028 (A) $^{*}P = 0.021$ (B) $^{**}P = 0.0001$ (AB) *vs.* unstimulated; $^{\#}P = 0.002$ (A) $^{\#}P = 0.004$ (B) *vs.* IFN-γ; $^{\&}P = 0.0001$(A) $^{\&}P = 0.007$ (B) *vs.* MPs (IFN-γ + LPS). MPs: microparticles

Effects of MPs on cytokine secretion by THP-1 monocytic cells

Concentrations of the pro-inflammatory cytokines TNF-α, IL-6, and MCP-1 were measured in the cell-free supernatants from THP-1 cells exposed to MPs derived from THP-1 cells treated with different stimuli. None of the MPs tested induced the secretion of TNF-α or IL-6 by THP-1 cells regardless of the type of stimulation the MP donor THP-1 cells received (data not shown). The secretion of MCP-1 was significantly enhanced in THP-1 cells treated with MPs isolated from IFN-γ plus LPS-stimulated donor THP-1 cells compared to the THP-1 cells treated with vehicle solution only (unstimulated) ($P = 0.004$, Figure 6).

DISCUSSION

There is increasing evidence that MPs are novel intercellular signaling agents that can be released by a variety of CNS cell types including microglia. MPs may exhibit immunomodulatory and biological properties similar to DAMPs, such as HMGB1 and TFAM.[13-16] Several studies have investigated the role of MPs as mediators of astrocyte-neuron communication;[15,16,27] however, to date the role of MPs as mediators in microglia communication with neurons, astrocytes, and other microglia remains less characterized.

The MP release and neurotoxicity assays employed in this study used human monocytic THP-1 cells and the SH-SY5Y human neuroblastoma cell line,

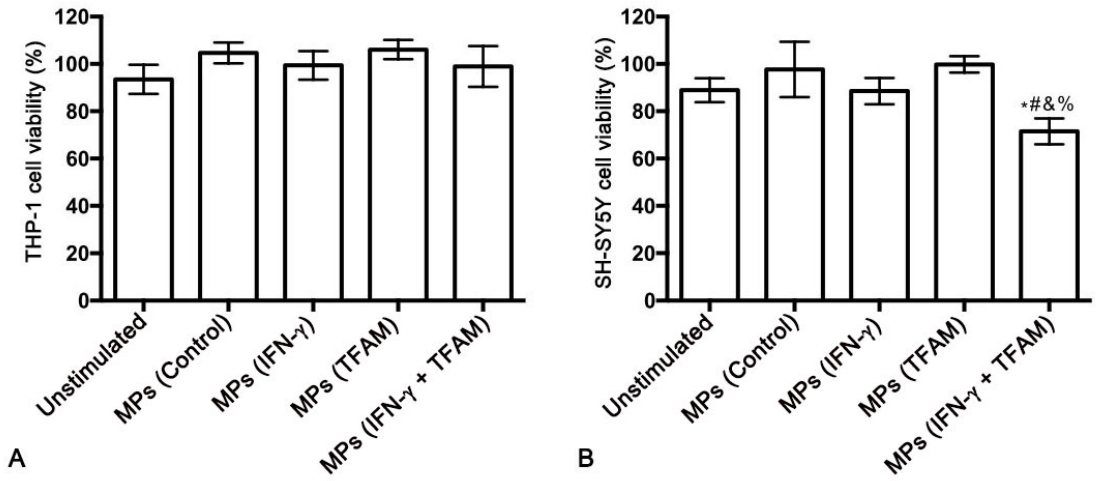

Figure 4: MPs isolated from human monocytic THP-1 donor cells stimulated with TFAM in combination with IFN-γ induce cytotoxicity of THP-1 cells towards human SH-SY5Y neuronal cells. $^{*}P = 0.01$ *vs.* unstimulated; $^{\#}P = 0.004$ *vs.* MPs (Control); $^{\&}P = 0.011$ *vs.* MPs (IFN-γ); $^{\%}P = 0.0001$ *vs.* MPs (TFAM). MPs: microparticles; TFAM: mitochondrial transcription factor A

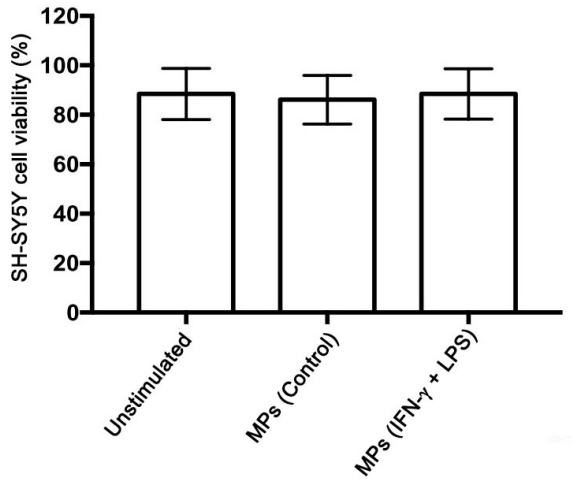

Figure 5: MPs isolated from IFN-γ plus LPS-stimulated human monocytic THP-1 donor cells are not directly toxic to human neuronal SH-SY5Y cells. MPs: microparticles; LPS: lipopolysaccharide

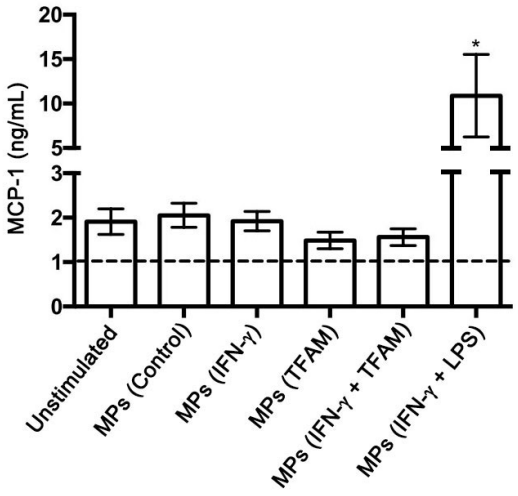

Figure 6: MPs isolated from IFN-γ plus LPS-stimulated THP-1 donor cells increase MCP-1 secretion by THP-1 cells. *P = 0.004 *vs.* unstimulated. MPs: microparticles; LPS: lipopolysaccharide

which have been widely used to model human macrophages (including microglia) and human neurons, respectively.[38-43] Moreover, the neurotoxicity induced by THP-1 cells in these experiments has been demonstrated to be very similar to that of human microglia derived from *post mortem* brain tissues.[44] Additionally, Combs *et al*.[45] showed that stimulated primary murine microglia cause toxicity toward mouse embryonic neurons in a manner very similar to the actions of stimulated THP-1 cells. Activation of THP-1 cells characterized by upregulated pro-inflammatory cytokine and cytotoxin secretion resulting in significantly reduced survival of SH-SY5Y cells has been achieved previously by stimulation with several different combinations of cytokines and inflammatory mediators including IFN-γ and LPS.[38,44,46] Our previous studies show that high concentrations of LPS (such as 0.5 μg/mL used in this study) in combination with IFN-γ induce maximum activation of THP-1 cells; however lower concentrations of LPS (e.g. 0.5 ng/mL) can also be used.[47,48]

First, to confirm that MPs could be released by our model of activated microglia, IFN-γ alone or in combination with LPS was added to human THP-1 monocytic cells for 24 h. Following incubation, the released MPs were isolated by differential centrifugation and analyzed by flow cytometry. Both unstimulated and stimulated

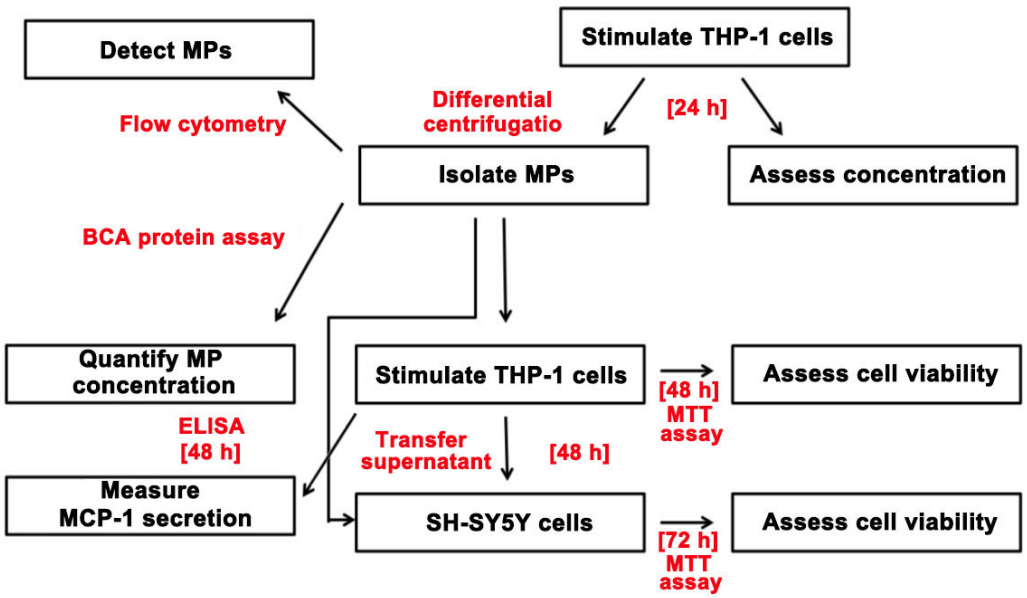

Figure 7: Flow diagram of the experimental procedure utilized in this study to determine whether MPs derived from THP-1 donor cells induce THP-1 cell neurotoxicity and cytokine secretion in an autocrine or paracrine manner. MPs: microparticles

THP-1 cells released MPs. This is consistent with previous publications showing the constitutive release of MPs by resting cells.[29,49] LPS in combination with IFN-γ was the only combination from the stimulants tested to induce the release of MPs above the levels released by the unstimulated control cells. This observation correlates well with other studies showing that activation can significantly upregulate MP release by a variety of cell types, including THP-1 cells.[15,27] It is important to note that IFN-γ on its own did not enhance the release of MPs, which is consistent with previous studies demonstrating that IFN-γ is not capable of inducing significant monocytic cell cytotoxicity in the absence of additional co-stimulatory molecule(s).[38,43]

Next, we investigated whether the isolated MPs possessed cytotoxic properties by conducting supernatant transfer experiments, which involved exposing THP-1 cells to MPs (10 μg protein/mL) isolated from THP-1 cells that had been stimulated with IFN-γ alone or in combination with LPS for 48 h. Our data indicate that MPs derived from IFN-γ plus LPS-stimulated THP-1 cells possess the ability to induce monocytic cell toxicity, while MPs derived from cells stimulated with IFN-γ only lack this ability. The cytotoxicity of THP-1 cells induced by MPs derived from IFN-γ plus LPS-stimulated THP-1 donor cells was enhanced by co-stimulation with IFN-γ. This finding was expected, as IFN-γ is a critical regulatory molecule involved in both innate and acquired immunity that has been shown to modulate the immune response of phagocytic cells.[50] Stimulation with IFN-γ on its own did not induce monocytic cell toxicity towards neuronal cells, reinforcing the idea that IFN-γ requires additional co-stimulatory molecules to achieve significant monocytic cell activation and secretion of cytotoxins. Since only the MTT assay was performed on SH-SY5Y cells, it is not known whether the neuronal cell death induced by supernatants from MP-stimulated THP-1 cells involved mainly apoptotic or necrotic mechanisms. Further studies are needed to address this research question.We also confirmed that the cytotoxic activity of the IFN-γ plus LPS-derived MPs towards the neuronal cells was indirect, as direct exposure of SH-SY5Y cells to MPs derived from either unstimulated or stimulated THP-1 cells did not induce any toxic effects [Figure 7].

Further studies will be required to determine the molecular content of the MPs isolated in these experiments and the mechanism of action of these MPs including the receptors involved in inducing the observed THP-1 cell toxicity towards the SH-SY5Y neuronal cells. A number of studies have shown that activated cells shed MPs containing inflammatory mediators such as IL-1β, which, in turn, is released to activate other immune cells and trigger an inflammatory response.[15] Our data demonstrate that the monocytic cell-derived MPs are able to act in an autocrine or paracrine manner. By recruiting additional monocytes, this activation pattern may then contribute to perpetuating the inflammation present in neuroinflammatory diseases such as Alzheimer's disease. Peroxisome proliferator-activated receptor gamma (PPAR-γ) has been shown to be one of the receptors targeted by MPs during autocrine activation of monocytes.[51] Identifying the receptors mediating the cellular effects of MPs may provide additional targets for attenuating the induced neuroinflammation.

TFAM, a novel DAMP, activates three different types of cultured human mononuclear phagocytes, including THP-1 cells, peripheral blood monocytes, and primary human microglia. It induces the expression of the pro-inflammatory cytokines IL-1β, IL-6, and IL-8.[12] Therefore, we decided to investigate an additional mechanism of action of TFAM by elucidating the role of MPs in TFAM-induced microglial toxicity towards neuronal cells. Moreover, to our knowledge, the role of DAMPs such as TFAM or HMGB1 as triggers of MP release has not yet been investigated. Similar to the results obtained for the IFN-γ plus LPS-derived MPs, the MPs derived from donor THP-1 cells stimulated with TFAM plus IFN-γ induced toxicity of THP-1 cells towards the SH-SY5Y neuronal cells, resulting in decreased cell viability.

MPs were also investigated for their ability to induce the release of pro-inflammatory cytokines by THP-1 cells. Only MPs derived from IFN-γ plus LPS-stimulated THP-1 cells induced the release of MCP-1 above the levels of the unstimulated control cells. MCP-1 is a potent chemotactic factor for innate immune cells that is produced by a variety of cell types including monocytes, astrocytes, and microglial cells, either constitutively or following activation by pro-inflammatory cytokines and oxidative stress.[52] Within the CNS, MCP-1 has been shown to facilitate the infiltration of peripheral blood monocytes across the blood-brain barrier and thereby amplify the neuroinflammatory state observed in neuropathologies with dysregulated microglial activation.[53] The CNS neurotoxicity associated with inflammatory mediators is often due to their action on microglia and astrocytes that leads to the secretion of reactive oxygen species or cytotoxins such as TNF-α. These mediators, in turn, can induce apoptotic or necrotic cell death of nearby neurons.[54-57] Yang *et al*.[58] showed that MCP-1 was not toxic towards cultures of primary cortical neurons. In the presence of microglia, however, MCP-1 was shown to cause neuronal death. Microglia express receptors for MCP-1, and MCP-1 was found to

increase microglial mRNA expression of TNF-α and IL-1β. Use of an MCP-1 neutralizing antibody decreased MCP-1 induced upregulated microglial expression of these pro-inflammatory mediators and also attenuated MCP-1 induced neuronal death in neuron/microglia co-cultures.[58] Accordingly, these properties of MCP-1 may provide an explanation for MP-mediated THP-1 cell toxicity towards SH-SY5Y neuronal cells.

MPs did not induce release of IL-6 and TNF-α (data not shown). These observations differ from results showing the release of IL-6 and TNF-α by human monocytes treated with MPs isolated from human monocytes stimulated with calcium ionophore A23187.[59] This study also demonstrated that the MPs induced nuclear factor-κB activation, which would explain the observed secretion of these pro-inflammatory cytokines. It is important to note that there are several factors that may influence the concentration and the type of MPs produced *in vitro* including: (1) the donor cell type;[60,61] (2) the stimulating agent used;[62] and (3) the incubation time with the stimulant.[63] These factors can modify the structure and content of the MPs released, thus affecting the physiological function of the MPs generated, leading to a different response elicited from the target cell. Therefore, additional experiments are required to fully characterize the MPs isolated in our experiment, to obtain a better understanding of the role that MPs play in microglia communication with other CNS cells.

Taken together, our data support previous observations that MPs could serve as intercellular signaling molecules in the brain. Since MPs can be released by a variety of activated cells, including microglia, and induce the release of additional inflammatory mediators from surrounding cells, they can perpetuate the inflammatory and neurotoxic environment observed in neuroinflammatory diseases. Therefore, further elucidation of the receptors and signaling pathways involved in MP-induced microglial cell activation is warranted as it may lead to the identification of additional targets for the development of therapeutic strategies aimed at preventing or treating the neuroinflammatory component characteristic of several CNS disorders.

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Conflict of interest

There are no conflicts of interest.

Patient consent

No patients involved.

Ethics approval

This article does not contain any studies with human participants or animals.

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Case Report Open Access

Cerebral venous thrombosis in patient of relapse of ulcerative colitis: report of a case

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ABSTRACT

Amongst the various systemic complications of ulcerative colitis, cerebral venous thrombosis (CVT) is an uncommon and serious neurological complication mainly associated during episodes of relapse of ulcerative colitis. CVT is suspected to be a consequence of hypercoagulable state occurring during the disease in genetic predisposed persons. Most patients present with rapid neurological deterioration. This devastating intracranial complication requires immediate medical intervention to avoid potentially life threatening consequences. The outcome is good, provided the disease is diagnosed on time and the treatment is started early. The authors present a patient of CVT, a rare complication seen during relapse of ulcerative colitis.

INTRODUCTION

Ulcerative colitis is an idiopathic chronic inflammatory bowel disease (IBD), which results from a complex relationship of environmental factors and genetic predisposition.[1] Ulcerative colitis (UC) is known to have extra intestinal central nervous system (CNS) complications. CNS features in UC are particularly severe and rarely reported due to their varied presentation. These include cerebrovascular disease, myelopathy and cerebral vasculitis.[2] The two most common thrombotic complications of UC are deep venous thrombosis and pulmonary thromboembolism.[3] IBD accounts for 1-6% of the total causes of cerebral venous thrombosis. The pathogenesis of CNS manifestations of IBD seems to be related to immune mechanisms or prothrombotic states; however the complete pathogenesis is yet to be elucidated. There are no major differences in clinical or radiological characteristics, prognosis, or treatment between patients of IBD-related cerebral venous thrombosis (CVT) and non-IBD related CVT.[4]

Quick Response Code:

CVT is a uncommon type of cerebrovascular disease that accounts for 0.5% of all strokes.[5] It usually presents as headache, seizures, focal neurological deficits, altered consciousness, and papilledema.[6] Due to its varied presentation and low incidence, CVT is not readily suspected, leading to delayed treatment and a poor impact on the prognosis.

We report a case of 39-year-old male, with 10 years long history of ulcerative colitis, who presented with cerebral venous sinus thrombosis following disease relapse. He was managed aggressively and the patient improved.

CASE REPORT

A 39-year-old young male, known case of ulcerative colitis for last 10 years (treated with tablet mesalamine and steroids), presented with complained of increased frequency of stools and abdominal pain for last 1 month. The stool frequency had increased to 6-8 times in a day, associated with blood and mucus. He also complained of new onset, severe daily headache for last 1 week. Headache was associated with nausea and vomiting. There was no history of photophobia or phonophobia. The evening preceding admission, patient went into a state of confusion and altered behaviour. There was no history of fever or seizure. There was no relevant similar past history and no family history of note.

On physical examination, he was afebrile; pulse was 94/min and blood pressure was 124/70 mmHg. There was no lymphadenopathy. On neurological examination, he was drowsy, but arousable and confused. Fundus examination showed signs of early papilledema. There was no limb weakness but the bilateral plantars were extensor response. Rest of neurological examination was within normal limits. Laboratory findings showed hemoglobin of 11.4 gm%; the white blood cell count was slightly elevated at 12,500/mm^3 and his serum albumin levels were 4.7 gm%. His erythrocyte sedimentation rate was 46 mm and C-reactive protein was 116 mg/L. His renal function tests, liver function and serum electrolytes were within normal range.

His contrast enhanced magnetic resonance imaging (MRI) brain revealed altered increased signal intensity in the anterior superficial cortical veins and the superior sagittal sinus on T1 weighted (T1W), T2 weighted (T2W) and fluid attenuated inversion recovery (FLAIR) images suggestive of their thrombosis. The high superficial cortical veins in the left fronto-parietal regions also show altered increased signal intensity on TIRM and T1W images suggestive of their occlusion. Similar altered signal intensity was visualized in the Torcula and the right sided transverse sinus. Also a note was made of a large left sided fronto-temporo-parietal and occipital infarct with hemorrhagic reconversion suggestive of a venous infarct [Figure 1]. Magnetic resonance venography of the brain was suggestive of thrombosis in the superior sagittal sinus and right transverse sinus [Figure 2]. Based on patient's history, clinical features and imaging findings a diagnosis of cerebral venous thrombosis was considered. The patient was managed with conventional heparin, anti oedema measures and other supportive treatment. On further work up for CVT, he was found to be Positive (Heterozygous)

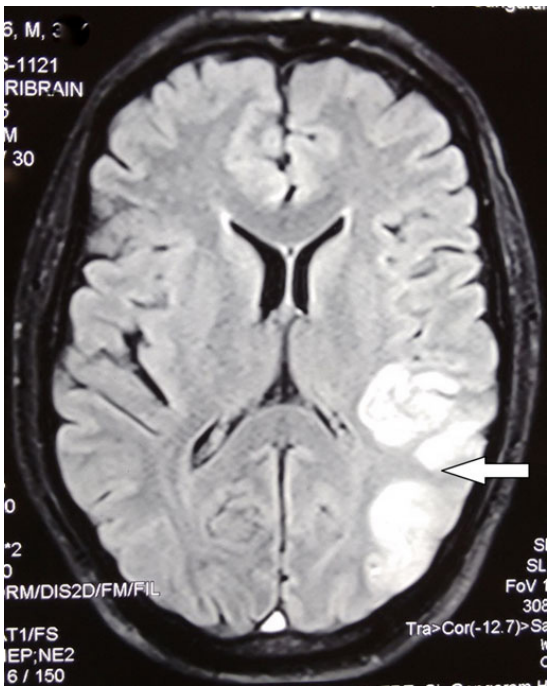

Figure 1: Contrast MRI brain, tirm image: left sided fronto-temporo-parietal and occipital infarct. Arrow shows large venous infarct in left parieto occipetal region. MRI: magnetic resonance imaging

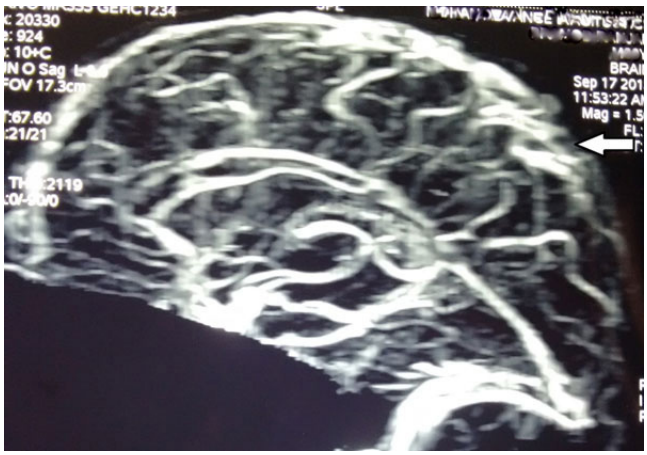

Figure 2: Magnetic resonance venography of the brain showing thrombosis in the superior sagittal sinus and right transverse sinus. Arrow shows occulusion of the superior sagittal sinus

for MTHFR mutation. Furthermore, he was also found to have serum homocysteine level of 20.5 μmol/L. His levels of anti-nuclear antibody, anti-double strand deoxyribonucleic acid, lupus anticoagulants, antiphospholipid antibodies, anticardiolipin antibodies, and anticyclic citrullinated peptides were negative.

The patient clinically improved in a period of five to seven days with the treatment. His symptoms had gradually subsided. He was then discharged.

DISCUSSION

Extensive thrombosis of venous sinuses is a serious complication of ulcerative colitis. Venous thrombosis is known to occur with a greater frequency than general population in patients with ulcerative colitis. Incidence of thrombosis is about 6.5% in patients with active IBD.[4] The relationship between thrombosis and UC has not been well defined. But recent evidence suggests that UC is an important factor for thrombotic complications.[7] The exact cause for the increased rate of thrombotic events in patients with IBD is still uncertain. However, it is most likely related to the interaction between acquired and inherited genetic risk factors. Also, the recent research has suggested it to be an interaction between the coagulation cascade in the body and cytokine mediators of chronic inflammation and also the inflammatory process can itself activate coagulation cascade.[8] The inflammatory process initiates clotting, impairs the fibrinolytic system and decreases the activity of natural anticoagulation mechanisms. Depression of anticoagulation mechanisms not only increases thrombosis, but also potentiates the inflammatory process. That is why, the majority of thrombotic events occur during the active phase of disease. Abnormalities in coagulation cascade such as elevated fibrinogen level, factor V, factor VIII, and increase in circulating thrombin-antithrombin complexes, decreased antithrombin III, thrombocytosis and increased platelet aggregation have been documented.[9] However, there is no significant evidence to associate hematological and coagulation abnormalities with CVT. Grainge *et al*.[10] analyzed 13,756 patients with IBD and 71,672 matched controls, and found that 139 patients and 165 controls developed venous thromboembolism. Overall, patients with IBD had a 3.4 times higher risk of developing venous thromboembolism than did controls, with risk increasing up to 8.4 times during a flare-up of IBD. Our patient presented with cerebral venous thrombosis, associated with relapse of UC.

MRI brain and a magnetic resonance venography (MRV) should be done immediately, once cerebral vein thrombosis is clinically suspected, so that the early diagnosis is not missed. MRI and MRV are considered the best tools for diagnosis and follow-up. The anticoagulation therapy for patients with CVT is similar in both with active and chronic UC, and has been associated with lower incidence of mortality, if started at appropriate time.

In patients with cerebral venous thrombosis, favourable results are possible with earlier diagnosis and appropriate treatment plan. If patients remain untreated, the mortality rate can be very high. Therefore, the present report highlights the value of considering the diagnosis of CVT, in patients with IBD, especially when the disease is in its relapse phase.

In conclusion, it is necessary to suspect CVT in a patient with relapse of UC, who presents with recent, unusual severe headache, stroke like features, seizures, or any other brain syndrome. The physician, neurologist or the gastroenterologist should be well aware of the increased risk of CVT in patients with relapse of UC. The gastroenterologists treating UC should be wise enough to suspect CVT, especially in genetically predisposed and immediately seek a neurologist's opinion for proper management. Also, it would be useful to investigate for genetic hypercoaguable state in patients of UC, in order to find out the at-risk group of UC patients.

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Conflicts of interest
There are no conflicts of interest.

Patient consent
Patient consent was obtained from the patient.

Ethics approval
Ethics approval was obtained prior to the commencement of the study.

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Commentary **Open Access**

Comments on "Loss of intranetwork and internetwork resting state functional connections with Alzheimer's disease progression"

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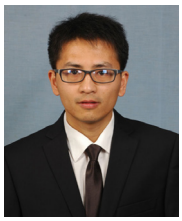

Dr. Jiu Chen is a Ph.D. researcher with the background of neurology and clinical psychology. His research focus is to investigate the pathogenesis of subjects at high risk to Alzheimer's disease by using the multi-modal magnetic resonance imaging (MRI) and event related potential (ERP) approaches.

Neuroimaging evidence of disconnection syndrome of Alzheimer's disease (AD) is extremely fascinating. In the study by Brier *et al*.,[1] they examined resting-state functional-connectivity magnetic resonance imaging (rs-fcMRI) in 5 functionally defined brain networks: default mode network (DMN), executive control network (CON), salience network (SAL), dorsal attention network (DAN), and sensory-motor network (SMN). Within a large sample size of human participants of either sex (n = 510), they divided subjects into three subgroups according to different AD severities, i.e. unaffected (clinical dementia rating, CDR 0), very mild (CDR 0.5), and mild AD (CDR 1). The major findings of this study were as follows. First, they found a loss of intra-network correlations in the DMN and other networks at CDR 0.5. Second, they found increases of intra-network correlations within the SAL between CDR 0 and CDR 0.5; however, they found reduced intra-network correlations within all networks at CDR 1. Third, they found that the three network pairs, DMN-DAN, DMN-SMN, and CON-SMN were preferentially affected at certain CDR stages. Finally, they found all inter-network correlations consistently reduced with advancing CDR stage.

Therefore, the authors concluded from their study that AD is associated with widespread loss of both intra- and inter-network correlations; these findings suggested

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that AD pathology may manifest focally in certain region of interest (ROI) pairs but not others. This is all good news for understanding AD pathophysiology and reinforces an integrative view of the brain's functional organization.

After reading the article with interest, I have some concerns about the study as follows:

First, in their study,[1] the authors defined 36 spherical (6 mm radius) ROIs by maximizing the topographic concordance between mapping derived by seed-based correlation and by spatial independent component analysis (ICA). A problem of ICA derived networks is they separate brain areas into subnetworks that show higher interconnectivity with each other. In addition, this approach can lead to overlapping anatomical areas that complicate the interpretation of the brain areas.

Second, the authors emphasized the notion that composite scores can capture the critical phenomenology. They performed global signal regression during preprocessing to reduce non-neuronal physiological noise in the fMRI BOLD time series (Raichle *et al*.[6]; Seeley *et al*.[7]), which could have markedly enhanced negative correlations (Dosenbach *et al*.[8]; Cole *et al*.[9]). Subsequently, those negative correlations would have been treated as neuronal signals in the composite scores. However, when calculating the mean effects of composite scores an offsetting effect of positive and negative correlations can occur. Therefore, care must be taken to explain thoroughly the composite scores.

Third, the authors performed a one-way analysis of variance and *post hoc t*-tests. However, they did not control the effects of age, education, or sex on the resting-state intra- and inter-network functional connections, which may have affected the results.

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There are no conflicts of interest.

Patient consent
No patient involved.

Ethics approval
This article does not contain any studies with human participants or animals.

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Current diagnosis and treatment of cryptococcal meningitis without acquired immunodeficiency syndrome

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ABSTRACT

Cryptococcal meningitis (CM) is a central nervous system infectious disease caused by *Cryptococcus*. It is the most common fungal infection in the central nervous system, accounting for about 48% of fungal infection. The disease occurs mainly in acquired immunodeficiency syndrome (AIDS) patients and concentrates in the immunocompromised people without AIDS. There are nearly one million new cases of CM each year, and about 70% of them died. In China, CM occurs mainly in people without AIDS and there is an increasing trend in recent years. Early diagnosis and treatment is the key to reducing morbidity and mortality associated with CM. The diagnosis mainly depends on laboratory examination such as morphological examination, fungal culture and antigen detection. History, clinical manifestation and imaging examination are the important parts of auxiliary examination. The initial combined antifungal treatment is emphasized, and the principle of fractional treatment including induction, consolidation and maintenance therapy should be followed. The high intracranial pressure must be reduced actively at the same time. In addition, it is proved that the novel immunotherapy combined with antifungal agents can improve the curative effect and limit the chance of antimicrobial resistance. Large-scale clinical trials are needed for further study.

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INTRODUCTION

Cryptococcal meningitis (CM) is the most common cause of fungal meningitis worldwide. Globally, there are approximately 957,900 new cases of CM each year, and about 624,700 of them died.[1] CM occurs mainly in the acquired immunodeficiency syndrome (AIDS) crowd abroad. In China, CM is sporadic, mainly in people without AIDS. In recent years, there is an increasing trend for the incidence of CM as a result of the wide application of antibiotic, hormone and immune inhibitors, organ transplantation in China. In developing countries, up to 70% of CM lead to death eventually.[2] The severity of disease and limited access to diagnostics and medications results in the high mortality of CM in resource-limited settings (RLS).[1] Early diagnosis and treatment is the key to reduce the morbidity and mortality.

No symptoms, hardship to select pathogen or lack of awareness in the early stages of the disease make the diagnosis difficult, particularly in RLS. The clinical manifestations and part of cerebrospinal fluid parameters such as fever, headache, high intracranial pressure, high protein and low glucose in cerebrospinal fluid (CSF) which are easily confused with tuberculous meningitis. Substantial resources, such as hospitalization, intravenous antifungal therapy, access to lumbar punctures, and strict monitoring are required in the process of CM treatment.[3] In this review, we mainly describe the available diagnostic methods and management of CM without AIDS.

DIAGNOSE OF CRYPTOCOCCAL MENINGITIS

The diagnosis of the CM is dependent on the medical history, clinical manifestations, imageological examination, cerebrospinal fluid parameters and laboratory tests. Among them, laboratory tests are the main methods to make a definite diagnosis. India ink staining and fungal cultures are regarded as the diagnostic gold standard. There is not much difference in diagnostic criteria of CM between patients with or without human immunodeficiency virus (HIV). Merely for patients with advanced HIV, World Health Organization (WHO) recomends early cryptococcal antigen (CrAg) screening and treatment in 2011.[4]

Medical history and clinical manifestation

The medical history of CM includes environment (the contact history of pigeons) and susceptible population with risk factors including long term treatment of immunosuppressant, broad-spectrum antibiotics and glucocorticoids, HIV infection and patients with immunodeficiency is important for providing initial clues and diagnostic evidence. The CM has a hidden onset and a slow course, mainly presenting the symptoms of increased intracranial pressure (headache, nausea, vomiting, and disturbance of consciousness), meningeal irritation sign (neck rigidity, Kernig sign and Brudzinski sign). Patients with altered mental status have high mortality.[5-7]

Patients with typical symptoms of the meningeal irritation are less than 20%.[6] Optic nerve damage is the most common among injury of cranial nerves caused by intracranial hypertension (optic nerve, oculomotor nerve, abducens nerve, facial nerve, vestibulocochlear nerve involvement). Forty percent of the patients with CM have visual involvement, including optic discedema and uveitis.[8-10] Second is the vestibulocochlear nerve damage. If there is parenchymal involvement, it would appear epilepsy seizures, hemiplegia, mental disorder, ataxia, *etc*.

Imaging examination

Computed tomography (CT) and magnetic resonance imaging (MRI) has limited effect on the diagnosis, but it is necessary to find the complications (intracranial mass and hydrocephalus).[11] Some professors divide the CM course into three periods.[12] Acute phase: cerebral edema is showed on CT or MRI. Brain parenchyma presents punctate low-density lesions and Long T1, long T2 signal area, it is similar to cerebral infarction, called “soap bubble damage”[12,13] which is caused by the expansion of the space (Robin Virchow) around the capillary. Subacute stage: Multifocal gelatinous pseudocysts formed in the deep white matter on both sides of the cerebral hemispheres, basal ganglia, thalamus and midbrain, *etc*. Chronic phase: intracranial single or multiple rounds, oval and sheet, *etc*., slightly higher or low density massive umbra, lesions surrounded by edema, may have mutual integration. Enhanced scan shows multiple small nodules ring, it is easy to be misdiagnosed as cerebral metastasis. Because of the correlation between CT/MRI and the disease progression or cerebrospinal fluid pressure, CT and MRI should be reviewed even if it was normal during the acute phase.

CSF parameter

The typical characteristic of cerebrospinal fluid for CM is high intracranial pressure (HICP) which is more than 350 mmH_2O or up to more than 900 mmH_2O. The reason for HICP is that *Cryptococcus* hinder the CSF to pass through the arachnoid villi which obstructs the CSF circulation channel.[14] Furthermore, the accumulation of capsular polysaccharide in arachnoid villi and subarachnoid spaces contributes to fluid retention by increasing the osmolarity of the CSF and

interstitial fluid.[15,16] The appearance of CSF is clear and transparent generally, and it can be slightly turbid if there is a large amount of *Cryptococcus*. Leukocyte count in CSF is increased (about 100-500 × 10^6/L) in the majority of people, or normal in the minority. In addition, the protein level rises (no more than 2 g/L usually), and the glucose and chloride decreases in CSF as the result of infection. The degree of decrease in glucose levels is significantly lower than that of other central nervous system infection.[17]

The characteristics of cerebrospinal fluid cytology: the total number of cells increases to different degrees, presenting mixed cell reaction or lymphocyte dominated mixed cell reaction. Monocyte constitutes the main ingredients in the most of the cerebrospinal fluid cytology of CM patients, the total number of cells decreases and the proportion of small lymphocytes also increases with the improvement of the disease. Therefore, cerebrospinal fluid cytology has also certain significance to the monitoring of the efficacy.

However, cerebrospinal fluid examination is normal in 10-17% other patients, especially in the patients with HIV.[18,19]

Other laboratory examination

The common laboratory examination for diagnosing of CM mainly includes morphological examination, fungal culture, and antigen detection.

Cryptococcus neoformans (*C. neoformans*) is a single-celled organism with a polysaccharide capsule. It exists in the blood, CSF, and tissues.[20] Morphological diagnosis depends on dyeing technology. India ink staining is considered to be one of the gold standards for diagnosis. It is a traditional method of identification of *C. neoformans*, especially in areas with limited resources because of its simple and rapid operation. Characteristic "starry night" phenomenon[20] would be observed by India ink staining: capsule is not shaded but surrounded dyed blue. Yet the sensitivity of india ink staining is only < 86%.[21,22] In addition, due to low fungal loads, India ink is insensitive for patients who presenting early after symptoms appear or being on initiating antiretroviral therapy (ART).[23] The detection rate of *C. neoformans* in the CSF is only 66% at first time, about 17.8% at second time, and others remain 3 to 20 times smear test under microscope to find positive.[24] May-Grunwald-Giemsa (MGG) staining has a relatively high positive detection rate, a small amount of *C. neoformans* can be detected after centrifugal precipitation, applying to patients with low amount of fungi. But the morphological characteristics of the fungi are not clear, it is easy to be confused with small lymph cells in CSF when the *C. neoformans* scattered in the distribution. Therefore, the detection method requires high skill levels from observers. Alcian blue staining is a special dye for the *C. neoformans*[25] and could dye the capsule to be deep blue, and the cell light blue, without the peripheral inflammatory cells being dyed. Therefore, the sensitivity of alcain blue staining is high and the fungi can be easily observed.[26] The combination of the above methods can improve the detection rate. Culture is considered the gold standard for diagnosis of cryptococcal meningitis.[27] But it is limited by culture conditions, culture time and the amount of cerebrospinal fluid and fungi, thus making the early diagnosis hampered. But it has important value for further drug sensitivity test and species classification.

The most reliable diagnostic method for cryptococcosis is to detect capsular polysaccharide antigen[20] which can be find in serum, CSF, and urine specimens. Serum CrAg is taken as an early biologic marker which is far more sensitive and rapid than direct detection of the pathogen, it highly predicts of the development of CM within one year.[28] Retrospective data suggests that CrAg screening in patients with late-stage human HIV ART may reduce cryptococcal disease and deaths.[29] However, presence of CrAg in CSF is more valuable for diagnosis of CM and serum CrAg assay can help to assist the diagnosis.

Main methods are consist of latex agglutination (LA) assays, enzyme immunoassays (EIAs), or the novel lateral flow assay (LFA).[20] LA or EIAs has been used for detecting CrAg for several years.[30] The sensitivity and specificity of the LA test for CSF is high. The sensitivity ranges from 93% to 100%, while the specificity ranges from 93% to 98%, which is significantly better than India ink staining and CSF cytology in the early diagnosis of CM. And the severity of the disease in patients with cryptococcal meningitis is correlated with the antigen titer of capsular polysaccharide, therefore, LA test also has the value of evaluating the severity of illness and the prognosis.[31] Although this method has high sensitivity, but may appear false positive results in patients with immunological diseases, such as rheumatoid.[32,33] Tedious manual operation and subjective intervention is the main weakness, in addition, this method needs equipment and refrigeration which restricts its application in resource limited area.[20] The sensitivity and specificity of EIAs test for capsular polysaccharide antigen is high which is 100% and 98% respectively for CSF samples.[34,35] However, EIAs test cannot be widely used because of the expensive detection kits.

The lateral flow assay (LFA) is developed in 2009, it could detect cryptococcal polysaccharide capsule

quickly by using gold-conjugated anti-cryptococcal monoclonal antibodies directed at *C. neoformans*.[30] It has higher sensitivity than LA and EIAs, and it is more sensitive for detecting the lower antigen in CSF.[22,30] The CrAg LFA which is low cost can be carried out at room temperature without refrigeration or complicate experimental equipment, takes just 10 min to get the results. Therefore, it is expected to reform the diagnosis of cryptcoccosis in the restricted area.[36]

Recently, molecular biological detection comprised of chromosome pulse electrophoresis, nucleic acid probe, DNA fingerprinting technique and polymerase chain reaction (PCR) has been carried out in some laboratories. PCR are often used at present. PCR which applicates specific primer aimed at conservative sequence of *C. neoformans* to the detection of fungi is rapidly and pecifically.[31] The primer used for multi-locus sequence typing of *C. neoformans* includes CAP59, GPD1, IGS1, LAC1, PLB1, SOD1, URA5.[37] The pathogenic fungi are identified as *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans* and hybrid strains by PCR. However, the requirements for the experimental technique of PCR is so high that this kind of test is not widely carried out in clinical practice at present.[31]

TREATMENT OF CRYPTOCOCCAL MENINGITIS

Cryptococcal meningitis without treatment is fatal in most cases. It is critical to diagnose early and treat promptly for the improvement of survival.[38]

Antifungal agents therapy

Antifungal drugs used commonly include amphotericin B (AmB), 5-Flurocytosine (5-FC) and fluconazole (FCZ).

AmB is a broad-spectrum antifungal agent. The mechanism of AmB is to combine with fungal cells membrane of ergosterol and interfere with cell metabolism and increase the cell membrane permeability aimed to bring about cell death. AmB is the first choice for the treatment of CM, and it has the best early fungicidal activity (EFA).

5-FC is a pyrimidine analogues, and its mechanism of action is to inhibit cell division by interfering with the synthesis of fungal DNA. Single drug treatment is easy to produce drug resistance. 5-FC is usually used incombination with AmB and is superior to the combination of AmB and FCZ.[39] The reason is that AmB has the ability to make the cell membrane permeability to increase, thus 5-FC is more susceptible to enter the fungus and appear synergistic fungicidal effect. Without use of 5-FC in induction therapy will lead to increased mortality, treatment failure[40] or recurrence.[41]

FCZ is one of the triazole antifungal agents and its mechanism of action is to destroy the cell membrane and promotes cell death by inhibiting the activity of cytochrome P450 by inhibiting the synthesis of ergosterol in fungal cell membrane.[42] FCZ is easy to go through the blood brain barrier (BBB) to reach a high concentration in CSF. However, it belongs to fungistat that the effect of killing *Cryptococcus* is weaker than that of AmB. Therefore, it can be used for sequential therapy after induction therapy. New drugs such as voriconazole and posaconazole have obvious anti-*Cryptococcus* activity *in vitro*.

Fractional treatment of the CM is recommended at present, consists of AmB plus 5-FC induction therapy, FCZ consolidation and maintenance therapy.[43]

Expert consensus of the diagnose and treatment of cryptococcal infection in China recommended combination therapy with AmB 0.5-1 mg/kg per day and 5-FC 100 mg/kg per day as induction treatment for non-HIV associated patients which earned widespread approval from experts.[44] The induction phase lasts at least 8 weeks which is different from the project of Infectious Diseases Society of America (IDSA), and this may be related to the use of the method in our country, namely it takes a period of time to begin with small dose to effective maintenance dose gradually. However, large-scale clinical trials are needed to demonstrate their validity. Then followed by consolidation therapy with FCZ or itraconazole 200-400 mg/day at least 12 weeks,[24] and maintenance therapy has not been mentioned in the Consensus [Table 1].

At present, there is a difference in the treatment of non-HIV associated patients, and its management is based on the characteristics other host and the pathogen. As a result of about 25% of the transplant patients are with renal dysfunction in the diagnosis of cryptococcal meningitis,[11] liposome amphotericin B

Table 1: Antifungal therapeutic schedule for non-HIV associated CM patients

Schedule	Course	
Induction period	AmB 0.5-1 mg/kg per day + 5-FC 100 mg/kg per day	≥ 8 weeks
Consolidation period	FCZ/Itraconazole 200-400 mg/day	≥ 12 weeks
Maintenance period	Not mentioned	

HIV: human immunodeficiency virus; CM: cryptococcal meningitis; AmB: amphotericin B; 5-FC: 5-Flurocytosine; FCZ: fluconazole

(LAmB)/amphotericin B lipid complexes (ABLC) with small renal toxicity are recommended in the induction period.[11,24] In 2010, IDSA[24] suggests induction therapy with LAmB (3-4 mg/kg per day i.v.) or ABLC (5 mg/kg per day i.v.) plus 5-FC (100 mg/kg per day i.v.) for at least 2 weeks, consolidation therapy with FCZ 400-800 mg (6-12 mg/kg) per day for 8 weeks. And maintenance therapy with FCZ 200-400 mg (3-6 mg/kg) per day lasts for 6-12 months [Table 2]. LAmB should be used at least 4-6 weeks without the use of 5-FC in induction therapy. Increasing dosage (6 mg/kg per day) should be conducted when the fungal load is higher or palindromia.

Treatment for non-HIV associated or non-transplant patients includes induction therapy with AmB 0.7-1.0 mg/kg per day or LAmB 3-4 mg/kg per day or ABLC 5 mg/kg per day plus 5-FC 100 mg/kg per day for 4-6 weeks. IDSA also recommends that it is essential to extend induction period if treated with AmB/LAmB monotherapy or treatment interrupted. In addition, consolidation therapy with FCZ 400-800 mg (6-12 mg/kg) per day lasts for 8 weeks, and maintenance therapy with FCZ 200 mg (3 mg/kg) per day lasts for 6-12 months [Table 3].

It is difficult to achieve effective concentration in the CSF for AmB or LAmB because of their poor ability to traverse BBB. Intravenous combined intrathecal administration of AmB can improve the drug concentration in CSF to inhibit the *C. neoformans* effectively, and observational studies suggest that it could be associated with improved survival.[45] However, it is necessary to prevent the occurrence of complications caused by intrathecal administration , such as paresthesias, radiculitis, or myelopathy.[46]

Previous studies in humans and animals indicate that intrathecal administration of lipid formulations of AmB is better tolerated than AmB.[47-49] Furthermore, there is an animal experiment suggesting that the combination of intravenous antifungal drugs with intrathecal administration of LAmB could be beneficial in terms of survival and reduction of fungal load in CSF.[47,50]

AmB is easy to combine with human cholesterol cell membrane,[48,49] so the adverse reactions are more and serious. AmB has high toxicity, especially liver and kidney toxicity. Renal toxicity which could lead to lowering glomerular filtration rate and electrolyte disturbances is the most common. Renal function can be restored by early termination of the use of AmB[51] or replacement of LamB.[11] In addition, some studies[11,52,53] support that preemptive hydration and electrolyte supplementation are the effective methods to minimize the toxicity in middle- and low-income countries (MLICs).[4] Anemia is another common side effect of AmB,[54] the reason is that the effect of the bone marrow on the synthesis of the erythropoietin.[55] 5-FC could get through the BBB easily and has slight adverse reactions, such as gastrointestinal reaction, rash, erythropenia, light degree damage of liver and kidney function, *etc*. Symptoms can be relieved after stopping taking the drug. The incidence rate of adverse reactions of FCZ is low, the symptoms mainly include gastrointestinal reaction, rash, and so on. Liver and kidney impairment are transient and would returned to normal after drug withdrawal generally.[56]

Treatment of high intracranial pressure

The incidence of HICP in patients with cryptococcal meningitis is more than 50%.[50] HICP is the leading cause of death and complications.[44] Therefore, effective control of intracranial pressure for improving clinical symptoms to gain enough time for the success of anti-fungal therapy is of crucial importance. Active treatment of HICP is crucial whether it is HIV-associated patients or not.[57] Methods used to reduce intracranial pressure commonly as follows:[58] (1) Drugs such as mannitol, glycerin fructose, corticosteroids, acetazolamide and so on. While the long-term effect of medical management is not clear that is not used routinely;[57,59] (2) Lumbar puncture. Patients whose intracranial pressure > 2.4 kPa are performed with regular lumbar paracentesis to maintain normal

Table 2: Antifungal therapeutic schedule for CM patients with renal dysfunction

Schedule	Course	
Induction period	LAmB 3-4 mg/kg per day/ABLC 5 mg/kg per day + 5-FC 100 mg/kg per day	≥ 2 weeks
Consolidation period	FCZ 400-800 mg/day or 6-12 mg/kg per day	≥ 8 weeks
Maintenance period	FCZ 200-400 mg/day or 3-6 mg/kg per day	6-12 months

CM: cryptococcal meningitis; LAmB: liposome amphotericin B; ABLC: amphotericin B lipid complexes; 5-FC: 5-Flurocytosine; FCZ: fluconazole

Table 3: Antifungal therapeutic schedule for non-HIV associated or non-transplant patients

Schedule	Course	
Induction period	AmB 0.7-1.0 mg/kg per day or LAmB 3-4 mg/kg per day or ABLC 5 mg/kg per day + 5-FC 100 mg/kg per day	4-6 weeks
Consolidation period	FCZ 400-800 mg/day or 6-12 mg/kg per day	8 weeks
Maintenance period	FCZ 200 mg or 6 mg/kg per day	6-12 months

HIV: human immunodeficiency virus; AmB: amphotericin B; LAmB: liposome amphotericin B; ABLC: amphotericin B lipid complexes; 5-FC: 5-Flurocytosine; FCZ: fluconazole

intracranial pressure. Release CSF 10-30 mL per day until the intracranial pressure has been normalized may be required for a few days. The treatment guideline of IDSA in 2010 points out that this method is the most effective and rapid way to reduce the pressure currently;[24] (3) Lumbar cistern drainage. This method could reduce the number of lumbar puncture and avoid patients' pain. In addition, it is a better method for patients whose intracranial pressure > 3.9 kPa and cannot be controlled effectively by frequent lumbar puncture. Make the open brain pressure fall to 50% by enough drainage of the cerebrospinal fluid. Regulate the drainage 300-400 mL per 24 h. It is best no more than 15 days for drainage each time in principle in case of CSF leakage or secondary infection; (4) Ommaya reservoir. This involves a device that allows for ventricle drainage invented by Sheldon and Ommaya[60] in 1963 and applicated as common treatment in adults with cryptococcal meningitis for relief of the symptoms of HICP. The anti-fungal drugs could be injected into ventricles using this device directly and reach effective concentration without influence of BBB. In addition, we could obtain CSF from the ommaya reservoir expediently and securely that it is useful in the evaluation of the state of illness changes and therapeutic effect advantageously. This method reduces the risk of exogenous infection due to the hermetic type structure. However, percutaneous puncture repeatedly may lead to the damage of reservoir or secondary infection; (5) Ventriculoperitoneal shunt. We should consider the ventriculoperitoneal shunt under following circumstances: the control of intracranial pressure is not ideal, recurrent cerebral hernia, occurrence of persistent or progressive cranial nerve defects. Anti-fungal therapy should be used at the same time to avoid peritoneal cavity infection; (6) Lateral ventricle drainage. If measures above mentioned cannot reduce the intracranial pressure effectively, or there is an expansion of the ventricles, lateral ventricle drainage should be performed in time. But the drainage time should not be too long (2-3 weeks), otherwise it is easy to cause infection. These surgical techniques above could not only reduce intracranial pressure but alsobe used to intrathecal or ventricular injection to improve the therapeutic effect.

Immune therapy

The main infection routes of *Cryptococcus* is through the respiratory tract, asymptomatic latent state is the most common infection state.[44] When there is immune function defect in people that could not resist the growth and reproduction of the fungi, *Cryptococcus* will proliferate and migrate through the blood to other organs in the body, leading to the CM eventually. Therefore, anti-*Cryptococcus* infection by immune regulation opens up a new way. Anti-fungal drugs combined immunotherapy has been put forward in recent years.

In a phase I clinical study overseas, twenty cases of cryptococcosis are treated with monoclonal antibody from mice aimed at capsule antigen, the result shows that high doses of monoclonal antibodies can reduce the level of polysaccharide antigen in serum temporarily, but there may appear allergic reactions and other side effects.[61]

It has been reported that clinical application of interferon in the treatment of fungal resistance in 2004.[62] Jarvis *et al*.[63] conduct a randomized controlled experiment and show that the rate of fungal clearance is accelerated by adding interferon on the basis of AmB combined with 5-FC, and there is no other side effects. But there is no statistical significance in mortality of patients between group with interferon 100 μg (Day 1, Day 3) and group with 100 μg (Day 1, Day 3, Day 5, Day 8, Day 10, Day 12).

The treatment guidelines set out by Infectious disease society of American in 2010 recommended to give formal antifungal agents combined with IFN-γ to patients with persistent infection (whose culture result of CSF is positive after 4 weeks of antifungal therapy with 100 μg/m^2 for more than 50 kg, 50 μg/m^2 for less than 50 kg 3 times a week for 10 weeks. Small-scale phase 2 clinical trials have shown the good curative effect. However, larger clinical trials are needed to verify.

In recent years, radioimmunotherapy has become an adjuvant immunotherapy for the treatment of *Cryptococcus* infection. The principle is to use the radioactive substances to label monoclonal antibodies, thus killing the fungi with cytotoxic radiation substance. Bryan *et al*.[64] have performed animal experiment, demonstrated that RIT is more effective to mice infected with *C. neoformans* than AmB. Besides, RIT could prevent the development of fungal resistance.[42]

CONCLUSION

Higher morbidity and mortality has caused great concern from scholars all over the world. How to make early diagnosis and effective treatment is the key point of the current study. Immunotherapy opens up a new way of treatment for CM. But it is in the bud. Therefore, the development of new drugs with effective antifungal activity and low toxicity as well as effective treatment is still a problem we need to solve.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

No patient involved.

Ethics approval

This article does not contain any studies with human participants or animals.

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Case Report **Open Access**

A case of anti-NMDA receptor encephalitis with ADEM-like clinical/MR findings

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ABSTRACT

In recent years, anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis overlapping with demyelinating disorders has attracted more and more attention. The case is about a 52-year-old woman with anti-NMDAR encephalitis presenting acute disseminated encephalomyelitis (ADEM)-like clinical/magnetic resonance (MR) findings. Here, the authors report this case and briefly review her MR evolution and the conditions of her prognosis. The recognition that patients with anti-NMDAR encephalitis may have demyelinating disorders, simultaneously or sequentially, is important. Otherwise, a high dose of steroid treatment with several courses could obtain good effect, even if given in the late phase.

INTRODUCTION

Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is a severe autoimmune encephalitis. Patients usually present with psychiatric/behavioral change, dyskinesia, memory deficit, autonomic instability, disorders of consciousness, and even life-threatening conditions.[1,2] Anti-NMDAR encephalitis has been recognized in patients of all ages, but more frequently in children, with or without teratoma, and young adults.[3] In recent years, anti-NMDAR encephalitis overlapping with demyelinating disorders has attracted more and more attention. However, anti-NMDAR encephalitis showing acute disseminated encephalomyelitis (ADEM)-like clinical/magnetic resonance (MR) findings is rare.

CASE REPORT

A 52-year-old Chinese woman presented with fever, headaches, neck rigidity, and apathy. She was treated successively with intravenous acyclovir and dexamethasone (DXM) in small amounts (10 mg/day) to control the symptoms as if they were from viral meningitis. Over the following weeks, the patient became progressively confused and had difficulty with walking and urinary and fecal incontinence. She was then transferred to our hospital. A clinical diagnosis

Quick Response Code:

of autoimmune encephalitis was considered, and she was treated with high doses of intravenous methylprednisolone (1 g/day for 3 days, 0.5 g/day for 2 days, 0.25 g/day for 1 day) with two courses of treatment.

The following tests were normal or negative: routine blood test, autoantibody screen, anti-neutrophil cytoplasmic antibody, anti-nuclear antibodies, antibodies related to paraneoplastic neurologic system diseases, acute flaccid paralysis, thyroid biochemistry and antibodies, HIV, CA-125, chest X-ray, electrocardiograms, abdomen and pelvic ultrasound, HBSAg (+), and PET-CT showed tumor-negative. Serum and cerebrospinal fluid (CSF) tested positive for anti-NMDAR (titers 1:10) (semiquantitative indirect fluorescent antibody, EUROIMMUN Laboratories). On admission, lumbar puncture (LP) initially showed CSF with a mildly raised protein of 0.51 g/L, with 60 × 10^6/L white blood cells, 2.16 mmol/L glucose. PCR for HSV, EBV, CMV in CSF, and bacterial cultures of the CSF were negative. On repeat testing 2 weeks later, LP showed the protein had normalized, a white cell count of 18 × 10^6/L, and 3.72 mmol/L glucose. Serum anti-neuromyelitis optica (NMO)/aquaporin-4 (AQP4) antibody was negative. Serum and CSF oligoclonal bands were positive. Magnetic resonance imaging (MRI) brain scans showed multiple areas of T2 hyperintensity within 1 month after onset and gradually evolving higher signals in the cerebral hemispheres, medulla, cerebellum, and C1-T6 spinal cord. MR scans showed disseminated demyelination lesions on T2 imaging within 1.5 months after onset [Figure 1A-D]. Two months following the onset of fever and headache she was given high doses of intravenous methylprednisolone (1 g/day for 3 days, 0.5 g/day for 2 days, 0.25 g/day for 1 day) for two periods of treatment. She improved significantly, could walk slowly and feed herself, had no fever, but had slow reactions and difficulty swallowing. After discharged from our hospital, within 3 months after onset, she had a very good recovery with only some residual dysphagia. Repeat serum and CSF NMDAR-Ab titers were 1:1. Repeat MR scans were clearly improved [Figure 1E-H].

DISCUSSION

Anti-NMDAR encephalitis is characterized by psychiatric disturbance, seizures, movement disorders, reduced consciousness, and positive NMDA receptor antibodies.[2] In recent years, several articles show that patients with anti-NMDAR encephalitis may develop concurrent or separate episodes of demyelinating disorders,[4] such as neuromyelitis optica spectrum disorder, multiple sclerosis and other demyelinating disorders. Attention should be paid when patients with demyelinating disorders have cooccurring mental disorders, and autonomic instability; because such unusual symptoms may overlap anti-NMDAR encephalitis. There are several articles about this.[5] Here, we report a middle-aged woman with NMDAR-Abs and clinical and radiologic evidence of ADEM, as well as follow-up after treatment.

Most patients with anti-NMDAR encephalitis will have complete or near-complete recovery. However,

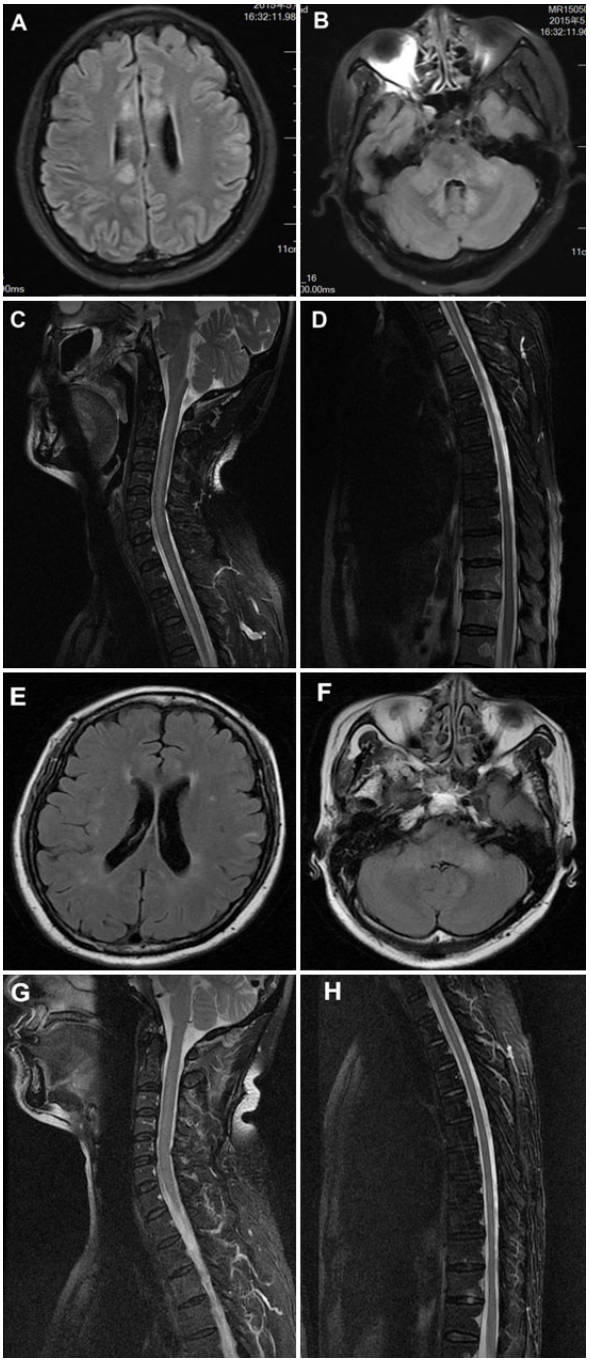

Figure 1: Magnetic resonance imaging (T2 flair) (A-D) disseminated demyelination lesions intracranial and spinal cord within 1.5 months after onset; (E-H) within 3 months after onset (in which she was treated with high dose intravenous methylprednisolone)

if it is concurrent with demyelination, there are more deficits, and more intense immunotherapies are often required.[2] Therefore, awareness that anti-NMDAR encephalitis and a demyelinating disorder may occur in the same patient is important for facilitating timely diagnosis and treatment. The pathogenic mechanism of anti-NMDAR encephalitis and demyelinating disorders coexisting is unknown. Several active immune mechanisms may likely play. One may be that functional NMDARs are composed of 5 different subunits as GRIN1, GRIN2A, GRIN2B, GRIN2C and GRIN2D, and these subunits may be associated with demyelinating disorder.[6] Additionally, Lipton's[7] work showed that oligodendrocytes and myelin contain NMDAR and that molecular mimicry or immune cross-reaction might be involved[8] in pathogenesis. However, the exact relationship between NMDAR antibodies and myelin dysfunction still needs more study.

Conventional treatments of anti-NMDAR encephalitis include corticosteroids, intravenous immunoglobulin (IVIG), and plasma exchange (TPE). These three treatments are considered "first-line" therapies for anti-NMDAR encephalitis.[9] But, in fact, IVIG and TPE are expensive or hard to get in some countries, so hormone therapy is very important.

Our case is notable for the simultaneous occurrence of anti-NMDA receptor encephalitis with ADEM-like clinical/MR features, where, after given a high dose of steroid treatment, the patient had a good prognosis. The patient's first signs were symptom of encephalitis: fever, headaches, and mental abnormalities that could not be controlled with DXM 10 mg/day and got worse. Gradually, she developed features of disseminated encephalomyelitis: confusion, difficulty walking, and urinary and fecal incontinence. With high doses of hormone therapy for two periods, the above symptoms were improved. Although early and aggressive therapy had not been given to this patient, a high dose of steroid treatment was given in the late phase[10] (1 month after onset), and had a good therapeutic effect. At 3 months after onset, her motor skills, responsiveness, and speech improved rapidly.

In conclusion, it is very important to recognize that patients with anti-NMDAR encephalitis may present as ADEM-like clinical/MRI features, simultaneously or sequentially. In addition, anti-NMDAR antibodies should be assessed in patients with encephalitis or psychiatric symptoms, even when the MRI suggests demyelinating disease. Future studies should pay more attention to these associations. In the mean time, a high dose of steroid treatment could be the first consideration. Even if given in the late phase, there may still be good effects.

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Conflicts of interest
There are no conflicts of interest.

Patient consent
Informed consent was obtained from all individual participants included in the study.

Ethics approval
Data collection in our study involving the patient is consistent with the ethical standards of the institution's ethics committee.

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Letter to Editor **Open Access**

Isolated unilateral chorea: a diagnostic challenge

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Movement disorders of paraneoplastic etiology are very rare, representing solely about 1% of all paraneoplastic neurological syndromes.[1] The most frequently associated carcinoma is small-cell lung cancer, although several others are reported such as hematologic, bowel, breast, prostate and bladder. It was reported an association with CV2/CRMP5, Anti-Hu/ANNA, CASPR2 and Yo antibodies, which are detectable in serum in most cases, isolated or in association.[1-4] However, in a minority of cases an autoantibody cannot be detected.[1] The antibody most frequently associated with paraneoplastic chorea is CV2/CRMP5, followed by Anti-Hu/ANNA. In a PNS EuroNetwork cohort[1] only 1/13 patients presented with unilateral chorea.

We present the case of a 62-year-old woman, smoker, who was observed in a neurology clinic because of involuntary movements of the left arm with one-month evolution, without any other complaint. At neurological examination, it was clear a unilateral chorea of the left limbs, worse in the left arm. The remaining examination was unremarkable. Treatment with risperidone and haloperidol were tried, without any symptom improvement. An etiology was extensively sought. The cerebral magnetic resonance imaging was normal. Hu/ANNA1-Ab and CV2/CRMP5-Ab in serum were negative. The patient refused a search for other antibodies and a lumbar puncture. A thoracic computed tomography revealed a mass in the superior lobe of the left lung. The biopsy allowed the diagnosis of a small-cell lung cancer. The patient started on chemotherapy seven months after the first symptoms and radiotherapy afterwards. Light respiratory complaints (dry cough) started only after the first chemotherapy cycle, approximately nine months after the beginning

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of the chorea. The chorea improved gradually along the first four cycles of treatment. She remained with occasional weak involuntary movements of the left hand. Unfortunately, eight months after the beginning of the oncological treatment, the disease worsened suddenly and she died a few weeks later.

Although the older age, subacute evolution of the symptoms and lack of response to neuroleptics pointed to a paraneoplastic etiology, the lack of other neurological or systemic symptoms and particularly the strict unilaterality were suggestive of other causes. There are only a few reported cases of isolated unilateral chorea of paraneoplastic etiology.[1,3,4]

Our patient started with chorea several months before the definitive diagnosis of lung cancer was made and manifested respiratory symptoms only a couple months after the beginning of chemotherapy. The most frequently associated antibodies were excluded in serum and we were unable to pursue the investigation because of patient refusal. A higher rate of detection of paraneoplastic autoantibodies in cerebrospinal fluid and serum when compared to serum alone is reported.[5] Therefore, in the present case we can't exclude an association of the hemichorea with an undetected antibody.

We would like to emphasize the great significance of an extensive and precocious search for an auto-immune and, particularly, paraneoplastic cause in patients with late-onset chorea, even when isolated and/or focal, in order to provide an early diagnosis and a precocious start of oncological treatment, to allow an improvement in the prognosis.

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There are no conflicts of interest.

Patient consent
Written consent was obtained from the patient versed in the article.

Ethics approval
This kind of article does not require document for ethics approval.

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Topic: Infectious Disease of Central Nervous System **Open Access**

Low antioxidant status of patients with central nervous system infections

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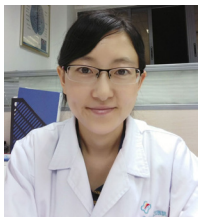

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ABSTRACT

Aim: The pathogenesis of central nervous system infections (CNSI) has not been fully understood; some studies indicated that reactive oxygen species may induce brain damage. The aim of our study was to investigate serum antioxidant status in patients with CNSI. **Methods:** The serum levels of uric acid (UA), bilirubin and albumin of 548 individuals were enrolled in our study, comprising of 114 healthy controls (HC) and 434 patients with five different kinds of CNSI, which including viral meningitis and/or meningoencephalitis, cysticercosis of brain, tuberculous meningitis and/or meningoencephalitis, cryptococcus meningitis and/or meningoencephalitis, and bacterial meningitis and/or meningoencephalitis. **Results:** The data suggested that there were reducing levels of oxidation state (serum UA, bilirubin and albumin) in CNSI patients when compared with HC. Likewise, similar results were observed when cohorts were divided into male and female subgroups. **Conclusion:** The authors demonstrated that serum antioxidant status in patients with CNSI was lower; the reason may be due to exhaustion of antioxidant capacity. Therefore, enhancing antioxidant power and keeping oxidative stress and antioxidants in balance may be beneficial to the patients with CNSI.

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INTRODUCTION

The pathogenesis of central nervous system infections (CNSI) has not yet been completely understood, but some scholars have indicated that oxidative stress and antioxidant imbalance may induce brain injury. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) may play a vital role in certain pathological processes acting as inflammatory agents causing intracranial complications.[1,2] ROS, such as superoxide anion, hydrogen peroxide or hydroxyl radicals, may result in damage to cellular DNA, proteins and lipids, and even causing cell death.[3] RNS, such as peroxynitrite (ONOO-), is a strong oxidant that can be cytotoxic including nitrotyrosine or lipidperoxidation in bacterial meningitis.[4] Oxidative stress, which plays a major role in disease pathogenesis, is defined as an imbalance in ROS and antioxidants. Cells use enzymatic and non-enzymatic antioxidants to control ROS levels to maintain an appropriate cellular redox balance. If antioxidant capacity is exhausted, an imbalance between oxidant and antioxidant status will lead to a state of oxidative stress, which could change the susceptibility of membranes and inactive biological molecules to damage.[5-7] Therefore, enhanced antioxidant protection and restored native homeostasis may be beneficial to the treatment of CNSI.

Uric acid (UA), as a scavenger of peroxynitrite, could inhibit central nervous system (CNS) inflammation and change blood-CNS barrier permeability, is considered to have a neuroprotective role.[8-10] Endogenous bilirubin converted from biliverdin, is an end product of heme degradation by biliverdinreductase (BVR).[11] Some studies confirmed that bilirubin could effectively block seizure-induced ROS and play a role in cerebroprotective effects.[12] Albumin, accounting for about 70% of the plasma colloid osmotic pressure, plays a vital role in maintaining the normal fluid distribution and constitutes the main circulating antioxidant system in the body. As potent scavengers of ROS derived from oxidative stress, albumin is the major source of extracellular reduced sulfhydryl groups (-SH).[13]

That is to say, UA, total bilirubin (Tbil) and albumin, are the major nonenzymatic antioxidant components of serum.[14] However, the situation of antioxidant status in different patterns of CNSI has not fully been investigated. Therefore, we retrospectively evaluated the antioxidant status of serum UA, bilirubin and albumin in patients with CNSI.

METHODS

We retrospectively reviewed the clinical data of 598 patients with CNSI who were hospitalized in the Third Affiliated Hospital of Sun Yat-sen University from January 2008 to June 2015. A total of 434 CNSI patients satisfying the diagnostic criteria were recruited in our study. Another 114 healthy subjects came to our hospital for check-ups at the same period and served as control patients. CNSI patients were divided into 5 groups: viral meningitis and/or meningoencephalitis (VM), cryptococcus meningitis and/or meningo-encephalitis (CM), tuberculous meningitis and/or meningoencephalitis (TM), bacterial meningitis and/or meningoencephalitis (BM), cysticercosis of brain (BC) [Figure 1]. For the diagnosis of meningitis and Neurocysticercosis, we took into account the patient history, symptomatology, along with regional epidemiology, and basic cerebrospinal fluid testing.[15,16]

Demographic and clinical features of patients with CNSI and healthy controls (HC) are shown in Tables 1 and 2.

Venous blood samples were drawn in the morning after patients' admission. Total serum UA, albumin and bilirubin concentrations were measured using a Clinical Analyzer 7180-ISE (Hitachi High-Technologies, Tokyo, Japan). Serum UA concentrations were measured using a UA assay kit based on the direct enzymatic oxidation of uric acid. Serum bilirubin concentrations were also measured by an enzymatic method with bilirubin oxidase.

The following subjects were excluded (1) patients

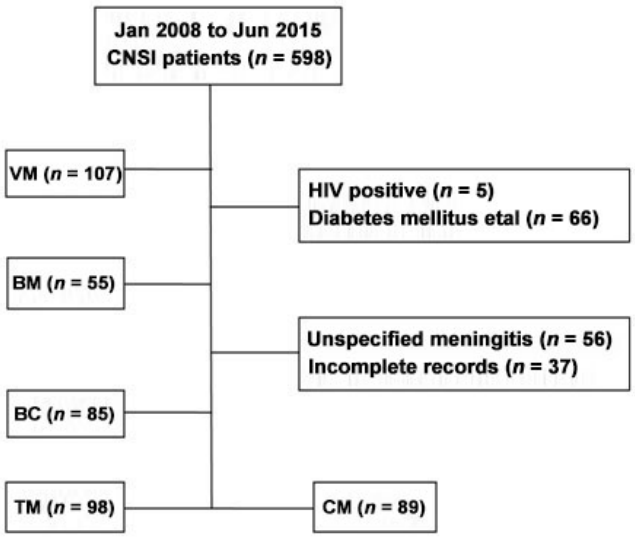

Figure 1: Enrollment process of patients. CNSI: central nervous system infections; VM: viral meningitis and/or meningoencephalitis; BC: cysticercosis of brain; TM: tuberculous meningitis and/or meningoencephalitis; CM: cryptococcus meningitis and/or meningoencephalitis; BM: bacterial meningitis and/or meningoencephalitis; HIV: human immunodeficiency virus

Table 1: Demographic of patients with CNSI and healthy control

Patients	No. of patients	Male	Female	Ages (mean ± SD)	*P*
Healthy control	114	71	43	37.76 ± 10.21	
Viral meningitis and/or meningoencephalitis	107	60	47	35.15 ± 13.38	0.061
Cysticercosis of brain	85	50	35	35.67 ± 12.75	0.231
Tuberculous meningitis and/or meningoencephalitis	98	56	42	35.98 ± 15.58	0.369
Cryptococcus meningitis and/or meningoencephalitis	89	62	27	37.81 ± 12.88	0.437
Bacterial meningitis and/or meningoencephalitis	55	40	15	35.64 ± 19.43	0.377

CNSI: central nervous system infections; *P*: ages of patients with CNSI *vs.* ages of healthy control

Table 2: Presenting clinical symptoms of CNSI (%)

Characteristics	Viral meningitis and/or meningoencephalitis	Cysticercosis of brain	Tuberculous meningitis and/or meningoencephalitis	Cryptococcus meningitis and/or meningoencephalitis	Bacterial meningitis and/or meningoencephalitis
Number	107	85	98	89	55
Headache	105 (98.1%)	46 (54.1%)	98 (100%)	89 (100%)	55 (100%)
Fever	103 (96.3%)	0	96 (97.9%)	79 (88.8%)	54 (98.2%)
Vomit	69 (64.5%)	12 (14.1%)	57 (58.2%)	71 (79.8%)	42 (76.4%)
Seizure	4 (3.7%)	53 (62.4%)	9 (9.18%)	6 (6.74%)	1 (1.82%)

CNSI: central nervous system infections

Table 3: Serum UA, bilirubin and albumin levels in patients with CNSI and HC (mean ± SD)

Patients	Total	Male	Female	$P^{1\dagger}$	$P^{2\dagger}$	$P^{3\dagger}$
UA (μmol/L)						
CNSI	253.91 ± 135.90	278.59 ± 146.33	214.97 ± 107.18			
HC	336.31 ± 109.36	389.00 ± 99.95	252.13 ± 88.43	< 0.001	< 0.001	0.018
Tbil (μmol/L)						
CNSI	13.67 ± 9.20	14.89 ± 10.41	11.69 ± 6.19			
HC	15.88 ± 6.03	16.92 ± 5.95	15.47 ± 5.97	0.015	0.099	0.001
Ibil (μmol/L)						
CNSI	10.18 ± 6.43	10.98 ± 7.23	8.87 ± 4.51			
HC	12.83 ± 4.51	13.48 ± 5.04	12.66 ± 4.46	< 0.001	0.005	< 0.001
Albumin (g/L)						
CNSI	41.80 ± 5.16	42.15 ± 5.33	41.26 ± 4.83			
HC	47.10 ± 2.83	47.64 ± 2.93	46.95 ± 2.39	< 0.001	< 0.001	< 0.001

$P^{1\dagger}$: CNSI *vs.* HC; $P^{2\dagger}$: male patients with CNSI *vs.* male HC; $P^{3\dagger}$: female patients with CNSI *vs.* female HC; UA: uric acid; CNSI: central nervous system infections; HC: healthy control; Tbil: total bilirubin; Ibil: indirect bilirubin

with diabetes mellitus, renal failure, malignancies, abnormal liver function or human immunodeficiency virus infection; (2) patients who were taking diuretics, aspirin or other drugs that could affect serum UA levels; and (3) individuals who refused to participate in the study.

Statistical analysis

Data were analyzed using SPSS statistical software (version 17.0, Chicago, IL, USA). Numerical variables were presented as mean ± standard deviation (SD), and categorical variables were expressed as percentage. A *P* value < 0.05 was considered significant. In order to reduce the effect of age, the differences between serum UA, bilirubin and albumin levels were analyzed using covariance analysis with age as covariant. Moreover, patients within each group were divided into male and female subgroups to eliminate the gender effect. Least significant difference *t*-test was used to test distinction between each two groups.

RESULTS

The analysis data for serum UA, bilirubin and albumin levels in patients with CNSI and HC are shown in Table 3. We observed that total serum UA levels of CNSI patients (253.91 ± 135.90 μmol/L) were significantly lower when compared with HC group (336.31 ± 109.36 μmol/L, *P* < 0.001). In each group of CNSI, serum UA levels were significantly lower than HC independent of the classification [Table 4]. Besides, in order to reduce the effect of gender, we divided each group into male and female subgroups. In male subgroups, serum total UA levels of CNSI (278.59 ± 146.33 μmol/L) were significantly lower than male HC (389.00 ± 99.95 μmol/L, *P* < 0.001) [Table 3]. In addition, in each male CNSI group, serum UA levels were also lower than those of male HC (*P* < 0.05) [Table 4]. In female groups, we found that levels of serum UA in female CNSI (214.97 ± 107.18 μmol/L) were also lower than female HC (252.13 ± 88.43 μmol/L, *P* = 0.018, Table 3). Equally, in each group of female CNSI,

Table 4: Serum UA, Tbil, Ibil, and albumin levels of patients with each group of CNSI (mean ± SD)

Patients	Total	Male	Female	$P^{1\dagger}$	$P^{2\dagger}$	$P^{3\dagger}$
Serum UA levels (μmol/L)						
Viral meningitis and/or meningoencephalitis	261.94 ± 107.88	285.89 ± 101.81	230.17 ± 108.56	< 0.001	< 0.001	0.227
Cysticercosis of brain	275.20 ± 92.02	319.87 ± 77.33	213.44 ± 73.63	0.001	0.005	0.066
Tuberculous meningitis and/or meningoencephalitis	286.77 ± 197.39	339.49 ± 220.30	215.23 ± 133.18	0.003	0.022	0.080
Cryptococcus meningitis and/or meningoencephalitis	224.46 ± 123.41	233.96 ± 127.26	201.82 ± 112.82	< 0.001	< 0.001	0.042
Bacterial meningitis and/or meningoencephalitis	197.27 ± 93.73	100.95 ± 96.98	186.22 ± 86.21	< 0.001	< 0.001	0.034
Serum Tbil levels (μmol/L)						
Viral meningitis and/or meningoencephalitis	15.75 ± 9.93	18.41 ± 11.78	12.29 ± 5.18	0.682	0.336	0.018
Cysticercosis of brain	10.91 ± 4.78	11.54 ± 4.89	10.01 ± 4.55	< 0.001	0.003	< 0.001
Tuberculous meningitis and/or meningoencephalitis	14.41 ± 11.15	15.19 ± 13.13	13.35 ± 7.73	0.111	0.350	0.111
Cryptococcus meningitis and/or meningoencephalitis	13.29 ± 9.22	14.51 ± 9.78	10.45 ± 7.10	0.011	0.164	0.001
Bacterial meningitis and/or meningoencephalitis	13.16 ± 7.74	13.87 ± 8.50	11.27 ± 4.96	0.025	0.121	0.025
Serum Ibil levels (μmol/L)						
Viral meningitis and/or meningoencephalitis	11.90 ± 7.43	14.06 ± 8.87	9.11 ± 3.68	0.181	0.550	0.001
Cysticercosis of brain	9.04 ± 3.93	9.40 ± 3.86	8.52 ± 4.02	< 0.001	0.001	< 0.001
Tuberculous meningitis and/or meningoencephalitis	9.93 ± 7.48	10.28 ± 8.78	9.47 ± 5.29	< 0.001	0.011	0.002
Cryptococcus meningitis and/or meningoencephalitis	9.49 ± 5.82	10.21 ± 6.02	7.81 ± 5.03	< 0.001	0.007	< 0.001
Bacterial meningitis and/or meningoencephalitis	10.11 ± 5.68	10.46 ± 6.00	9.17 ± 4.79	0.003	0.028	0.017
Serum albumin levels (g/L)						
Viral meningitis and/or meningoencephalitis	42.65 ± 5.03	42.73 ± 5.69	42.54 ± 4.05	< 0.001	< 0.001	< 0.001
Cysticercosis of brain	42.82 ± 3.08	43.18 ± 3.05	42.29 ± 3.10	< 0.001	< 0.001	< 0.001
Tuberculous meningitis and/or meningoencephalitis	41.40 ± 5.24	42.34 ± 4.81	40.13 ± 5.58	< 0.001	< 0.001	< 0.001
Cryptococcus meningitis and/or meningoencephalitis	41.47 ± 5.27	42.52 ± 4.95	39.02 ± 5.27	< 0.001	< 0.001	< 0.001
Bacterial meningitis and/or meningoencephalitis	39.90 ± 6.87	39.11 ± 7.16	41.99 ± 5.71	< 0.001	< 0.001	< 0.001

$P^{1\dagger}$: CNSI *vs.* HC; $P^{2\dagger}$: male patients with CNSI *vs.* male HC; $P^{3\dagger}$: female patients with CNSI *vs.* female HC; UA: uric acid; CNSI: central nervous system infections; HC: healthy control; Tbil: total bilirubin; Ibil: indirect bilirubin

we found serum UA levels were lower than those of female HC, as shown in Table 4.

For serum Tbil and indirect bilirubin (Ibil), we found that serum total levels in CNSI were significantly lower than HC (13.67 ± 9.2 μmol/L, $P = 0.015$ and 10.18 ± 6.43 μmol/L, $P < 0.001$) respectively. In each group of CNSI, serum Tbil levels of patients with BC, CM, and BM were significantly lower when compared with HC. For serum Ibil levels, patients with CNSI were also lower, except for VM [Table 4]. Besides, we further divided each group into male and female subgroups. In both subgroups, compared to HC, we found that serum Tbil and Ibil levels in CNSI were significantly lower, except for male serum Tbil ($P = 0.099$, Table 3).

For albumin, we found that total serum levels in CNSI were lower than HC (41.80 ± 5.16 and 47.10 ± 2.83, $P = 0.000$, Table 3). We came to a similar conclusion in each group of CNSI. Interestingly, similar results have been observed in male and female subgroups as well [Table 4].

DISCUSSION

Our study showed that there were reducing serum levels of UA, bilirubin and albumin in CNSI patients. Interestingly, these results were also observed when we divided cohorts into male and female groups. Our studies have shown that patients with CNSI have lower serum UA, which were consistent with previous reports.[17] Similar results that CNSI patients with lower serum albumin and bilirubin levels than HC groups were also observed. Similar relevant results were not

reported before. In conclusion, our findings suggest that there were low serum levels of UA, bilirubin and albumin in patients with CNSI.

The mechanisms of CNS damage during meningitis have not been conclusively identified. Increasing evidence shows a massive production of ROS and RNS could lead to significant collateral damage in pneumococcal meningitis, because of their toxic actions, such as lipid peroxidation, DNA strand breakage and production of inflammatory cytokines. Therefore, a potential use of oxidative inhibitors as an adjunctive treatment could be beneficial in treating meningitis.[18] Our work showed that patients with CNSI had low serum UA, Tbil, Ibil and albumin levels. The notion is that oxidative damage plays a crucial role in CNSI, which may be because of the low antioxidant status.

UA is a natural antioxidant in the blood and brain, which has been shown that exogenous administration of urate is protective in experimental bacterial meningitis.[19] UA is capable of scavenging free radicals and chelating transitional metal ions by preventing peroxynitrite induced protein nitrosylation, lipid and protein peroxidation, and inactivation of tetrahydrobiopterin.[20] Low levels of UA are detrimental to neurons, while high levels of UA contribute to neuroprotection.[21,22]

Our study showed that serum levels of UA in patients with CNSI were lower, therefore, increasing in UA concentration has been suggested as one of the possible mechanisms as a replacement therapy. Generally speaking, serum UA levels are highly sex and age dependent. In this study, we further divided each group into female and male subgroups,[23] we found that in all groups, men had higher serum UA levels than women which is consistent with previous studies.[17,24]

Bilirubin, the end product of heme metabolism, is formed from biliverdin. As the products of Hemeoxygenase (HO)-catalyzed heme breakdown, it has an essential cerebroprotective role.[11,12] There are two major forms of HO, existing HO-1 and HO-2. It is generally accepted that the elevated HO-1 levels represent an attempt to restore redox homeostasis and to down-modulate inflammation.[25] HO-2, the constitutively active isoform, has an essential cerebroprotective role against seizure-induced loss of endothelial vasodilator function in newborn piglets.[26] Some observations suggested that bilirubin, as a potent antioxidant, could reduce cerebrovascular complications in the seizures of newborn babies.[12] Some scholars provided that bilirubin could be as neuroprotectant against oxidative stress injury.[27] Moreover, increasing evidences suggested that bilirubin also possessed multiple biological activities, including potential immunomodulatory properties.[28] Our results supported the finding that serum bilirubin levels in CNSI were lower than the control group, in spite of there being no apparent difference when compared with multiple sclerosis (MS). Furthermore, gender differences also existed, and we found that women also had lower mean serum bilirubin values when compared with men.

Otherwise, some researchers demonstrated that serum albumin, specifically block echovirus by inhibiting the uncoating step in the virus replication cycle. That is to say that in man, echovirus infection may be modulated by serum albumin.[29]

Imbalanced metabolism and excess free radical generation could cause oxidative stress, which has been defined as a principle pathological cause of neurodegenerative disorders, such as MS,[23] Parkinson's disease.[30] Therefore, it could be favorable for increasing antioxidant levels, such as UA, bilirubin and albumin levels in CNSI, which should not focus only on anti-infective therapy, but also on the antioxidant effects.

In conclusion, low serum UA, bilirubin and albumin levels in CNSI patients were found in our study, the reason may be due to exhaustion of antioxidant capacity. An imbalance between oxidant and antioxidant activities will result in the development of intracranial inflammation and destruction of neurons. These findings may increase our understanding of pathophysiology of CNSI. Therefore, to enhance antioxidant power and keep oxidative stress and antioxidant in balance may be beneficial to patients with CNSI, that is to say, improving the clinical and laboratory results in CNSI, by elevating serum UA, bilirubin and albumin could be considered.

In the future, we plan to measure the antioxidant levels in cerebrospinal fluid (CSF) of CNSI patients, and we may compare the antioxidant status of blood and CSF in these patients.

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Conflicts of interest
There are no conflicts of interest.

Patient consent
Informed consent was obtained from all individual participants included in the study.

Ethics approval

Data collection in our study involving the patient is consistent with the ethical standards of the institution's ethics committee.

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Editorial **Open Access**

Endocannabinoid metabolism in neurodegenerative diseases

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Dr. Chu Chen is a tenured professor in the Neuroscience Center of Excellence, Louisiana State University Health Sciences Center in New Orleans, USA. The research programs in Dr. Chen's laboratory focus on neuroinflammation in health and disease. His current research interests are in endocannabinoid signaling in neurodegenerative diseases.

Endocannabinoids are endogenous lipid mediators contributing to a variety of physiological, pharmacological, and pathological processes primarily through acting on cannabinoid receptors (CB1R and CB2R), which are targets of Δ^9-tetrahydrocannabinol (Δ^9-THC), the major psychoactive ingredient in marijuana.[1] Although N-arachidonoyl ethanolamide is the first identified endocannabinoid, 2-arachidonoylglycerol (2-AG), the second identified endocannabinoid, is the most abundant ligand produced in our body and a full agonist for CB1R and CB2R.[2] It has been well recognized that 2-AG is a retrograde messenger modulating synaptic transmission and plasticity at both inhibitory GABAergic and excitatory glutamatergic synapses in the brain.[2-4] In particular, augmentation of 2-AG signaling by inhibition of its metabolism has been attracted attention recently due to its profound anti-inflammatory and neuroprotective properties.[4,5]

2-AG is synthesized largely from diacylglycerol by diacylglycerol lipase α and β and primarily hydrolyzed by the enzyme monoacylglycerol lipase (MAGL) to glycerol and arachidonic acid (AA), a precursor of prostaglandins and leukotrienes [Figure 1]. And 2-AG is also degraded by the enzymes α/β hydrolase domain-containing protein 6 and 12 and metabolized oxidatively by cyclooxygenase 2 (COX-2). Apparently, 2-AG is a very unstable bioactive lipid mediator, and it is easily and rapidly degraded by these enzymes upon its synthesis.[2] It has been estimated that 85% of 2-AG is metabolized by MAGL in the brain.[6] Arachidonic acid-delivered prostaglandins and leukotrienes are generally proinflammatory and neurotoxic, whereas 2-AG is capable of resolving neuroinflammation and protecting neurons from harmful insults.[4,5,7] This suggests that

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disruption of MAGL would be a promising strategy to enhance anti-inflammatory and neuroprotective 2-AG signaling, while reducing proinflammatory and neurotoxic eicosanoid (e.g. PGE_2) levels [Figure 1].

Alzheimer's disease (AD) is the most common cause of dementia in elderly. While the etiology of AD is multifactorial, accumulating evidence implicates traumatic brain injury (TBI) as an epigenetic risk factor in AD development and dementia. Chronic traumatic encephalopathy (CTE) is the most recently defined TBI-caused neurodegenerative disease, and neuropathology and neurocognitive deficits in CTE are similar to those in AD.[4,8-10] The significant similarities and overlap in the spectrum of changes in neuropathology, neurobiology, synaptic and neurocognitive deficits between CTE and AD suggest that CTE, in essence, is a TBI-triggered AD-like neurodegenerative disease. Repetitive or multiple brain injuries may lead to AD-like neuropathology, impairments in synaptic and cognitive functions, and dementia.[8-10] However, there are currently no effective therapies to prevent and treat AD and TBI-caused AD-like neurodegenerative disease or to halt progression of diseases. Earlier studies show that 2-AG protects neurons against brain trauma in a mouse model of closed head injury.[7] Recent studies provide evidence that inactivation of MAGL reduces neuroinflammation, Aβ accumulation and deposition, tau phosphorylation, and neurodegeneration and improves synaptic and neurocognitive functions in several animal models of neurodegenerative diseases, including AD, Parkinson's disease (PD), and TBI.[8,11-14] This suggests that manipulations of 2-AG metabolism may provide novel pharmacotherapies for these intractable neurodegenerative diseases.[4,8,11,12]

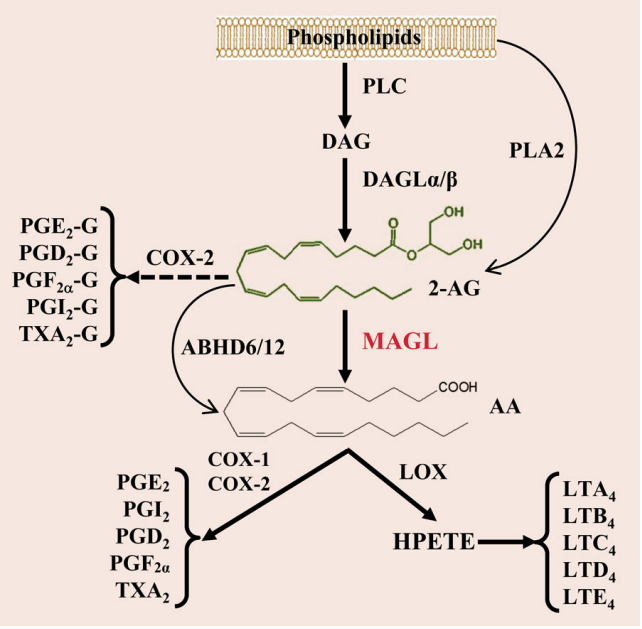

Figure 1: Pathways of 2-AG synthesis and metabolism. 2-AG is largely synthesized from DAG, which is formed from membrane phospholipids through PLC, by DAGLα/β. 2-AG in the brain is primarily hydrolyzed by MAGL to glycerol and AA. It is also hydrolyzed by ABHD6/12 to AA and metabolized oxidatively by COX-2 to form a new type of prostaglandin glycerol esters when expression and activity of COX-2 are excessively elevated during inflammation. AA is a precursor of prostaglandins through the enzymes COX-1/2 and HPETE through the enzyme arachidonate 5-LOX to form leukotrienes (LTA4 to E4). 2-AG: 2-arachidonoylglycerol; PLC: phospholipase C; DAG: diacylglycerol; DAGLα/β: diacylglycerol lipase α and β; PLA2: phospholipase A2; AA: arachidonic acid; COX-2: cyclooxygenase-2; MAGL: monoacylglycerol lipase; ABHD6/12: α/β hydrolase domain-containing protein 6 and 12; HPETE: hydroperoxyeicosatetraenoic acid; LOX: lipoxygenase

Although disruption of MAGL ameliorates neuropathology and prevent synaptic and cognitive declines in animal models of neurodegenerative diseases,[4,8,11-14] the signaling pathways that mediate these beneficial effects produced by MAGL inhibition are still unclear. MAGL loss-of-function enhances anti-inflammatory and neuroprotective 2-AG signaling and decreases proinflammatory and neurotoxic prostaglandins and leukotrienes [Figure 1]. It is possible that the beneficial effects produced by MAGL inhibition are mediated either by enhanced 2-AG signaling through CB1R or CB2R-depdendent mechanism or by reduced eicosanoid levels through cannabinoid receptor-independent mechanisms.[4,8,11-14] It has been proposed that peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor that displays significant anti-inflammatory properties, is a target of endocannabinoids.[15,16] Recent studies demonstrated that suppression of neuroinflammation and alleviation of AD neuropathology by 2-AG or inactivation of MAGL are mediated via PPARγ,[5,11,16] suggesting that PPARγ is likely a downstream signaling molecule of 2-AG. Despite the fact that the mechanisms by which inhibition of 2-AG metabolism alleviates neuroinflammation and neuropathology and prevents deterioration in synaptic and cognitive functions in animal models of AD, PD, and TBI remain to be elucidated, MAGL is a promising therapeutic target for neurodegenerative diseases.

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Editorial **Open Access**

On the need to unify neuroscience and physics

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Neuroscience is a relatively new research field that, so far, has resulted in important progress in understanding the physiology, biochemistry, pharmacology, and structure of the vertebrate brain.[1] Because of this progress, spectacular technological developments,[2] i.e. positron emission tomography, functional magnetic resonance imaging, transcranial magnetic stimulation (TMS), diffusion tensor imaging, magneto-encephalography, electro-encephalography, *etc*., and new treatments based on them, such as high-frequency repetitive TMS (rTMS),[3] deep brain stimulation,[4] *etc*., have been of great use. However, despite those technical and clinical successes in neuroscience, in which the advances in physics[5] have played a substantial role, one fundamental problem is still unsolved, namely, how to unify neuroscience and physics?[6] As we will discuss in the present editorial, not only is this problem important from a purely fundamental, theoretical perspective, but it is also vital for the development of more optimal treatments in clinical neuroscience.

In recent years, we have seen fascinating new discoveries in the field of neuroscience, such as the brain's dark energy,[7] the existence of a default mode network,[8] *etc*., and as a result of those discoveries, views on the human brain's processing and functioning have been evolving. For a long time, brain function was studied by investigating physiological responses to environmental demands.[9] However, although this

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is an interesting approach, this is only a small part of the story because this theoretical framework does not take into account that a large part of the brain's energy is devoted to intrinsic neuronal signaling instead of extrinsic demands.[10] Here, worthy of important note is that a miswiring of brain regions involved in the default mode has been suggested to play a significant role in various neurological diseases, such as Alzheimer's disease, schizophrenia, *etc*.[10] Last year, the existence of a so-called "extrinsic mode network" was proposed.[11] This network is considered to be complementary to the default mode network; when one of them is up-regulated, the other is down-regulated. In this case, the extrinsic mode network would be responsible in tasks whereas the default mode network would be activated in task-absent situations.[11] The extrinsic mode network is conceptualized to be a cortical network for "extrinsic" neuronal activity while the default mode network is considered to be a cortical network for "intrinsic" neuronal activity. Despite the fact that these frameworks are theoretically useful, they can only explain one part of brain functioning because they do not explain how the human brain (and organism) is embedded in and connected to the world around it. In other words, a complete neurological theory of human (un)conscious brain processing should always be explainable and describable by the laws of physics because the human species is part of nature and is subject to nature's underlying fundamental laws.

In other words, to date, the link between physics and neuroscience has been missing, so now is the time to find this missing link.[6] Many attempts have been made to build a bridge between physics and neuroscience, but somehow the puzzle remains incomplete. For instance, one of those attempts is the so-called "Orchestrated objective reduction (Orch-OR)" hypothesis,[12] but proving the existence of quantum processing in microtubules turned out to be difficult,[13] which made neuroscientists skeptical about this possible solution. In addition, many neuroscientists consider it to be a poor model of brain physiology.[14] Nevertheless, Penrose and Hameroff seem correct in their assumption[12] that a theory of everything[15] should not only fit within the laws of classical and quantum mechanics but also fit within the fundamental laws of neuroscience. Note that in fact, the human brain is a conglomeration of atomic constituents, and as a result, it should be subject to the laws of physics.[16]

However, perhaps the solution to this apparently unsolvable problem is simple; it might have to do with the way in which we describe nature and with the difference in human "conscious" versus "unconscious" brain processing. Although at the conscious level, we do not have the impression that our brain processing is connected to and interacting with the environment surrounding us, this might be the case at the unconscious level (e.g. think about the special role of the observer's[17] (un)conscious brain processing and interaction in physics[6]). At the conscious level, we have the impression that we can only consciously interact with the environment that surrounds us, for instance, when we kick a ball, the ball will move. Humans observe themselves as an individual information processing entity not continuously interacting with and not continuously connected to the information around them (for instance, we do not notice this when we are asleep). Only the laws of classical mechanics seem to apply to us, but this cannot be the reality; it must be a human illusion. Moreover, we tend to overemphasize the importance of conscious human information processing[18] because as a matter of fact, like an iceberg where on average only 10% is visible above the surface of the water, most of the information processing during the day, and particularly during the night,[19] is done unconsciously.

To conclude, neuroscience is a relatively new research field, so many discoveries are still to be made. Moreover, further technical advancements are required because the temporal resolution of the neuroimaging techniques available today is way too slow[20] to detect any kind of quantum processing in the human brain. To date, we do not fully understand the underlying physics of the brain; consequently, we are influencing processes (for instance, when we use brain stimulation for clinical purposes in neuroscience) that we do not completely understand. A better understanding of the brain's underlying processes is needed before those brain stimulation treatment techniques can be applied without any risks.[21] Finally, perhaps the time has come to re-think physics,[22] and although classical and quantum mechanics have helped us to describe nature; in fact, what is really needed to take the next step is to consider the whole universe/nature as simple information[23] in which the human brain/organism is only a tiny information processing system embedded in and interacting with that universe/nature.[6]

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Patient consent

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Topic: Infectious Disease of Central Nervous System **Open Access**

Screening of genetic loci predisposing to herpes simplex virus infection on mouse chromosome 17

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Herpes simplex virus, susceptibility to infection, microsatellite site, association analysis, mouse chromosome 17

ABSTRACT

Aim: The herpes simplex virus (HSV), one of the most common viruses infecting humans, is featured by a high infection rate and usually causes complex disorders difficult to diagnose and treat. Disease progression is always combined with the specific interaction between organism and environment, but genetic factors play a decisive role in most pathogenic processes. Like most human disorders, individual difference has also been involved in the pathogenesis of HSV infection. The present study aimed to screen the potential gene loci that regulates human predisposition to HSV infection. **Methods:** With reference to previous studies, inbred mouse lines with significantly distinct predisposition to HSV infection were chosen for gene loci screening. Gene sites on mouse chromosome 17 associated with susceptibility to HSV infection were then identified by correlation analysis and genome-wide scanning technique. **Results:** Genes affecting the vulnerability of mice to HSV infection were mapped to three regions on the 17th mouse chromosome, D17MIT51.1, D17MIT39.1 and the region between D17MIT180.1 and D17MIT184. **Conclusion:** The results suggest that the mouse genetic background plays an important role in its susceptibility to HSV-1 infection, which might be regulated by multiple predisposing quantitative trait loci.

INTRODUCTION

As an infection relapse could confer severe consequence in the pathogenicity of herpes simplex virus (HSV) infection, avoiding infection and preventing recurrence after treatment is of great importance. Individual differences involved in the pathogenesis of HSV infection in mice have been long studied. Several reports related to HSV infection susceptibility further pointed to the role of genetic background in the HSV infection process.[1-5] To further analyze the susceptibility to HSV infection in different inbred mouse lines, we scanned the 17th mouse chromosome for gene sites associated with it using the correlation analysis and

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genome-wide scanning technique.

METHODS

Genomic DNA extraction

Firstly, 50-100 frozen tissue samples were weighed, grinded into powder in liquid nitrogen using a grinding bowel and pestle, and then immediately mixed with 1 mL Tripure. Tissue sample powder was then further homogenized 10 times using a homogenizing drill on ice for 20 min until no tissue fluid particles was visible, centrifuged at the speed of 12,000 *g* at 4 ℃, for 10 min. The homogenate was then kept at room temperature for 5 min to make sure the nuclear protein was totally separated. Each milliliter homogenate was mixed with 0.3 mL chloroform and shaken vigorously at 4 ℃ for 15 s, kept at room temperature for 2-15 min, then Centrifuged at 12,000 *g* at 4 ℃, for 15 min. To get high quality DNA, the upper layer of colorless aqueous liquid after centrifugation was removed. Each milliliter homogenate was mixed with 0.2 mL 100% ethanol which was stored at 4 ℃, mixed completely by rotating the bottle upside down several times and kept at room temperature for 2-3 min for DNA precipitation. After centrifuging at 2,000 *g* for 5 min at 4 ℃, the upper layer liquid was removed with a pipette carefully. Each milliliter sample solution was then mixed with 1 mL of 0.1 mol/L sodium citrate dissolved in 10% ethanol, kept at room temperature for 30 min with frequent mixing, and centrifuged at 4 ℃ for 5 min at 2,000 *g* again. The upper layer liquid was collected, mixed with 75% ethanol, and kept at room temperature for 30 min with frequent mixing. Then, after centrifuging at 4 ℃ and 2,000 *g* for 5 min, the upper layer liquid was removed and the DNA sample was dried in the air or vacuum for 5-10 min. Finally, we dissolved the DNA with 50 uL TE solution, pipetted out 1 uL sample for color comparison and another 10 uL for electrophoresis, and the residual was stored at -20 ℃ for further analysis. DNA samples were diluted with 90 uL MQ water and analyzed with ultraviolet spectrophotometer. The OD260 value, OD280 value and OD260/280 value were used for calculating the concentration of DNA.

Primer design and synthesis

Primer design referenced information from the mouse genome program (detailed information can be viewed on the website of Jackson Laboratory), which was designed by Shanghai Jikang Biotechnology Limited Company [Table 1]. The detailed information about PCR reaction system and PCR reaction condition can be seen in Table 2.

Microsatellite loci detection

First, 1 mL Hi-Di Formamide was mixed with 50 uL GeneScan -500 LIZ Size Standard, then mixed with 9.5 uL polymerase chain reaction (PCR) product, which was diluted 20 times. Tubes containing the above solution were placed in the PCR instrument for degeneration at 95 ℃ for 5 min, kept on ice for more than 5 min. Using an ABI PRISMTM 310 genetic analyzer of the ABI Company for electrophoresis, the voltage was set to 15 KV and run at 60 ℃ for 28 min. The samples were then collected for further analysis.

Electrophoresis data processing

By using the software Genescan (311) and Genetyper (3.7), we could get the detected fragment size. The equipment was provided by ABI Company; the PCR amplification reagents by Baosheng Bioengineering Limited Company; and the fluorescent primers by Shanghai Jikang Biotechnology Limited Company.

RESULTS

Scanning 12 microsatellite spots on the 17th chromosome of two mouse lines

The differences of 12 microsatellite loci between two inbred mouse lines BALB/C and C57BL/6 were first scanned. Among the loci scanned, seven of them were significantly different: D17MIT245.1, D17MIT46, D17MIT51.1, D17MIT180.1, D17MIT20. 1, D17MIT184 and D17MIT39.1 [Table 3; Figure 1].

Scanning microsatellite loci on the 17th chromosome using three inbred mouse lines

To minimize false-positives among the above seven sites obtained using the two inbred mouse lines, we further searched the literature and found that another inbred mouse line, DBA-2, had similar susceptibility to HSV infection as BALB/C mice. Therefore, scanning these three inbred mouse lines for microsatellite loci led to the exclusion of two of the above seven loci, D17MIT245.1 and D17MIT46. Our updated scanning results showed that D17MIT51.1, D17MIT39.1 and the genomic region between D17MIT180.1 and D17MIT184 were mouse microsatellite regions affecting susceptibility to HSV infection [Table 4; Figure 2].

Bioinformatic analysis of genes in the HSV infection susceptibility regions on chromosome 17

For identifying potential genes involved in the susceptibility of mice to HSV infection, we used bioinformatics to analyze the genes localized in these regions. Based on the above results, bioinformatic analysis found approximately 140 genes in the positive sites D17MIT51.1, D17MIT39.1 and the region between D17MIT180.1 and D17MIT184 [Tables 5-7]. Among those genes, there were about 33 human

Table 1: Primer sequences for scanning microsatellite loci on the 17th chromosome of mouse genome

Loci name		Primer sequence	Genetic distance (cM)
D17MIT245.1	Forward	FAM-TGTGCTCTGGCTAGGGAGTT	3
	Reverse	CACATTCATATGTACACACACATGC	
D17MIT143.2	Forward	FAM-CTTACAAGCATCCTGTGGAACTC	5
	Reverse	GAGGACCAACAGTCAAACATAGC	
D17MIT46	Forward	FAM-TCCACCCCACTACCTGACTC	11.7
	Reverse	CCCTTCTGATGACCACAGGT	
D17MIT146.1	Forward	FAM-CTGTCAGCAGAACGTTCCTTAGT	17.1
	Reverse	CCAACTCAAGCCTTACATAGTGG	
D17MIT51.1	Forward	FAM-TCTGCCCTGTAACAGGAGCT	22.9
	Reverse	CTTCTGGAATCAGAGGATCCC	
D17MIT10.1	Forward	FAM-TGCACTTGCATAAGGAAAAC	24.5
	Reverse	GACTTTGGGGCCTACTTATG	
D17MIT180.1	Forward	FAM-AGACACTGTCTAAAAACACAAGATGG	29.4
	Reverse	TTGTGTTCATATGCATGTGTGC	
D17MIT20.1	Forward	FAM-AGAACAGGACACCGGACATC	34.3
	Reverse	TCATAAGTAGGCACACCAATGC	
D17MIT184	Forward	FAM-TGCACTACCCAAACATGCAT	38.5
	Reverse	ACTTCTGACAGGAAGCATCCA	
D17MIT93.1	Forward	FAM-TGTCCTTCGAGTGTTTGTGTG	44.5
	Reverse	TCCCCGGTGAATGAGTTATC	
D17MIT39.1	Forward	FAM-CCTCTGAGGAGTAACCAAGCC	45.3
	Reverse	CACAGAGTTCTACCTCCAACCC	
D17MIT122.1	Forward	FAM-TCTCTTCACTGCAATGGAACA	51.9
	Reverse	GAACCTATAGGCTCTTGAATAGATGG	

homologous genes that showed some of the following characteristics: (1) containing many quantitative trait loci, such as epididymal fat pad weight quantitative trait loci (QTL) 3, subcutaneous fat pad weight QTL 4, spleen weight QTL 9, *etc*.; (2) containing some genes related to the important physiological functions of the body such as Mut methylmalonyl-Coenzyme A mutase; (3) containing genes related to the developmental and physiological function such as early growth adjusted QTL 2, early growth QTL 5, *etc*.; (4) containing genes associated with some diseases such as the Down syndrome critical region gene 1-like 1, MSM lymphoma resistance 1, *etc*.; (5) containing mouse tissue associated antigen H-2.

Table 2: PCR reaction system and PCR reaction condition

PCR reaction system (total volume: 10 uL)	
Non-enzyme water	5.4 μL
10 × PCR buffer	1.0 uL
Mg^{2+} (25 mmol/L)	0.5 uL
dNTP (each 2.5 mmol/L)	1.0 μL
P1 (5 pM)	0.5 uL
P2 (5 pM)	0.5 uL
Template DNA (30-50 ng/uL)	1.0 uL
Ex-Taq enzyme (5 U/μL)	0.1 μL
PCR reaction condition	
95 ℃	5 min
94 ℃	30 s
Time	30 s
72 ℃	30 s
Repeat the 2nd to 4th steps for totally 38 cycles	
72 ℃	10 min
Store at 4 ℃	

PCR: polymerase chain reaction

DISCUSSION

It has been widely observed that different species or even individuals of the same species show differences in response to infection, but the explanation for this phenomenon is still rather controversial. There have

Table 3: Microsatellite loci scanning using BALB/C and C57BL/6 inbred mice

Microsatellite loci	BALB/C susceptible	C57BL/6 tolerant
D17MIT245.1	**194**	**202**
D17MIT143.2	112	112
D17MIT46	**218**	**236**
D17MIT146.1	166	166
D17MIT51.1	**152**	**154**
D17MIT10.1	155	155
D17MIT180.1	**139**	**137**
D17MIT20.1	**163**	**175**
D17MIT184	**126**	**128**
D17MIT93.1	155	155
D17MIT39.1	**86**	**104**
D17MIT122.1	141	141

Microsatellite loci with difference were marked in bold

Table 4: Microsatellite loci scanning using BALB/C, DBA-2 and C57BL/6 inbred mice

Microsatellite loci	Susceptible		Tolerant
	BALB/C	DBA-2	C57BL/6
D17MIT245.1	194	200	202
D17MIT143.2	112	112	112
D17MIT46	218	208	236
D17MIT146.1	166	166	166
D17MIT51.1	**152**	**152**	**154**
D17MIT10.1	155	149	155
D17MIT180.1	**139**	**139**	**137**
D17MIT20.1	**163**	**163**	**175**
D17MIT184	**126**	**126**	**128**
D17MIT93.1	155	169	155
D17MIT39.1	**86**	**86**	**104**
D17MIT122.1	141	123	141

Microsatellite loci with difference were marked in bold

been reports suggesting that the genetic background might play an important role.[1,6-8] The causative factors for different responses to infection, the possible ways of intervention, the revolutionary changes of infection prevention, and the control that resulted from those changes have aroused great interest among the scientific community. In this study, we analyzed the genetic background that contributes to the HSV infection susceptibility.

The Herpes virus genus (Herpesviridae) is among the enveloped, linear, double-stranded DNA viruses that widely exist in nature. Approximately 100 HSV species have already been identified or partially identified. Among them, two HSV species, SV-1 and HSV-2, that share 50% homology, have been closely associated with humans. According to statistics provided by the WHO, approximately 70% of the total population worldwide carries HSV antibodies and more than one-third suffers from recurrent HSV infection. Along with its high prevalence rate, a variety of human diseases are closely related with HSV infection, including human herpes labialis, herpes conjunctivitis, 20 herpes zoster encephalitis and other diseases causing great harm to human health. HSV encephalitis is the most common, sporadic, viral encephalitis, accounting for 10-20% of acute, viral, encephalitis and 60-80% of natural mortality. Understanding the complex and specific characteristics of HSV infection-related diseases has been a scientifically and socially pressing need that has led to overcoming the recent difficulties in diagnosis and treatment.

Table 5: Bioinformatics of genes in microsatellite loci D17MIT51.1 region

No.	Mouse genome	Corresponding human genes	Functions
1	Epididymal fat pad weight QTL 3		QTL
2	Subcutaneous fat pad weight QTL 4		QTL
3	Spleen weight QTL 9		QTL
4	Early growth adjusted QTL 2		QTL
5	Early growth QTL 5		QTL
6	Pulmonary adenoma susceptibility 12		QTL
7	Weight 6 weeks QTL 11		QTL
8	Weight 10 weeks QTL 12		QTL
9	DNA segment, Chr 17, Hunter 19		
10	DNA segment, Chr 17, Hunter 20		
11	DNA segment, Chr 17, Hunter 21		
12	Down syndrome critical region gene 1-like 1	*DSCR1L1*	
13	Fat pad 7		QTL
14	Mandibular morphogenesis 1		QTL
15	MSM lymphoma resistance 1		QTL
16	Bystin-like	*BYSL*	
17	DNA segment, Chr 17, ERATO Doi 191, expressed		
18	DNA segment, Chr 17, ERATO Doi 763, expressed		
17	Methylmalonyl-Coenzyme A mutase	*MUT*	
18	Neuroscience mutagenesis facility, 318		
19	Cysteine-rich secretory protein 2	*CRISP2*	
20	DNA segment, Chr 17, Tubingen 37		
21	DNA segment, Chr 17, Tubingen 16		
22	Ventral midbrain iron content 9		QTL
23	Soft tissue heal 11		QTL
24	DNA segment, Chr 17, Tubingen 37		
25	Gastritis type A susceptibility locus 4		QTL
26	H2 (histocompatibility-2, MHC)		Complex/cluster/region
27	Histocompatibility 2, Q region		Complex/cluster/region
28	Long bones 10		QTL
29	Lymphoma latency acceleration		QTL
30	Leishmaniasis resistance 1		QTL
31	Locomotor activity 2		QTL
32	T cell receptor beta variable 4, control 1		QTL
33	T-cell receptor induced activation 3		QTL
34	Modifier of Odc1		QTL
35	UVB induced immunosuppression 2		QTL
36	Cleidocranial dysplasia		Complex/cluster/region detail
37	Ectonucleotide Pyrophosphatase/phosphodiesterase 5	*ENPP5*	
38	T-complex-associated testis expressed 1	*TCTE1*	
39	Xenotropic murine leukemia virus 57		

QTL: quantitative trait loci

Table 6: Bioinformatics of genes in microsatellite loci D17MIT39.1 region on the 17 mouse chromosome

No.	Mouse genome	Corresponding human genes	Functions
1	Laminin receptor 9		
2	Proteoglycan induced arthritis 20		QTL detail
3	Ribosomal protein L19, related sequence 8		pseudogene
4	T-cell integration locus		
5	Xanthine dehydrogenase	*XDH*	xanthine dehydrogenase
6	Sine oculis-related homeobox 2 homolog (Drosophila)	*SIX2*	
7	Sine oculis-related homeobox 3 homolog (Drosophila)	*SIX3*	
8	MutS homolog 2 (E. coli)	*MSH2*	DNA mismatch repair protein, eukaryotic MSH2 type
9	Carcass protein in high growth mice 3		QTL
10	DNA segment, Chr 17, XREFdb 57		

QTL: quantitative trait loci

Table 7: Bioinformatics of genes in microsatellite loci from D17MIT180.1 to D17MIT184 region

No.	Mouse genome	Corresponding human genes	Functions
1	High mobility group nucleosomal binding domain 1, related sequence 8		
2	Cyclin D3	*CCND3*	
3	Ecotropic viral integration site 14		
4	High mobility group nucleosomal binding domain 2. related sequence 4		
5	DNA segment, Chr 17, Roswell Park 11, expressed		
6	Transplantation-specific integration cluster 1		
7	Body weight 2		
8	DNA segment, Chr 17, CEPH 9		
9	DNA segment, Chr 17, Le Roy 1		
10	DNA segment, Chr 17, Tubingen 40		
11	P300/CBP-associated factor	*PCAF*	
12	Progastricsin (pepsinogen C)	*PGC*	
13	Thymus specific insertion locus		
14	Meprin 1 alpha ;MGI:96963	*MEP1A*	
15	DNA segment, Chr 17, Seldin 7		
16	Macrophage migration inhibitory factor, pseudogene 8		Pseudogene
17	Cerebellar cAMP 8		QTL
18	DNA segment, Chr 17, John C. Schimenti 39		
19	DNA segment, Chr 17, National Cardiovascular Center, Shionogi 7		
20	DNA segment, Chr 17, National Cardiovascular Center, Shionogi 34		
21	DNA segment, Chr 17, XREFdb 556		
22	abdominal fat weight 3		QTL
23	DNA segment, Chr 17, Abbott 3		
24	DNA segment, Chr 17, Birkenmeier 8		
25	DNA segment, Chr 17, Tubingen 20		
26	Heligmosomoides polygyrus nematode resistance 7		QTL
27	Heligmosomoides polygyrus nematode resistance 7		QTL
28	Obesity and body weight QTL 4		QTL
29	RAB5A, member RAS oncogene family	*RAB5A*	
30	Skin tumor susceptibility 10		QTL
31	DNA segment, Chr 17, Brigham Young University 2		
32	High density lipoprotein (HDL) level 4		QTL
33	Vav 1 oncogene	*VAV1*	
34	Nrtn ;neurturin;	*NRTN*	
35	Creatine kinase, brain, related sequence 2		
36	Epstein-Barr virus induced gene 3	*EBI3*	
37	DNA segment, Chr 17, Hunter 24		
38	Ephrin A5	*EFNA5*	
39	RAS-like, family 2, locus 3		
40	Protein tyrosine phosphatase, receptor type, S	*PTPRS*	
41	Abdominal fat percentage 1		QTL
42	P. chabaudi malaria resistance QTL 7		
43	Caseinolytic peptidase, ATP-dependent, proteolytic subunit homolog (E. coli)	*CLPP*	
44	Plasmacytoma susceptibility 5		
45	Ribosomal protein L32, related sequence 7		Pseudogene
46	Sulfotransferase family, cytosolic, 1C, member 1		
47	DNA segment, Chr 17, Tubingen 23		
48	Complement component 3	*C3*	
49	CD86 expression in activated macrophages		QTL
50	DNA segment, Chr 17, Hunter 15		
51	DNA segment, Chr 17, University of California at Los Angeles 2		
52	EGF-like module containing, mucin-like, hormone receptor-like sequence 1	*EMR1*	
53	EGF-like module containing, mucin-like, hormone receptor-like sequence 4	*EMR4*	
54	Modifier of obesity 4		QTL
55	Fer (fms/fps related) protein kinase, testis specific 1		

Continued...

No.	Mouse genome	Corresponding human genes	Functions
56	tubulin, beta 4	*TUBB4*	
57	DNA segment, Chr 17, ERATO Doi 599, expressed		
58	DNA segment, Chr 17, Hunter 16		
59	SH3-domain GRB2-like 1	*SH3GL1*	
60	Thin fur		
61	Abdominal fat percent QTL 6		QTL
62	Early somite stage arrest 15a		
63	HDL QTL 29		QTL
64	KH-type splicing regulatory protein	*KHSRP*	
65	Regulatory factor X, 2 (influences HLA class II expression)	*RFX2*	
66	Skeletal muscle weight 5		QTL
67	DNA segment, Chr 17, Wayne State University 104, expressed	*C19orf10*	
68	DNA segment, Chr 17, XREFdb 173		
69	Immune response 5		
70	Feminization 1 homolog a (C. elegans)	*FEM1A*	
71	DNA segment, Chr 17, Indiana University Medical 1		
72	DNA segment, Chr 17, John C. Schimenti 20		
73	DNA segment, Chr 17, XREFdb 181		
74	Laminin, alpha 1	*LAMA1*	
75	RalA binding protein 1	*RALBP1*	
76	Twisted gastrulation homolog 1 (Drosophila)	*TWSG1*	
77	Protein tyrosine phosphatase, receptor type, M	*PTPRM*	
78	Age related hearing loss 3		QTL
79	Stathmin 1, related sequence 2		pseudogene
80	Abdominal fat weight QTL 7		QTL
81	DNA segment, Chr 17, Birkenmeier 9		
82	DNA segment, Chr 17, Brigham and Women's Genetics 1496 expressed		

QTL: quantitative trait loci

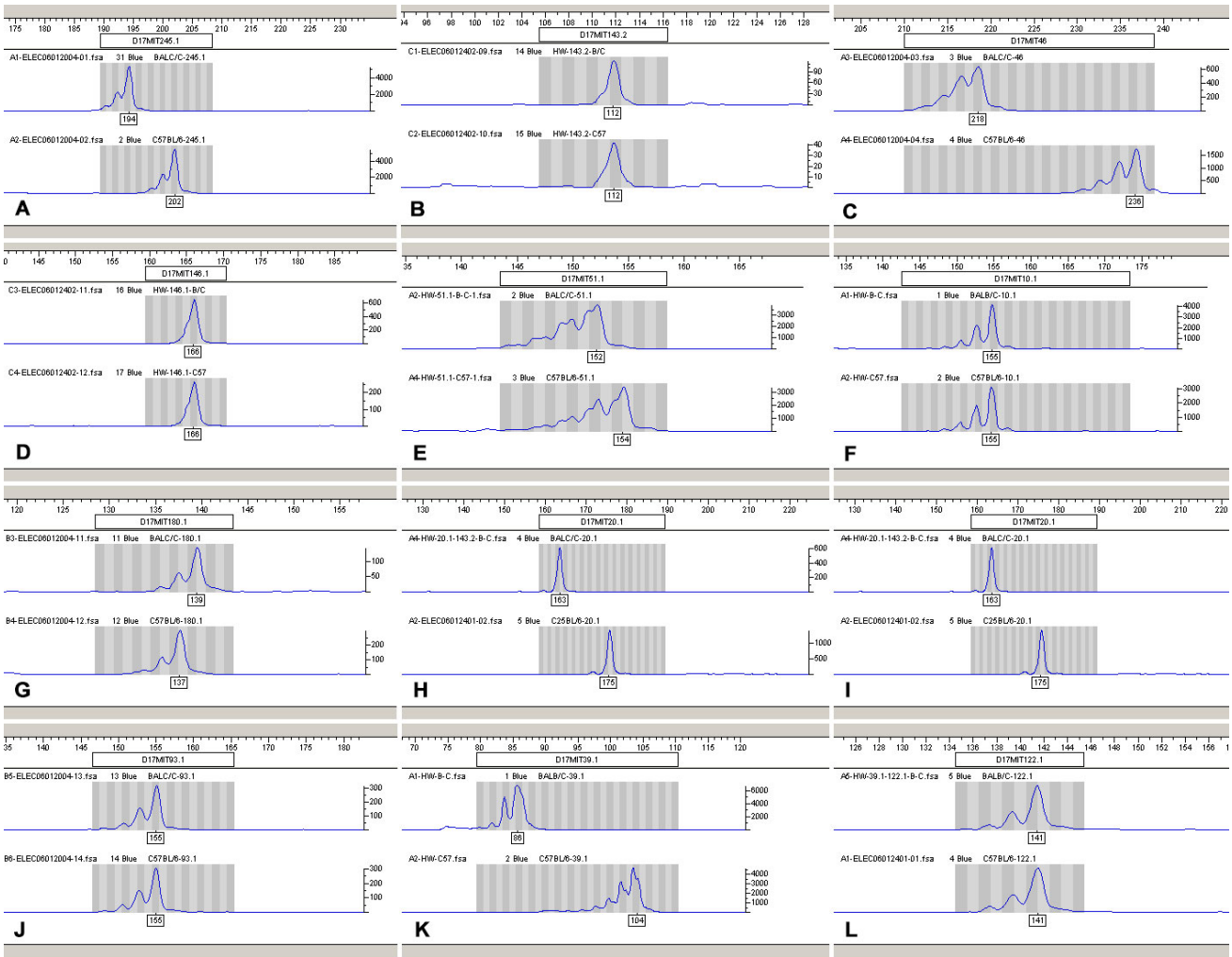

Figure 1: The microsatellite loci scanned using two mouse lines. A: D17MIT245.1; B: D17MIT143.2; C: D17MIT46; D: D17MIT46.1; E: D17MIT51.1; F: D17MIT10.1; G: D17MIT180.1; H: D17MIT20.1; I: D17MIT18; J: D17MIT93; K: D17MIT39.1; L: D17MIT122.1

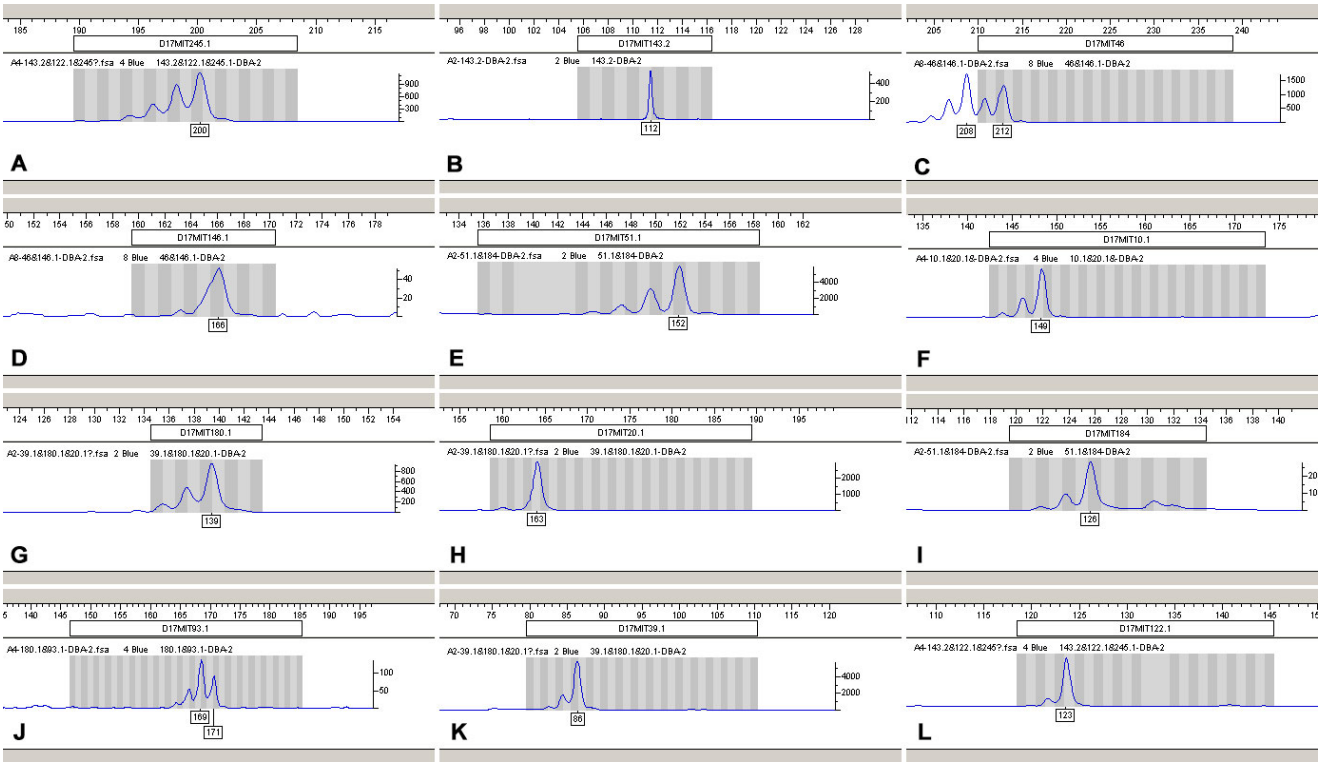

Figure 2: The microsatellite loci scan results of two DBA-2 mouse lines. A: D17MIT245.1; B: D17MIT143.2; C: D17MIT46; D: D17MIT46.1; E: D17MIT51.1; F: D17MIT10.1; G: D17MIT180.1; H: D17MIT20.1; I: D17MIT18; J: D17MIT93; K: D17MIT39.1; L: D17MIT122.1

As a viral disease seriously affecting human health with an increasing incidence in recent years, herpes simplex virus 1 (HSV-1) infection typically generates uncomfortable, watery blisters on the skin or mucous membranes of the mouth and lips,[9,10] potentially leads to encephalitis with remarkable sequelae, or vesicle eruption on genital organs.[11] More importantly, the eruption of these blisters and vesicles are frequently attributed to the long-term latent infection of HSV-1 in the nervous system.[12] Although the human HSV infection rate is very high, it is difficult to fully attract people's attention, so it's difficult for us to associate HSV infection with genetic background. Therefore, most of the previous HSV infection studies focused on the acquired immune response after infection, showing that T cell-mediated immune response plays an important role in resisting HSV-1 infection,[13,14] and immune suppressed or immune deficient individuals are vulnerable to opportunistic herpes virus infection.[15] Furthermore, recent studies[16,17] suggested that innate immune response plays a key role in limiting the spread of the virus. The development of innate immune germ line occurs earlier than acquired immune response system,[18] and these two mechanisms function differently. This is undoubtedly an important point that genetic backgrounds play an important role in HSV infection. Meanwhile, the clinical symptoms of acute infection, as well as the long-term pathologic processes induced by recurrent latent infection, have been shown to closely correlate with the complex viral genome structures and the molecular mechanism of viral gene transcriptional regulation and replication.[19,20]

In fact, as early as 1975, Lopez[1] reported that there are significant differences in the genetic backgrounds of inbred mice that had significantly different reactions to the same or similar HSV infection, which undoubtedly suggested that genetic background might be an important factor for susceptibility to infection. This mechanism revealed by Lopez has also been confirmed by other studies.[2,3] Also, there was a follow-up study on the relationship between the genetic background and susceptibility to HSV infection. Zawatzky *et al*.[21] showed that compared with susceptible DBA/2 mice, the relatively tolerant C57BL/6 mice could produce more interferons in the immune response when it comes to HSV-1 infection. But Brenner *et al*.[22] showed that there is no significant difference in the immune response to HSV infection among those two mouse lines. If the findings in mortality after infection phenotype, Simmons *et al*.[23] reported that only one gene loci functions in this process, while Kastrukoff *et al*.[24] reported that there were two loci separated in the role. We are inclined to believe that from the viewpoint of a gene associated with genetic background, it is undoubtedly that genetic background plays an important role in the phenotypic susceptibility to HSV infection. Since the genetic

background is polygenic, and HSV has no apparent genetic background, the susceptibility of HSV infection itself is very likely regulated by more than one gene controlling quantitative traits.

By bioinformatics analysis, our results suggest that approximately 140 genes were found in the area of D17MIT51.1, D17MIT39.1 and the genomic region between D17MIT180.1 and D17MIT184, and functions of the majority of those genes are not fully revealed. There is a possibility that the above sites are related to the susceptibility to HSV infection, especially the growth-related genes which are highly suggestive of the importance of genetic background. Among these 140 genes, there were about 33 genes homologous to human genes. Their main functions include binding with other partners, regulating a variety of physiological processes, and the modulation of the phosphorylation process of various enzymes and coenzymes.

Based on our experimental results and bioinformatics analysis, the genetic background might play an important role in susceptibility to HSV infection, which is also consistent with most previous studies. It is worth noting that our finding is likely to be a quantitative trait locus, and may not be a particular system or population-specific mechanism. It is just a hint of this phenomenon in a particular system or population which has a relative higher or lower incidence in another race or ethnic groups. This is not consistent with some previous research.

In summary, the biological information and related data analysis suggest that these genes have important physiological and pathological functions. However, up to now, their associations with HSV susceptibility infection have not been reported, suggesting that they could be potential candidate genes that contributed to the different susceptibility to HSV infection.

Because HSV infection phenotypes have not been clearly defined yet, some issues are far from a consensus. For instance, whether the differences in response to HSV infection really exist and whether the genetic background plays a role in it. All these issues will undoubtedly limit the objectivity of this research. It is also necessary to validate the results in the present study by expanding the sample size, further investigating the role of the regulatory regions in regulating susceptibility to HSV infection. Furthermore, the functions of genes near these microsatellite loci, as well as their functions in regulating susceptibility, deserve further investigation.

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Conflicts of interest
There are no conflicts of interest.

Patient consent
There is no patient involved.

Ethics approval
Ethics approval was obtained prior to the commencement of the study.

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If the manuscript is contributed to a special issue, please also mention it in the cover letter.
If the manuscript was presented partly or entirely in a conference, the author should clearly state the background information of the event, including the conference name, time and place in the cover letter.

2.2 Types of Manuscripts

There is no restriction on the length of manuscripts, number of figures, tables and references, provided that the manuscript is concise and comprehensive. The journal publishes Original Article, Review, Meta-Analysis, Case Report, Commentary, etc. For more details about paper type, please refer to the following table.

Manuscript Type	Definition	Abstract	Keywords	Main Text Structure

Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
Meta-Analysis	A Meta-Analysis is a statistical analysis combining the results of multiple scientific studies. It is often an overview of clinical trials.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Technical Note	A Technical Note is a short article giving a brief description of a specific development, technique or procedure, or it may describe a modification of an existing technique, procedure or device applied in research.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Commentary	A Commentary is to provide comments on a newly published article or an alternative viewpoint on a certain topic.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Editorial	An Editorial is a short article describing news about the journal or opinions of senior editors or the publisher.	None required	None required	/
Letter to Editor	A Letter to Editor is usually an open post-publication review of a paper from its readers, often critical of some aspect of a published paper. Controversial papers often attract numerous Letters to Editor	Unstructured abstract (optional). No more than 250 words.	3-8 keywords (optional)	/
Opinion	An Opinion usually presents personal thoughts, beliefs, or feelings on a topic.	Unstructured abstract (optional). No more than 250 words.	3-8 keywords	/
Perspective	A Perspective provides personal points of view on the state-of-the-art of a specific area of knowledge and its future prospects. Links to areas of intense current research focus can also be made. The emphasis should be on a personal assessment rather than a comprehensive, critical review. However, comments should be put into the context of existing literature. Perspectives are usually invited by the Editors.	Unstructured abstract. No more than 150 words.	3-8 keywords	/

2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or

protein names are included, the abbreviated name rather than full name should be used.

2.3.1.2 Authors and Affiliations

Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

2.3.1.3 Abstract

The abstract should be a single paragraph with word limitation and specific structure requirements (for more details please refer to Types of Manuscripts). It usually describes the main objective(s) of the study, explains how the study was done, including any model organisms used, without methodological detail, and summarizes the most important results and their significance. The abstract must be an objective representation of the study: it is not allowed to contain results which are not presented and substantiated in the manuscript, or exaggerate the main conclusions. Citations should not be included in the abstract.

2.3.1.4 Keywords

Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

2.3.2 Main Text

Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

2.3.2.1 Introduction

The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

2.3.2.2 Methods

Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

2.3.2.3 Results

This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

2.3.2.4 Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

2.3.2.5 Conclusion

It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

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Each author is expected to have made substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data, or the creation of new software used in the work, or have drafted the work or substantively revised it.

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In order to maintain the integrity, transparency and reproducibility of research records, authors should include this section in their manuscripts, detailing where the data supporting their findings can be found. Data can be deposited into data repositories or published as supplementary information in the journal. Authors who cannot share their data should state that the data will not be shared and explain it. If a manuscript does not involve such issue, please state "Not applicable." in this section.

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References should be numbered in order of appearance at the end of manuscripts. In the text, reference numbers should be placed in square brackets and the corresponding references are cited thereafter. Only the first five authors' names are required to be listed in the references, other authors' names should be omitted and replaced with "et al.". Abbreviations of the journals should be provided on the basis of Index Medicus. Information from manuscripts accepted but not published should be cited in the text as "Unpublished material" with written permission from the source.

References should be described as follows, depending on the types of works:

Types	Examples
Journal articles by individual authors	Weaver DL, Ashikaga T, Krag DN, Skelly JM, Anderson SJ, et al. Effect of occult metastases on survival in node-negative breast cancer. N Engl J Med 2011;364:412-21. [PMID: 21247310 DOI: 10.1056/NEJMoa1008108]
Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. Hypertension 2002;40:679-86. [PMID: 12411462]

Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. J Urol 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. J Appl Clin Pediatr 2012;27:1903-7. (in Chinese)
Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. BJOG 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. Proc Natl Acad Sci U S A. Forthcoming 2002.

For other types of references, please refer to U.S. National Library of Medicine.
The journal also recommends that authors prepare references with a bibliography software package, such as EndNote to avoid typing mistakes and duplicated references.

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Additional data and information can be uploaded as Supplementary Material to accompany the manuscripts. The supplementary materials will also be available to the referees as part of the peer-review process. Any file format is acceptable, such as data sheet (word, excel, csv, cdx, fasta, pdf or zip files), presentation (powerpoint, pdf or zip files), image (cdx, eps, jpeg, pdf, png or tiff), table (word, excel, csv or pdf), audio (mp3, wav or wma) or video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv). All information should be clearly presented. Supplementary materials should be cited in the main text in numeric order (e.g., Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2, *etc*.). The style of supplementary figures or tables complies with the same requirements on figures or tables in main text. Videos and audios should be prepared in English, and limited to a size of 500 MB or a duration of 3 minutes.

2.4 Manuscript Format

2.4.1 File Format

Manuscript files can be in DOC and DOCX formats and should not be locked or protected.

2.4.2 Length

There are no restrictions on paper length, number of figures, or amount of supporting documents. Authors are encouraged to present and discuss their findings concisely.

2.4.3 Language

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Figures should be cited in numeric order (e.g., Figure 1, Figure 2) and placed after the paragraph where it is first cited;
Figures can be submitted in format of tiff, psd, AI or jpeg, with resolution of 300-600 dpi;
Figure caption is placed under the Figure;

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Labels, numbers, letters, arrows, and symbols in figure should be clear, of uniform size, and contrast with the background;
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Tables should be cited in numeric order and placed after the paragraph where it is first cited;
The table caption should be placed above the table and labeled sequentially (e.g., Table 1, Table 2);
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Abbreviations and symbols used in table should be explained in footnote;
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Abbreviations should be defined upon first appearance in the abstract, main text, and in figure or table captions and used consistently thereafter. Non-standard abbreviations are not allowed unless they appear at least three times in the text. Commonly-used abbreviations, such as DNA, RNA, ATP, *etc*., can be used directly without definition. Abbreviations in titles and keywords should be avoided, except for the ones which are widely used.

2.4.8 Italics

General italic words like *vs*., *et al., etc*., *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

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SI Units should be used. Imperial, US customary and other units should be converted to SI units whenever possible. There is a space between the number and the unit (i.e., 23 mL). Hour, minute, second should be written as h, min, s.

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Numbers appearing at the beginning of sentences should be expressed in English. When there are two or more numbers in a paragraph, they should be expressed as Arabic numerals; when there is only one number in a paragraph, number < 10 should be expressed in English and number > 10 should be expressed as Arabic numerals. 12345678 should be written as 12,345,678.

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