all MS, the single susceptible haplotype contained all of the *DRB1*15:01* alleles. However, because this haplotype also tagged several other *DRB1* alleles well, it is not possible to determine which of the *DRB1* alleles are responsible for the association. The most highly associated resistant haplotype (G–G) mostly contained **01:01*, **13:02* and **09:01* alleles. *DRB1*01* and **09* alleles have previously been shown to confer resistance to MS and AQP4− MS,[14] and it is likely the same association identified in this data set.

For the AQP4− haplotype analyses, G–G and A–G haplotypes made up 90% of the total haplotypes. The most strongly associated haplotype was G–G ($P = 9.04 \times 10^{-8}$; OR = 2.32). Seventy-five percent of this haplotype contains **04* alleles. The association of this haplotype is particularly striking when considering that it excludes **15:01*, and considering that the **15:01* effect is included in the non-GG haplotypes in the analysis (**15:01* is found exclusively with the A–G haplotype). This haplotype is tagging a non-**15:01* allele, which has a large effect, which could be larger than the **15:01* effect, on AQP4− MS susceptibility in the Japanese population. The very modest associations between **15:01* presence/absence and general MS and AQP4− MS also support the observation that the **15:01* haplotype may have a reduced role in Japanese MS as compared with MS in western populations. Isobe *et al.*[14] also identified a **04* association with AQP4− MS, and it is therefore this allele group that is the likely source of the susceptible association or part of a susceptible haplotype.

Although an association of *DRB1*15:01* with conventional Japanese MS has been reported previously,[10,34,35] a lack of a *DRB1*15* association, except when stratifying by **09* or **12*, has also been reported in Japanese MS.[14] The results of the present study clarify the **15* association. In this study, with a larger sample, presence/absence of **15:01* was found to be modestly associated with MS and AQP4− MS. However, the SNP that tags both **15:01* and **15:02* (rs9271366) was not associated with either disease classification. This result indicates that the non-associated **15:02* alleles are diluting the modest association of **15:01* when only two-digit genotypes are available. If **15:01* is indeed the causative allele in western populations, this finding is surprising considering that **15:02* proteins are expected to present the same antigens as proteins derived from **15:01*.[36] However, **15:02* was also identified as MS non-associated in two very small previously published studies,[37,38] and it has been shown that the two alleles may have differing effects with regard to aplastic anemia.[36] The difference in effect of the two alleles may be due to the single amino-acid difference between proteins from the two alleles, LD with another associated mutation or differences in expression between the two alleles. Because of the large association of **15:01* with MS in individuals of European descent and moderate association in other ethnicities, determining the mechanism for the difference in association between **15:01* and **15:02* may greatly increase our understanding of the molecular causes of MS.

Through conditional analysis, Lincoln *et al.*[39] found that the Class II haplotype block and *DRB1* accounted for most of the MHC-associated MS susceptibility in two populations of European descent, and that Class III associations could be explained by LD with Class II genes. Other studies have suggested, through haplotype analysis, that *DRB1*15:01* interacts with other genes in the Class II region to cause susceptibility, or that *DRB1*15:01* is part of a susceptible haplotype, but itself is not the causative genetic factor for the strongest genetic association with MS in individuals of European descent. This study also finds that in the Japanese MS population, the *DRB1*15:01* allele is not part of the major MS susceptible haplotype in AQP4− patients.

In conclusion, the objective of this study was to elucidate the effects of the HLA in Japanese MS. This study is the largest study of the HLA's contribution to MS in Japanese individuals. Haplotype analysis revealed a large susceptible association, likely *DRB1*04* or a locus in LD with *DRB1*04* alleles, with AQP4− MS, which excluded *DRB1*15:01* and other loci sharing a haplotype with *DRB1*15:01*. Several resistant haplotypes were identified, but it is difficult to say whether these haplotypes truly harbor resistant alleles or whether they only appear resistant when opposed to the susceptible haplotypes. Finally, although a very modest association of *DRB1*15:01* with MS was observed, *DRB1*15:02* was not associated. Because of the similarities of the proteins from these two alleles, differing only at a single amino acid, further studies to understand the nature of this difference, whether it be functional or haplotypic, could greatly increase our understanding of the molecular mechanisms leading to MS.

Materials and methods

Subjects

All samples from Japanese cases were collected in the Neurology Departments of the University Hospitals of the South Japan MS Genetics Consortium, which comprises the following six universities, all located in southwestern Japan: Kyushu University, Yamaguchi University, Ehime University, Hiroshima University, Kinki University and Osaka University. The final data set consisted of DNAs from 280 control (HC) and 204 patients with MS (193 cases who fulfilled the revised McDonald criteria[28] and 11 cases who at least met the criteria of clinically isolated syndrome[41] and were suggestive of MS), genotyped on a custom Infinium iSelect HD Custom Genotyping BeadChip (Illumina Inc., San Diego, CA, USA) for 6040 MHC region SNPs. SNPs were selected by previously described methods,[27,42,43] and included an additional 4431 non-chromosome six SNPs genotyped for assessment of population stratification. Anti-AQP4 antibody was measured in all patients using green uorescent proteinAQP4 fusion protein-transfected human embryonic kidney cells as described previously.[14] All participants gave written informed consent. This study was approved by the UCSF institutional review board, and the institutional ethical committees at each university of the South Japan MS Genetics Consortium.

DRB1 genotypes were available for 218 Japanese individuals. The *HLA-DRB1* alleles of the subjects were determined by hybridization of sequence-specific oligonucleotide probes in specific amplicons, as described elsewhere.[44] In addition, 264 controls and 203 patients were typed for *DRB1*15:01* presence/absence using validated gene-specific TaqMan assays as described by Caillier *et al.*[25]

Quality control

SNPs. SNPs were removed from the data set for missing genotypes greater than 5%, violation of HardyWeinberg equilibrium ($P<0.001$), or having a minor allele frequency less than 5%. SNPs were also removed if they were significantly ($P<0.001$) differently missing between all patients and controls (PLINK—test-missing) or if they were non-randomly missing ($P<0.001$) with respect to their expected genotypes derived from nearby SNPs in LD (PLINK—test-mishap). After all QC, 3534 HLA region SNPs remained. All genomic positions reported correspond to NCBI SNP build 129.[45]

Samples. All individuals with 10% or more missing genotypes were removed from analysis. To verify ethnicity, multidimensional scaling was used to cluster the experimental sample with data from 12 HapMap populations. Data for 705 non-chromosome six SNPs were common between all data sets and used for the analysis. Plots were generated for the first component onto the second component, and for each informative component (components 1–7) separately.

To identify population stratification, principal components were calculated for each individual using the 3668 non-chromosome six SNPs remaining after QC. Based on the scree plot, the first three components were considered informative (Supplementary Figure 4). Visual examination of a three-dimensional plot of the first three components identified three obvious outliers (Supplementary Figure 5). T^2 statistic analysis (Supplementary Figure 6) identified six outliers (including the three identified by visual examination), and data for all six samples were removed from the study.

Statistical analyses

Association analyses. Logistic regression (PLINK—logistic) was used to determine the association between HLA SNPs and MS, AQP4+ MS and AQP4− MS. An additive genetic model was assumed and gender included as a covariate for all analyses. Following the methods of McElroy *et al.*,[27] for each trait multiple rounds of analyses were performed, with each successive round including the most significant SNPs from the previous rounds, until no SNPs were significant at an FDR[46] of 0.1. This method facilitates the identification of multiple associated SNPs that are not redundantly associated with the trait through LD.

Haplotype analyses. Haplotypes were estimated for the significant SNPs (PLINK—hap). Haplotype effects were determined by weighted logistic regression of disease status onto haplotype dosage, with gender as a covariate to identify specific haplotypes that may be tagging trait-associated loci. Four-digit *HLA-DRB1* genotypes were available for a subset of individuals ($n=218$). To investigate how haplotypes of significant SNPs relate to *HLA-DRB1* allelic polymorphism, haplotype frequencies were estimated by maximum-likelihood implemented in an Expectation Maximization algorithm.[47] Positive predictive values and sensitivities of the SNPs to predict *HLA-DRB1* alleles at the haplotype level were computed. All analyses were completed using PLINK (version 1.07),[48] JMP Genomics 4.1 (SAS Institute Inc., Cary, NC, USA) and R (version 2.9),[49] unless otherwise noted.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank the MS patients and controls who participated in this study. We thank RR Lincoln, R Guerrero, H Mousavi and A Santaniello for sample and database management. This work was supported by grants from the National Institute of Health U19AI067152, RO1NS46297 and National Multiple Sclerosis Society RG3060C8. JPM is a post-doctoral fellow supported by the National MS Society. NI is a post-doctoral fellow supported by The Association for Preventive Medicine of Japan. The members of the South Japan Multiple Sclerosis Genetic Consortium are J Kira, N Isobe, T Matsushita and S Yoshimura (Kyushu University), S Kusunoki and K Miyamoto (Kinki University), S Sakoda and Y Nakatsuji (Osaka University), T Kohriyama, K Ochi and M Matsumoto (Hiroshima University), T Kanda (Yamaguchi University) and T Miki and Y Kawano (Ehime University).

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Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)

Neurology Asia 2011; 16(2) : 139 – 142

T cell reactivities to myelin protein-derived peptides in neuromyelitis optica patients with anti-aquaporin-4 antibody

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Abstract

We previously reported the establishment of major myelin protein-derived T cell lines from 11 patients with multiple sclerosis. In the present study, we determined anti-aquaporin-4 (AQP4) antibody status in these patients and classified them into five patients with anti-AQP4 antibody who met the criteria for neuromyelitis optica (NMO) or NMO spectrum disorders, and six patients without anti-AQP4 antibody who fulfilled the revised McDonald criteria for multiple sclerosis. T cell lines reactive to myelin oligodendrocyte glycoprotein, proteolipid protein and myelin basic protein were detected in 5/5, 3/5 and 3/5 of the anti-AQP4 antibody-positive patients, respectively, and in 5/6, 4/6 and 4/6 of the anti-AQP4 antibody-negative ones, respectively. T cell lines from most of these patients showed inter- or intra-molecular epitope spreading, irrespective of anti-AQP4 antibody status. These findings suggest that T cells are stimulated in vivo against major myelin proteins in anti-AQP4 antibody-positive patients with NMO/NMO spectrum disorders.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that is generally considered to be mediated by myelin-autoreactive T cells. By contrast, neuromyelitis optica (NMO) is characterized by severe and selective involvement of the optic nerves and spinal cord. Recently, a specific IgG against NMO, designated NMO-IgG, targeting aquaporin-4 (AQP4), was described.[1,2] Because of the high specificity of NMO-IgG/anti-AQP4 antibody and the selective loss of AQP4 from acute lesions in autopsied NMO spinal cord specimens[3], NMO has been claimed to be a distinct disease entity with a fundamentally different causal mechanism from MS. The demyelination in NMO is proposed to be secondarily produced following damage to the astrocyte foot process, where AQP4 is localized.[3]

In Asians, selective and severe involvement of the optic nerves and spinal cord is characteristic, and there are two distinct subtypes of MS: the opticospinal form of MS (OSMS), which has similar features to the relapsing-remitting form of NMO in Western populations[4], and the conventional form of MS, which is associated with disseminated lesions in the CNS[4], similar to classical MS in Western populations. Because a fraction of OSMS patients also have anti-AQP4 antibodies[1,5], OSMS is suggested to be the same disease as NMO. Although both NMO and OSMS are claimed to be primary astrogliopathies, it remains unknown how anti-AQP4 antibody present in the peripheral blood enters into the CNS across the blood brain barrier (BBB) to initiate parenchymatous inflammation, and how astrocyte foot process damage produces extensive demyelination.

We previously reported the establishment of T cell lines (TCLs) reactive to major myelin proteins, such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG), by stimulating peripheral blood T cells from MS patients with a myelin peptide mixture, before the discovery of NMO-IgG.[6] Therefore, in the present study, we aimed to

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clarify anti-AQP4 antibody status in these patients and compare differences in T cell reactivities to myelin proteins between anti-AQP4 antibody-positive and -negative conditions.

METHODS

Subjects and antigen-specific responses of established T cell lines

T cell lines were originally established from 11 patients with MS (three men and eight women) according to the McDonald criteria and seven healthy controls.[6] The median age was 48 years (range 28–64 years), while the median disease duration was 8.5 years (range 1–23 years). Briefly, T cell lines specific to the myelin self-peptides were established from peripheral blood mononuclear cells (PBMCs)[6], using 64 overlapping peptides of 16- to 21-amino acids in length, corresponding to the primary sequences of ^{196}MBP (amino acids 1–196), ^{276}PLP (amino acids 1–276), and ^{218}MOG (amino acids 1–218), including the exon 1–3 and exon 4–6 junctions of MBP.[6] Antigen-specific proliferation of the T cell lines was determined using peptide-pulsed PBMCs, as follows: The T cell lines ($3x10^4$) was cultured with irradiated (3,000 cGy) PBMCs for 72 h and pulsed with 1 μCi/well of [^{3}H] thymidine for the last 16 h. Test wells were considered to be positive with a stimulation index >2.0 and with a Δcpm (antigen-stimulated cpm minus non-stimulated cpm) >1,000 and at least three standard deviations above the mean cpm of unstimulated control wells. Blocking of the proliferative response was investigated by adding the following anti-HLA class II monoclonal antibodies (mAbs): Hu-4 (anti-HLA-DRB1+DRB5 monomorphic), 1a3 (anti-HLA-DQ monomorphic) and B7/21 (anti-HLA-DP monomorphic). The original T cell reactivity data, which have been previously described[6], were used for the present analyses.

Detection of anti-AQP4 antibody

Anti-AQP4 antibody was detected by an indirect immunofluorescence method using green fluorescent protein (GFP)-AQP4 fusion protein-transfected human embryonic kidney cells (HEK-293), as previously described.[5,7]

RESULTS

Anti-AQP4 antibody was detected in five of the eleven patients; these patients fulfilled the criteria for NMO[8] or NMO spectrum disorders.[9] Reactivities to MOG, PLP and MBP were detected in T cell lines established from 5/5, 3/5 and 3/5 of the anti-AQP4 antibody-positive patients with NMO/NMO spectrum disorders, respectively, and from 5/6, 4/6 and 4/6 of the anti-AQP4 antibody-negative patients with MS, respectively (Table 1). T cells reactive for myelin antigens from four of the five anti-AQP4 antibody-positive patients showed inter- or intra-molecular epitope spreading, while the same was true for five of the six anti-AQP4 antibody-negative MS patients (Figure 1).

DISCUSSION

Intramolecular epitope spreading is defined as a phenomenon in which T cells initially react only to a major or dominant antigenic epitope of an immunized antigen, and later show reactivity to other secondary or cryptic epitopes of the same immunogen. Intermolecular epitope spreading is defined as a phenomenon in which T cells initially reactive to only the immunized antigen molecule later demonstrate reactivity to other non-immunized molecules. Thus, reactivity of T cell lines to multiple sites of an antigenic molecule is regarded as intramolecular epitope spreading, while reactivity of T cell lines to multiple antigenic molecules is regarded as intermolecular epitope spreading. Inter- and intra-molecular epitope spreading of T cell lines is usually observed

Table 1: Proliferative responses to each myelin protein

Myelin protein	Anti-AQP4 antibody-positive NMO/NMO spectrum disorders	Anti-AQP4 antibody-negative MS	Healthy control
MBP	3/5	4/6	2/7
PLP	3/5	4/6	2/7
MOG	5/5	5/6	3/7

MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein

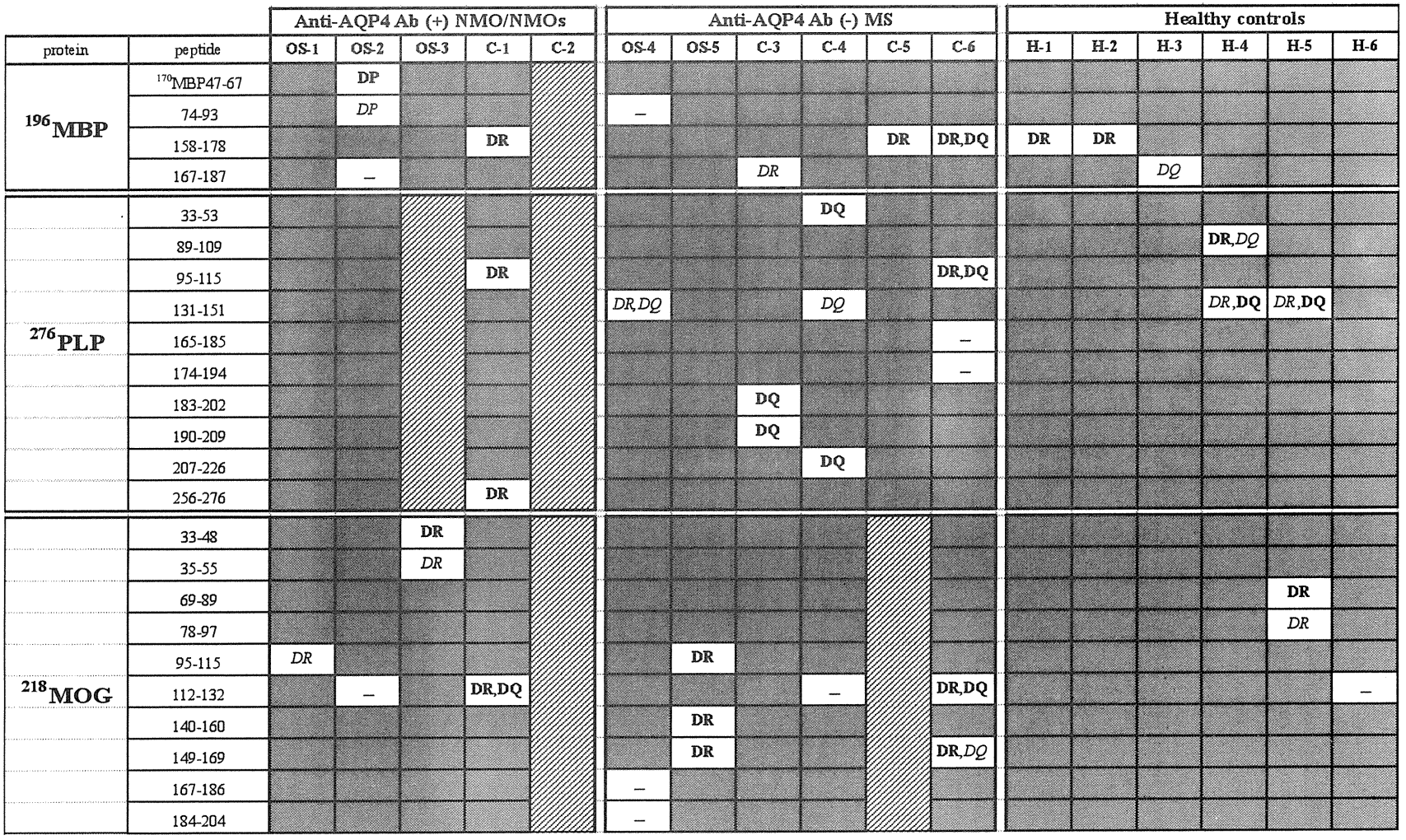

Figure 1. Myelin protein-reactive T cell lines characterized for peptide specificity and HLA restriction. T cell lines demonstrating either proliferative responses (open box) or no response (closed box) to peptides are shown. The hatched box indicates that the relevant epitope of T cell lines could not be determined because of a low response to the myelin peptide mixture. The restriction by HLA class II molecules of T cell lines is indicated in bold letters when anti-HLA class II mAb inhibited the proliferative response of T cell lines by ≥80%. Italic letters and (-) indicate that the mAb directed against the corresponding HLA class II molecules inhibited T cell proliferation by 50–80% and ≤50%, respectively.

Anti-AQP4 Ab: anti-aquaporin-4 antibody; HLA: human leukocyte antigen; mAb: monoclonal antibody; TCL: T cell lines; (+): positive; (-): negative.

in individuals whose T cells are stimulated or sensitized in vivo by specific antigen(s), whereas T cell lines stimulated or sensitized in vitro by antigen(s) during culture only react to neither multiple epitopes of the same antigen nor multiple antigens.

In the present study, T cell lines from healthy subjects also showed some reactivity to myelin proteins, but this was limited to one or two site(s) of a single myelin protein (five out of six cases demonstrated such a pattern). By contrast, T cell lines from four of five anti-AQP4 antibody-positive NMO/NMO spectrum disorders patients and five of six anti-AQP4 antigen-negative MS patients showed reactivity to multiple sites of multiple myelin proteins. It is therefore suggested that inter- and intra-molecular epitope spreading of T cell lines against myelin proteins occurs in most anti-AQP4 antibody-positive patients with NMO/NMO spectrum disorders and most anti-AQP4 antibody-negative ones with MS, but not in healthy subjects. The inter- and intra-molecular epitope spreading observed in anti-AQP4 antibody-positive patients indicates that T cells are already sensitized in vivo against major myelin proteins.

Recently, it was shown that sera from NMO patients with anti-AQP4 antibody can damage astrocytes in vivo following induction of experimental autoimmune encephalomyelitis by MBP-specific T cells.[10-12] It thus appears that encephalitogenic T cells are required for anti-AQP4 antibodies to exert their effects efficiently. Accordingly, the myelin protein-specific T cells found in anti-AQP4 antibody-positive NMO/NMO spectrum disorders patients may contribute to the initiation of CNS inflammation, and thereafter, anti-AQP4 antibody may invade the CNS and damage astrocytes in the presence of complement.

However, our T cell lines were established from the patients at certain periods after disease onset. Thus, myelin protein-specific T cell responses may have been secondarily developed after disease onset as a result of intermolecular epitope spreading. It will therefore be critical to study which CNS antigens T cells target at the time of initial attack, to elucidate the mechanisms underlying CNS anti-AQP4 autoimmunity. Nevertheless, it is possible that the myelin protein-specific T cells found in anti-AQP4 antibody-positive patients contribute to the development of inflammatory demyelination either primarily or secondarily.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Research Committee of Neuroimmunological Diseases, the Ministry of Health, Labour and Welfare, Japan and from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Journal of the Neurological Sciences 311 (2011) 69–77

Contents lists available at SciVerse ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Autoimmunity in neuromyelitis optica and opticospinal multiple sclerosis: Astrocytopathy as a common denominator in demyelinating disorders

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ARTICLE INFO

Article history:
Received 30 May 2011
Accepted 29 August 2011
Available online 29 September 2011

Keywords:
Multiple sclerosis
Neuromyelitis optica
Baló's disease
Opticospinal multiple sclerosis
Aquaporin-4
Astrocyte

ABSTRACT

Neuromyelitis optica (NMO) selectively affects the optic nerves and spinal cord. In Asians, multiple sclerosis (MS) is rare; however, when it appears, the selective and severe involvement of the optic nerves and spinal cord is characteristic. This form, termed opticospinal multiple sclerosis (OSMS), has similar features to the relapsing form of NMO in Westerners. The discovery that NMO-IgG, an NMO-specific IgG, targets aquaporin-4 (AQP4), suggested that NMO is a distinct disease entity with a fundamentally different etiology from MS. Because NMO-IgG is present in 30–60% of OSMS patients, OSMS in Asians is suggested to be the same entity as NMO. Pathologically, perivascular immune complex (IgM, IgG and C9neo) deposition and extensive loss of AQP4 in active lesions are reported hallmarks of NMO. However, we found that some autopsied NMO cases showed selective AQP4 loss while others showed preservation of AQP4, despite extensive tissue destruction. Vasculocentric deposition of complement and immunoglobulin was detected only in NMO patients, with less than 30% of actively demyelinating lesions showing AQP4 loss. Such heterogeneity of AQP4 expression and immunoglobulin deposition suggests a heterogeneous disease process in NMO. We recently reported that AQP4 was extensively lost in glial fibrillary acidic protein-positive hypertrophic astrocytes, both in demyelinated and myelinated layers of actively demyelinating lesions in Baló's disease, a variant of MS. We also found that in some acute MS lesions, AQP4 was lost extensively far beyond the areas of myelin loss. Active demyelinating lesions involved perivascular lymphocyte cuffings, consisting mainly of T cells in Baló's disease and MS, while the same was true for approximately half of the active lesions in NMO. This review proposes that anti-AQP4 antibody-dependent AQP4 loss occurs in some NMO patients while antibody-independent AQP4 astrocytopathy can occur in heterogeneous demyelinating conditions, including Baló's disease, NMO and MS. The latter may be mediated by T cells and other cell-mediated mechanisms, and should be tested in future experimental studies.

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS), which is thought to occur following autoimmune attack of CNS myelin. On the other hand, neuromyelitis optica (NMO) is an inflammatory disease of the CNS selectively affecting the optic nerves and spinal cord. In this condition, longitudinally extensive spinal cord lesions (LESCLs) extending three or more vertebral segments are regarded to be characteristic hallmarks following magnetic resonance imaging (MRI) [1]. Pathologically, both axons and myelin are involved, resulting in necrotic cavitation.

In Asians, MS is rare; however, when it appears the selective but severe involvement of the optic nerves and spinal cord is characteristic [2]. This form, termed opticospinal MS (OSMS), has similar features to the relapsing form of NMO in Westerners [1]. The nosological position of NMO has long been a matter of debate; however, the recent discovery of a specific IgG against NMO, designated NMO-IgG, suggests that NMO is a distinct disease entity with distinct etiology from MS [3,4]. Because NMO-IgG has been reported to be present in approximately 50–60% of OSMS patients [3,5], OSMS in Asians is claimed to be the same entity as NMO. However, the observation that NMO-IgG is not found in all cases of NMO or OSMS [5–7], and that 5–10% of classical MS patients also carry the antibody [3,5,7] cast doubt on the homogeneity of NMO and the simple dichotomy of categorizing human demyelinating disease into MS and NMO. In Asians, the mechanism underlying the formation of LESCLs appears to be heterogeneous, and the disease condition in those with NMO-IgG does not completely overlap with OSMS in Asians [6,7].

On the other hand, Baló's concentric sclerosis is relatively frequently reported among Asians, especially in Filipinos, Southern Han Chinese and Taiwanese [8]. This disease is said to be a rare variant of MS with huge brain lesions showing concentric rings of alternating demyelination and preserved myelin layers. Although the mechanism

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doi:10.1016/j.jns.2011.08.043

of Baló's disease still remains to be elucidated, we revealed extensive aquaporin-4 (AQP4) loss in demyelinated and myelinated layers of Baló's lesions without perivascular immunoglobulin and complement deposition [9]. Recently, Graber et al. [10] reported the occurrence of concentric rings of Baló in the brainstem in an NMO patient with NMO-IgG. These findings collectively suggest a link exists among NMO, Baló's disease and MS. Therefore, we recently reappraised AQP4 expression patterns in NMO and MS and found selective loss of AQP4 without vasculocentric deposition of complement and immunoglobulins, which is also detected in a fraction of MS and NMO patients [11], suggesting a common underlying mechanism among the three conditions. In this review, from an immunohistopathological, and humoral and cellular autoimmune point of view, possible underlying mechanisms of human demyelinating diseases, encompassing MS, NMO and Baló's disease, are discussed.

2. Recent immunohistopathological studies on NMO and MS

The pathological hallmark in MS is sharply demarcated demyelinating plaques with axons relatively preserved. By contrast, in NMO, intense demyelination, a great loss of axons, perivascular lymphocytic infiltration, microglial proliferation and vascular proliferation are seen in optic nerve and spinal cord lesions; these can occasionally lead to cystic cavities in severely involved areas [12–15]. Astrocytosis is scarce in some necrotic lesions but considerable in others. On the other hand, the neuropathological features of MS in Asians are as follows: (1) preferential occurrence of lesions in the optic nerves and spinal cord; (2) necrotizing lesions with occasional cavity formation not only in the spinal cord and optic nerves but also in the cerebrum; (3) poor gliosis; and (4) poor perivascular cuffing in the necrotic form [16–19]. Perivascular cuffing and gliosis varied regionally. Spinal cord lesions were usually most severe in the lower cervical to the mid thoracic cord. Polymorphonuclear leukocyte infiltration was occasionally seen in severe lesions in Asian MS patients, but eosinophil infiltration, as described in Western NMO patients [20], was not reported in early [16–19] or more recent literature [21].

More recently, Lucchinetti et al. [20] described perivascular immune complex deposition (IgM, IgG and C9neo) in a rim or rosette pattern. Misu et al. [22] reported that extensive loss of AQP4 was accompanied by decreased glial fibrillary acidic protein (GFAP) staining in active perivascular lesions where myelin basic protein (MBP) staining was relatively preserved in postmortem Japanese NMO cases. MBP loss with an AQP4 preservation pattern was not observed in any of the 22 active inflammatory lesions. Based on the presence of immunoglobulin and complement deposition in active perivascular lesions, Misu et al. [22] postulated that astrocytic impairment associated with the loss of AQP4 by humoral immunity is the primary event in NMO, suggesting a primary role for the anti-AQP4 antibody in NMO pathology. Roemer et al. [23] made similar observations regarding novel NMO lesions in the spinal cord, optic nerves and medullary tegmentum extending to the area postrema where the blood–brain barrier (BBB) is absent.

3. Questions relating to AQP4 immunohistopathology in NMO

Contradictory to the pivotal reports by Misu et al. [22] and Roemer et al. [23], Kobayashi et al. [24] reported an autopsied case of NMO showing preservation of AQP4 in severe lesions in the spinal cord and medulla, and in the demyelinated lesions in the optic nerve. In MS plaques, according to Misu et al. [22], AQP4 was never lost but was somewhat upregulated, reflecting astrogliosis, while in Roemer's report [23], some chronic MS plaques showed selective AQP4 loss. Therefore, there are considerable inconsistencies concerning AQP4 expression among pathology reports on NMO and MS. With respect to AQP4 pathology in NMO, I raise the following questions (Table 1): (1) Is AQP4 loss specific to NMO? (2) Is AQP4 uniformly lost in NMO lesions? (3) Is AQP4 loss always accompanied with vasculocentric deposition of immunoglobulin and complement? (4) Are there any T cell components in NMO? (5) How does such a tiny perivascular deposition of immunoglobulin and complement produce such a large lesion in the spinal cord and occasionally in the brain [25]?

Table 1
Questions and our answers relating to AQP4 immunohistopathology in NMO.

No.	Questions	Answers
1	Is AQP4 loss specific to NMO?	No
2	Is AQP4 uniformly lost in NMO lesions?	No
3	Is AQP4 loss always accompanied with vasculocentric deposition of immunoglobulin and complement?	No
4	Are there any T cell components in NMO?	Yes
5	How does such a tiny perivascular deposition of immunoglobulin and complement produce such a large lesion in the spinal cord and occasionally in the brain?	Astrocytopathy may play a role.

4. Reappraisal of AQP4 immunohistopathology in NMO, Baló's disease and MS

To address the above-mentioned questions, we first assessed AQP4 expression in the concentric demyelinating lesions of Baló's disease [9]. We evaluated AQP4 expression relative to another astrocytic marker (GFAP), the extent of demyelination, and lesion staging and perivascular deposition of complement and immunoglobulin in four Filipino cases with Baló's disease. All cases with Baló's disease demonstrated extensive AQP4 loss in demyelinated and myelinated layers of all actively demyelinating lesions, with perivascular lymphocytic cuffings of T cells but no deposition of immunoglobulins or complement around vessels. None of the patients with MRI-confirmed Baló's disease were seropositive for anti-AQP4 antibody [26]. AQP4 loss is thus supposed to be induced independently from the anti-AQP4 antibody. We therefore proposed autoantibody-independent AQP4 astrocytopathy in Baló's disease [26].

Second, we reappraised AQP4 expression patterns in NMO and MS [11]. We evaluated AQP4 expression relative to GFAP, the extent of demyelination, lesion staging (CD68 staining for macrophages), and perivascular deposition of complement and immunoglobulin in 11 patients with NMO and NMO spectrum disorders (NMOSD), five with MS, and 30 with other neurological diseases. Six NMO/NMOSD and two MS cases showed preferential AQP4 loss beyond the demyelinated areas, irrespective of lesion staging. Importantly, even in the same NMO patients, AQP4 loss was observed in some active lesions while AQP4 was preserved in other active lesions where numerous myelin-laden macrophages had infiltrated. This was true even for an NMO patient who was confirmed to be seropositive for the anti-AQP4 antibody. The other five NMO and three MS cases showed AQP4 preservation even in actively demyelinating lesions, despite grave tissue destruction. Vasculocentric deposition of complement and immunoglobulin was detected only in NMO/NMOSD patients, with less than 30% of actively demyelinating lesions showing AQP4 loss. These findings collectively suggest that AQP4 loss without perivascular complement and immunoglobulin deposition can occur in heterogeneous demyelinating conditions, including NMO, Baló's disease and MS (Fig. 1).

From the above findings, AQP4 down-modulation does not seem to be specific for NMO, and the mechanisms underlying AQP4 down-modulation could be heterogeneous (Table 1). Thus, an answer to question 1 is NO. AQP4 loss does not occur uniformly throughout NMO lesions, even in actively demyelinating lesions, suggesting that the mechanism of lesion formation is heterogeneous, even in the same patient. Consequently, the answer to question 2 is also NO. AQP4 loss was less frequently observed in optic nerve lesions as compared with lesions in other CNS sites (Fig. 2) [11]. Because AQP4 loss was not always accompanied with vasculocentric deposition of immunoglobulin and complement, the answer to question 3 is again

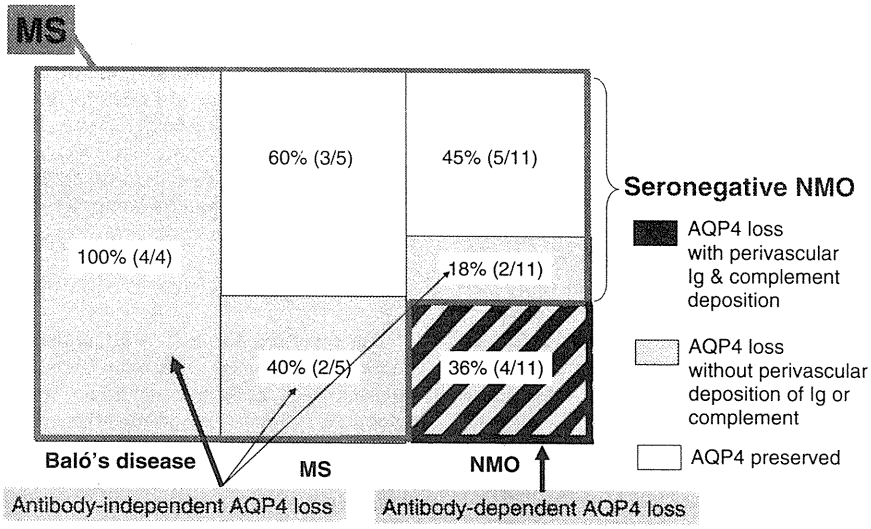

Fig. 1. AQP4 expression pattern in MS, NMO and Baló's disease. Even NMO cases presenting AQP4 loss with perivascular immunoglobulin and complement deposition in active lesions demonstrated AQP4 loss without perivascular immunoglobulin and complement deposition in other lesions. NMO cases having AQP4 loss without perivascular immunoglobulin and complement deposition may correspond to anti-AQP4 antibody-seronegative NMO or could be OSMS with LESCLs. Number of cases positive/number of cases examined in parenthesis. AQP4 = aquaporin-4; LESCLs = longitudinally extensive spinal cord lesions; MS = multiple sclerosis; NMO = neuromyelitis optica; OSMS = opticospinal multiple sclerosis.

NO. We did find T cell infiltration in half of the NMO lesions showing more or less AQP4 down-modulation. Therefore, the answer to question 4 is YES.

5. Humoral immunity in NMO, MS and Baló's disease

The high specificity of NMO-IgG for NMO suggests that NMO is a distinct disease entity from MS [3,4]. Because NMO-IgG recognizes AQP4 on astrocyte endfeet, astrocytic destruction by anti-AQP4 antibodies, which fix and activate complement, is thought to be the primary pathogenic process in NMO [4].

5.1. Specificity of anti-AQP4 antibody

NMO-IgG has not been described in other inflammatory diseases in Westerners; however, 9% of MS cases in Lennon's original series did have the antibodies [3]. To date, 5–15% of tested MS cases were found to be positive for NMO-IgG or anti-AQP4 antibody [3,5–7,27] while 10% of NMO-IgG-positive patients had brain lesions that were indistinguishable from MS lesions [28]. This indicates the existence of considerable overlap between NMO and MS, which should not be ignored.

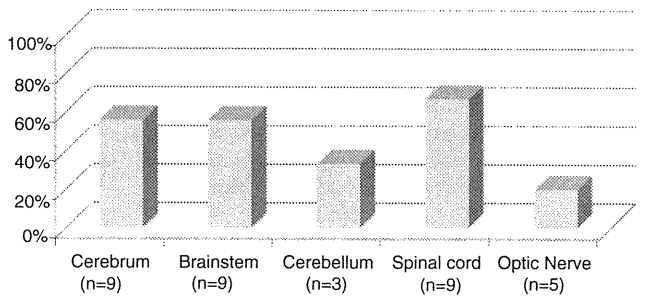

Fig. 2. Frequency of AQP4 loss according to CNS sites of active and chronic active lesions in nine autopsied NMO cases. AQP4 = aquaporin-4; CNS = central nervous system; NMO = neuromyelitis optica.

5.2. Sensitivity of the anti-AQP4 antibody

One of the confounding problems is that NMO-IgG is not detected in all NMO patients (Table 2). In Caucasians, 73% were positive in Lennon's original report [3] and similar figures have also been reported elsewhere: 61.1% in Jarius et al. [29] and 56.72% in Paul et al. [27]. Recently, Fazio et al. [30] conducted immunofluorescence, flow cytometry and radioimmunoprecipitation assays in Italian patients with NMO and found up to 47% were positive. In Africans and their descendants, much lower positivity rates were reported: 33.3% in Caribbean patients with NMO [31] and 5% in African-American patients with OSMS [32]. In Japan, Nakashima et al. [5] reported the detection of NMO-IgG in 63% of OSMS patients and 15% of CMS patients. Recently, the same group also reported that 20 of 22 NMO patients had the anti-AQP4 antibody, while none of the 53 MS patients tested positive (90% vs. 0%) [33]. Tanaka et al. [34], in their selected series of MS patients, independently reported that the anti-AQP4 antibody positivity rate was 61.5% in OSMS patients with LESCLs and 0% in CMS patients without LESCLs. We reported that the positivity rate for the anti-AQP4 antibody was 27.1% (13/48) in OSMS patients, 5.6% (3/54) in CMS patients, 0% (0/52) in patients with other neurological diseases and 0% (0/35) in healthy controls [6]. Among OSMS patients, the antibody positivity rate was highest (55.6%) in OSMS patients with both LESCLs and MS-like brain lesions fulfilling the Barkhof criteria for MS [35], although NMO-IgG was originally described in patients with exclusive optic nerve and spinal cord lesions.

Table 2
Positivity rates for NMO-IgG/anti-AQP4 antibody among races.

Race	Disease	NMO-IgG/anti-AQP4 antibody (%)
Caucasians [3,27–30]	NMO	30–73[a]
Northern Japanese [5]	OSMS	63[a]
Southern Japanese [6]	OSMS	27[a]
Caribbean [31]	NMO	33
African-American [32]	OSMS	5

[a] Measured by Mayo Clinic.

There are obvious discrepancies in the detection rates among the above-mentioned series. The reasons for these may relate to differences in the subjects used: selected versus consecutive patients; NMO versus OSMS patients with LESCLs; Northern versus Southern Japanese patients that have been shown to have somewhat distinctive features in clinical phenotype by a recent nationwide survey [36,37]. They could also relate to the methods used: AQP4-transfected versus GFP-AQP4 fusion protein-transfected; fixed transfected cell specimens versus unfixed ones; 1:4 dilution versus 1:400 dilution. However, even in the studies done by the Mayo clinic, there are considerable differences in positivity rate, indicating that the difference is partly attributable to differences among subjects (Table 2) [3,5,6,27–32]. It remains to be elucidated whether the 30–70% of NMO patients who fulfilled the NMO diagnostic criteria and did not carry the antibodies are truly seronegative NMO patients or false negatives due to the low sensitivity of the assay employed.

In the case of Baló's disease, the anti-AQP4 antibody in sera was negative in six MRI-confirmed Baló's cases by standard cell-based immunofluorescence and flow cytometric assays [26], suggesting that AQP4 loss occurs through anti-AQP4 antibody-unrelated mechanisms in this condition.

5.3. Titers and IgG subclass

Takahashi et al. [33] claimed that anti-AQP4 antibody titers showed a very strong positive correlation with spinal cord lesion length ($R = 0.9108$), while others have not confirmed this result [6,7,38,39]. Although the NMO-IgG/anti-AQP4 antibody usually appears in the early course of the disease [40], seroconversion of NMO-IgG/anti-AQP4 antibody during the course of illness is observed in some patients [6,7]. This may indicate the possibility that the antibody is produced after tissue destruction on some occasions, as seen in MS patients in whom various autoantibodies emerge during the clinical course; some of them target even neural antigens, such as neurofascin, and are shown to be functional in vivo [41]. Considering that OSMS patients with a low titer of anti-AQP4 antibody showed similar clinical and immunological features to those of OSMS patients without the antibody, it is possible that a low titer anti-AQP4 antibody is secondary to severe tissue destruction [7]. However, once it appeared, even though secondarily, it could exert pathogenic effects in vivo.

To clarify the clinical relevance of anti-AQP4 antibody titers and the IgG subclass, we developed a bridging enzyme-linked immunosorbent assay (ELISA) and a flow cytometric assay [42]. In the latter, each IgG subclass was quantitated using the mean fluorescence intensity ratio of AQP4-transfected and -untransfected human embryonic kidney cells. By ELISA, levels of the anti-AQP4 antibody were observed to positively correlate with the number of relapses presenting with optic neuritis while they showed a steady rise over time without concomitant relapse in seven of twelve longitudinally studied patients over an average of 8 years. Anti-AQP4 antibody levels determined by standard immunofluorescence assay, ELISA and flow cytometry had no correlation with disease severity, such as Expanded Disability Status Scale (EDSS) scores.

By flow cytometry, IgG1, 2, 3 and 4 anti-AQP4 antibodies were found in 97.8, 39.1, 13.0 and 8.7%, respectively. In patients not receiving corticosteroids, the levels of IgG1 anti-AQP4 antibody correlated positively with disease duration, while those of IgG2 antibodies correlated negatively with maximum spinal cord lesion length, being lower in patients with longitudinally extensive spinal cord lesions compared with those without, but higher in patients with anti-SS-A/B antibodies than in those without. Moreover, IgG2 anti-AQP4 antibody carriers showed a younger age of onset and a lower Progression Index than those with other subclasses. We hypothesize that a longer disease duration and a higher number of relapses increase total anti-AQP4 antibody levels, especially IgG1, possibly through affinity maturation. A similar observation was made by other authors, in which total anti-AQP4 antibody titers increased as relapse numbers increased and disease duration became longer [43,44]. There also exists a unique subgroup of IgG2 anti-AQP4 antibody carriers with a younger age of onset and humoral autoimmune background who demonstrate a relatively benign course, in spite of having high levels of total anti-AQP4 antibody. Such IgG2 anti-AQP4 antibody carriers may correspond to cases with so-called "benign NMO" in the literature [45,46] and may not require a long-term administration of low dose corticosteroids and immunosuppressants.

5.4. Autoimmune background for anti-AQP4 antibody production

The relapsing form of NMO with the anti-AQP4 antibody is frequently associated with other autoantibodies and autoimmune diseases, such as Sjögren syndrome, systemic lupus erythematosus, autoimmune thyroiditis and myasthenia gravis, in Westerners [1,47]. Even in Asian patients with anti-AQP4 antibodies, other autoantibodies, such as SSA and SSB, as well as other autoimmune diseases, such as Sjögren syndrome, are frequently present [6,7,48–50]. Therefore, an autoimmune-prone background, especially heightened humoral autoimmunity, seems to be an important factor in the production of the anti-AQP4 antibody. We found that, among anti-AQP4 antibody-positive individuals, Th1 cell percentage showed a significant negative correlation with anti-AQP4 antibody titer, and that those with SSA/SSB antibody had significantly higher titers of the anti-AQP4 antibody [7]. Therefore, a high titer anti-AQP4 antibody seems to be produced in those with a heightened humoral autoimmune background, a Th2-prone condition; however, an anti-AQP4 antibody titer itself does not necessarily correlate with severity of the disease, rather curiously tends to show an inverse correlation with EDSS scores [6]. This result is probably due to the existence of NMO patients with mild disability but with a high anti-AQP4 antibody titer.

5.5. Pathogenicity of NMO-IgG/anti-AQP4 antibody

Sera and IgG from NMO patients with NMO-IgG/anti-AQP4 antibody induce astrocyte damage and death in primary cultures only in the presence of complement [51–53], while in the absence of complement they do not affect AQP4 water channel function in astrocytes [54]. IgG containing the anti-AQP4 antibody from NMO-IgG-seropositive NMO patients reproduces astrocyte loss in vivo only when MBP-specific T cells are transferred to cause experimental autoimmune encephalomyelitis (EAE) [55–57]. However, when the AQP4 antibody was injected into young rats with a leaky BBB, or after transfer of non-encephalitogenic T cells, it did not induce any disease or neuropathological alterations in the CNS [57]. Direct injection of NMO-IgG together with human complement into the brain of mice induced AQP4 loss with extensive inflammatory infiltrates, having some similarity to human NMO lesions [58]. Inflammation might be exaggerated by the fact that mice complement inhibitor existing in the CNS has no prohibiting effects on human complement and it still implies some factor might trigger breakdown of the BBB at first. Recently, Kinoshita et al. [59] reported that following pre-treatment with complete Freund's adjuvant, injection of anti-AQP4 antibody-containing IgG can induce inflammation and astrocyte damage in the CNS. Thus, if non-specific inflammation renders the BBB leaky, anti-AQP4 antibody may enter into the CNS and cause astrocyte destruction. However, animals never demonstrated any clinical signs and pathological changes never became extensive as seen in human NMO cases.

6. Cellular immunity in NMO and MS

6.1. AQP4-specific T cell response

By immunizing overlapping pentadecameric peptides of AQP4, Kalluri et al. [60] found that the N-terminal region of AQP4 is highly

immunogenic in mice, and that the intracellular epitope AQP4 (22–36) was a major immunogenic determinant. AQP4 (22–36) and AQP4 (289–303)-specific T cells were present in the natural T cell repertoire of C57BL/6 mice and T cell lines could be raised. However, active immunization with these AQP4 peptides did not produce any signs of disease, despite induction of antigen-specific T cells [60]. These observations imply that AQP4 is not encephalitogenic. Human T cell epitopes on AQP4 remain to be elucidated.

6.2. Myelin protein-specific T cell response

We reported the establishment of major myelin protein-derived T cell lines (TCLs) from anti-AQP4 antibody-seropositive NMO patients as well as anti-AQP4 antibody-seronegative MS patients [61,62]. TCLs from most of these patients reacted with multiple epitopes on the plural myelin proteins, such as MBP, proteolipid protein and myelin oligodendrocyte glycoprotein, which was not seen in TCLs from healthy donors that were established in the same way. Therefore, this indicates that inter- or intra-molecular epitope spreading against myelin proteins occurs in MS and NMO patients, irrespective of anti-AQP4 antibody status. These findings suggest that T cells are stimulated in vivo against major myelin proteins even in anti-AQP4 antibody-positive patients with NMO/NMOs, like anti-AQP4 antibody-negative MS patients. Therefore, after myelin-specific T cells initiate CNS inflammation, antibodies recognizing various components of CNS antigens might modify the clinicopathological features of demyelinating diseases (Fig. 3).

6.3. Th17 and Th1 cytokines

In peripheral blood, OSMS shows a pronounced T-helper-1 (Th1) and T-cytotoxic-1 (Tc1) shift, where interferon-γ (IFNγ)-producing T cells predominate over IL-4-producing T cells throughout the relapse and remission phases [63,64]. We previously reported that IL-17 is upregulated in the cerebrospinal fluid (CSF) of OSMS patients and that levels of both IL-17 and the downstream cytokine IL-8 in CSF show a significant positive correlation with spinal cord lesion length [21]. Recently, by simultaneously measuring the levels of 27 cytokines and chemokines in CSF from patients with various causes of myelitis, we found that IL-17, IFNγ and granulocyte-colony stimulating factor were specifically elevated in OSMS patients, irrespective of the presence or absence of the anti-AQP4 antibody [65]. IL-17 (IL-17A) is exclusively produced by Th17 cells, which are CD4+ T cells recently shown to be a distinct lineage from Th1 and Th2 cells [66]. Increasing evidence suggests that Th17 cells, but not Th1 cells, are responsible for organ-specific autoimmune diseases, such as EAE [66,67]. IL-8 is a chemokine for neutrophils. In OSMS patients, CSF neutrophilia and infiltration of neutrophils in severe lesions are characteristic [21]. Hence, elevated IL-8 may be partly responsible for such neutrophil activation and mobilization in OSMS. Indeed, the level of myeloperoxidase, an activated neutrophil product, is increased in sera from OSMS patients, especially in those with LESCLs at relapse [68]. Th17 cells carrying granzyme B have recently been shown to efficiently disrupt BBB tight junctions and loosen the BBB [69]. Therefore, autoimmune Th17 cells may initiate BBB disruption and inflammation in OSMS, causing vasogenic edema in the CNS, regardless of the anti-AQP4 antibody status.

IL-17 has also been shown to induce vascular endothelial growth factor (VEGF) production in target tissues [70]. Regarding other factors with possible effects on vascular permeability, we previously reported that the levels of VEGF in sera were significantly elevated in OSMS patients, showing a significant positive correlation with spinal cord lesion length [71]. Given that AQP4 knockout mice showed prolonged vasogenic edema [72], but a decrease in the level of cytotoxic edema [73], the anti-AQP4 antibody produced either by a heightened humoral autoimmune background or secondarily by tissue breakdown may prolong resolution of tissue edema, thereby contributing to further tissue destruction in NMO and OSMS patients. Prolonged vasogenic edema at sites where the surrounding space is tight (the bony optic canal portion of the optic nerves) or where vascular supply (thoracic spinal cord) is poor may cause poor recovery from tissue damage in patients with the anti-AQP4 antibody.

6.4. Infections

Relapsing NMO is associated with other autoimmune disorders whereas monophasic NMO is associated with preceding infection [1]. Recently, it was reported that 88% of parainfectious NMOs are monophasic [74]. Hypercomplementemia and elevation of C-reactive protein are seen in anti-AQP4 antibody-positive patients with NMO spectrum disorders at relapse; however, such a systemic inflammatory reaction is rare in classical MS [75]. Considering its relapsing

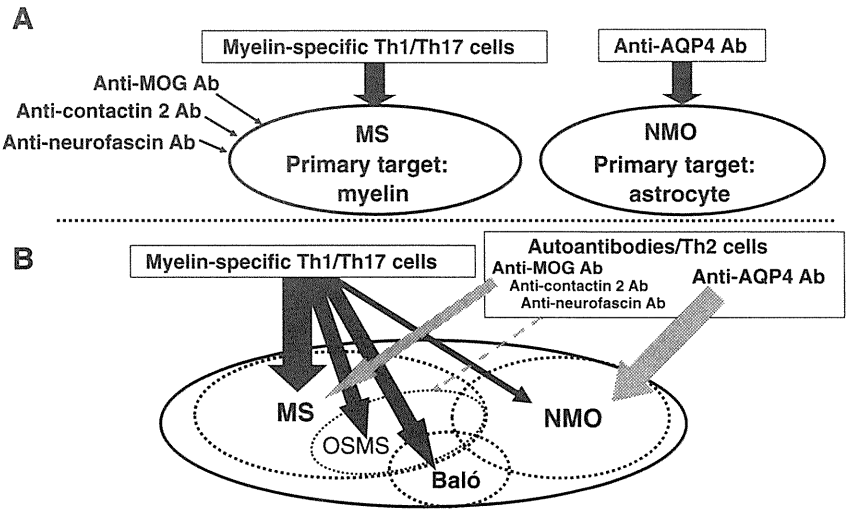

Fig. 3. Two hypothetical mechanisms of MS and NMO. In panel (A), myelin is a primary target of T cells and autoantibodies in MS, whereas in NMO, astrocytes are a primary target of the anti-AQP4 antibody. In panel (B), myelin-specific T cells initiate CNS inflammation and autoantibodies recognizing various components of CNS antigens modify clinicopathological features. In (A), NMO takes a uniform disease process presenting AQP4 loss in all active lesions whereas in (B) heterogeneous disease processes take place even in the same patient, showing heterogeneous pathological features. Ab = antibody; AQP4 = aquaporin-4; CNS = central nervous system; MOG = myelin-oligodendrocyte glycoprotein; MS = multiple sclerosis; NMO = neuromyelitis optica; OSMS = opticospinal multiple sclerosis; Baló = Baló's disease.

nature, specific acute infection is less likely to play a role in causing relapsing NMO with the anti-AQP4 antibody. However, we found that *Helicobacter pylori* infection is more prevalent in anti-AQP4 antibody-positive NMO than anti-AQP4 antibody-negative CMS patients and healthy controls [76]. In such patients, the antibody response to *Helicobacter pylori* neutrophil activating protein (NAP) was found to be markedly exaggerated and anti-NAP antibody titers showed a positive correlation with disease severity (EDSS scores) [77]. Therefore, it is possible that persistent *Helicobacter pylori* infection potentiates Th17/Th1 responses, thereby contributing to the autoimmune response of Th17/Th1 cells to CNS antigens. Alternatively, chronic persistent infection may in part contribute to the development of NMO through molecular mimicry between bacterial AQP and human AQP4, and the products of infectious agents, such as lipopolysaccharides, may render the BBB leaky.

7. Autoimmune mechanisms of NMO, MS and Baló's disease

7.1. Proposed mechanism

Based on the high specificity of the anti-AQP4 antibody and the selective loss of AQP4 in NMO lesions, it is postulated that the complement activating anti-AQP4 antibody plays a pivotal role in the development of NMO lesions [78]. Once the anti-AQP4 antibody gets across the BBB, it binds to AQP4 molecules on the astrocyte foot processes and activates complement. Activated complement mobilizes

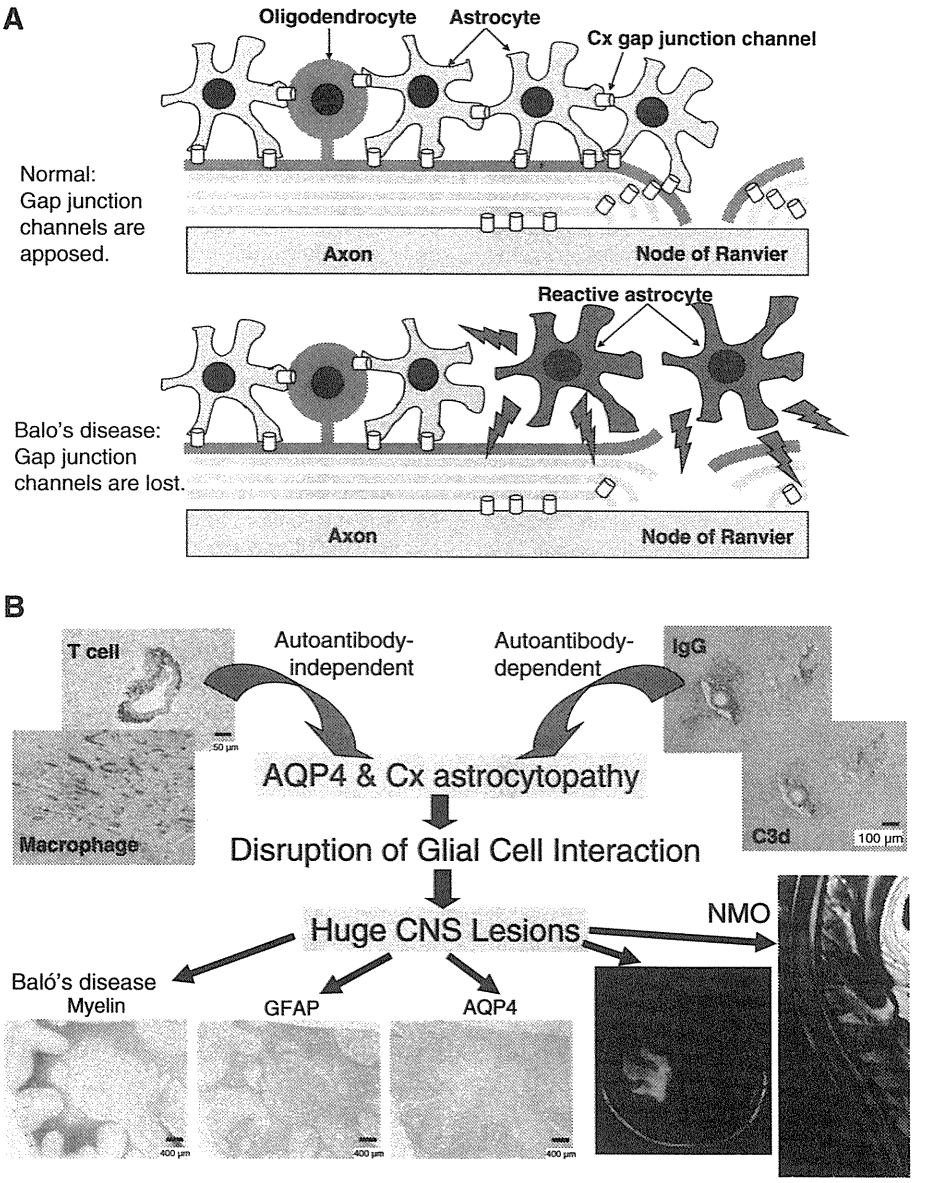

Fig. 4. (A) Hypothetical mechanism of connexin astrocytopathy causing secondary demyelination. Loss of astrocytic connexins, such as connexin 43, may disrupt interactions between astrocyte–astrocyte and astrocyte–oligodendrocyte. Once disruption of connexin gap junction channels occurs in the perivascular areas by either autoantibody-mediated or T cell-mediated mechanisms, disturbance of glial intercellular communications may extensively propagate along with the fiber tracts. (B) Hypothetical mechanism of huge CNS lesions in demyelinating diseases. Connexin loss may cause a widespread disruption of intercellular communications among glial cells and axons while AQP4 loss exacerbates vasogenic edema associated with inflammation. AQP4 and connexin astrocytopathy culminating in huge CNS lesions occurs in Baló's disease, and could also develop in a fraction of MS and NMO patients (light green-colored areas in Fig. 1). AQP4 = aquaporin-4; CNS = central nervous system; Cx = connexin; NMO = neuromyelitis optica.

neutrophils and eosinophils that then facilitate tissue destruction. The observation that the anti-AQP4 antibodies so far examined by immunofluorescence assays are all from the IgG1 subclass [7] and can efficiently fix complement is compatible with such a hypothesis. Here, demyelination is secondary to destruction of astrocytes, which is supposed to be fundamentally distinct from the primary demyelinating mechanism executed by myelin antigen-specific T cells and anti-myelin autoantibodies in MS.

7.2. Concerns about the proposed mechanism of NMO

I have several concerns surrounding the above-mentioned hypothesis based on the anti-AQP4 antibody. First, in the presence of high titers of anti-AQP4 antibodies, some patients remain in remission [7], and there are cases where patients who harbor the anti-AQP4 antibody do not show signs of NMO for a long time [79]. Because AQP4 is present in astrocyte foot processes behind the BBB, additional factors that disrupt the BBB and render the antibody able to enter the CNS across the BBB may be required to initiate relapse. In addition, the fact that anti-AQP4 antibody titers [6,7,38,39] do not strictly correlate with the occurrence of relapse in most studies to date further supports the prerequisite for some additional factor to induce relapse. Indeed, in animal models, myelin antigen-specific T cells are required for the anti-AQP4 antibody to cause extensive lesions in vivo in the CNS [56,57]. Our observation that in anti-AQP4 antibody-positive NMO patients' peripheral blood T cells reactive to major myelin proteins showed intra- and inter-molecular epitope spreading [62] suggests that T cells are already stimulated with myelin antigens in vivo in these patients. Second, AQP4 is present in the retina, distal collecting tubules, gastric mucosa, muscle and lung, and NMO-IgG binds to these structures [4,78]; however, no impairments in these organs have been observed to date. In particular, although Müller cells, which are equivalent to astrocytes, abundantly express AQP4 in the endfeet adjacent to blood vessels in the retina, no severe inflammation has ever been reported in anti-AQP4 antibody-positive NMO patients, suggesting that the presence of the complement-fixing anti-AQP4 antibody is not sufficient to produce tissue damage. Moreover, AQP4 expression is ubiquitous throughout the CNS, although its expression level varies, being high in the gray matter of the spinal cord [11,22]. Cerebral gray matter and the cerebellum abundantly express AQP4 [11,22]; however, these sites are seldom involved in NMO [80]. Such a ubiquitous presence of AQP4 cannot explain the selectiveness of lesion distribution, namely in the optic nerves and spinal cord. Third, the deposited immunoglobulins in postmortem NMO lesions are mainly IgM [20] while the anti-AQP4 antibodies described are all IgG. We observed that some NMO lesions show perivascular deposition of complement and IgG in acute lesions, while no AQP4 loss is found [11], suggesting that perivascular complement and IgG deposition does not strictly correlate with AQP4 loss.

7.3. Mechanism of huge CNS lesions

Anti-AQP4 antibody-positive NMO patients occasionally develop huge brain lesions, such as LESCLs seen in the spinal cord. Such extensive white matter lesions in anti-AQP4 antibody-positive NMO patients demonstrate high signal intensity on apparent diffusion coefficient maps and low or isointensity on diffusion-weighted MRI images, suggesting the nature of the lesions to be vasogenic edema [6,25]. Using magnetic resonance spectroscopy, a high choline peak and a low n-acetyl aspartate peak are observed, compatible with acute demyelination [6,25]. These findings strongly suggest that the nature of the lesions in anti-AQP4 antibody-positive MS patients is inflammatory demyelination with vasogenic edema. However, interestingly, even in such extensive brain lesions, gadolinium enhancement of the lesions is absent or scant [25,81], except for cases complicated with other systemic autoimmune diseases, suggesting largely preserved integrity of the BBB in this condition. Contrarily, Ito et al. [82] reported that multiple patchy enhancing lesions with blurred margins, described as "cloud-like enhancement", are found in 90% of NMO patients with contrast enhancement, suggesting breakdown of the BBB in this condition. Thus, the mechanism underlying lesion formation could be heterogeneous, even among individuals with the anti-AQP4 antibody.

7.4. Our hypothesis

The idea that astrocytopathy induces secondary demyelination or that such a tiny deposition of immunoglobulin and complement around vessels produces huge lesions is ill-defined and requires further clarification. We recently found that connexins, which form gap junction channels between glial cells and between glia and axons, were entirely lost in the AQP4-diminished lesions in Baló's disease, NMO and MS (submitted for publication). Connexin gap junction channels not only appose glial cells but also have a critical role in intercellular communication between glia. Therefore, we hypothesize that once glial connexins are disrupted by either autoantibodies, such as the anti-AQP4 antibody, or T cells in the perivascular areas, dysfunction of glial cell interaction may spread along with neural fibers (Fig. 4A). Connexin loss may cause widespread disruption of intercellular communication while AQP4 loss exacerbates vasogenic edema. AQP4 and connexin astrocytopathy occurs in Baló's disease and a fraction of MS and NMO, culminating in huge CNS lesions (Fig. 4B). Such a hypothesis should be tested by future experimental studies.

Acknowledgments

I would like to thank Drs. Takeshi Matsuoka, Satoshi O Suzuki, Katsuhisa Masaki, Noriko Isobe, Tomomi Yonekawa and Takuya Matsushita for their help with neuropathological and immunological examinations. This work was supported in part by grants from the Research Committees of Neuroimmunological Diseases, the Ministry of Health, Labour and Welfare, Japan and from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Review

MULTIPLE SCLEROSIS JOURNAL | MSJ

Multiple Sclerosis Journal
17(7) 771–779

DOI: 10.1177/1352458511400475
msj.sagepub.com
SAGE

Astrocytopathy in Baló's disease

Jun-ichi Kira

Abstract

Baló's disease is characterized by alternating rings of demyelination and preserved myelin. As additional multiple sclerosis (MS)-like lesions often coexist in Baló's cases, Baló's disease is regarded as a variant of MS. In demyelinated areas, many hypertrophic astrocytes are present in close contact with oligodendrocytes, which often show apoptotic features. In the outermost layer of preserved myelin, stress proteins involved in tissue preconditioning are abundant in oligodendrocytes. The peri-plaque perimeter is thus assumed resistant to subsequent attack, thereby leaving a layer of preserved myelin. In some cases, Baló's concentric rings develop step by step in a centrifugal direction, whereas many other cases show simultaneous enhancement of multiple rings. Therefore tissue preconditioning and successive ring formation does not fully describe the mechanism of the disease. We recently reported that in four Filipino Baló's patients, aquaporin-4 (AQP4) was extensively lost in glial fibrillary acidic protein-positive hypertrophic astrocytes, both in demyelinated and myelinated layers of all actively demyelinating lesions. None of six further patients with MRI-confirmed Baló's disease were seropositive for anti-AQP4 antibody. I propose that AQP4 astrocytopathy, in the absence of anti-AQP4 antibody, is characteristic of Baló's disease. This hypothesis should be tested in future experimental studies.

Keywords

aquaporin-4, astrocytopathy, Baló's disease, concentric lesions, multiple sclerosis (MS), neuromyelitis optica (NMO)

Date received: 26th September 2010; revised: 8th December 2010; accepted: 17th January 2011

Introduction and methods

Baló's disease was first described by Marburg[1] more than a century ago, under the name 'encephalitis periaxialis scleroticans'. Baló, a Hungarian neuropathologist, was the first to stress a concentric pattern of lesions, and he named the disease 'encephalitis periaxialis concentrica', based on the observation that 'the white matter of the brain is destroyed in concentric layers in a manner that leaves the axis cylinders intact'.[2] The condition has since been named after him.

Baló's disease is diagnosed histologically, using autopsied tissues, by the presence of alternating layers of demyelination and relatively preserved myelin. Historically, the disease was very rarely reported; however, since the introduction of magnetic resonance imaging (MRI) into clinical practice, the number of reported cases with Baló-like concentric lesions has increased. Such neuroimaging studies, together with recent immunohistochemical analyses, have given important insight into the pathophysiology of Baló's disease. Although the peculiar concentric nature of the lesions has attracted a lot of attention, the mechanism producing this pathology remains to be elucidated. We recently reported extensive aquaporin-4 (AQP4) loss in Baló's disease lesions and pointed out the importance of AQP4 astrocytopathy in the absence of anti-AQP4 antibodies.[3] Here, I have conducted a literature search on Baló's disease using PubMed and reviewed recent progress in research on Baló's disease. I propose a novel mechanism for concentric demyelinating ring formation.

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Clinical findings of Baló's disease

Baló's disease is rare in White populations but is relatively frequently reported in certain Asian populations, such as Filipinos, southern Han Chinese and Taiwanese.[4] The disease most commonly shows an acute onset, although some cases have a subacute onset. Initial symptoms are usually mental and behavioral changes and these symptoms progress into major deficits within a few months, presenting paralysis, hyperreflexia, seizure, dysphagia, sphincter impairment, and akinetic mutism. The severity of symptoms is in accord with lesion expansion determined by MRI. Without treatment, the disease culminates in death or severe disability. Cerebrospinal fluid (CSF) is normal or has mild inflammatory changes.[4,5] Oligoclonal bands (OBs) are usually absent in CSF, while some recent cases, demonstrating a transition to multiple sclerosis (MS), were reported to have OBs.[6–10]

A monophasic course and poor prognosis were previously thought to be common; however, MRI has made clinical diagnosis of Baló's concentric rings possible, and early intervention with high-dose corticosteroids and other immunotherapies has markedly improved its prognosis.[11] Cases showing spontaneous recovery have even been reported.[6] Some Baló's disease cases have recovered from the initial insult, but have had a relapsing course and demonstrated the coexistence of Baló-like concentric lesions and MS-like lesions.[11] Also, some cases initially diagnosed as MS have later developed Baló's concentric lesions, as determined by MRI.[7,12] Since the clinical introduction of MRI, a considerable number of reports have described a transition of these two conditions.[8–12] In these articles, some cases have been reported as Baló's disease while others have been described as MS with concentric lesions.[8–12] Hence, it is difficult to completely differentiate Baló's disease from MS with concentric aspects, and some authors regard Baló's disease as a variant of MS.[11]

Pathological findings of Baló's disease

Early pathological studies revealed that the most commonly affected site is the cerebral white matter. The pons, cerebellar white matter and basal ganglia were also affected in some autopsied cases.[5,13] Cortical gray matter and subcortical U fibers are usually spared, as distinct from MS.[4,5,14] The spinal cord was long considered to be unaffected in Baló's disease; however, recent MRI-diagnosed or pathologically proven cases have shown this to be untrue.[15,16] Concentric rings are typically parallel rings with a 'storm center',[5] but there are cases showing various other patterns, such as distorted rings, a rosette or carnation pattern, a mosaic pattern, and parallel bars.[5] Demyelinated layers are consistently wider than myelinated ones.[14] Within the demyelinated bands, the inner border is sharp whereas the outer border is blurred and gradually transitions to the white matter at the outermost edge, which appears normal.[14]

Moore et al.[15] noted in a Baló's case that the bands of intact myelin comprised mainly remyelinated fibers, and they proposed the possibility of remyelination for the myelinated layers. However, Yao et al.[17] and others[4] reported that in the demyelinated areas, where Bodian staining showed axons to be relatively preserved, there were no thinly myelinated fibers, indicating that remyelination is not responsible for the occurrence of the myelinated rings. Yao et al.[17] found that the number of oligodendrocytes was markedly reduced in the demyelinated layers, and, after long disease duration, oligodendrocyte numbers were considerably decreased, even in the relatively preserved myelin layers. These authors also observed that there were many bizarrely shaped giant astrocytes that stained with glial fibrillary acidic protein (GFAP) in the demyelinated as well as the myelinated areas, which closely associated with oligodendrocytes in the demyelinated areas.[17]

Lucchinetti et al.[18] proposed four basic demyelinating patterns of MS lesions. Among them, the so-called pattern III lesions show a characteristic disturbance of oligodendroglia, defined as: distal dying back oligodendrogliopathy with oligodendroglia apoptosis. In this condition, apoptotic nuclear changes in oligodendrocytes and preferential loss of myelin-associated glycoprotein and cyclic-nucleotide phosphodiesterase are characteristic. Lucchinetti et al.[18] described 8 of 22 MS cases with pattern III lesions showing concentric layering of myelinated and demyelinated tissues. Most of these cases had an acute disease course of less than 8 weeks before biopsy or autopsy, while longer surviving cases showed a disease consistent with relapsing–remitting MS.[18] These cases might thus be regarded as MS with concentric aspects, while Baló's disease has some histopathological features of pattern III lesions.[19,20]

Features of pattern III lesions can be seen in a variety of white matter pathologies, such as white matter hypoxia/ischemia, progressive multifocal leukoencephalopathy (PML), and cuprizone intoxication, which interferes with cellular energy metabolism.[19] Hypoxia-like tissue injury has, therefore, been proposed to be critical in Baló's disease.[19,20] Stadelmann et al.[20] found high levels of stress proteins that are involved in tissue preconditioning, such as hypoxia-inducible factor 1α and heat-shock protein, in oligodendroglia in the outermost layer of preserved myelin. Based on the neuroprotective effects of these proteins, the

perimeter layer of peri-plaque tissue is assumed to be resistant to subsequent attack, thereby leaving a layer of preserved myelin.[20]

Vascular and inflammatory components also exist in Baló's concentric lesions. Courville[5] described capillary degeneration in the demyelinated layers but relative capillary preservation in the myelin-preserved layers, suggesting impairment of circulation, especially in the demyelinated areas. Additional small lesions, showing perivascular concentric demyelination, can be seen in some cases, further supporting an important role of vascular factors disrupting the blood–brain barrier (BBB).[14] Many foamy macrophages containing myelin debris infiltrate Baló's lesions, being most numerous around vessels and in the demyelinated areas.[17] Small foci of perivascular mononuclear cell cuffing, which included lymphocytes, was observed in partly myelinated layers, as well as in white matter of normal appearance.[17] We revealed that perivascular lymphocyte cuffing consists mainly of T cells and a few B cells in Baló's concentric lesions, but did not observe any vasculocentric deposition of immunoglobulin or complement.[3]

Neuroimaging findings of Baló's disease

Cases with MRI-proven Baló's concentric lesions have repeatedly been reported after the introduction of MRI into clinical practice. Most of these cases had Baló's concentric lesions in the cerebral hemisphere, although the occurrence of Baló's concentric lesions in the brainstem has also been described.[21,22] Chen et al.[23] proposed that Baló's concentric rings do not arise simultaneously, but develop step by step in a centrifugal direction. These authors observed that enhancement of the outer layers relatively devoid of demyelination developed first, and was followed by progressive demyelination occurring at the inner aspect of the enhancement.[23] Thereafter, new enhancement appeared at the periphery while the previously enhanced rings disappeared. Recurrence of this process forms alternating demyelinated and relatively preserved myelin bands.

Such findings seem to fit well with the preconditioning hypothesis based on the pathological observation of upregulated protective factors in the outermost layers, and support a mechanism of tissue preconditioning and outward expansion of the concentric lesions. By contrast, Kastrup et al.[24] reported that contrast enhancement of T2 hyperintense rings developed synchronously rather than successively. Simultaneous multiple layers of ring enhancement or serpentine ring enhancement are described by many other authors.[8–11] Wang et al.[11] concluded that enhancement patterns of Baló's concentric lesions are heterogeneous: synchronous enhancement of most concentric rings, heterogeneous enhancement of different rings, and confined enhancement of the outermost ring, in part depending on the timing of MRI scans. Baló's concentric rings are already present at the time of initial presentation in many MRI-confirmed cases.[8–11] These observations suggest that tissue preconditioning and successive ring formation cannot fully explain the mechanisms of Baló's disease in all cases.

Although some authors have claimed that Baló's lesions show increased diffusion,[9] Wiendl et al.[25] reported diffusion-weighted MRI abnormalities in a case of Baló's disease on the first day that neurological symptoms appeared. They found markedly restricted diffusion on an apparent diffusion coefficient (ADC) map of a very acute lesion, which MRI showed to have developed Baló's concentric lesions at day 14 after the disease onset. Kavanagh et al.[26] also reported alternating bands of restricted and unrestricted diffusion in a case with Baló's disease. Restricted diffusion is seen in a variety of conditions showing cytotoxic edema, such as brain infarction, PML, acute disseminated encephalomyelitis (ADEM), vasculitis, herpes simplex encephalitis, and Creutzfeldt-Jakob disease.[27–31] MS lesions are usually described as showing an increased ADC due to vasogenic edema following BBB damage and acute demyelination by inflammation.[32] However, MS cases demonstrating restricted diffusion in the acute phase are increasingly being reported.[33–37] Some authors noted increased diffusion in the MS lesion center but restricted diffusion in the periphery,[38] while others even described a conversion of restricted diffusion to a vasogenic edema pattern on serial MRI scans.[35] Such restricted diffusion indicates the presence of cytotoxic edema in acute inflammatory demyelinating lesions, including MS and ADEM, which could be produced by myelin and oligodendroglia swelling, energy failure, and ischemia. It is conceivable that very acute lesions of Baló's disease may have restricted diffusion due to similar mechanisms.

Using serial proton magnetic resonance spectroscopy (MRS), Chen[39] reported a decrease of the *N*-acetyl-aspartate (NAA)/creatine (Cr) ratio, an increase of the choline (Cho)/Cr ratio, and emergence of a lactate peak in Baló's concentric lesions, which are consistent with axonal loss, demyelination, and inflammatory cell infiltration, respectively, as seen in autopsied samples.

Coexistence of neuromyelitis optica and Baló's concentric lesions

Graber et al.[22] reported a 29-year-old Afro-Caribbean female with neuromyelitis optica (NMO) presenting Baló's concentric lesions. She had recurrent episodes

of transverse myelitis and acute optic neuritis, and NMO IgG was positive. At the third attack, she developed visual loss and brainstem signs attributable to Baló's concentric lesions in the brainstem. Kreft et al.[16] also described a similar case of a 57-year-old White female who had episodes of transverse myelitis and bilateral optic neuritis, with normal brain MRI and no OBs in the CSF. She later developed Baló's concentric lesions in the cerebral hemisphere. In both cases, plasma exchange was not effective, suggesting factors other than humoral ones were involved.

Pathologically, occurrence of Baló-type concentric lesions in NMO cases has also been reported in early autopsy studies, under the names of acute disseminated sclerosis[40] and concentric lacunar leukoencephalopathy.[41] Itoyama et al.[42] described concentric lamellar demyelinated lesions in Japanese patients with atypical MS; one had bilateral optic neuritis and transverse myelitis, and the other showed transverse myelitis and brainstem signs. Both showed marked CSF pleocytosis exceeding 300 cells/mm^3. The latter had Baló's concentric lesions in the spinal cord, while the former had Baló-like concentric lesions in the optic chiasm. Both cases might be regarded as cases with NMO or NMO spectrum disorder. These findings collectively suggest that Baló's concentric lesions could emerge in NMO.

Aquaporin-4 astrocytopathy in the absence of anti-aquaporin-4 antibodies in Baló's disease

NMO was also thought to be a variant of MS; however, the recent discovery of NMO IgG targeting the AQP4 water-channel protein expressed in astrocyte foot processes suggests that NMO is distinct from MS.[43,44] In NMO, extensive AQP4 and GFAP loss in areas of relatively preserved myelin, together with vasculocentric deposition of IgM, IgG and activated complement, were reported to be characteristic.[45,46] Therefore, in NMO, it is hypothesized that anti-AQP4 IgG1 antibodies bound to AQP4 expressed on the astrocytes fix complement and initiate an inflammatory cascade, and that the primary target is astrocytes, with demyelination occurring secondarily.

Considering the occurrence of Baló's concentric lesions in NMO cases, we immunohistochemically investigated four autopsied Filipino cases with Baló's disease, aiming to clarify AQP4 expression in this condition.[3] We found that in Baló's concentric lesions, AQP4 was extensively lost in both the demyelinated and myelinated layers of all six actively demyelinating lesions. Numerous CD68-positive macrophages that phagocytose myelin debris had infiltrated the lesions, even though the lesions were entirely and strongly stained with GFAP.[3] In the lesions, hypertrophic astrocytes showed intensive staining for GFAP, while AQP4 expression was totally lost (Figure 1). By contrast, in normal-appearing white matter adjacent to the lesions, AQP4 staining was seen in the perivascular astrocyte foot processes. Only in the outer portions of the concentric lesions were there one or two bands showing loss of GFAP and neurofilament in addition to AQP4 loss. These bands are thus considered to be necrotic, and look similar to 'concentric lacunar leukoencephalopathy' (Baló rings in an NMO case).[41] In the demyelinated layers, Bodian staining revealed relative preservation of axons, whereas neurofilament staining was markedly diminished, suggesting axonal impairment, which is in accord with the decreased NAA/Cr ratio determined by MRS.[39] In demyelinated as well as myelinated layers, connexin 43 immunostaining was also lost in Baló's disease while its expression is usually unregulated in chronic astrogliotic scar.[47] Since connexin 43 forms heteromeric gap junctions among astrocytes and oligodendrocytes, widespread disturbance of astrocyte–astrocyte and astrocyte–oligodendrocyte interaction is suggested in Baló's lesions.

We observed perivascular lymphocyte cuffing by CD45RO-positive T cells, but only by a few CD20-positive B cells in Baló's concentric lesions.[3] Interestingly, there was no deposition of immunoglobulins (IgG and IgM) or complement (C3 and C9neo) around vessels.[3] In the normal-appearing white matter adjacent to the outer edge of the lesions,[47] lamellar infiltration of macrophages was seen, while T cells infiltrated distant areas, which is consistent with the early pathological reports describing lymphocyte infiltration in normal-appearing white matter.[17]

In another six patients with MRI-confirmed Baló's concentric lesions,[11] we examined anti-AQP4 antibody in sera by a conventional immunofluorescence method and a more sensitive flow cytometric assay, and found that none of the cases were positive for anti-AQP4 antibody.[47] These findings collectively suggest that AQP4 astrocytopathy in the absence of anti-AQP4 antibody is characteristic of Baló's disease.

Hypothetical mechanisms for the formation of Baló's concentric rings

A variety of mechanisms have been proposed since the original description of the rings. The current dominant hypothesis is distal oligodendrogliopathy and tissue preconditioning.[19,20] Here, initial insult induces oligodendrocyte apoptosis, and in the lesion edges, oligodendrocytes are preconditioned to produce stress proteins, which work protectively at the second insult