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Anti-aquaporin-4 antibody induces astrocytic cytotoxicity in the absence of CNS antigen-specific T cells

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ABSTRACT

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease of the central nervous system (CNS). Anti-aquaporin-4 antibody (AQP4-Ab) is a highly specific serum autoantibody that is detected in patients with NMO. Several lines of evidence indicate that AQP4-Ab not only serves as a disease marker but also plays a pivotal role in the pathogenesis of NMO. Although the pathogenicity of AQP4-Ab *in vivo* has recently been demonstrated, the presence of CNS antigen-specific T cells is recognized as a prerequisite for the antibody to exert pathogenic effects. Thus, it remains unclear whether AQP4-Ab is the primary cause of the disease or a disease-modifying factor in NMO. Here we report that pre-treatment with complete Freund's adjuvant (CFA) alone is sufficient for AQP4-Ab to induce astrocytic damage *in vivo*. Our results show the primary pathogenic role of AQP4-Ab in the absence of CNS antigen-specific T cells, and suggest that danger signals provided by nonspecific inflammation can be a trigger for those who harbor the autoantibody to develop NMO.

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1. Introduction

Neuromyelitis optica (NMO) has been considered a demyelinating disease of the central nervous system (CNS), and whether NMO is a variant of multiple sclerosis (MS) or not has been disputed [1]. However, the recent identification of a highly specific serum autoantibody, anti-aquaporin-4 antibody (AQP4-Ab), in NMO patients has led us to presume a different pathological mechanism in NMO [1,2]. AQP4 is one of the most abundant water channels in the CNS, and it is predominantly expressed on the perivascular endfeet of astrocytes [3].

Several lines of evidence indicate that AQP4-Ab not only serves as a disease marker but also plays a pivotal role in the pathogenesis of NMO. The loss of AQP4 and glial fibrillary acid protein (GFAP) is reported in the active lesions of NMO where myelin components are relatively preserved [4]. The cytotoxic effect of AQP4-Ab on astrocytes has been demonstrated *in vitro* by our group and others [5–8]. Most importantly, animal models have recently provided evidence of the pathogenicity of AQP4-Ab *in vivo* [9–11]. We and others have shown that AQP4-Ab-positive IgG reproduces pathological changes strikingly similar to those of NMO patients when passively transferred to rats with experimental autoimmune encephalomyelitis (EAE) [9–11].

Although these observations suggest the pathogenicity of AQP4-Ab in NMO, it still remains unclear whether the antibody is a disease-modifying factor or the primary cause of the disease [12]. Naive rats are reported to be resistant to the transfer of NMO with AQP4-Ab-positive IgG [10]. The compromise of the blood–brain barrier (BBB) is not likely to be the reason AQP4-Ab failed to exert its pathogenic effect in this model, because juvenile rats with the leaky BBB were also resistant to the transfer of AQP4-Ab-positive IgG [10]. As co-injection of CNS antigen-specific T cells with AQP4-Ab-positive IgG could exert the pathogenicity in these rats, the presence of CNS antigen-specific T cells was suggested to be a prerequisite for the pathogenicity of AQP4-Ab [10].

Interestingly, Saadoun et al. recently showed the pathogenicity of AQP4-Ab without pre-existing inflammation [13]. In that report, direct co-injection of AQP4-Ab-positive IgG with human complement into mouse brains induced loss of astrocytes [13]. However, it is important to take into account that complement-regulatory proteins on target cells are unable to fully play their protective role against complement derived from different species [14]. Considering the fact that astrocytes are the largest component of the CNS cells producing complement, and are therefore endowed with various types of complement-regulatory proteins [15], it is especially

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important to examine the effect of AQP4-Ab *in vivo* utilizing complement of the same species as the recipients. This speculation is supported by the fact that Saadoun et al. failed to reproduce the pathological changes with mouse complement in their report [13].

In the present study, we show that when rats are pre-treated with complete Freund's adjuvant (CFA) alone, AQP4-Ab induces its pathogenic effect in the absence of CNS antigen-specific T cells. The findings of this study strongly support the primary role of AQP4-Ab in the pathogenesis of NMO, and suggest that danger signals provided by nonspecific inflammation can be a trigger for those who harbor the antibody to develop NMO [16,17].

2. Materials and methods

2.1. Patients and immunoglobulin preparations

Human samples were collected from three patients with NMO [18] who tested positive for AQP4-Ab, and two patients with clinically definite MS [19] who tested negative for the autoantibody. A pooled human polyclonal immunoglobulin preparation (Rockland) was also used as an additional control. Purified IgG of the NMO or the control group was prepared as previously described [9]. Informed consent was obtained from each patient and this study was approved by the ethics committees of Osaka University Hospital and Hyogo College of Medicine.

2.2. Passive IgG transfer experiments

Experimental procedures were approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine. Eight-week-old female Lewis rats were injected with CFA (Difco) containing 400 μg of H37Ra *Mycobacterium tuberculosis* (Difco) into the plantar surface of each hind paw. The rats were then given daily injections of 20 mg human IgG intraperitoneally for four consecutive days from three days after the CFA injection.

2.3. Cell viability assay

Primary astrocytes were obtained from Sprague–Dawley rats as previously described [7]. The astrocytes were incubated with 10% purified IgG (2 mg/ml) and with 20% sera obtained from a healthy volunteer as the source of viable complement. After the 5-h incubation with purified IgG, the viability of cultured astrocytes was assessed by 4-[3-(2-methoxy-4-nitrophenyl)-2-[4-nitrophenyl]-2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-8) assay using a Cell Counting Kit-8 (Dojin East). The relative cell viability of each group was assessed by comparison with untreated astrocytes.

2.4. AQP4-Ab assay

The detection of AQP4-Ab in patients' sera was performed according to the previously described method [20]. The AQP4-Ab titer was assessed by the final concentration that showed positive staining in serial 4-fold dilutions. Briefly, human embryonic kidney cells (HEK) transfected with or without M23-human AQP4 (Genecopoeia) were seeded on chamber slides and fixed with 4% paraformaldehyde (PFA). After a 1-h incubation with diluted samples, the cells were incubated with a biotin-conjugated anti-human IgG (1:200; Rockland), followed by an Alexa Fluor 488-conjugated streptavidin (1:200; Molecular Probes) for 30 min each at room temperature. The AQP4-Ab titer was determined by comparing the fluorescence intensity of HEK cells with or without AQP4.

2.5. Histology and immunohistochemistry

One day after the final injection of human IgG, the rats were killed by terminal anesthesia. Spinal cords were embedded in Tissue Tek OTC compound and snap frozen. The 10-μm thick frozen tissue sections were fixed in ice-cold acetone for the staining with anti-C5b-9 antibody or in 4% PFA for staining with other antibodies. After incubation with 1% bovine serum albumin for 30 min, the sections were incubated overnight at 4 °C with primary antibodies, followed by appropriate secondary antibodies for 2 h at room temperature. The antibodies used in this study were as follows: goat anti-fibrinogen (1:50; MP Biomedicals), goat anti-AQP4 (1:50; Santa Cruz), rabbit anti-GFAP (1:100; DAKO), mouse anti-GFAP (clone DC47; 1:50; rPeptide), mouse anti-granulocyte (clone HIS48; 1:20; Abcam), mouse anti-CD68 (1:50; Serotec), mouse anti-CD3 (1:20; Serotec), rabbit anti-C5b-9 (1:660; Abcam), biotin-conjugated anti-human IgG (1:100; Rockland), rhodamine-conjugated anti-goat IgG (1:50; Santa Cruz), rhodamine-conjugated anti-mouse IgM (1:25 for CD3 and 1:50 for GFAP; Cappel), rhodamine-conjugated anti-mouse IgG (1:25; Sigma), fluorescein-conjugated anti-rabbit IgG (1:50; Cappel) antibodies, and Alexa Fluor 488-conjugated streptavidin (1:200; Molecular Probes). Images were captured by a LSM510 laser scanning microscope (Zeiss), an AX80T microscope (Olympus), or a BZ9000 microscope (Keyence).

2.6. Statistical analysis

One-way analysis of variance and Bonferroni's multiple comparison tests were used for the statistical analysis.

3. Results

3.1. AQP4-Ab induces astrocytic cytotoxicity in the absence of CNS antigen-specific T cells

To investigate whether AQP4-Ab can induce astrocytic cytotoxicity in the absence of CNS antigen-specific T cells, IgG purified from patients with NMO (IgGnmo) or from control patients (IgGcon) were administered to rats pre-treated with CFA alone (CFA-rats). The CFA-induced compromise of the BBB was examined immunohistochemically with an anti-fibrinogen antibody. While the serum fibrinogen was restricted within the vasculature in normal rats (Fig. 1A), the breakdown of the BBB was confirmed in the spinal cords of CFA-rats (Fig. 1B) as previously reported [21]. In addition, T-cell infiltration into the spinal cord was hardly observed by immunohistochemical analysis in CFA-rats (data not shown), which is consistent with a previous report [22]. Although rats injected with neither IgGcon nor IgGnmo showed apparent clinical symptoms, all of the three IgGnmo obtained from different NMO patients induced remarkable swelling of astrocytes especially around the blood vessels (Fig. 1C and D). Perivascular loss of GFAP- and AQP4-immunoreactivities (IRs) was also observed in rats given one of the three IgGnmo (Fig. 1E and F). Neither the swelling of astrocytes nor the loss of GFAP- or AQP4-IRs was observed with IgGcon. These results strongly suggest that when nonspecific inflammation is induced, AQP4-Ab can exert its pathogenic effect without CNS antigen-specific T cells.

3.2. Pathogenic effect of IgGnmo in vivo correlates with AQP4-Ab titer and astrocytic cytotoxicity in vitro

As the loss of GFAP-IR was observed only with one of the three IgGnmo, we investigated whether the discrepancy of the pathogenic effect could be explained by the difference in AQP4-Ab titer

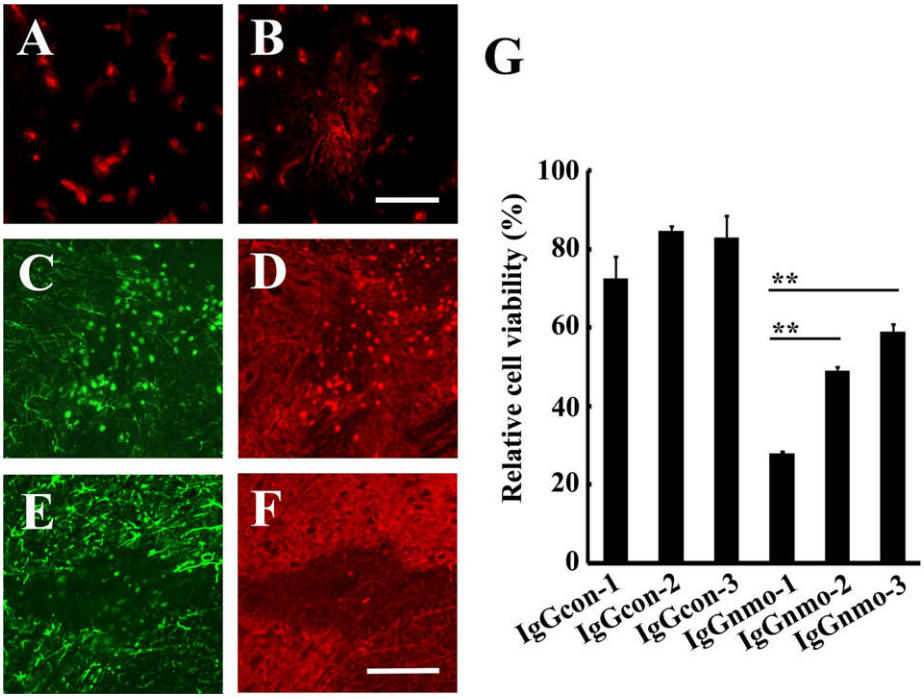

Fig. 1. AQP4-Ab induces astrocytic cytotoxicity in the absence of CNS antigen-specific T cells. Purified human IgG was administered to rats for four consecutive days from day 3 after the injection with CFA alone. (A–F) Spinal cord sections immunostained with anti-fibrinogen Ab (A, B), anti-GFAP Ab (C, E) and anti-AQP4-Ab (D, F) are shown. (C) through (F) are from the rats given IgGnmo. (A, B) The leakage of serum fibrinogen into the parenchyma was observed in the spinal cords of the CFA-rats (B), whereas fibrinogen-IR was restricted to the vasculature in normal rats (A). (C, D) Remarkable swelling of GFAP- and AQP4-positive cells was observed especially around blood vessels only in the NMO group. (E, F) The perivascular loss of GFAP- and AQP4-IRs was also observed in the rats injected with IgGnmo of the highest AQP4-Ab titer. Scale bars: (A, B) 200 μm; (C–F) 200 μm. (G) Cytotoxic effect of IgGnmo on astrocytes. Rat primary astrocytes were incubated with 10% purified IgG prepared from each patient, supplemented with 20% normal human serum. After the 5-h incubation, the relative cell viability compared with untreated astrocytes was assessed by WST-8 assay ($n = 3$ per each sample). Each column represents the mean ± SEM. $^{**}P < 0.01$ for IgGnmo-1 versus IgGnmo-2 and -3.

of each sample. Consistent with our speculation, IgGnmo that produced the loss of GFAP-IR showed the highest AQP4-Ab titer (IgGnmo-1 in Table 1). To further examine the pathogenic effect of each IgGnmo *in vitro*, we assessed the astrocytic cytotoxicity of each sample by WST-8 assay. Rat astrocytes were exposed to the purified IgG of each patient, together with human sera as the source of viable complement for 5 h. All of the three IgGnmo showed a higher pathogenic effect on astrocytes compared with those treated with IgGcon (Fig. 1G). In addition, IgGnmo that produced the loss of GFAP-IR *in vivo* exhibited significantly the highest pathogenic effect on astrocytes among the three IgGnmo (Fig. 1G and Table 1).

3.3. Nuclear fragmentation is observed in the majority of balloon-like astrocytes

To investigate the form of death of the astrocytes in our current model, we next examined the features of ballooned astrocytes observed in the NMO group by the triple labeling of GFAP, AQP4, and DAPI. A strong staining pattern of AQP4 was observed in the cytoplasm of ballooned astrocytes (Fig. 2B), indicating that internaliza-

Table 1
AQP4-Ab titer and relative astrocyte cytotoxicity *in vitro*.

Sample	AQP4-Ab titer	Relative cell viability (%)	*P*-Value
IgGnmo-1	3840	27.8 ± 0.5 ($n = 3$)	
IgGnmo-2	960	49.0 ± 0.9 ($n = 3$)	0.01
IgGnmo-3	240	58.9 ± 2.0 ($n = 3$)	0.01

Astrocyte cytotoxicity *in vivo*	IgGcon	IgGnmo
Ballooned Astrocytes	0/6 (0%)	5/8 (62%)
Loss of GFAP	0/6 (0%)	2/8 (25%)
Loss of AQP4	0/6 (0%)	2/8 (25%)

Data are shown as the means ± SEM. The *P*-value was calculated for IgGnmo-1 versus IgGnmo-2 and -3. CFA-rats were injected either with AQP4-Ab-negative IgG ($n = 6$; 2 each with 3 different IgGcon), or AQP4-Ab-positive IgG ($n = 8$; 2 with IgGnmo-1, 3 with IgGnmo-2 and IgGnmo-3).

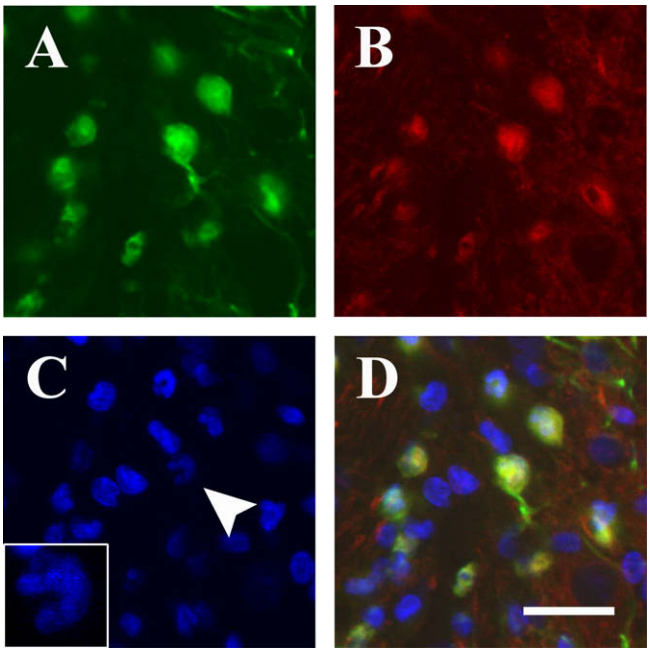

Fig. 2. Nuclear fragmentation is exhibited in the majority of balloon-like astrocytes. The spinal cord lesions in the NMO group were immunostained with anti-GFAP Ab (A), anti-AQP4-Ab (B) and DAPI (C). A merged image is shown in (D). (A, B, D) Intensive staining of AQP4 was observed in the cytoplasm of the ballooned GFAP-positive cells. (A, C, D) The majority of the ballooned GFAP-positive cells had fragmented nuclei (arrowhead, inset). Scale bar: 40 μm.

tion of AQP4 is taking place in these cells [6]. Although necrotic processes are suggested to occur in dying astrocytes of NMO [5,7–10], the majority of the ballooned astrocytes (88 out of 100 cells) unexpectedly showed fragmented nuclei, the primary feature of apoptosis, in our current model (Fig. 2C).

3.4. Perivascular deposition of immunoglobulin and activated complement is observed in the NMO group

Since a pivotal role of classical complement pathway has been suggested in the pathogenesis of NMO [4,5,7,23], we next examined whether there is a deposition of immunoglobulin and activated complement in our current model. In the rats given IgGnmo, remarkable deposition of immunoglobulin and C5b-9, the terminal product of activated complement, was observed especially at the perivascular lesions where the loss of astrocytes was observed (Fig. 3A–F). The rosette-stone-like pattern of C5b-9 deposition closely resembling the characteristic pathology of NMO was also observed at glia limitans where GFAP-IR was relatively decreased (Fig. 3G–I, dotted area) [4,23]. These observations further support the pivotal role of classical complement pathway in the AQP4-Ab-mediated astrocyte damage [4,5,7,23].

3.5. Predominant infiltration of granulocytes is observed in the spinal cord of the NMO group

In contrast to MS, active lesions of NMO are marked by remarkable infiltration of granulocytes and activated macrophages/microglia [24]. Therefore, we investigated the cellular infiltration into the lesions in our current model. Histological analysis showed remarkable infiltration of inflammatory cells only in the rats given IgGnmo (Fig. 3K). In addition, immunohistological analysis re-

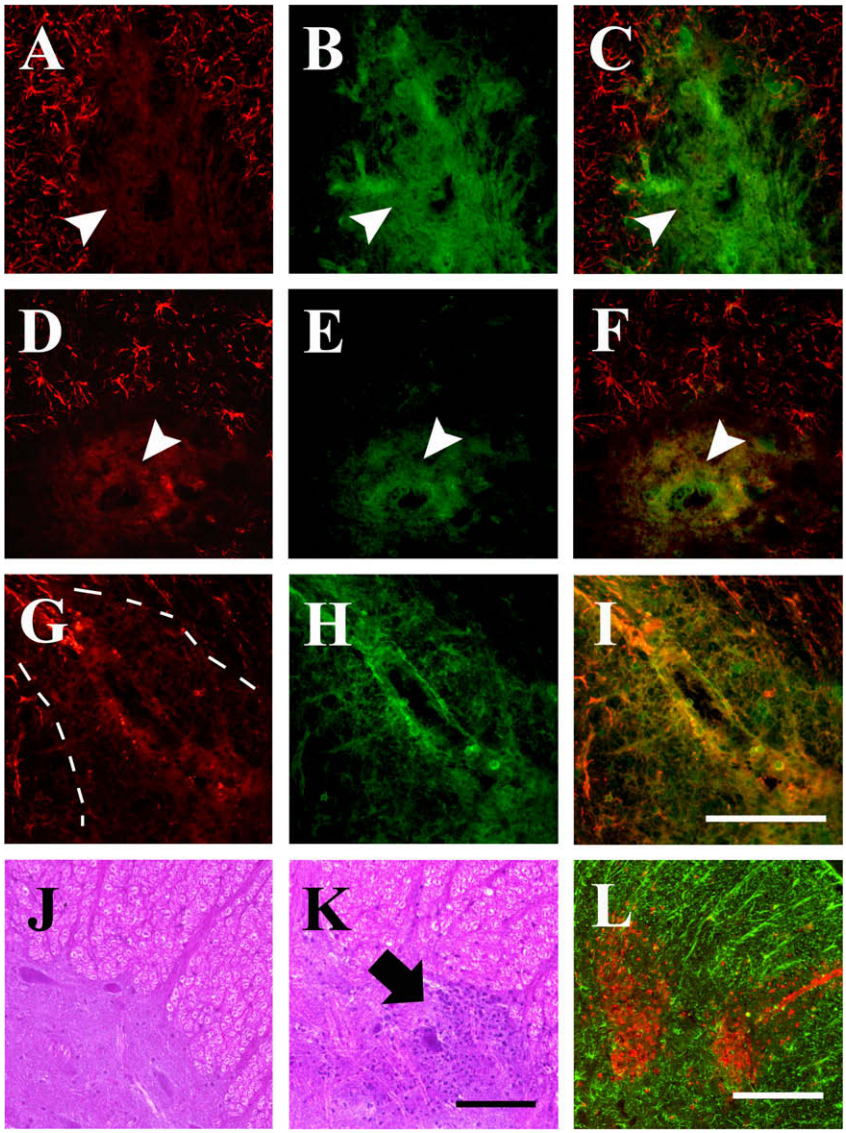

Fig. 3. Complement deposition and recruitment of granulocytes are observed in rats of the NMO group. (A–I) Spinal cord lesions in the NMO group were immunostained with anti-GFAP Ab (A, D, G), anti-human IgG (B), and anti-C5b-9 Ab (E, H). Merged images are shown in (C), (F) and (I). Arrowheads in (A–F) indicate vascular lumens. (A–F) Remarkable deposition of human IgG and C5b-9 was observed at the perivascular lesion where the loss of GFAP-positive cells was observed. (G–I) C5b-9 deposition of a rosette-stone-like pattern was also observed at the glia limitans where GFAP-IR was decreased (dotted area). (J, K) Hematoxylin and eosin sections of spinal cords in CFA-rats given IgGcon or IgGnmo. Massive infiltration of inflammatory cells (arrow) was observed especially in the gray matter of the NMO group (K), whereas cellular infiltration was not observed in the control group (J). (L) Spinal cord sections of the NMO group immunostained with anti-GFAP (green) and anti-granulocyte (red) antibodies. Infiltrating cells were mainly composed of granulocytes. Scale bars: (A–I) 200 μm; (J, K) 250 μm; (L) 500 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

vealed that these infiltrates are predominantly composed of granulocytes (Fig. 3L). There were only a few macrophages and lymphocytes observed in the lesions (data not shown). The predominant recruitment of granulocytes was reminiscent of the pathology in NMO [24], indicating that a pathogenic mechanism similar to that of NMO is taking place in our current model.

4. Discussion

We and others have recently shown that when AQP4-Ab-positive IgG is passively transferred to rats with EAE, it reproduces pathological changes similar to those of NMO [9–11]. However, it has remained unclear whether AQP4-Ab can be pathogenic in the absence of CNS antigen-specific T cells, because the pathogenicity of AQP4-Ab was observed only when EAE was induced in the recipient rats [9–11]. Bradl et al. reported that the transfer of AQP4-Ab to naive rats or even to juvenile rats with the leaky BBB failed to produce any pathological changes, while co-injection of CNS antigen-specific T cells with AQP4-Ab-positive IgG could induce the pathogenic effects [10]. Thus, the presence of CNS antigen-specific T cells has been suggested to be a prerequisite for the pathogenicity of AQP4-Ab [10].

In contrast to the previous hypothesis, we showed here that pre-treatment of recipient rats with CFA alone is sufficient for AQP4-Ab to exert its pathogenic effect. Remarkable swelling and the loss of astrocytes were observed in CFA-rats injected with IgGnmo. Although we cannot totally exclude the possibility that nonspecifically activated T cells in the periphery played a supportive role in the present study, the scarcity of T cells in the lesions, in addition to the absence of CNS-specific antigen in our immunizing protocol, suggest that CNS antigen-specific T cells are not required for the AQP4-Ab-mediated astrocytic cytotoxicity.

Besides being often used as a potent adjuvant for inducing antigen-specific immune reaction, CFA is also known to stimulate danger signals in the host [25]. It induces a leukocyte population shift in the periphery without the elevation in the levels of inflammatory cytokines [21,26]. On the other hand, microglia and astrocytes are activated within four days after the injection of CFA [27]. These activated glial cells are accompanied by production of inflammatory cytokines such as TNF-α or IL-6 [27], which are known to disrupt the integrity of the BBB [28]. Although it still remains to be clarified which effect of CFA is actually important to potentiate the pathogenicity of AQP4-Ab, our present model highlights the importance of nonspecific inflammation as a trigger to develop NMO or to induce relapses in NMO patients. This observation is consistent with the previous report showing high frequency of acute febrile illness preceding with neurological symptoms in NMO [17].

In this present study, the loss of astrocytes was observed only with one of the three IgGnmo. That discrepancy might have been caused either by a difference in AQP4-Ab titer or by a different pathogenic capacity of AQP4-Ab contained in each sample. Our *in vitro* results support both possibilities. This speculation is also supported by the recent reports showing the correlation of the clinical disease activity of NMO with AQP4-Ab titer or with the cytotoxic effect assessed *in vitro* [29–31].

In addition to the astrocyte damage, the active lesions in our current model were marked by specific deposition of immunoglobulin and C5b-9, along with infiltration of a large number of granulocytes. All of these pathological changes are strikingly similar to those observed in NMO patients [24]. This observation not only supports the pivotal role of the complement pathway in AQP4-Ab-mediated inflammation [4,5,7,23], but also suggests that C3a and C5a released from the activated complement might be potent inducers to recruit granulocytes in NMO [6]. Whether or not these infiltrating granulocytes are actively involved in the AQP4-Ab-mediated astrocyte damage needs to be examined in the future investigations [6].

With regard to the form of death that the dying astrocytes exhibited in our present study, we unexpectedly observed a remarkable number of ballooned astrocytes with fragmented nuclei. This observation seems to conflict with the previous reports showing astrocytic necrosis in NMO [5,7–10]. One possible explanation is that during necrotic processes, leakage of extracellular DNase into the cytoplasm induced the fragmentation of nuclei in these cells [32,33]. This speculation is supported by the recent report showing positive TUNEL staining in ballooned astrocytes of an NMO animal model [10]. Another possibility is that C5b-9 formed on astrocytes induced apoptosis of these cells. C5b-9 can also induce apoptosis of target cells when the amount of C5b-9 formation is relatively small [32]. Therefore, it remains to be clarified whether necrosis or apoptosis is the primary form of astrocyte death in NMO.

In conclusion, we showed here that when nonspecific inflammation co-exists, AQP4-Ab can exert its pathogenic effect without CNS antigen-specific T cells. The present study strongly supports the primary role of AQP4-Ab in the pathogenesis of NMO, and suggests that danger signals provided by nonspecific inflammation can be a trigger for those who harbor the antibody to develop NMO [16,17].

Acknowledgments

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