Neuromyelitis Optica: Pathogenicity of Patient Immunoglobulin In Vivo

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Objective: Severe inflammation and astrocyte loss with profound demyelination in spinal cord and optic nerves are typical pathological features of neuromyelitis optica (NMO). A diagnostic hallmark of this disease is the presence of serum autoantibodies against the water channel aquaporin-4 (AQP-4) on astrocytes.

Methods: We induced acute T-cell–mediated experimental autoimmune encephalomyelitis in Lewis rats and confronted the animals with an additional application of immunoglobulins from AQP-4 antibody–positive and –negative NMO patients, multiple sclerosis patients, and control subjects.

Results: The immunoglobulins from AQP-4 antibody–positive NMO patients are pathogenic. When they reach serum titers in experimental animals comparable with those seen in NMO patients, they augment clinical disease and induce lesions in the central nervous system that are similar in structure and distribution to those seen in NMO patients, consisting of AQP-4 and astrocyte loss, granulocytic infiltrates, T cells and activated macrophages/microglia cells, and an extensive immunoglobulin and complement deposition on astrocyte processes of the perivascular and superficial glia limitans. AQP-4 antibody containing NMO immunoglobulin injected into naïve rats, young rats with leaky blood–brain barrier, or after transfer of a nonencephalitogenic T-cell line did not induce disease or neuropathological alterations in the central nervous system. Absorption of NMO immunoglobulins with AQP-4–transfected cells, but not with mock-transfected control cells, reduced the AQP-4 antibody titers and was associated with a reduction of astrocyte pathology after transfer.

Interpretation: Human anti–AQP-4 antibodies are not only important in the diagnosis of NMO but also augment disease and induce NMO-like lesions in animals with T-cell–mediated brain inflammation.

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Neuromyelitis optica (NMO) has originally been defined as an acute inflammatory demyelinating disease, predominantly affecting the spinal cord and optic nerves.[1] It was for a long time regarded as a variant of multiple sclerosis (MS), although with major distinctive pathological features.[2] A major breakthrough in our understanding of the disease was the discovery of an autoantibody response with high diagnostic sensitivity and specificity for this disease.[3] These autoantibodies can be detected in more than 75% of all patients[4] and are directed against aquaporin-4 (AQP-4), a water channel expressed on astrocytes.[5,6] Using these antibodies as a diagnostic marker allowed researchers to define the clinical spectrum of the disease,[7–9] which in essential aspects differs from that seen in classic MS.[10–12]

The presence of autoantibodies in the serum does not necessarily mean that they are pathogenic. In paraneoplastic encephalomyelitis, autoantibodies are excellent diagnostic markers of the disease,[13] although in most variants of the disease, the lesions in the central nervous system (CNS) are induced by cytotoxic T cells irrespective of the presence or absence of antibodies.[14] On the other hand, autoantibodies against components of the neuromuscular junction, seen in myasthenia gravis, Lambert–Eaton syndrome, or Isaac syndrome, are directly involved in the induction of clinical disease and structural damage, and disease can be transferred by the antibodies alone into recipient animals.[15–19] Requirements for autoantibodies to be pathogenic in the nervous system are that they can reach their specific target epitope from the extracellular space, that they

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can enter the target tissue in sufficient concentration, and that activated effector mechanisms, such as complement or activated macrophages, are present in the target organ in sufficient amount.[20]

Several observations suggest that AQP-4 autoantibodies may be partly responsible for the distribution and cellular composition of lesions in the CNS of NMO patients.[21] Regions of the brain and spinal cord with high AQP-4 expression are preferential targets for lesions in NMO patients.[11,22,23] Active NMO lesions show a selective loss of AQP-4 immunoreactivity and of glial fibrillary acidic protein (GFAP) containing astrocytes.[22–24] At sites of active tissue injury, immunoglobulin (Ig) and activated complement is deposited in a rosette-like perivascular manner,[2] consistent with a binding to astrocyte processes. Human NMO sera recognize an extracellular determinant of AQP-4; they can induce AQP-4 endocytosis in transfected cell lines in vitro[23,25,26] and destroy them in a complement-dependent manner.[6,27] Last, and probably most importantly, removing circulating antibodies from NMO patients by plasma exchange is beneficial.[6,28] On the other hand, AQP-4 antibodies are low in titer or absent in the cerebrospinal fluid (CSF) of NMO patients,[29] and transfer of disease by these antibodies into normal animals has not been achieved so far.[21] Here we show that human NMO Ig, which contains AQP-4 antibodies, augments clinical disease in T-cell–mediated autoimmune encephalomyelitis and induces lesions in the CNS, which reflect characteristic pathological features of NMO.

Materials and Methods

Source of Immunoglobulin Fractions

Ig fractions were obtained from sera or plasma exchange material of six patients with AQP-4 antibody–positive NMO or transverse myelitis, two patients with AQP-4 antibody–negative NMO, five patients with MS, one patient with noninflammatory neurological disease, and three healthy control subjects, essentially as described previously[30] (Table 1; see supplemental information 1). As a further control, we used a commercial human Ig (Subcuvia). The use of the patient's plasma for this study was approved by the Ethics Committee of Tohoku University School of Medicine (No. 2007-327)

Table 1. **Source of Immunoglobulin Used in the Transfer Experiments**

Patient No.	Age (yr)/Sex	Disease	Disease Duration (yr)	MRI	AQP-4 Antibody Titer	Other Autoantibodies
J 0	41/F	RTM	4	LETS	1:8,192	ana−, ssa−, ssb−
J 1	77/F	NMO	29	LETS, BL	1:65,536	ana−, ssa+, ssb−
J 2	55/F	NMO	17	LETS	1:16,384	ana+, ssa−, ssb−
I GF	54/F	NMO	9	LETS, BL	1:20,480	ana+
I RE	68/F	NMO	n.a.	LETS	1:10,240	n.d.
I RK	50/F	NMO	4	LETS, BL	1:640	n.d.
J 3	61/F	NMO	28	LETS	Negative	ana−
J 4	61/F	NMO	39	LETS	Negative	ana+, ssa+, ssb−
I TH	35/M	MS	5	SL, BL	Negative	n.d.
I KT	25/F	MS	Acute	BL	Negative	n.d.
J 5	34/F	MS	3	BL	Negative	ana−
J 6	34/F	MS	6	BL	Negative	ana−, ssa−, ssb−
J 7	44/F	MS	11	BL	Negative	ana−
I SN	24/M	PNP	n.a.	—	Negative	n.d.
J 8	37/M	Control	—	—	Negative	n.d.
J 9	34/F	Control	—	—	Negative	n.d.
J 10	58/F	Control	—	—	Negative	n.d.

MRI = magnetic resonance imaging; AQP-4 = aquaporin-4; RTM = relapsing transverse myelitis; LETS = longitudinally extensive lesion over three vertebral segments; ana = antinuclear antibody; ssa = anti–SS-A antibody; ssb = anti–SS-B antibody; NMO = neuromyelitis optica; BL = brain lesion fulfilled by the international panel's criteria of multiple sclerosis; n.a. = not available; n.d. = not done; MS = multiple sclerosis; SL = spinal cord lesions; acute = fulminant acute episode; PNP = polyneuropathy.

and the Ethics Committee of Innsbruck Medical University (No. AN3041, 257/4.8, 21.09.2007)

Anti–aquaporin-4 Antibody Assay

We detected and titrated human anti–AQP-4 antibodies by the method described in our previous reports.[25,29]

Absorption of Neuromyelitis Optica Immunoglobulin with Aquaporin-4 Molecules of Transfected Cells

Human embryonic kidney 293 cells were seeded in 75cm^2 flasks at a density of 1.6×10^6 cells. The cells were transiently transfected after 24 hours to overexpress an AQP-4/Emerald Green Fluorescent Protein (Invitrogen, La Jolla, CA) fusion protein or EmGFP without AQP-4 as a control. For these transfections, 24μl Fugene (Roche, Mannheim, Germany) was added to 776μl serum-free medium containing 8μg plasmid DNA, and the resulting complexes were added to the cells. Seventy-two hours after transfection, the purified and concentrated plasma exchange samples of two NMO patients (Patients I RE and I GF) were diluted with phosphate-buffered saline (PBS)/20% serum-free CellGro DC Medium (CellGenix, Freiburg, Germany) to a final concentration of 1mg/ml IgG and added to the transfected cells. After an overnight incubation at 37°C/5% CO_2 with gentle shaking, the patients' sera were removed and centrifuged for 15 minutes at 13,000 rpm. Then the absorption was repeated. Afterward, the IgG fractions were concentrated via Amicon Centriplus Centrifugal Filter Devices (Millipore, Bedford, MA) to a final concentration of 11 (Patient GF) or 7mg/ml (Patient RE) IgG and screened for the presence of anti–AQP-4 antibodies. One milliliter of the resulting IgG fractions were injected into one rat each.

Animals

Lewis rats were obtained from Charles River Wiga (Sulzfeld, Germany) and used at an age of 8 weeks (approximately 170gm body weight) or 3 weeks (approximately 45gm body weight). They were housed in the Decentral Facilities of the Institute for Biomedical Research (Medical University Vienna) under standardized conditions. The experiments were approved by the ethic commission of the Medical University Vienna and performed with the license of the Austrian Ministry for Science and Research.

Experimental Autoimmune Encephalomyelitis Induction and Tissue Preparation

T-cell lines against myelin basic protein (MBP) and ovalbumin were produced as described in detail previously.[31] Autoimmune encephalomyelitis was induced in Lewis rats by passive transfer of 1×10^6 MBP-reactive T cells.[32] Weight loss as earliest clinical sign of experimental autoimmune encephalomyelitis (EAE) started 4 days after the transfer. At this time point the animals were injected intraperitoneally with 1ml PBS containing either 10mg human Igs from NMO patients or control subjects, 10mg normal human IgG (Subcuvia), or no further additives (Tables 1, 2, and 3). The amount of IgG transferred is well tolerated by rats[33] (data from our own observations as well). In addition, normal animals were injected with the same amount of human patient–derived Ig or control IgG in the absence of T-cell

Table 2. **Transfer of Aquaporin-4 Immunoglobulin (J 0) Containing and Control Immunoglobulin (Subcuvia) in Rats with and without T-Cell–Mediated Experimental Autoimmune Encephalomyelitis**

T Cells	hIg	Animals Injected (n)	Clinical Score, mean ± SD	hIg S/CSF	α-AQP-4 Serum	α-AQP-4 CSF	T Cells/mm^2 in Lesions, mean ± SD	Macrophages/mm^2 in Lesions, mean ± SD	Granulocytes/mm^2 in Lesions, mean ± SD	Area of AQP-4 Loss in Perivascular Areas (×1,000μm^2), mean ± SD	Area of GFAP Loss in Perivascular Lesions (×1,000μm^2), mean ± SD
MBP	AQP-4 +	6[a]	3 ± 0.4[b]	2.5[c]	1:512-1:1,024	0-1:8	232 ± 41[b]	1,157 ± 256[b]	479 ± 152[b]	57 ± 11[b]	25 ± 10[b]
OVA	AQP-4 +	3[a]	0	n.d.	1:512	0	2 ± 1	5 ± 1	0	0	0
0	AQP-4 +	6[a]	0	254.1	1:512-1:1,024	0	4 ± 5	7 ± 4	0	0	0
MBP	Subcuvia	6[a]	2 ± 0.4[c]	3.0[c]	0	0	184 ± 61[c]	366 ± 74[c]	20 ± 6[c]	0	0
OVA	Subcuvia	3[a]	0	n.d.	1:512	0	3 ± 1	6 ± 1	0	0	0
0	Subcuvia	6[a]	0	91.0	0	0	2 ± 2	9 ± 3	0	0	0
MBP	AQP-4 +	3[d]	n.d.	n.d.	1:64-1:1,024	n.d.	367 ± 78	1,561 ± 435	127 ± 122	43 ± 30	19 ± 14
MBP	Subcuvia	3[d]	n.d.	n.d.	0	n.d.	507 ± 55	1,058 ± 267	13 ± 3	0	0

[a]Animals had a body weight of approximately 170gm. [b]$p < 0.01$ in comparison with MBP and Subcuvia. [c]$p < 0.01$ in comparison with control subjects without T-cell transfer. [d]Animals had a body weight of approximately 45gm.
hIg = human immunoglobulin; SD = standard deviation; hIg S/CSF = serum cerebrospinal fluid ratio of human immunoglobulin; α-AQP-4 serum = α-aquaporin-4 antibody titer in rat serum after transfer; CSF = cerebrospinal fluid; GFAP = glial fibrillary acidic protein; MBP = T-cell transfer with myelin basic protein (MBP) cells; OVA = T-cell transfer with ovalbumin cells; n.d. = not done.

Table 3. **Transfer of Human IgG from Neuromyelitis Optica Patients and Control Subjects in Rats with Myelin Basic Protein T-Cell–Induced Experimental Autoimmune Encephalomyelitis**

Source	Rats with Transfers (n)	α-AQP-4 Serum	hIg Serum (ng/ml)	Path. AQP Loss	Path. GFAP Loss
J 0 RTM	7	1:512-1:1,024	16,370	++	++
J 1 NMO	2	1:8,192	8,081	++	++
J 2 NMO	2	1:512	8,401	+	+/−
I GF NMO	2	1:512	31,218	++	++
I RE NMO	2	1:16,384	23,004	++	++
I RK NMO	2	1:16	16,995	+	+/−
AQP-4 antibody–negative NMO (n = 2)	4	—	8,792-21,975	—	—
MS (n = 5)	10	—	7,793-11,653	—	—
PNP (n = 1)	2	—	13,345	—	—
Healthy control subjects (n = 3)	6	—	5,603-12,124	—	—
Subcuvia	7	—	14,081	—	—

α-AQP4 serum = antibody titers against aquaporin-4 in rat sera at time of death; hIg serum = concentration of human immunoglobulin in rat serum at time of death; path. AQP loss = inflammatory spinal cord lesions with loss of aquaporin-4; path GFAP loss = inflammatory spinal cord lesions with loss of glia fibrillary acidic protein; RTM = relapsing transverse myelitis; ++ = extensive perivascular loss of aquaporin-4 or glia fibrillary acidic protein; NMO = neuromyelitis optica; + = loss of aquaporin-4 or glia fibrillary acidic protein at the glia limitans around inflamed vessels; +/− = segmental loss of aquaporin-4 or glia fibrillary acidic protein at the glia limitans of inflamed vessels; MS = multiple sclerosis; PNP = polyneuropathy.

transfer (see Table 2). Testing of human Ig from NMO and control patients was performed in a blinded fashion without knowledge of AQP-4 antibody titers or clinical diagnosis.

Twenty-four hours later, the animals were killed with CO_2. Blood samples were taken by cardiac and CSF samples by suboccipital puncture. Afterward, the animals were perfused with either 4% phosphate-buffered paraformaldehyde (PFA; n = 5) or with PBS (n = 2). From animals that had been perfused with PFA, brains, spinal cord tissue, and peripheral organs (kidney, lung, liver, muscle, intestine, spleen, and thymus) were dissected and immersed for another 18 hours in PFA. From animals that had been perfused with PBS, the spinal cord was cut in several pieces. Some of these pieces were frozen in liquid nitrogen; the others were fixed for 18 hours in PFA. PFA-fixed material was routinely embedded in paraffin. Tissue sections were stained with hematoxylin and eosin, with Luxol fast blue myelin stain, and with Bielschowsky silver impregnation for detection of axons and neurons. In addition, tissue blocks from the spinal cord were further fixed in 3% phosphate-buffered glutaraldehyde for 24 hours, osmicated, and routinely embedded in epoxy resin. Plastic sections of 0.5μm in thickness were cut on an ultramicrotome and stained with toluidine blue.

Immunohistochemistry

All tissue blocks were cut in serial sections, and all stainings were done essentially as described previously,[34] using the following antibodies: W3/13 (T cells and granulocytes; mouse monoclonal; Serotec, Kidlington, United Kingdom); ED1 (macrophages, microglia; mouse monoclonal; Serotec); commercial anti–AQP-4 (rabbit polyclonal; Sigma, Vienna, Austria); anti-GFAP (rabbit polyclonal; Dako, Glastrup, Denmark; or mouse monoclonal; Neomarkers, Fremont, CA); anti–S-100β (mouse monoclonal; Sigma); anti–proteolipid protein (mouse monoclonal; Serotec); anti-NG-2 (glial progenitor cells; rabbit polyclonal; Chemikon, Austria); anti–human Ig (biotinylated donkey; polyclonal; GE Healthcare/Amersham, Vienna, Austria); anti-complement C9 (rabbit polyclonal[35]), and antiactivated caspase (CM-1; Idun Pharmaceuticals, New York, NY). Immunohistochemistry was completed by using appropriate biotinylated secondary antibodies (sheep anti–mouse, donkey anti–rabbit, donkey anti–sheep/–goat; all from GE Healthcare/Amersham, Vienna, Austria) and subsequent incubation of the sections with peroxidase-labeled avidin (Sigma). DNA fragmentation was analyzed by terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling staining as described previously.[36]

Confocal Laser Microscopy and Double Staining

Fluorescence immunohistochemistry was performed on paraffin sections, using primary antibodies from different species (commercial α-AQP-4 [rabbit], α-GFAP [mouse], or α-S100β [mouse]; biotinylated anti–human Igs [donkey]). The antibodies were applied simultaneously at 4°C overnight. After washing with PBS, secondary antibodies consisting of goat anti–mouse Cy2 (1:200; Jackson ImmunoRe-

search, West Grove, PA) and biotinylated anti–rabbit (1:200; Amersham Pharmacia Biotech) were applied simultaneously for 1 hour at room temperature. The staining was finished by application of streptavidin-Cy3 (1:75; Jackson ImmunoResearch) for 1 hour at room temperature. Fluorescent preparations were examined as described previously.[37]

Quantitative Evaluation of Immunostained Sections

Quantification was done by manual counting, using a morphometric grid placed within the ocular lens. We first determined the total area of the spinal cord section by counting the number of grid points, which were located within the spinal cord section. We then determined the number of grid points, which were located within areas of AQP-4 or GFAP loss. The areas of AQP-4 or GFAP loss are given in $1{,}000\mu m^2$ per spinal cord cross section. For inflammation a morphometric grid of 0.25×0.25mm was superimposed over the lesion with the inflamed vessel being located in the center of the grid. We then counted the number of T cells, macrophages, and granulocytes located within the entire area of the grid. The values were then recalculated as cells per square millimeter.

Statistical Evaluation

Statistics were calculated with the Statgraphics Plus program. The Mann–Whitney (Wilcoxon) W test (comparison of medians) was always used.

Results

Anti–aquaporin-4 Antibody Containing Neuromyelitis Optica IgG Exacerbates Experimental Autoimmune Encephalomyelitis

In a first step, we analyzed whether intravenous injection of NMO IgG, containing high titers of AQP-4 antibodies, can modulate EAE induced by passive transfer of MBP-reactive T cells. Ten milligrams of purified NMO IgG derived from plasma exchange extracts of a patient with transverse myelitis and high AQP-4 antibody titer or of commercially available human Ig was injected at the onset of clinical disease. The human anti–AQP-4 antibodies were reactive to human and rat AQP-4, bound to the surface of rat astrocytes (data not shown), and were dominantly of the IgG1 isotype (see supplementary information 1). The injection resulted in high serum concentrations/titers of human IgG and human anti–AQP-4 antibodies (1:512 to 1:1,024, comparable with those seen in human NMO patients[29]). Animals with T-cell–mediated EAE showed profound blood–brain barrier leakage, reflected by high concentrations of human Ig in the CSF. However, similar as in NMO patients,[29] AQP-4 antibodies in the CSF were very low in titer or undetectable, suggesting absorption of the specific antibodies by the excess of the cognate antigen in the CNS tissue. All T-cell–injected animals receiving human anti–AQP-4 antibodies developed more severe symptoms of EAE than their control IgG or vehicle-injected counterparts (see Table 2). Disease exacerbation was already evident within the first 24 hours after injection of the human anti–AQP-4 antibodies.

Anti–aquaporin-4 Antibody Containing Human Neuromyelitis Optica IgG Induces Lesions in the Central Nervous System, Which Resemble Early Lesions of Neuromyelitis Optica

When human anti–AQP-4 antibodies were injected at the onset of MBP-specific T-cell–induced CNS inflammation, profound changes of perivascular astrocytes became apparent (Figs 1 and 2; see Table 2). In the center of the lesions, typical astrocyte markers such as AQP-4, GFAP, and S-100β protein were no longer detectable, suggesting destruction of astrocytes and their foot processes (see Figs 1A, C, E). Loss of AQP-4 was also observed at the lesion edge, where GFAP or S-100β protein were still present in numerous astrocyte processes (see Fig 2B). In spite of the presence of GFAP or S-100β, the respective astrocytes looked damaged. They were swollen (see Fig 2E), showed enlarged nuclei, and had less reactivity for S-100β protein than normal astrocytes in the adjacent tissue (see Fig 2A). DNA fragmentation, as demonstrated by terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling staining, was seen in some astrocytes around the lesions (see Fig 2A, inset). The cells, however, did not express activated caspase-3 (data not shown), nor did they show nuclear condensation or fragmentation, suggestive for apoptosis (see Fig 2A). Massive human IgG and rat complement C9 reactivity was seen at sites of AQP-4 and GFAP loss (see Figs 1G, I). Confocal microscopy showed colocalization of human Ig and AQP-4 in perivascular lesions (see Fig 2C), and appeared to be precipitated as insoluble immune complexes (see Supplementary Fig 1). Furthermore, as in human NMO lesions, C9 was precipitated on perivascular and subpial astrocyte processes (see Fig 2D). In contrast with the profound astrocyte pathology, no pathological changes were found in neurons, oligodendrocytes, NG-2 reactive progenitor cells, or myelin (data not shown). Hence, mature astrocytes and their processes are the selective target in animals receiving MBP-specific T cells and human anti–AQP-4 antibody containing NMO Ig. The pathological picture seen in animals receiving T cells and control human IgG was radically different: Here, the astrocytes at sites of inflammation showed profound GFAP and AQP-4 immunoreactivity (see Figs 1B, D, F). In particular, astrocytic foot processes of the glia limitans were clearly visible at all sites of inflammation (see Figs 1D, F) and no perivascular astrocytic swelling was seen (see Fig 2F).

Injection of human anti–AQP-4 antibodies at the onset of MBP-specific T-cell–induced CNS inflammation also led to a dramatic increase in the number of

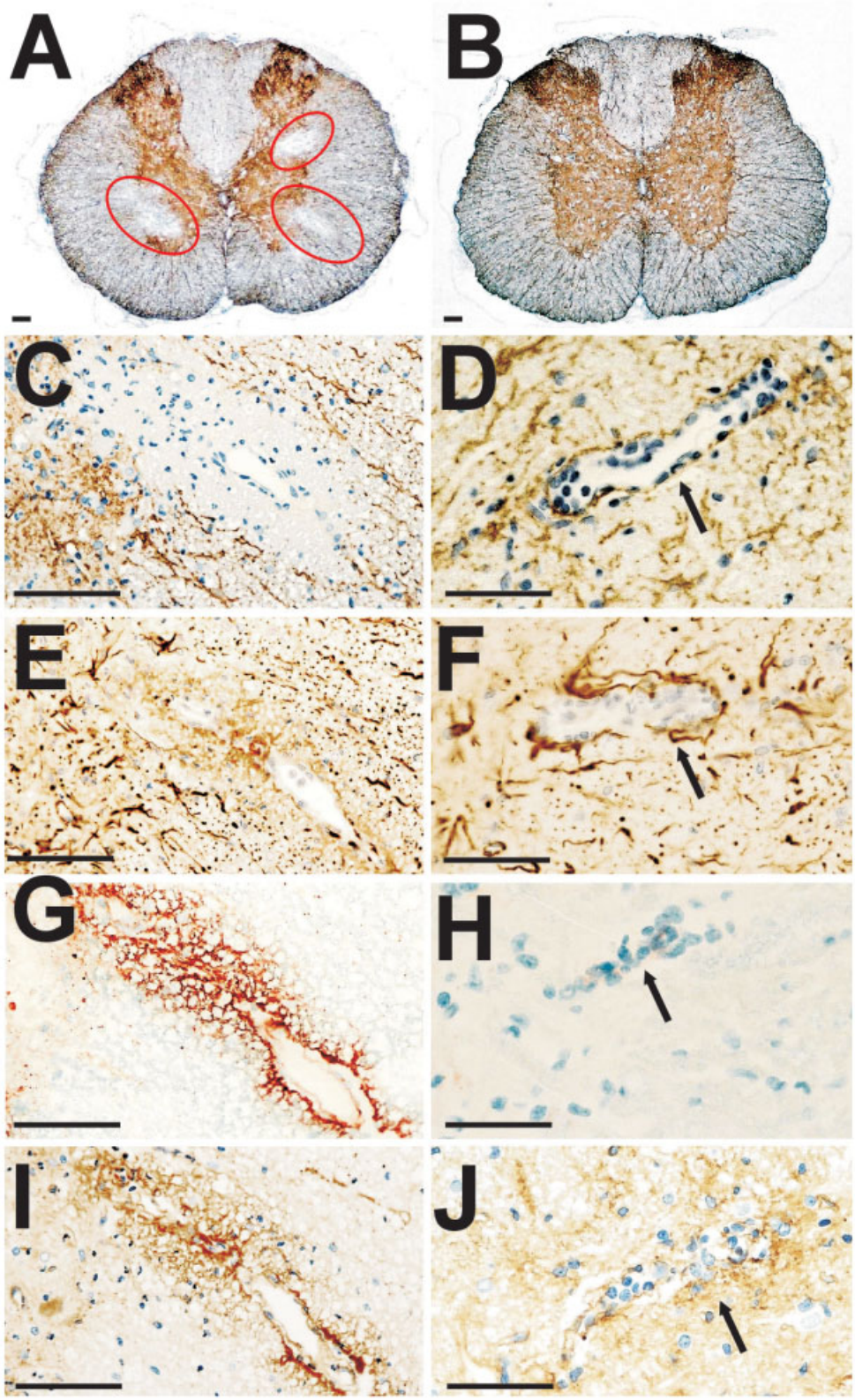

Fig 1. Spinal cord cross sections of animals injected with T cells and human anti–aquaporin-4 (anti–AQP-4) antibodies (A, C, E, G, I) or T cells and control IgG (B, D, F, H, J). The sections were reacted with the commercial anti–AQP-4 antibody (A, B; detailed in C, D), or antibodies against glial fibrillary acidic protein (GFAP; E, F), C9 (G, H), and human IgG (I, J). Areas of AQP-4 loss are encircled in red. Arrows *point to a perivascular inflammatory cuffs. Transfer of neuromyelitis optica (NMO) immunoglobulin in experimental autoimmune encephalomyelitis (EAE) results in profound loss of AQP-4 (A, C) and GFAP (E), as well as massive C9 (G) and immunoglobulin deposition (I) around inflamed vessels predominantly in the spinal cord gray mater. In contrast, transfer of control immunoglobulin shows inflammation with preservation of perivascular AQP-4 (B, D) and GFAP (F) reactivity, no C9 deposition (H), and diffuse immunoglobulin staining (J) around inflamed vessels. Scale bars = 300μm.*

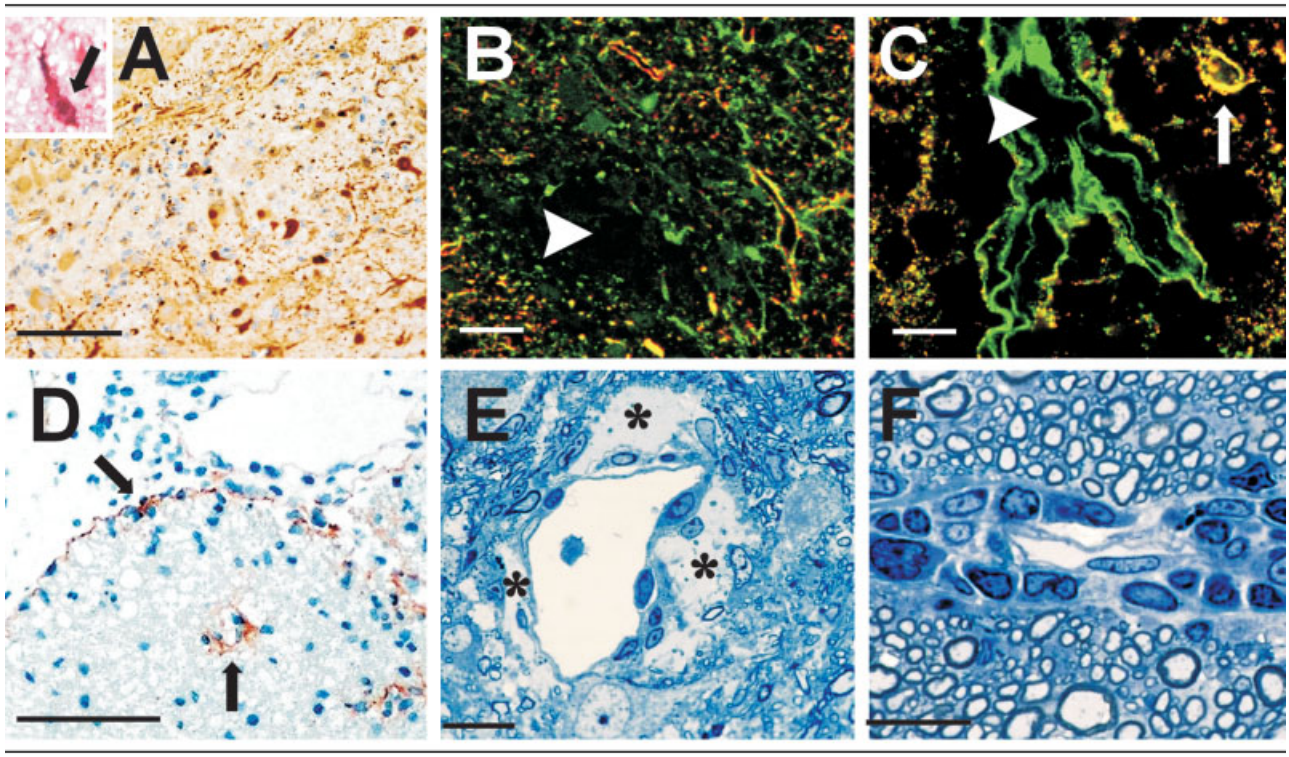

Fig 2. Astrocytes in spinal cord lesions of animals injected with myeline basic protein (MBP)–specific T cells in the presence (A–E) or absence (F) of human anti–aquaporin-4 (anti–AQP-4) antibodies. (A) S-100β staining (brown) is almost completely lost in the center of T/hα AQP-4–induced perivascular lesions. (A, inset) Some astrocytes around these perivascular lesions are still S-100β–positive (red) but show signs of DNA fragmentation (black staining of nucleus [arrow] *and spillover of fragmented DNA [black] in the cytoplasm, indicative of necrosis). (B) Confocal microscopy shows that the loss of AQP-4 (red) in perivascular lesions of T/hαAQP-4–treated animals is even present when glial fibrillary acidic protein (GFAP)–reactive astrocytes (green) are still preserved.* White arrowhead *indicates an inflamed blood vessel. (C) Human IgG (green) is found in the wall of blood vessels, on the AQP-4+ (red) astrocytic glia limitans* (white arrow), *and on AQP-4+ astrocyte processes or in macrophage granules in the tissue (note that because of colocalization, AQP-4+ cells binding human IgG appear yellow). (D) Rat complement C9 reactivity (red; highlighted by* black arrows*) is seen on subpial and perivascular astrocyte processes. (E) Semithin plastic sections show astrocyte swelling in early lesions (astrocytes highlighted by* asterisks*). (F) T/coIgG-treated animals show inflammation in the absence of astrocyte damage. Scale bars = 250μm (A); 100μm (B, C); 300μm (D); 30μm (E, F).*

granulocytes and macrophages in the lesions (see Table 2). Also, in this aspect, the lesions looked strikingly similar to those in NMO patients,[2] and differed from lesions in animals treated with MBP-specific T cells and control IgG or PBS, which contained comparable numbers of T cells but significantly less granulocytes and macrophages (see Table 2).

To further confirm the specificity of pathological changes induced by the human anti–AQP-4 antibodies, we analyzed CNS inflammation provoked by cotransfer of MBP-specific T cells and antibodies against myelin oligodendrocyte glycoprotein (MOG; Fig 3). To directly compare this with our results in NMO Ig–induced lesions, we specifically selected lesions in areas with high AQP-4 expression, such as the spinal cord gray matter. Also after injection of anti-MOG antibodies, the presence of antibodies increased inflammation with granulocyte infiltration of the lesions, as well as with Ig and complement deposition.[32] However, in marked contrast with the situation in their human anti–AQP-4 antibody–injected counterparts, anti-MOG IgG–injected animals displayed a selective loss of myelin, but no loss of AQP-4, GFAP, or S-100β protein (see Fig 3).

Neuromyelitis Optica Immunoglobulin Precipitates Lesions Predominantly in Brain Areas, Which Are Targeted by the Autoreactive T Cells and Express High Levels of Aquaporin-4

The location of inflammatory lesions in the brain and spinal cord depends on the antigen specificity of autoimmune T cells.[38] Thus, MBP-specific T cells predominantly target the spinal cord and the brainstem, whereas lesions in the forebrain and the cerebellum are infrequent.[38] In addition, AQP-4 is not evenly distributed within the brain tissue. Overall, in normal animals, expression is more pronounced in gray than in white matter areas, and particularly high expression is

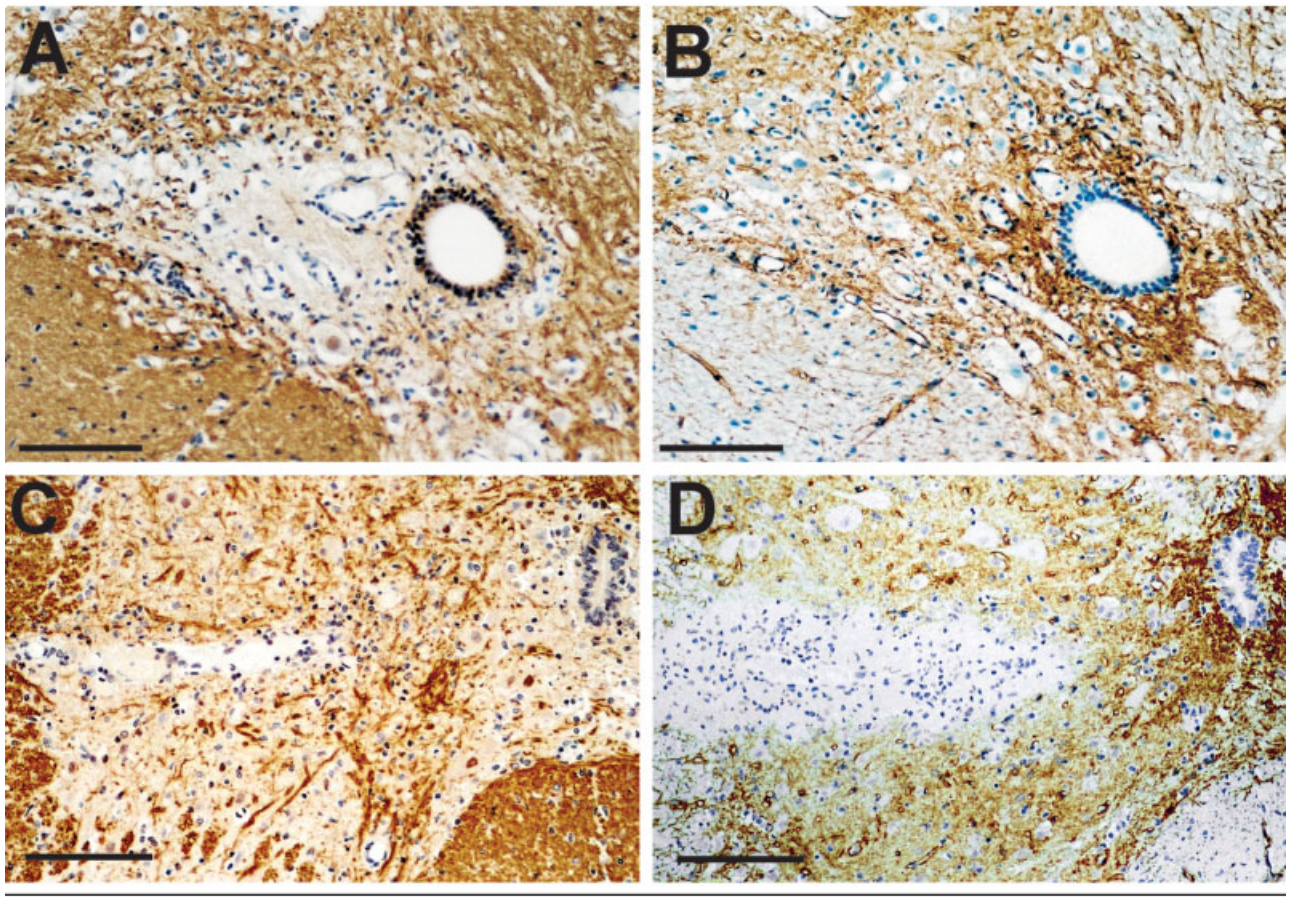

Fig 3. Differences in lesion pathology in animals injected with T cells and anti–myelin oligodendrocyte glycoprotein (anti-MOG) antibodies or human anti–aquaporin-4 (anti–AQP-4) antibodies. (A, B) Lesions in T/α MOG–injected animals show loss of MBP staining indicative for demyelination (brown, A) but preservation of AQP-4 in these lesions (brown, B). (C, D) Lesions in T/hα AQP-4–injected animals show preservation of myelin (MBP staining, brown, C) and loss of AQP-4 (brown, D). Scale bars = 200μm.

seen in periventricular areas and around the central canal of the spinal cord. In our experimental model, inflammatory lesions with perivascular loss of AQP-4 were more frequently seen in the spinal cord gray matter than in the white matter (see Fig 1A). In addition, large and frequent lesions were found at the floor of the IVth ventricle (Fig 4A), in the basal hypothalamus, adjacent to the third ventricle, and in the optic nerve

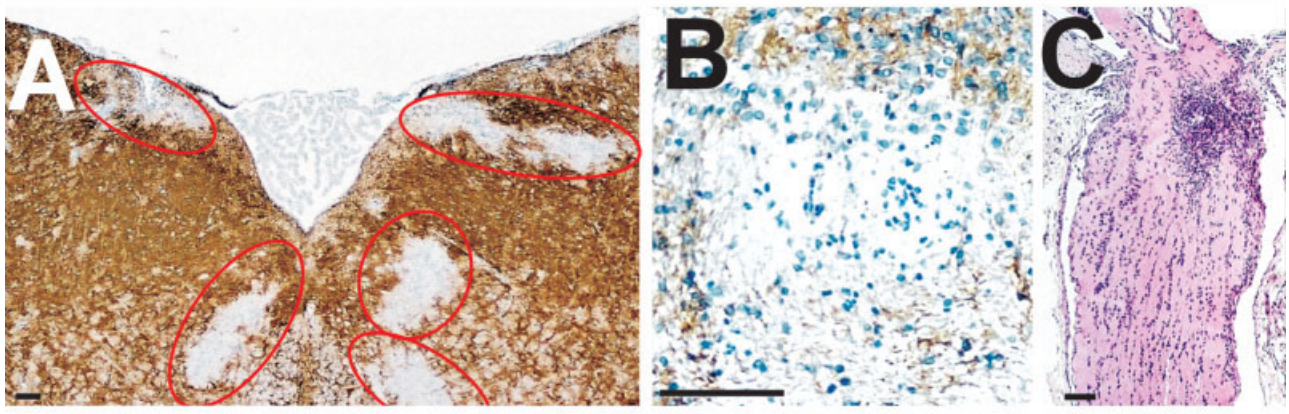

Fig 4. Aquaporin-4 (AQP-4) staining of inflammatory lesions from T/hα AQP-4–injected animals. Profound loss of AQP-4 (brown) is observed in the medulla, as indicated by red circles *(A), and in the optic chiasma (B). Note massive inflammation at the optic nerve head (C). Scale bars = 100μm*

Table 4. **Transfer of Neuromyelitis Optica IgG from Aquaporin-4 Antibody$^+$ Neuromyelitis Optica Patients after Preabsorption with Aquaporin-4/EmGFP or EmGFP Expressing Cells**

Patient No.	NMO Ig Absorbed with	α-AQP-4 Titer in NMO Ig Preparation	α-AQP-4 Titers in Rat Serum	Perivascular Infiltrates	Perivascular Infiltrates with AQP-4 Loss	Perivascular Area with AQP-4 Loss
I RE	AQP-4/EmGFP	1:5,120	1:280	4.6	1.5	5.1
I RE	EmGFP	1:10,240	1:360	4.7	3.0	13.5
I GF	AQP-4/EmGFP	1:640	b.d.	4.6	0	0
I GF	EmGFP	1:1,280	1:40	4.7	0.3	1.4

The numbers of perivascular infiltrates were counted on 22 spinal cord sections per animal along the entire neuraxis and represent the average numbers of infiltrates per spinal cord section. The determinations of the α aquaporin-4 (AQP-4) antibody titers in the rats and the histological evaluations of AQP-4 loss in these animals were made by S.M. and H.L., respectively, who were both blinded to the experimental protocol.
NMO = neuromyelitis optica; Ig = immunoglobulin; AQP-4 loss = area of AQP-4 loss in perivascular areas ($\times 1{,}000 \mu m^2$); b.d. = below the limit of detection.

and chiasm (see Figs 4B, C). Lesions in other brain areas were less consistently seen and, when present, much smaller. This topographical pattern of lesions is similar to that described in NMO patients. Interestingly, circumventricular organs, such as the area postrema and the subfornical organ, were free of inflammation and lesions with AQP-4 loss, despite the presence of AQP-4 immunoreactivity and a leaky blood–brain barrier. No pathology was seen in peripheral organs, such as muscle, intestine, and kidney, despite the presence of AQP-4.

Central Nervous System Lesions with Loss of Aquaporin-4 and Glial Fibrillary Acidic Protein Are Specifically Induced by Aquaporin-4 Antibody Containing Neuromyelitis Optica IgG

We have described so far that intravenous transfer of AQP-4 antibody containing Ig into animals with T-cell–mediated EAE augments disease and induces CNS lesions with selective loss of AQP-4 and GFAP. To test whether this effect is specific for AQP-4 antibody containing NMO Ig, we tested in a blinded fashion five additional AQP-4 antibody–positive Ig fractions from different NMO patients in comparison with Ig fractions from AQP-4 antibody–negative NMO patients (n = 2), MS patients (n = 5), other neurological disease patients (n = 1), and control subjects without neurological disease (n = 3; see Table 3). In comparison with control subjects, Ig fractions from three further NMO patients, which contained high titers of AQP-4 antibodies predominantly of the IgG1 isotype, induced lesions with widespread loss of AQP-4 and GFAP. Two other NMO Ig fractions with low AQP-4 antibody titers also produced lesions with subtle loss of AQP-4 and GFAP. None of the other Ig fractions induced CNS pathology with AQP-4 or GFAP loss (see Table 3; see Supplemental Fig 2). We then determined whether the AQP-4 antibodies in the NMO Ig were indeed responsible for the loss of AQP-4 reactivity in the lesions. For this purpose, we preabsorbed the NMO Ig from two different AQP-4 antibody+ NMO patients with cells expressing AQP-4/EmGFP or with cells expressing EmGFP only, and transferred these different NMO Ig preparations at the onset of EAE. In both cases, preabsorption with AQP-4/EmGFP cells led to a pronounced decrease in the numbers of perivascular lesions with, and the areas of, AQP-4 loss (Table 4; Fig 5).

Human Anti–aquaporin-4 Antibodies Need the Presence of Autoreactive, Central Nervous System Antigen-Specific T Cells to Become Pathogenic

In the absence of CNS antigen-specific T cells, the IgG preparations from NMO patients with anti–AQP-4 antibodies tested in this study or control IgG did not induce inflammation or tissue injury in the brain or spinal cord of recipient animals (Figs 6A–D), despite similar titers of human anti-AQP-4 antibodies and similar concentrations of human IgG in their circulation (see Table 2). The serum/CSF ratio of human IgG in these animals was 250:1 (see Table 2), suggesting limited access of transferred antibodies into the CNS compartment. In the brain, weak human IgG and traces of complement C9 immunoreactivity were found in both human anti–AQP-4 antibody– and control IgG–injected animals in the optic nerve head and the circumventricular organs. This was not associated with inflammatory lesions, astrocyte pathology, or AQP-4 loss.

To further address the question whether NMO Ig may induce pathology in conditions of impaired blood–brain barrier permeability, we injected the AQP-4 antibody containing Ig into juvenile rats 3 weeks after birth. In such animals, focal areas of increased blood–brain barrier permeability are seen, reflected by endothelial reactivity for dysferlin, a marker

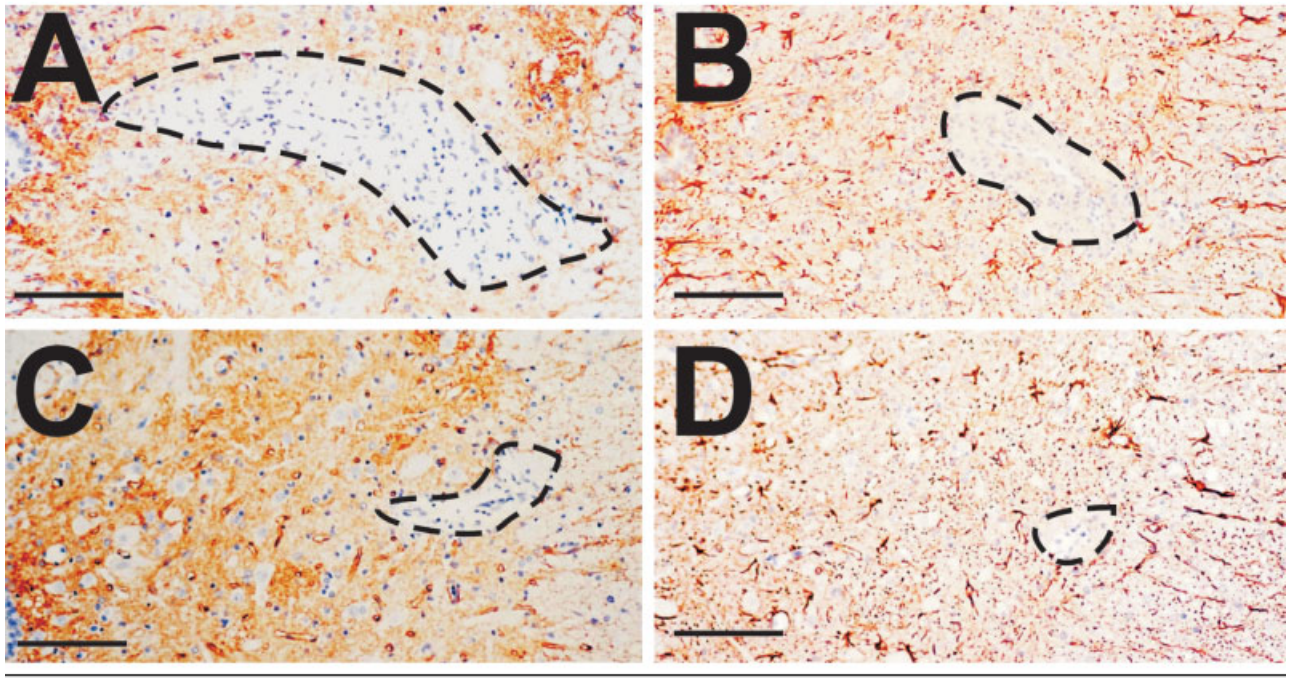

Fig 5. The anti–aquaporin-4 (anti–AQP-4) antibodies found in the neuromyelitis optica (NMO) Ig of Patient I RE are responsible for the loss of AQP-4 reactivity in the tissue. The degree of AQP-4 (A, C) and glial fibrillary acidic protein (GFAP) loss (B, D) in the tissue is reduced after the prior absorption of NMO Ig with transfectants expressing AQP-4/EmGFP fusion proteins (C, D), as compared with NMO Ig absorbed with transfectants expressing EmGFP only (A, B). Note that we obtained identical results with preabsorbed NMO Ig from two of two patients studied. Scale bars = 100μm.

for leaky endothelial cells,[39] and by leakage of serum proteins into the perivascular tissue (see Fig 6F). Furthermore, AQP-4 expression in such animals is comparable with that seen in adult rats (see Fig 6G). Despite pronounced vascular leakage of human Ig into the tissue, we found no signs of inflammation, deposition of activated complement, or loss of AQP-4 or GFAP (see Figs 6E–G). Similarly, the increase of circulating human Ig by injections of 10mg AQP-4 containing NMO Ig on 3 consecutive days in adult rats did not induce inflammation or lesions with AQP-4 or GFAP loss (data not shown).

To determine whether the pathogenic epitope of AQP-4, recognized by NMO Ig, is indeed present in juvenile rats, we induced inflammation by MBP-reactive T cells in these animals before the transfer of AQP-4 antibody containing NMO Ig. We found lesions with augmented inflammation and loss of AQP-4 and GFAP, which were similar in size and distribution as described earlier in adult rats. Despite the much greater dose of NMO Ig in relation to body weight in these animals, no further increase in lesion size and frequency was observed. In these animals, serum concentrations of human Ig and serum anti-AQP 4 antibody titers were similar compared with those seen in adult rats (see Table 2).

Discussion

The presence of serum antibodies against AQP-4 is a diagnostic hallmark for NMO. We show here that Ig fractions containing these antibodies are pathogenic in vivo. If present in serum titers comparable with those seen in NMO patients, they augment clinical disease in EAE and induce lesions within the CNS, which closely reflect those seen in the respective human disease.[2,22,23] Similarities include an inflammatory reaction composed of T cells, activated macrophages/microglia, and granulocytes. Extensive Ig and complement deposition on astrocyte processes of the perivascular and superficial glia limitans is associated with selective loss of the astrocyte markers GFAP and S-100β. Even more pronounced is the loss of AQP-4 itself, which is also seen in areas adjacent to the lesions, which still contain GFAP-reactive cell processes. In initial stages of lesion formation, as analyzed in this study, astrocytes are targeted in a highly specific manner, leaving neurons, axons, oligodendrocytes, myelin, and NG-2–positive progenitor cells intact. These findings are in line with studies on acute inflammatory lesions of NMO patients, where AQP-4 and GFAP were completely lost, whereas MBP remained relatively preserved,[40] and with studies demonstrating an increase of GFAP in the CSF of NMO patients during relapse.[41,42]

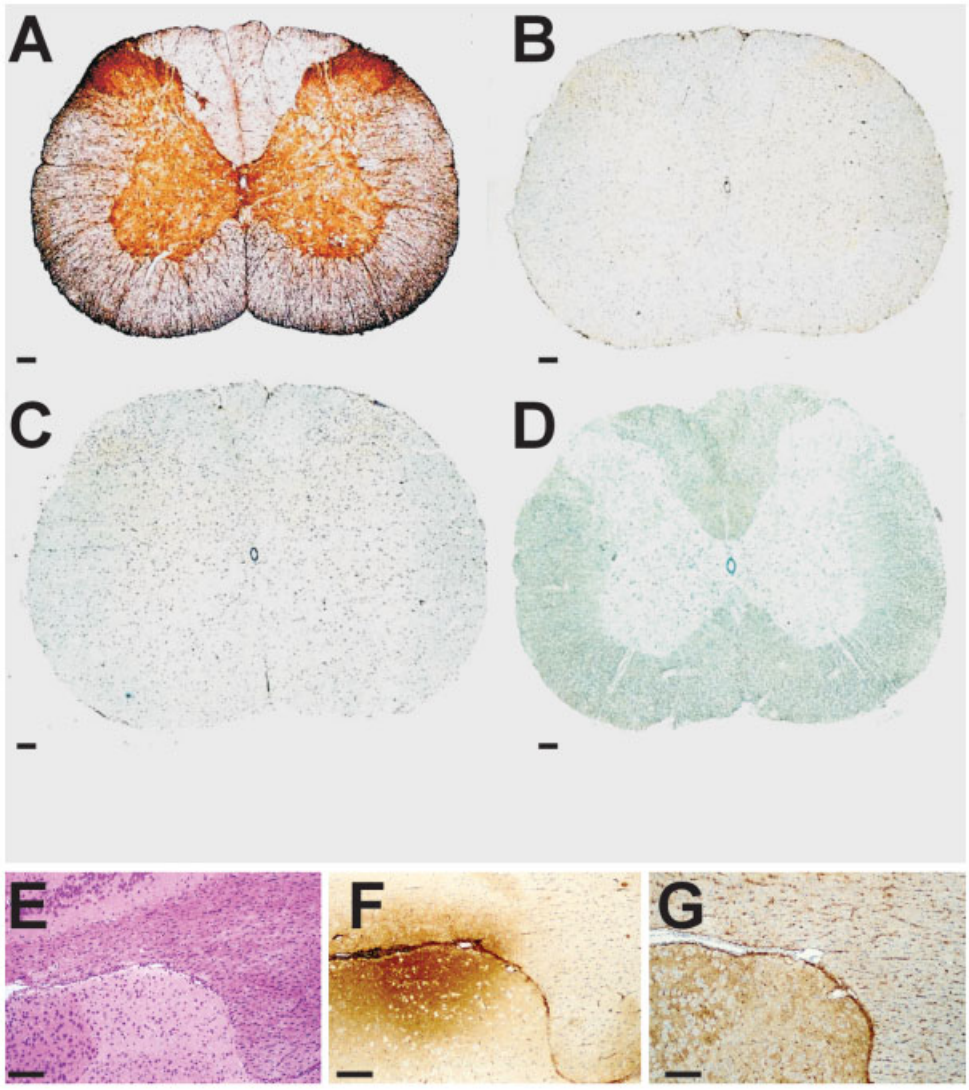

Fig 6. Aquaporin-4 (AQP-4) is not targeted by human anti–AQP-4 antibodies in the absence of T cells. (A–D) Spinal cord cross sections of a representative 0/hα AQP-4–treated animal. AQP-4 staining pattern is normal (brown, A), there is no evidence for inflammation (lack of staining with the macrophage/activated microglia cell–specific antibody ED1; B), absence of human IgG (C) or complement C9 (D). (E–G) There is no evidence for cellular infiltrates (E) or AQP-4 loss (G) in the central nervous system of 3-week-old rats injected with neuromyelitis optica (NMO) Ig containing anti–AQP-4 antibodies only, despite focally pronounced leakage of serum proteins into the perivascular tissue (F). Scale bars = 100μm.

There are, however, some differences in the pathology between our new experimental model and NMO in humans. Although initial tissue alterations seen in the CNS of NMO patients closely resemble those seen here, in more advanced stages, the lesions are larger and less specifically directed against astrocytes, reflected by additional demyelination and neuronal and axonal destruction.[2] This discrepancy is not surprising, because in our experiment, the tissue was analyzed 24 hours after the transfer of human anti–AQP-4 antibodies. We have chosen this time point because the differences between NMO and MS lesions are most characteristic in initial lesions, and because in the passive transfer EAE model, the opening of the blood–brain barrier is temporally restricted for a very short time window. This prevents a long-standing destruction of astrocytes that, at least in transgenic models, may secondarily lead to increased inflammation and myelin or neuronal injury.[43] Another difference was that in NMO, the inflammatory infiltrates contain many eosinophils,[2,44] which were absent in the rat model. This may be because of the strain of rats used for our experiments, because the combination of MOG-specific T cells and MOG-specific antibodies lead to a recruitment of eosinophils to CNS lesions in BN and DA rats, but not in Lewis rats, the animals used in our study.[45,46]

A key observation of our study was that human

anti–AQP-4 antibodies were pathogenic only when they reached the CNS at sites of brain inflammation. This is similar to what was observed in previous experiments on the pathogenicity of demyelinating anti-MOG antibodies.[32] This may have several explanations. Under normal conditions, the blood–brain barrier restricts entry of serum proteins into the CSF compartment, which makes it unlikely that autoantibody concentrations are reached that are high enough to initiate lesions. Furthermore, in the CNS, autoantibodies can become pathogenic only in the presence of activated effector cells such as macrophages[47] or of sufficient amounts of complement.[35,48] In vitro studies have shown that NMO Ig is able to lyse rat astrocytes in a complement-dependent manner.[27] All these conditions are fully fulfilled in a T-cell–mediated inflammatory environment in the CNS. Our studies in juvenile animals suggest that antibody leakage through the blood–brain barrier alone is not sufficient to induce NMO lesions, but the amount of pathogenic antibody and complement reaching the target tissue may be lower than in the context of an inflammatory lesion.

The availability of the AQP-4 epitope on astrocytes is not only regulated by the cell cycle or the differentiation stage of these cells, but also by CNS inflammation[49–51] and by AQP-4 clustering in orthogonal arrays.[52] Thus, expression of AQP-4 in a given location alone does not necessarily imply that this molecule is recognized by pathogenic NMO antibodies. The absence of pathological alterations in the area postrema or in peripheral organs, such as the kidney, despite the presence of AQP-4 and its accessibility for serum proteins indicates that clustering of AQP-4 leading to the expression of the pathogenic epitope does not occur at these sites.

The lack of pathogenicity of human AQP-4 autoantibodies under noninflammatory conditions is probably reflected in NMO patients. Although it has been shown that the disease is ameliorated by plasma exchange[28] or depletion of B cells,[53] patients can also recover from disease attacks under antiinflammatory therapy alone.[54] In particular, patients may show relapses and remissions of the disease in spite of unchanged or even high AQP-4 antibody titers in their serum.[29,55] Furthermore, it has recently been shown in a case report that serum antibodies against AQP-4 can be present already several years before clinical onset of NMO,[56] that NMO IgG binding to AQP-4 molecules in orthogonal arrays does not affect the function of this water channel,[52] and that interleukin-17, interferon-γ, and other proinflammatory cytokines are markedly increased in the CSF from NMO patients, regardless of the presence or absence of anti–AQP-4 antibody.[57] Cumulatively, these findings suggest that the presence of anti–AQP-4 antibody within the CNS cannot induce any inflammation unless T-cell inflammatory components do exist there.

The need for T cells to trigger active disease in NMO raises the question of their antigen specificity. From the experimental paradigm of MOG antibody transfer, it is well established that the antigen specificity of the T cells is irrelevant for autoantibodies to become pathogenic, provided they recognize an antigen within the CNS and are able to induce encephalomyelitis in a region where the target antigen for the autoantibodies is expressed.[32,58] Alternatively, the strong IgG response against AQP-4 in NMO patients suggests that they may mount a potentially encephalitogenic T-cell response against this antigen itself. Whether this is the case is currently unresolved, but clonal expansion of T cells, previously described to occur in the peripheral blood of NMO patients,[59] indicates that T-cell responses may be involved in the pathogenesis of this disease.

In conclusion, we show here that, just like monoclonal α AQP-4 antibodies,[60] AQP-4 antibody containing Ig isolated from the serum of NMO patients, but not control subjects, augments disease in the presence of CNS antigen-specific T cells and induces CNS lesions strikingly similar to those seen in NMO patients. Thus, the mechanism of tissue injury resembles one postulated to occur in a subset of MS patients.[48] However, this study strongly suggests a direct role of AQP-4 antibodies in causing astrocytopathy, a feature clearly distinguishing NMO from MS. It further suggests that anti–AQP-4 antibodies are modifying factors of NMO, like the anti-MOG and anti-neurofascin antibodies in MS. It is necessary to know the triggers of CNS inflammation or autoimmune responses, or both, in NMO and MS patients to finally settle the question whether NMO and MS are disease variants or separate diseases.

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