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A role for humoral mechanisms in the pathogenesis of Devic's neuromyelitis optica

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Summary

Devic's disease [neuromyelitis optica (NMO)] is an idiopathic inflammatory demyelinating disease of the CNS, characterized by attacks of optic neuritis and myelitis. The mechanisms that result in selective localization of inflammatory demyelinating lesions to the optic nerves and spinal cord are unknown. Serological and clinical evidence of B cell autoimmunity has been observed in a high proportion of patients with NMO. The purpose of this study was to investigate the importance of humoral mechanisms, including complement activation, in producing the necrotizing demyelination seen in the spinal cord and optic nerves. Eighty-two lesions were examined from nine autopsy cases of clinically confirmed Devic's disease. Demyelinating activity in the lesions was immunocytochemically classified as early active (21 lesions), late active (18 lesions), inactive (35 lesions) or remyelinating (eight lesions) by examining the antigenic profile of myelin degradation products within macrophages. The pathology of the lesions was analysed using a broad spectrum of immunological and neurobiological markers, and lesions were defined on the basis of myelin protein loss, the geography and extension of plaques, the patterns of oligodendrocyte destruction and the immunopathological evidence of complement activation. The pathology was identical in all nine patients. Extensive demyelination was present across multiple spinal cord levels, associated with cavitation, necrosis and acute axonal pathology (spheroids), in both grey and white matter. There was a pronounced loss of oligodendrocytes within the lesions. The inflammatory infiltrates in active lesions were characterized by extensive macrophage infiltration associated with large numbers of perivascular granulocytes and eosinophils and rare $CD3^+$ and $CD8^+$ T cells. There was a pronounced perivascular deposition of immunoglobulins (mainly IgM) and complement C9neo antigen in active lesions associated with prominent vascular fibrosis and hyalinization in both active and inactive lesions. The extent of complement activation, eosinophilic infiltration and vascular fibrosis observed in the Devic NMO cases is more prominent compared with that in prototypic multiple sclerosis, and supports a role for humoral immunity in the pathogenesis of NMO. Based on this study, future therapeutic strategies designed to limit the deleterious effects of complement activation, eosinophil degranulation and neutrophil/macrophage/microglial activation are worthy of further investigation.

Keywords: Devic's syndrome; eosinophils; humoral immunity; neuromyelitis optica; neuropathology

Abbreviations: ADEM = acute disseminated encephalomyelitis; BBB = blood–brain barrier; EAE = experimental allergic encephalomyelitis; EG MBP = eosinophil granule major basic protein; GFAP = glial fibrillary acidic protein; MAG = myelin-associated glycoprotein; MOG = myelin oligodendrocyte glycoprotein; NMO = neuromyelitis optica; ON = optic neuritis

Table 1 *Antibodies used for immunocytochemistry*

Antigen	Ab type	Target	Source
Complement components			
C1s	mAb	C1s deposits	Dept Biochemistry, Cardiff, UK
C3	Polyclonal	C3 deposits	Dept Biochemistry, Cardiff, UK
C4	Polyclonal	C4 deposits	Dept Biochemistry, Cardiff, UK
C5b	Polyclonal	Activated C-component C5, C5b	Dept Biochemistry, Cardiff, UK
C6	Polyclonal	C6 deposits	Dept Biochemistry, Cardiff, UK
C7	Polyclonal	C7 deposits	Dept Biochemistry, Cardiff, UK
C8	Polyclonal	C8 deposits	Dept Biochemistry, Cardiff, UK
C9	Polyclonal	C9 deposits	Dept Biochemistry, Cardiff, UK
C9neo	mAb	Activated lytic C-complex	Dept Biochemistry, Cardiff, UK
Other cell markers and immunoglobulins			
CD3/CD8	mAb	T cells	Dako, Glostrup, Denmark
CD20	mAb	B cells	Dako, Glostrup, Denmark
GFAP	Polyclonal	Astrocytes	Dako, Glostrup, Denmark
IgG	mAb	IgG deposits, plasma cells	Amersham, Buckinghamshire, UK
IgM	Polyclonal	IgM	Dako, Glostrup, Denmark
KiM1P	mAb	Monocytes, microglia	Dr W. Bruck, Berlin, Germany
27E10	mAb	Activated macrophages	BMA Biomedicals, Augst, Switzerland
MRP 14	mAb	Activated macrophages	BMA Biomedicals, Augst, Switzerland
CCR3	Polyclonal	Chemokine receptor	Santa Cruz Biotechnology, Santa Cruz, Calif., USA
CXCR3	mAb	Chemokine receptor	Leukosite
CCR5	mAb	Chemokine receptor	R&D Systems, Minneapolis, Minn., USA
EG MBP	Polyclonal	Eosinophil granules	Dr Gerry Gleich, Mayo Clinic, Rochester, Minn., USA
Myelin proteins			
MBP	mAb	Myelin	Boehringer, Mannheim, Germany
PLP	mAb	Myelin	Serotec, Oxford, UK
MAG B11F7	mAb	Myelin	Doberson *et al*. (1985)
MAG D7E10	mAb	Myelin	
MAG	Polyclonal	Myelin	Matthieu *et al*. (1990)
MOG 8–18C5	mAb	Myelin/oligodendrocytes	Dr S. Piddlesden, Cardiff, UK
MOG Y10	mAb	Myelin/oligodendrocytes	Dr S. Piddlesden, Cardiff, UK
MOG Z12	mAb	Myelin/oligodendrocytes	Dr S. Piddlesden, Cardiff, UK
CNPase	mAb	Myelin/oligodendrocytes	Affinity Research Products, UK

mAb = monoclonal antibody.

Introduction

Devic's disease [neuromyelitis optica (NMO)] is an idiopathic inflammatory demyelinating disease of the CNS characterized by attacks of optic neuritis (ON) and myelitis (Devic, 1894, 1895; Gault, 1894). Although, historically, NMO has been regarded as a monophasic disease characterized by nearly simultaneous onset of bilateral ON and myelitis, previous studies suggest that this disease may pursue a relapsing–remitting course (Mandler *et al*., 1993). A recent review of the Mayo Clinic experience with NMO found that approximately two-thirds of patients in a clinic-based large series had a relapsing form of NMO (Wingerchuk *et al*., 1999). A number of distinctive characteristics of NMO were identified, including the following: normal MRI scan of the head (occasionally abnormal in a small percentage of early cases), longitudinally extensive signal abnormality in the spinal cord during acute attacks, typically extending over three or more vertebral segments; occasional prominent CSF pleocytosis that may be associated with a polymorphonuclear predominance, and generally poor outcome of attacks, some of which lead to respiratory failure and ventilator dependence, a complication virtually unknown in multiple sclerosis. Unfortunately, no long-term effective treatment has been established for this disease.

The basic histopathological features of NMO have been described previously (Cloys and Netsky, 1970; Mandler *et al*., 1993; Prineas, 1997): acute spinal cord lesions demonstrate diffuse swelling and softening extending over multiple spinal segments, and occasionally may involve the entire spinal cord in a patchy or continuous distribution. These lesions are characterized by extensive macrophage infiltration associated with myelin and axonal loss, and necrosis of both the grey and white matter of the spinal cord. Perivascular inflammation is variable. Chronic lesions are characterized by gliosis, cystic degeneration, cavitation and atrophy of the spinal cord and optic nerves. An apparent increase in the number and prominence of blood vessels with thickened and hyalinized

walls have been described in necrotic and peri-necrotic spinal cord areas (Mandler *et al*., 1993).

The immunopathological mechanisms responsible for the necrotizing and demyelinating spinal cord and optic nerve lesions in NMO are unknown. Furthermore, whether NMO is a subtype of multiple sclerosis or a distinct disease entity remains controversial. Clinical and serological clues suggest the possibility of B cell dysregulation. We investigated the possible role of humoral mechanisms in producing the necrotizing demyelination of the spinal cord and optic nerves in autopsy material of nine previously well-characterized NMO patients. Lesions were analysed on the basis of myelin protein loss, the geography and extension of plaques, the patterns of axonal and oligodendrocyte destruction, the nature of vascular alterations, the character and distribution of the inflammatory infiltrate, and the immunopathological evidence of complement activation.

Material and methods

Clinical history of NMO patients

This study was performed on archival material of nine previously well-characterized autopsy cases of NMO (Mandler *et al*., 1993, 1998). Material was collected in the Department of Neuropathology at the Mayo Clinic ($n = 5$) and the Department of Neuropathology at the University of New Mexico ($n = 4$). Detailed clinical histories were available on all nine cases (eight females; one male; average age 50 years, range 16–80 years). The clinical course was relapsing in eight patients and monophasic in a single case. Mean disease duration ($\pm$ standard error of the mean) was 2.4 ± 0.8 years. All patients died from respiratory compromise. The presenting syndrome was ON in four patients and myelopathy in five patients, with a mean interval between optic neuropathy and myelopathy of 19 months (range 4–41 months). Three patients had a history of one or more associated autoimmune disorders: hypothyroidism (two), pernicious anaemia (one), thrombocytopenic purpura (one).

Neuropathological controls

Neuropathological controls included other inflammatory demyelinating CNS disorders such as acute disseminated encephalomyelitis (ADEM; $n = 3$ autopsy cases), pathologically defined as demyelination limited to perivenular areas (Hart and Earle, 1975); and 73 previously well-characterized biopsy ($n = 51$) and autopsy ($n = 22$) cases of active multiple sclerosis (Lucchinetti *et al*., 2000). These controls were included to determine whether the pathological observations found in NMO were unique to this disorder, or rather features representative of the family of related idiopathic inflammatory demyelinating disorders. In addition, three cases of acute spinal cord infarction were included in order to determine whether complement was non-specifically activated in other necrotic inflammatory disorders restricted to the spinal cord.

Neuropathological techniques and immunocytochemistry

All cases underwent assessment of one to six blocks per biopsy case, and up to 20 blocks per autopsy case. All tissue blocks were classified with regard to lesional activity (Brück *et al*., 1995). Paraffin-embedded 5-μm sections were stained with haematoxylin–eosin, Luxol fast blue (LFB) myelin stain, periodic acid–Schiff (PAS) reaction, and Bielschowsky's silver impregnation axonal stain.

Immunocytochemistry was performed without modification on paraffin sections using an avidin–biotin or an alkaline phosphatase/anti-alkaline phosphatase technique as previously described in detail (Vass *et al*., 1990) with the antibodies listed in Table 1. The primary antibodies were omitted in controls. Sections were further analysed for the presence of intact and/or degranulated eosinophils by an indirect immunofluorescence method using specific eosinophil granule major basic protein (EG MBP) antibody as reported previously (Filley *et al*., 1982).

In situ hybridization was performed using digoxigenin-labelled riboprobes specific for proteolipid protein (PLP). The source and specificity of the probes, the labelling techniques and the methods of *in situ* hybridization have been described in detail previously (Breitschopf *et al*., 1992). Following *in situ* hybridization, the sections were either counterstained with haematoxylin or processed for immunocytochemistry with anti-PLP antibodies as described above. To visualize degenerating cells in tissue sections, DNA fragmentation within cell nuclei was determined using the method of *in situ* tailing (Gold *et al*., 1994). The sections were then processed for immunocytochemistry with antibodies against myelin oligodendrocyte glycoprotein (MOG), glial fibrillary acidic protein (GFAP), T cells and macrophages as described above. Apoptotic oligodendrocytes were defined by nuclear condensation and fragmentation in cells stained by either MOG or 2′,3′-cyclic nucleotide phosphodiesterase (CNPase) antibodies.

Lesional staging

Eighty-two lesions were examined from nine autopsy cases of clinically confirmed NMO. Lesions were classified with respect to demyelinating activity, as described previously (Brück *et al*., 1995). *Early active demyelinating lesions* were diffusely infiltrated by macrophages immunoreactive for all myelin proteins including MOG (Fig. 1C and D). *Late active demyelinating lesions* were more advanced with respect to myelin degradation, and were immunoreactive for the major myelin proteins major basic protein (MBP) and PLP, but not for MOG. *Remyelinating lesions* were characterized by uniformly thin and irregularly arranged myelin sheaths. *Inactive demyelinated lesions* were completely demyelinated without signs of active demyelination. Demyelinating activity in the NMO lesions was immunocytochemically classified as early active in 21 lesions, late active in 18 lesions, inactive in 35 lesions and remyelinating in eight lesions.

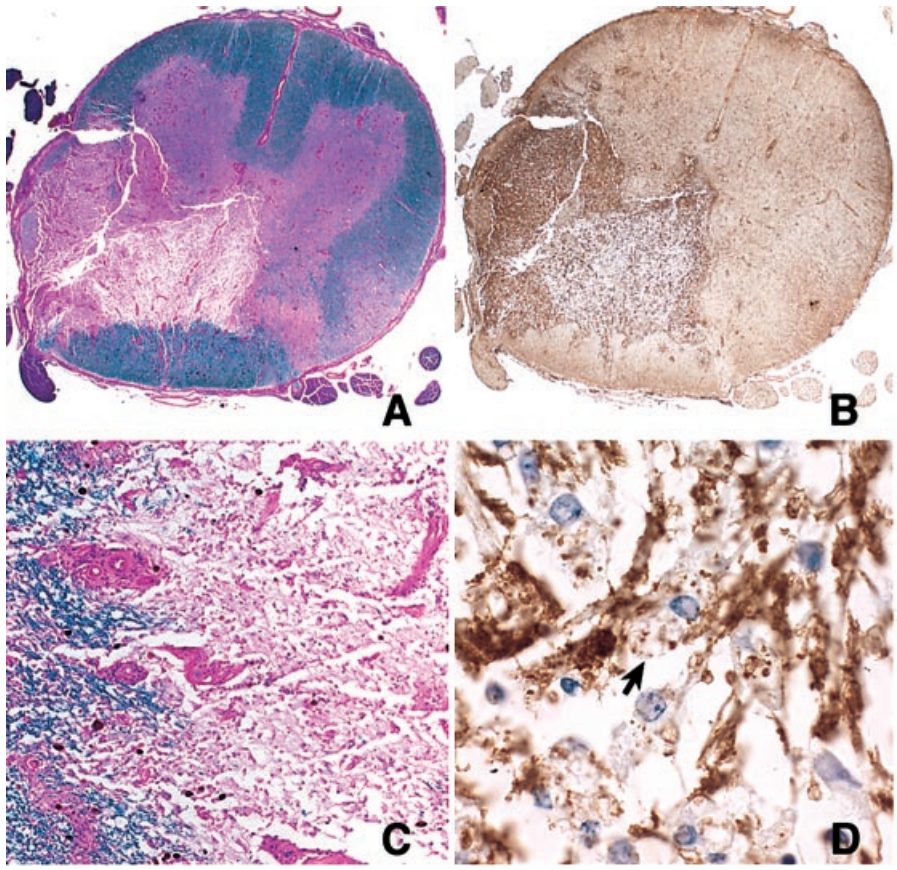

Fig. 1 Histopathology of NMO. (**A**) Spinal cord cross-section demonstrating extensive demyelination involving both the grey and white matter (Luxol fast blue and PAS myelin stain, mag ×10). (**B**) The lesion is filled with numerous macrophages (KiM1P pan-macrophage stain). (**C**) There is a sharp demarcation between the periplaque white matter and the plaque edge (Luxol fast blue and PAS myelin, mag ×100). (**D**) Macrophages within the actively demyelinated lesion contain myelin debris within the cytoplasm (arrow; immunocytochemistry for MOG, mag ×600).

Quantitative morphometry of labelled cells

The number of cells stained by immunocytochemistry or *in situ* hybridization per square unit of tissue was determined on serial sections. A topographical map was established for each lesion outlining the periplaque white matter, zone of active myelin destruction, inactive plaque centre and region of remyelination. The number of cells was determined in each of these distinct plaque areas in 10 standardized microscopic fields of 25 000 μm^2, each defined by an ocular morphometric grid. Values in tables and figures represent number of cells per square millimetre.

Statistical analysis

Non-parametric group tests were used to compare groups. All values are expressed as means ± standard error of the mean.

Results

The basic structural pathology was identical in all nine NMO cases. Extensive demyelination was present across multiple spinal cord levels, associated with cavitation, necrosis and acute axonal pathology (spheroids), in both grey and white matter (Figs 1A and 2C). Lesions were typically located within the central portions of the spinal cord, with peripheral rims of myelin preservation. There was a pronounced loss of oligodendrocytes within all the lesions (Fig. 2D), with rare evidence of Schwann cell remyelination (four lesions). No oligodendrocyte apoptosis was found, and comparative immunocytochemistry of active lesions revealed no selective loss of myelin-associated glycoprotein (MAG; a marker of oligodendrocyte dystrophy). Optic nerves and/or chiasm demonstrated inactive demyelination or partial remyelination in all subjects, with no evidence of ongoing demyelinating activity.

The inflammatory infiltrates in actively demyelinating NMO spinal cord lesions were characterized by extensive macrophage/microglial infiltration (Fig. 1B), numerous B lymphocytes, and few $CD3^+$ and $CD8^+$ T-lymphocytes (Fig. 2A and B). This was associated with a prominent eosinophilic and granulocyte perivascular infiltrate in early active demyelinating NMO lesions (Fig. 3A and B). The numbers

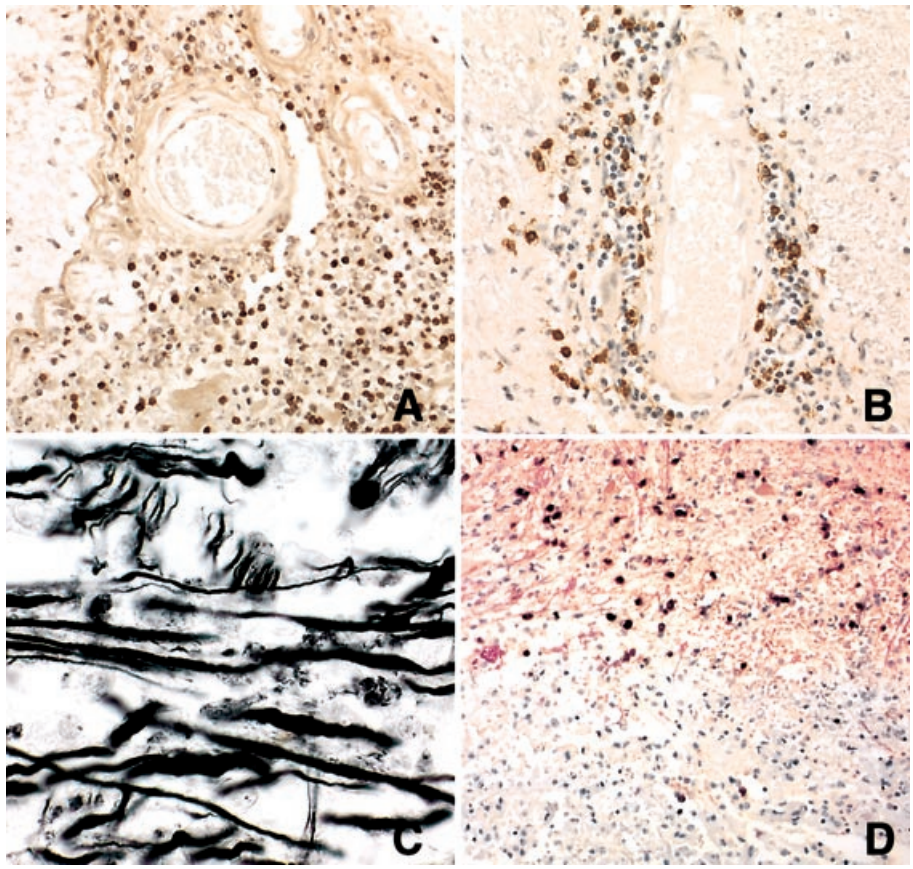

Fig. 2 Histopathology of neuromyelitis optica. (**A**, **B**, mag ×200) The perivascular infiltrate contains numerous $CD3^+$ T lymphocytes (**A**) and $CD8^+$ T lymphocytes (**B**). (**C**). There is a marked reduction in axonal density and acute axonal pathology consisting of swellings and spheroids (Bielschowsky silver impregnation, mag ×400). (**D**) There is a marked reduction in oligodendrocytes within the lesion [PLP mRNA *in situ* hybridization (black); double-labelled with immunocytochemistry for PLP protein (red, mag ×200)].

of eosinophils ranged from 172 to 215/mm^2, and granulocytes ranged from 64 to 688/mm^2. Immunofluorescence staining for antibody to EG MBP confirmed the presence of both intact and degranulated eosinophils in both early active lesion areas and within the meninges (Fig. 3C–F).

Scattered CCR3 immunoreactivity was present on small round cells resembling T lymphocytes in all NMO early active lesions containing perivascular eosinophils, as well as in two early active eosinophil rich Marburg cases of acute multiple sclerosis. Immunoreactivity for the chemokine receptors CXCR3 and CCR5 observed in the NMO cases was similar to those in multiple sclerosis lesions (Sorensen *et al*., 1999), with CXCR3 immunoreactivity on predominantly perivascular leukocytes and abundant CCR5 staining on mononuclear phagocytes and lymphocytes in areas of ongoing and completed demyelination.

All early active NMO lesions demonstrated a pronounced perivascular Ig and C9neo reactivity in two distinct patterns (Fig. 4A). Total immunoglobulin (Ig) was deposited in a characteristic perivascular rosette pattern, and along the outer rim of thickened vessel walls (Fig. 4B and C). When Ig isotypes were analysed, immunoreactivity was most pronounced for IgM. The Ig reactivity co-localized with a similar pattern of C9neo staining (a marker of complement-mediated tissue injury) in both a rosette and rim perivascular pattern (Fig. 4D and E). Other complement components such as C1q, C3, C4, C6, C7, C8 and C9 were also accentuated in a similar perivascular pattern, but were also present in a relatively diffuse manner. This observation suggests that besides complement activation, diffuse leakage of complement proteins most likely occurred across an impaired blood–brain barrier (BBB). Only C9neo was restricted to sites where complement was activated. A similar rosette and rim pattern of perivascular macrophage staining was also observed (Fig. 4F and G). In addition, there was evidence of vascular fibrosis and hyalinization together with an apparent increase in the density of blood vessels in active spinal cord NMO lesions. There was, however, no evidence of fibrinoid necrosis or granulocyte infiltration of the vessel wall, as seen in acute lesions of necrotizing vasculitis (Fig. 5A and B).

Table 2 summarizes the neuropathological findings in NMO cases compared with control cases of active multiple sclerosis, ADEM and acute spinal cord infarction. T cells and

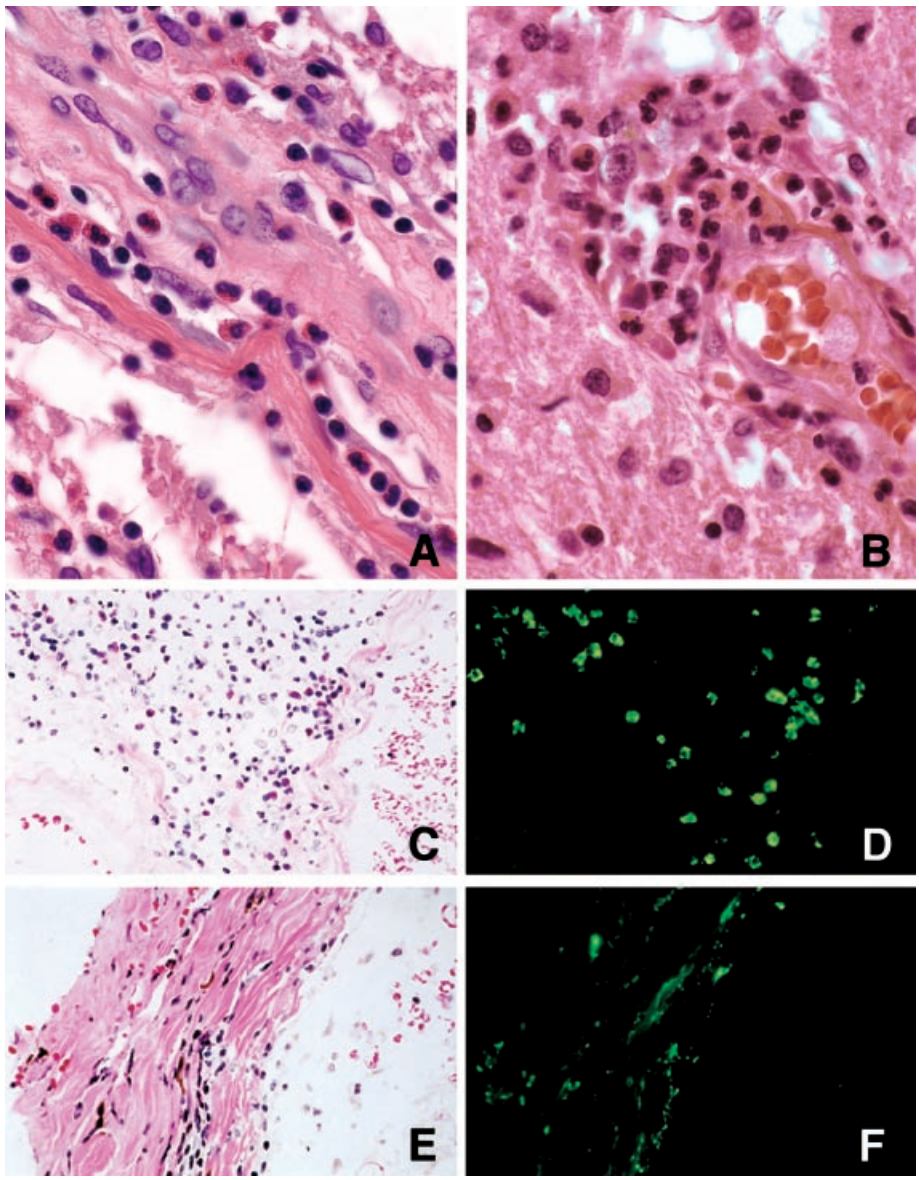

Fig. 3 Inflammatory infiltrate in NMO. Numerous perivascular eosinophils (**A**) and granulocytes (**B**) are located within the lesion (haematoxylin–eosin, mag ×600). Intact perivascular and parenchymal eosinophils are present within the lesion (haematoxylin–eosin, mag ×100) (**C**). Eosinophil MBP immunofluorescence of corresponding serial section (mag ×200) (**D**). Eosinophils are also present within the meninges with evidence of degranulation demonstrated by the irregular punctate granular staining [haematoxylin–eosin stain (**E**) with eosinophil MBP immunofluorescence of corresponding serial section (**F**), mag ×100].

Table 2 *Comparison of structural and immunopathological features of actively demyelinating NMO, multiple sclerosis, ADEM and spinal cord infarction lesions (percentage of cases)*

	T cells	MO	Ig	C9neo	E	N	Hyalinized vessels
NMO (n = 8)*	100	100	100	100	56†	56	100
Multiple sclerosis (n = 73)	100	100	52	52	4	4	0
ADEM (n = 3)	100	100	0	0	0	0	0
Spinal cord infarction (n = 3)	100	100	0	0	0	0	0

*The single autopsy case with no actively demyelinating lesions was not included in this analysis; †21 of 21 early active NMO lesions contained eosinophils. MO = macrophages; Ig = immunoglobulin deposition; E = eosinophils; N = neutrophils.

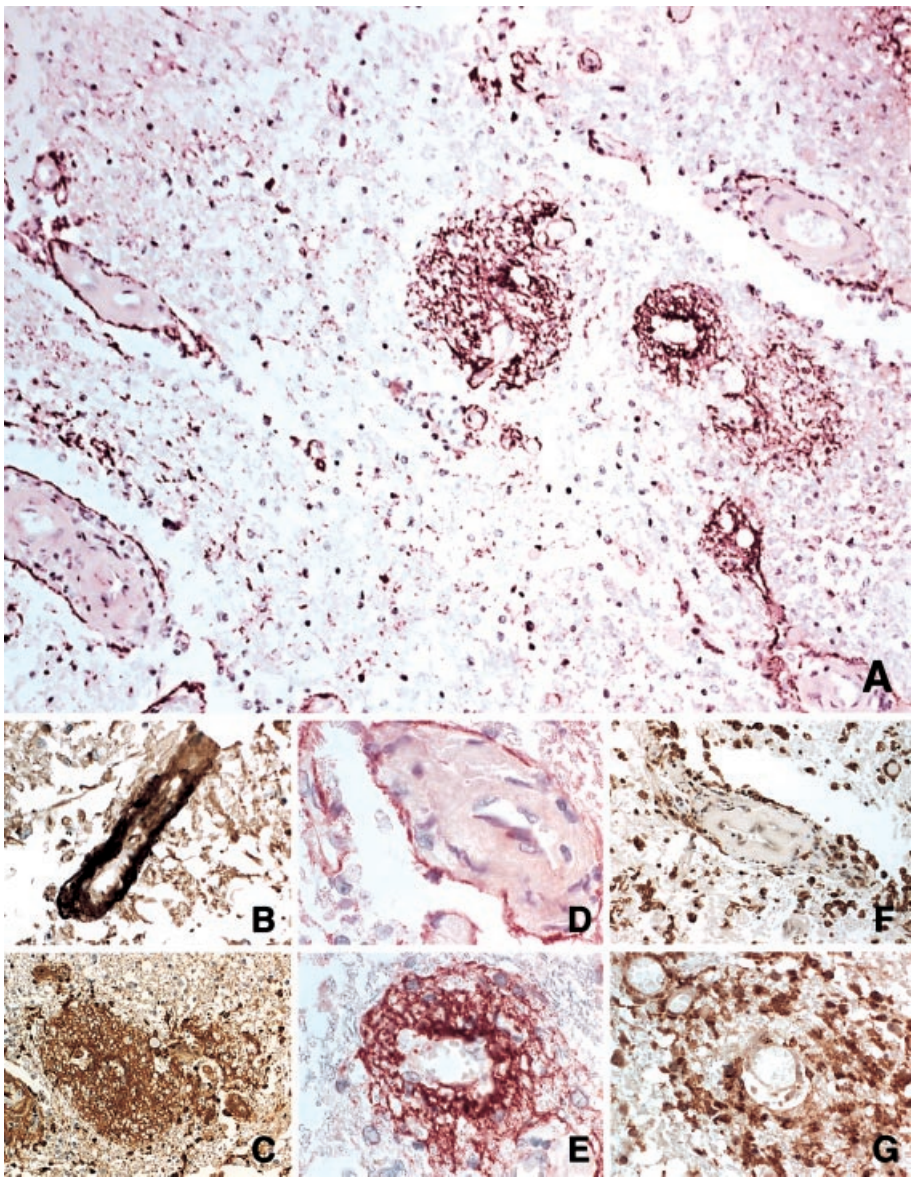

Fig. 4 Evidence for humoral immunity in NMO. (**A**) Actively demyelinating NMO lesion shows massive deposition of complement C9neo-antigen (red staining) in a rim pattern on the outer surface of thickened blood vessels, as well as in a rosette perivascular pattern (mag ×200). (**B**) There is pronounced perivascular immunoglobulin reactivity (human Ig). (**C**) Immunocytochemistry for IgM demonstrates a rosette perivascular staining pattern. Higher power view of staining for complement activation with C9neo-antigen (red) demonstrates this rim (**D**) and rosette (**E**) pattern of staining. Macrophages co-localize in a similar rim (**F**) and rosette (**G**) pattern (KiM1P pan-macrophage stain) (mag ×400).

macrophages were present to a variable degree in all active multiple sclerosis, ADEM and acute spinal cord infarct lesions. Fifty-two per cent of active multiple sclerosis cases (38 of 73) also demonstrated the deposition of immunoglobulin and activated complement (C9neo reactivity). However, Ig and complement deposition in multiple sclerosis was distributed in a pattern quite distinct from the striking perivascular rosette and rim pattern of Ig deposition co-localizing with complement activation observed in NMO cases. In active multiple sclerosis lesions, the degree of complement activation was less pronounced, and was present on degenerating myelin sheaths, within macrophages and on oligodendrocytes along the active plaque edge (Lucchinetti *et al*., 2000), as opposed to the perivascular pattern described in NMO lesions. No complement activation or immunoglobulin reactivity was observed in acute spinal cord infarction or ADEM cases. Eosinophils and granulocytes were observed in rare cases of fulminant Marburg acute multiple sclerosis (4% of cases; three of 73), compared with 56% of NMO cases (five of nine cases). Furthermore, all early active NMO lesions (21

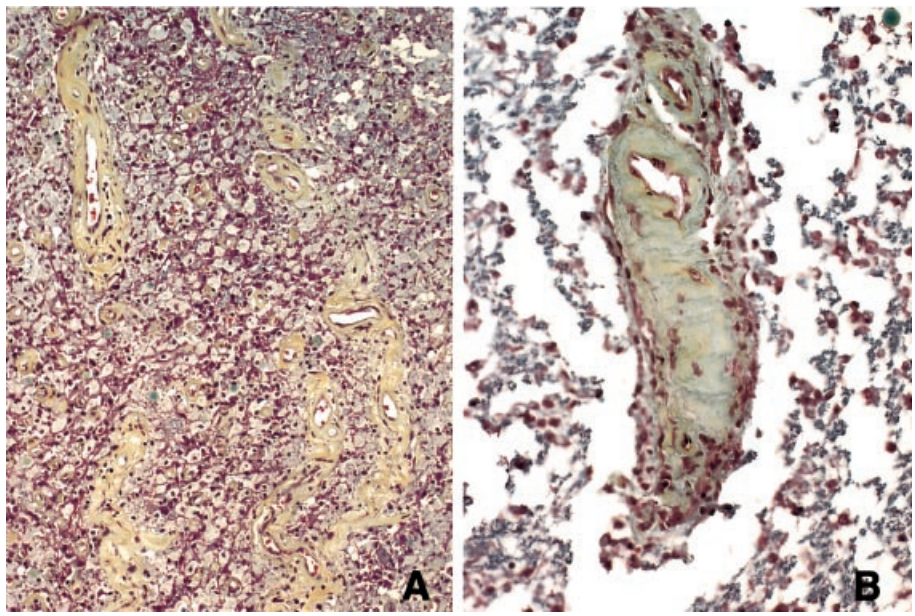

Fig. 5 Vessel pathology in NMO. (**A**) There is an apparent increase in numbers and prominence of thickened hyalinized blood vessels within the lesion (Movat, mag ×100). (**B**) Higher power view emphasizes collagen infiltration of vessel wall (green with Movat, mag ×400).

of 21) contained eosinophils. No eosinophils were present in ADEM or acute spinal cord infarction cases. Hyalinized vessels were present in all nine NMO cases, but absent from active multiple sclerosis, ADEM and acute spinal cord infarction cases.

Discussion

Unique pathological features of NMO

The pattern of tissue inflammation included extensive complement activation, eosinophil/neutrophil infiltration and vascular fibrosis in early active demyelinating NMO autopsy lesions. These attributes were more prominent and in a unique perivascular pattern compared with typical multiple sclerosis, and supported a role for humoral autoimmunity in the pathogenesis of NMO. We found a uniform pathological pattern in all nine cases of NMO. The co-localization of Ig, C9neo antigen and activated macrophages in the perivascular region, coupled with the prominent vascular hyalinization observed in active NMO lesions, suggests that the CNS vasculature may be an early and specific target of the disease process. These findings also emphasize an important role for complement activation along the classical pathway (Morgan, 1995). Activation of the classical complement cascade is initiated by the binding of complement component C1q to its receptor in the Fc portion of the antibody molecule. This interaction initiates the classical complement pathway cascade with resultant production of chemo-attractants for macrophages and the eventual production of the membrane attack complex, which inserts itself into cell membranes eventually causing lysis and cell death. Receptor-mediated docking of macrophages to the Fc portion of bound IgG leads to their activation, and to the release of cytokines and oxygen radicals, all of which may contribute to tissue injury. The presence of C9neo in NMO lesions clearly demonstrates that the terminal lytic complement complex (i.e. membrane attack complex) is activated preferentially at perivascular sites. Interestingly, we found that precipitated Ig staining in NMO lesions was most prominent for IgM, which is especially effective in fixing complement (Heyman, 2000). Furthermore, macrophages cannot dock to bound IgM via Fc receptors. Therefore, in NMO, complement must initiate the destruction of myelin, which is then secondarily taken up by macrophages, possibly in complexes with IgM and complement components. Taken together, our observations argue for a primary role of complement activation in mediating tissue injury in NMO.

Relevant experimental animal models

Pathological comparisons between the lesions of one select variant of MOG-induced experimental allergic encephalomyelitis (EAE) and NMO reveal striking similarities (Storch *et al*., 1998*b*). The balance of cellular and humoral factors in this model greatly impacts the topography and immunopathology of the lesions. Interestingly, those genetic strains or immunization protocols that favour humoral immune mechanisms reproduce the pathological hallmarks of NMO in the animal. Brown Norway rats sensitized with soluble MOG in incomplete Freund's adjuvant mount a very prominent antibody response, and develop a chronic disease with pronounced demyelination, most frequently in the spinal cord and optic nerve. Active demyelination in these experi-

mental lesions is associated with the deposition of immunoglobulin and complement activation, and the acute inflammatory infiltrates contain neutrophilic and eosinophilic granulocytes. The parallels in lesional topography, complement activation and eosinophilic inflammation observed between NMO and those specific models of MOG-induced EAE that drive a humoral response further supports a role for humoral immunity in the pathogenesis of NMO.

Proposed pathogenic mechanism in NMO

Based on these novel immunopathological observations, we speculate on several mechanisms that may be involved in NMO pathogenesis. The pronounced Ig reactivity co-localizing with complement activation at sites of vessel damage suggest the perivascular space may be the primary site of injury in NMO. This may be due to a specific antibody targeted to a vascular antigen. Alternatively, antigen liberated within the CNS in the course of the destructive process may reach the perivascular space and be recognized there by antibodies derived from the circulation. Finally, a non-specific inflammatory reaction initiated by the deposition of circulating immune complexes may be involved. In either scenario, the classical complement pathway is activated, and leads to the recruitment of activated macrophages to these perivascular sites where they bind either via a receptor for complement components or Ig/Fc receptor. Activated macrophages, together with eosinophils and neutrophils, locally generate cytokines, proteases and oxygen/nitrogen free radicals, which may contribute to both vascular and parenchymal damage, resulting in non-selective bystander destruction of both grey and white matter, including axons and oligodendrocytes. Increased vascular permeability and oedema may contribute to parenchymal damage via secondary ischaemia, and might account for the typical central location of NMO plaques within the spinal cord (Prineas and McDonald, 1997). A similar central location is found in severe cases of MOG-EAE, and this phenomenon is most likely due to oedema-induced ischaemia (Lassmann, 1983). Novel antigens liberated during the destructive process may further amplify the destructive immune response in NMO.

Complement may be activated non-specifically in response to tissue necrosis. Given the necrotic nature of NMO lesions, we considered that complement activation might be a secondary phenomenon occurring as a result, rather than as a cause, of necrosis. This possibility seems unlikely given the fact that we did not observe complement activation in the acute spinal cord infarction cases. In addition, C9neo antigen deposition was not observed in acute or chronic white matter brain infarcts (H. Rauschka, B. Kornek, C. Stadelmann, A. Steffenl, W. Brück, C. Lucchinetti *et al*., unpublished results).

Role of eosinophils in NMO

One of the most striking novel features we describe regarding the histopathology of active NMO lesions is the intense perivascular and meningeal infiltration of the spinal cord with eosinophils and neutrophils. Activated eosinophils release basic granule proteins such as MBP, eosinophil-derived neurotoxin, eosinophil cationic protein and eosinophil peroxidase (Kaneko *et al*., 1997). These granules have cytotoxic properties and serve as markers for eosinophil activation (Venge, 1995). In addition to elevated numbers of eosinophils within the NMO lesions, we have confirmed the presence of eosinophil degranulation in spinal cord tissue of NMO patients. We have also demonstrated evidence for CCR3 expression in NMO lesions. CCR3 has been shown to be the principal receptor for the chemokine eotaxin, a potent eosinophil chemo-attractant. CCR3 is expressed primarily by eosinophils in humans (Ponath *et al*., 1996). Eotaxin signalling through CCR3 is an important mediator of eosinophil recruitment (Daugherty *et al*., 1996). CCR3 is selectively expressed by human T helper 2 (Th2) cells and therefore is associated with Th2 responses (Sallusto *et al*., 1998). Taken together, our data provide morphological evidence that eosinophils are increased in number, functionally active and likely contribute to the destructive inflammatory process in NMO.

The cytotoxicity of eosinophil granule proteins has been well established (Corrigan and Kay, 1996). Eosinophils are the dominant source of interleukin-4, which may cause a Th1 to Th2 shift in cytokine profile (Rumbley *et al*., 1999). Eosinophils are thought to play a major role in a variety of human diseases, including allergic inflammation, asthma, malignancy and host defense against helminth infections (Rothenberg *et al*., 1996). Eosinophil granule MBP is highly toxic to endothelial cells in a dose-dependent manner and is thought to contribute to vessel damage in necrotizing vasculitis associated with eosinophil infiltration (Chen *et al*., 1996). There is a single report of CSF eosinophilia in a child presenting with recurrent transverse myelitis (Snead and Kalavsky, 1976), as well as a report of eosinophilic vasculitis, pericarditis and hypocomplementaemia in a patient presenting with a disorder resembling NMO (Tanphaichitr, 1980). More recently, a retrospective study on multiple sclerosis in Aboriginals revealed an increased frequency in optic nerve and spinal cord involvement compared with non-Aboriginals (Mirsattari *et al*., 2001). They described a single autopsy case characterized by necrosis and eosinophil infiltrates within the spinal cord lesion that they attributed to chronic β-interferon treatment. However, none of the NMO patients included in our series received β-interferon therapy, and therefore this does not sufficiently explain the presence of eosinophils in NMO lesions.

The exact role of eosinophils in NMO immunopathology is unclear. It remains to be determined whether eosinophil activation is a primary or secondary event in NMO lesion formation. Activation of complement generates several biologically active peptides that have potent chemo-attractant potential (Asghar, 1998). The most clinically relevant complement-derived chemotactic factor is C5a, a cleavage product of the fifth component of complement. In addition to

its chemotactic activity, C5a is a potent factor for activating eosinophils, including the release of eosinophil granule proteins (Kernen *et al*., 1991). Complement activation within the lesions may have induced the production of eosinophil chemotactic factors, resulting in the secondary activation of eosinophils and the subsequent release of eosinophil granule proteins in vessel walls.

Possible explanations for the restricted topography of NMO lesions

The reasons why the spinal cord and optic nerve are preferentially affected in NMO are unknown. There are several possible explanations. It is possible, although unlikely, that these sites harbour a restricted CNS or vascular antigen. Alternatively, it may be that the spinal cord and optic nerve are particularly vulnerable to antibody-mediated injury due to the inherent weakness of the BBB at these sites. The normal BBB is highly impermeable to plasma proteins and circulating leukocytes, and thus it can protect the CNS against an immunological reaction. However, in those areas lacking an effective BBB, such as the spinal nerve roots, it may be proposed that circulating pathogenic antibodies could gain access to the CNS via these structures and diffuse out into the immediate vicinity. In EAE models, active lesions predominantly affect the spinal cord (Lassmann, 1983). ON in EAE also tends to predictably occur in the retrobulbar optic nerve (Guy and Rao, 1984). Lesions at these two sites are thought to reflect the higher degree of BBB permeability in these regions compared with the brain (Rao, 1981; Guy and Rao, 1984; Butter *et al*., 1991). The increased BBB permeability in the spinal cord may also be due to the inherent vascular properties of this region, where capillaries are larger than those in the brain. Thus, on a background of an inflammatory process in the presence of extremely high antibody titres, lesions might preferentially, but not exclusively, affect the spinal cord and optic nerve. This hypothesis would be compatible with the observation that in late stages of NMO, lesions often disseminate into other CNS regions (Wingerchuk *et al*., 1999).

Clinical associations with NMO

Several clinical associations with NMO suggest this disorder is distinct from multiple sclerosis. NMO patients often have circulating auto-antibodies at frequencies that exceed those seen in classical multiple sclerosis (Wingerchuk *et al*., 1999). The pathogenicity of these auto-antibodies is unknown: they might cause damage directly through the recognition of epitopes on normal cells, or indirectly through the formation of immune complexes that deposit in normal tissue and activate the complement cascade. Their presence in Devic patients may also reflect a more widespread B cell response. Prominent antibody responses can also be found with respect to endogenous myelin antigens, such as MOG. A recent study analysed antibody responses to MOG, MBP and S100β in the serum of four cases of Devic's disease (Haase and Schmidt, 2001). The authors reported a prominent anti-MOG response in all patients, MBP antibodies in two and S100β antibodies in one patient, and, similar to our data, concluded their findings were consistent with a widespread B cell immune response in Devic patients.

In contrast to classical multiple sclerosis, there are numerous reports of inflammatory opticospinal disease associated with connective tissue as well as other autoimmune disorders (April *et al*., 1976; Kinney *et al*., 1979; Goldman *et al*., 1984; Nambu *et al*., 1988; Lindsey *et al*., 1992; Simeon-Aznar *et al*., 1992; Bonnet *et al*., 1999; Mochizuki *et al*., 2000). Unlike multiple sclerosis, there is a racial predilection for NMO in non-whites (O'Riordan *et al*., 1996). Japanese patients with 'opticospinal' multiple sclerosis behave similar to relapsing NMO patients, and differ immunogenetically from Japanese with Western multiple sclerosis, consistently being HLA-DR2 negative (Shibasaki *et al*., 1981). Furthermore, plasma exchange has been reported to be effective for the management of acute relapses in NMO patients with or without associated connective tissue disorders (Konttinen *et al*, 1987; Fletchner and Baum, 1994; Weinshenker *et al*., 1999; Biliciler *et al*., 2001). Plasma exchange is known to reduce the amount of circulating autoantibodies and immune complexes (Clark *et al*., 1991), which might explain its effectiveness in some NMO patients.

Conclusions

This study suggests four separate lines of evidence supporting a role for humoral mechanisms in the pathogenesis of NMO: the lesion pathology, similarities with a select variant of MOG-induced EAE, clinical association with antibody-mediated collagen vascular disorders and the response to plasma exchange. Although complement activation is observed in a subset of classical multiple sclerosis patients (Storch *et al*., 1998*a*; Lucchinetti *et al*., 2000), the pattern and pronounced perivascular distribution of complement activation seen in NMO is unique. Despite differences in the clinical manifestations, imaging findings, CSF biochemistries and pathology between NMO and multiple sclerosis, the distinction between these disorders remains controversial (Mandler *et al*., 1993; Wingerchuk *et al*., 1999). Reduced levels of the neuro-inflammatory metalloproteinase marker MMP-9 in the CSF of patients with NMO supports the possibility of a different pathogenic mechanism of lesion production, since MMP-9 is markedly elevated in acute multiple sclerosis CSF (Mandler *et al*., 2001). Moreover, tissue inhibitor of metalloproteinases (TIMP-1) was significantly reduced in relapsing–remitting multiple sclerosis CSF, but not in NMO CSF (Mandler *et al*., 2001). These CSF biochemical differences might well be related to differences in the tissue inflammatory reaction in multiple sclerosis and in Devic's NMO. However, since the entire spectrum of multiple sclerosis pathology, including optico-spinal disease,

can be reproduced in the MOG-induced EAE rat model using the same MOG antigen but different strain/sensitization regimens (Storch *et al*., 1998*b*), it is still not clear whether NMO is pathogenetically distinct from multiple sclerosis, or rather reflects the effects of distinct host immunogenetic and environmental factors at play.

NMO is an aggressive and disabling inflammatory demyelinating disease, and treatment for this condition is often ineffective. Based on this study, future therapeutic strategies designed to limit the deleterious effects of complement activation, eosinophil degranulation and neutrophil/macrophage/microglial activation are worthy of further investigation.

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