

Cerebrospinal Fluid Aquaporin-4 Antibody Levels in Neuromyelitis Optica Attacks

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To elucidate immunopathogenetic roles of aquaporin-4 antibodies in the cerebrospinal fluid (CSF) of neuromyelitis optica spectrum disorders (NMOSD), we analyzed aquaporin-4 antibody titers, cellular and inflammatory markers in the CSF collected from 11 aquaporin-4 antibody seropositive patients. The CSF aquaporin-4 antibody levels during attacks (but not in sera) closely correlated with pleocytosis, inflammatory cytokines including interleukin-6 that can regulate antibody-producing plasmablasts, and glial fibrillary acidic protein levels in the CSF. The amount of aquaporin-4 antibodies present in the central nervous system may have therapeutic implications, as it is associated with astrocyte injury and inflammatory responses during NMOSD attacks.

ANN NEUROL 2014;76:305–309

Neuromyelitis optica (NMO) and the limited forms known as NMO spectrum disorders (NMOSD) are characterized by severe attacks of optic neuritis and/or longitudinally extensive transverse myelitis (LETM).¹ A significant proportion of NMOSD patients are seropositive for aquaporin-4 (AQP4) antibodies.² The primary target for these autoantibodies is the AQP4, a water channel richly expressed in the foot processes of astrocytes.³ Immunoglobulin G (IgG) deposits with complement activation in perivascular areas, associated with astrocyte loss, are observed in acute NMOSD lesions.^{4,5}

We previously reported a remarkable increase in the cerebrospinal fluid (CSF) of glial fibrillary acidic protein

(GFAP), an astrocyte-specific marker during NMOSD attacks.⁶ The GFAP elevation was far more pronounced than myelin and neuronal markers, indicating that astrocytes are the primary target cells in these patients with AQP4 antibodies. Previous studies have found elevated interleukin (IL)-6 and other cytokine levels in the CSF of NMOSD patients compared to multiple sclerosis (MS) patients and controls, and that the concentration of IL-6 seems to correlate with GFAP levels.^{7–9} However, AQP4 antibody titers in the serum did not correlate with GFAP levels in 1 study,⁸ and no study has evaluated AQP4 antibody titers in the CSF. In this pilot study, we evaluated AQP4 antibody levels measured directly in the CSF during attacks using our highly sensitive cell-based assay (CBA) for AQP4 antibodies,¹⁰ and correlated these levels with astrocyte damage and the cytokine profile.

Patients and Methods

We enrolled a total of 11 consecutive NMOSD patients (10 females; 1 male) with a median age of 50 years (range = 24–71) seen at Hospital das Clinicas, Faculty of Medicine, University of Sao Paulo (Brazil) with detectable AQP4 antibody in sera and CSF seen during 2011 and 2012—6 patients with definitive NMO who met Wingerchuk's 2006 revised diagnostic criteria,¹ and 5 NMOSD patients with LETM (3 monophasic and 2 relapsing). For simplicity, we use the term NMOSD to encompass both NMO and NMOSD. We included NMOSD cases with paired serum and CSF samples (at least 2 aliquots of 500 µl) stored at –80°C for analysis. Seven sera/CSF samples were collected during attacks (before intravenous methylprednisolone or plasmapheresis) and 4 during remission (for diagnostic purposes without signs suggestive of NMOSD attacks in the past 30 days, all receiving oral prednisone and 2 in combination with azathioprine). Samples were shipped on dry ice and stored again at –80°C until analysis.

All sera and CSF samples were analyzed at Tohoku University to detect AQP4 antibodies using a CBA with HEK-293 living cells stably transfected with the M23 isoform of AQP4 as

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Received Apr 14, 2014, and in revised form May 27, 2014. Accepted for publication Jun 23, 2014.

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.24208

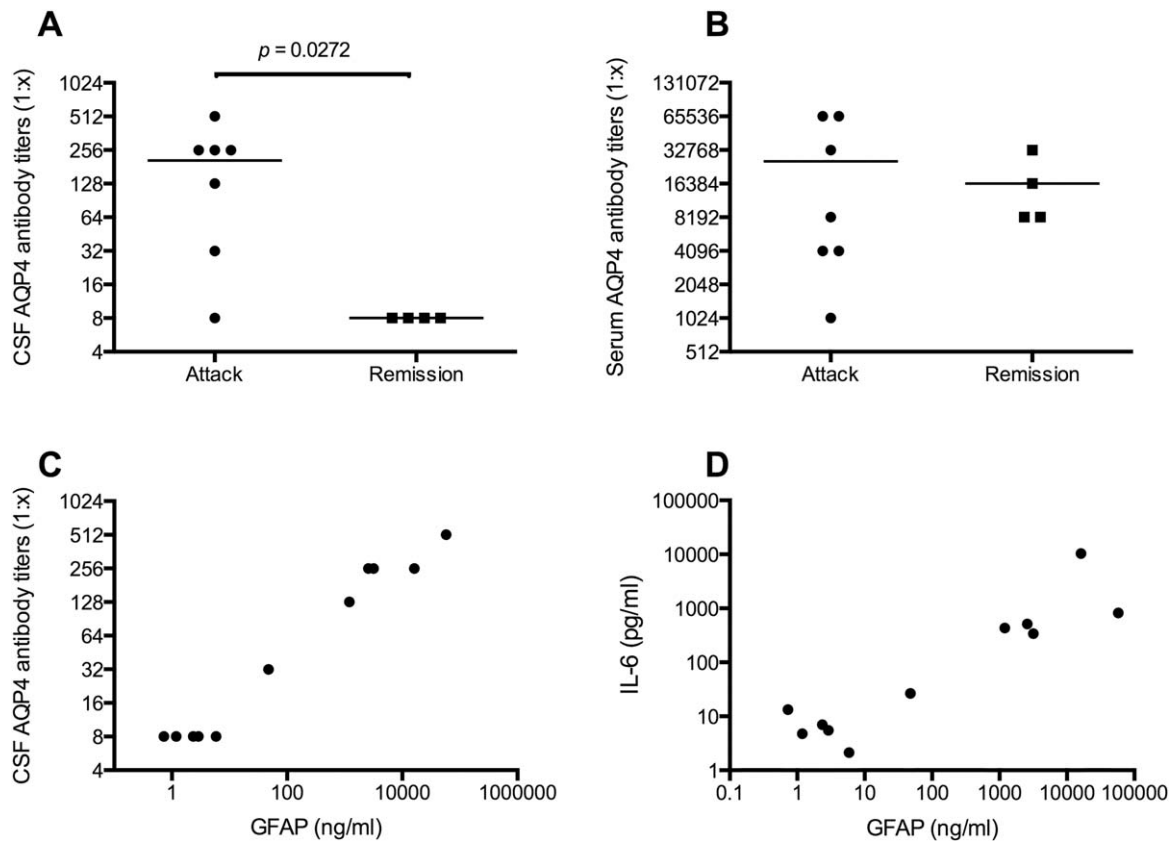


FIGURE 1: Aquaporin-4 (AQP4) antibody titers in (A) the cerebrospinal fluid (CSF) and (B) serum during attacks and remission. (C) CSF AQP4 antibody titers strongly correlate with glial fibrillary acidic protein (GFAP; Spearman $\rho = 0.9439$, $p < 0.0001$), and (D) GFAP levels correlate with interleukin (IL)-6 (Spearman $\rho = 0.8091$, $p = 0.0026$).

previously described.¹⁰ The AQP4 antibody titers were calculated using endpoint dilutions.

Astrocyte damage was evaluated measuring GFAP levels in the CSF with a commercially available enzyme-linked immunosorbent assay (A05188; SPI-Bio, Montigny-le-Bretonneux, France) as previously described following manufacturer's instructions.⁶ In addition, we measured the following cytokine levels in the CSF: IL-1 β , IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, interferon (IFN)- γ , soluble CD40 ligand, and tumor necrosis factor- α . Cytokines were measured using a multiplexed fluorescent magnetic bead-based immunoassay (Bioplex Pro Human Th17 Cytokine Panel 15-Plex, 171-AA001M; Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions. Only cytokines with detectable levels in at least one-third of the CSF samples were considered for statistical analysis.

We have compared the measurements of samples obtained during attacks and remission with Mann-Whitney *U* test, Spearman correlation coefficient rank was used to evaluate correlation between 2 variables, and 2-tailed *p* values < 0.05 were considered significant. We considered correlations to be of interest with $r > 0.6$. Results of measurements are shown in mean and standard deviation unless otherwise indicated.

This study was approved by the ethics committee of each center and conducted in accordance with internationally

recognized ethical standards. All study participants provided written consent.

Results

The serum AQP4 antibody titers were found at high levels in all patients during attacks and remission. In contrast, AQP4 antibody titers in the CSF were remarkably higher only in samples collected during attacks, as shown in the Figure. Consequently, the CSF:serum ratio found during remission ($1:2,048 \pm 1:1,448$) was higher than during NMO attacks ($1:204 \pm 1:175$), $p = 0.0030$, and accompanied by elevation of other CSF parameters indicative of inflammation such as pleocytosis (61 ± 88 vs 2 ± 1 cells/ μ l, $p = 0.0152$) and protein (74.0 ± 41.9 vs 27.0 ± 4.5 mg/dl, $p = 0.0273$). Furthermore, the levels of IL-6 ($1,784.97 \pm 3,788.05$ vs 6.42 ± 4.81 pg/ml, $p = 0.0121$), IL-1 β (0.35 ± 0.34 vs 0.06 ± 0.02 pg/ml, $p = 0.242$), and IL-10 (1.33 ± 0.71 vs 0.77 ± 0.17 pg/ml, $p = 0.0424$) were also elevated in CSF samples collected during NMO attacks compared to those collected during remission. Similarly to AQP4 antibody levels and cytokines in the CSF, the GFAP levels were remarkably elevated during attacks compared to samples collected

TABLE 1. Correlations between CSF and Serum AQP4 Antibody Titers and Cytokine Levels in the CSF of NMOSD Patients (n = 11)

Cytokine	CSF AQP4 Antibody Titers		Serum AQP4 Antibody Titers	
	Spearman rho	<i>p</i>	Spearman rho	<i>p</i>
IL-1 β	0.7916	0.0037	0.0069	0.1125
IL-4	-0.2343	0.4881	-0.2140	0.9838
IL-6	0.9054	0.0001	0.0970	0.7766
IL-10	0.8321	0.0015	-0.1624	0.6333
IL-17A	-0.1673	0.6229	-0.2408	0.4757
IL-23	-0.4364	0.1796	-0.3274	0.3257
IL-25	0.3919	0.2333	0.1810	0.5943
IL-31	0.6386	0.0345	-0.2738	0.4152
IFN- γ	0.5992	0.0514	-0.0794	0.8164
sCD40L	0.5008	0.1166	-0.1986	0.5582
TNF- α	0.5406	0.0860	-0.3936	0.2311

AQP4 = aquaporin-4; CSF = cerebrospinal fluid; IFN = interferon; IL = interleukin; NMOSD = neuromyelitis optica spectrum disorders; sCD40L = soluble CD40 ligand; TNF = tumor necrosis factor.

during remission ($11,578.00 \pm 21,188.00$ vs 2.66 ± 2.32 ng/ml, $p = 0.0242$).

We found a very strong correlation of CSF AQP4 antibody titers with GFAP ($r = 0.9439$, $p < 0.0001$), cell counts ($r = 0.7679$, $p = 0.0058$), and protein levels ($r = 0.9460$, $p < 0.0001$) in the CSF. However, there was no correlation between serum AQP4 antibody titers and any of those parameters in the CSF (GFAP, $r = 0.1860$, $p = 0.5860$; cell counts, $r = -0.2891$, $p = 0.3886$; protein $r = 0.0532$, $p = 0.8764$). Interestingly, the serum AQP4 antibody titers did not correlate with CSF AQP4 antibody titers ($r = 0.1860$, $p = 0.5840$), even if we analyze only the samples collected during attacks ($r = 0.3397$, $p = 0.4560$).

Cytokines such as IL-1 β , IL-6, IL-10, and IL-31 also correlated with CSF AQP4 antibody levels, but there was no correlation with serum AQP4 antibody titers, as summarized in the Table. We also found correlation between astrocyte injury measured by GFAP levels and concentrations of IL-1 β ($r = 0.7517$, $p = 0.0076$), IL-6 ($r = 0.8091$, $p = 0.0026$), IL-10 ($r = 0.8082$, $p = 0.0026$), IL-31 ($r = 0.6986$, $p = 0.0168$), and IFN- γ ($r = 0.6391$, $p = 0.0343$).

Discussion

The identification of serum AQP4 antibodies exclusively in patients with NMOSD clearly distinguished it from MS.² NMOSD-like lesions observed in experimental

models with passive transfer or direct injection of AQP4 antibodies strongly suggest the pathogenicity of these autoantibodies.^{11,12} Astrocytes express AQP4, and their damage is evident with remarkable increase of GFAP in the CSF during NMOSD attacks.⁶ Moreover, we previously reported that GFAP levels correlate with incapacity measured by expanded disability status scale and spinal cord lesion length. However, the serum AQP4 antibody titers usually do not correlate with GFAP levels,^{6,8} limiting the value of measuring AQP4 antibody titers during relapses in the serum. In this pilot study, we have shown that the amount of AQP4 antibody present directly in the CSF strongly correlates with astrocyte damage represented by elevated GFAP in the CSF, and this occurs together with signals of blood-brain barrier (BBB) breakdown and release of inflammatory cytokines. Elevation of IL-1 β may promote BBB disruption,¹³ other proinflammatory molecules are also increased in the CSF of NMOSD,^{8,14} and they may promote the recruitment of neutrophils and eosinophils found in acute NMOSD lesions.⁴ We recognize that further studies including a larger number of patients with attack and remission samples are required to confirm these findings, but our results indicate that the AQP4 antibody levels found in the central nervous system (CNS) are a critical element to promote astrocyte injury in a dose-dependent manner.

BBB disruption correlated with CSF anti-AQP4 levels, and this may represent a significant source of

AQP4 antibodies in the CSF during attacks. However, we cannot distinguish the AQP4 antibodies produced intrathecally in our study. Interestingly, we found that IL-6 and IL-10 correlated with AQP4 antibody levels in the CSF. IL-6 can promote B-cell survival, including IgG producing plasmablasts,¹⁵ and IL-10 can promote maturation of memory B cells and immunoglobulin class switch.¹⁶ Previous studies reported that B cells could migrate and differentiate to AQP4 antibody producing plasmablasts,^{17,18} so >1 mechanism might be involved in the CSF AQP4 antibody levels. Patients treated with monoclonal antibodies blocking IL-6 receptor (tocilizumab) have a reduction in the number of circulating plasmablasts and experience a reduction in the number of attacks,¹⁹ but it is unclear whether the drug effects have some extension into the CNS.

NMOSD attacks are usually severe and patients have a high risk of disability. Understanding the mechanisms that promote the BBB disruption and/or the migration of pathogenic memory B cells resulting in the presence of large amounts of AQP4 antibodies in the CNS can provide new therapeutic strategies to treat NMOSD patients in a more tailored approach with less of the risks and side effects found in B-cell depletion with rituximab, immunosuppressive drugs, and corticosteroids. Furthermore, strategies blocking the binding of pathogenic AQP4 antibodies to their antigen in the CNS²⁰ or preserving BBB integrity may reduce the astrocyte damage and permanent disability due to NMOSD attacks.

Acknowledgment

This study was supported by KAKENHI (22229008) of the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the Health and Labor Sciences Research Grant on Intractable Diseases (Neuroimmunological Diseases) from the Ministry of Health, Labor, and Welfare of Japan. The funding sources had no role in the design, collection, analysis, or interpretation of the study, nor in the writing of the article or decision to submit.

Authorship

D.K.S.: study conception, data analysis, and drafted the manuscript. D.C.: design of the study, data analysis, and edited the manuscript. F.M.d.H.J.: design of the study and data analysis. I.N.: study conception, data analysis, and drafted the manuscript. S.N.: design of the study and data analysis. T.T.: design of the study, data analysis, and reviewed the manuscript. R.F.S. and S.L.A.-P.: design of the study and data analysis. T.M.: design of

the study and reviewed the manuscript. L.S. and M.A.: study conception, data analysis, and reviewed the manuscript. K.F.: study conception, data analysis, and drafted the manuscript.

Potential Conflicts of Interest

D.K.S.: scholarship, Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan; grant, Ichiro Kanehara Foundation; speaking fees, Novartis; associate editor, *Arquivos de Neuropsiquiatria*. I.N.: personal fees, Mitsubishi Tanabe Pharma Corporation, Biogen Idec Japan, Novartis Pharmaceuticals Japan, Bayer Yakuhin; grant, LSI Medience Corporation. S.N.: grants, Japan Society for the Promotion of Science, MEXT of Japan, Ministry of Health, Labor, and Welfare of Japan. R.F.S.: scholarship, Genzyme; grants, Biogen Idec, personal fees, Biogen Idec, Bayer. T.M.: speaker honoraria, Bayer Schering Pharma, Biogen Idec Japan, Mitsubishi Tanabe Pharma, Astellas Pharma, Asahi Kasei Medical; research support, Bayer Schering Pharma, Biogen Idec Japan, Chemo-Sero-Therapeutic Research Institute, Mitsubishi Tanabe Pharma, Asahi Kasei Kuraray Medical, Teva Pharmaceutical, Teijin Pharma; grants, Bayer Schering Pharma, Biogen Idec Japan, Mitsubishi Tanabe Pharma, Asahi Kasei Medical, Teva Pharmaceutical, Teijin Pharma, Kaketsuken Co; grants-in-aid for scientific research, Ministry of Education, Science, and Technology of Japan, Ministry of Health, Labor, and Welfare of Japan. L.S.: consulting, Novartis, Teva, MedImmune, Pfizer. K.F.: advisory board, Bayer Schering Pharma, Biogen Idec Japan, Mitsubishi Tanabe Pharma, Novartis Pharma, Chugai Pharmaceutical, Ono Pharmaceutical, Nihon Pharmaceutical, Merck Serono, Alexion Pharmaceuticals, Medimmune, *Medical Review, Sri Lanka Journal of Neurology*; editorial board, *Clinical and Experimental Neuroimmunology*; speaker honoraria, travel support, Bayer Schering Pharma, Biogen Idec Japan, Mitsubishi Tanabe Pharma, Novartis Pharma, Eisai, Astellas Pharma, Takeda Pharmaceutical, Asahi Kasei Medical, Daiichi Sankyo, Nihon Pharmaceutical; research support, Bayer Schering Pharma, Biogen Idec Japan, Mitsubishi Tanabe Pharma, Chugai Pharmaceutical, Ono Pharmaceutical, Nihon Pharmaceutical, Asahi Kasei Medical, Chemo-Sero-Therapeutic Research Institute, Teva Pharmaceutical, Teijin Pharma, Genzyme Japan; grants-in-aid for scientific research, Ministry of Education, Science, and Technology of Japan, Ministry of Health, Welfare, and Labor of Japan.

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