Related B cell clones populate the meninges and parenchyma of patients with multiple sclerosis

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In the central nervous system of patients with multiple sclerosis, B cell aggregates populate the meninges, raising the central question as to whether these structures relate to the B cell infiltrates found in parenchymal lesions or instead, represent a separate central nervous system immune compartment. We characterized the repertoires derived from meningeal B cell aggregates and the corresponding parenchymal infiltrates from brain tissue derived primarily from patients with progressive multiple sclerosis. The majority of expanded antigen-experienced B cell clones derived from meningeal aggregates were also present in the parenchyma. We extended this investigation to include 20 grey matter specimens containing meninges, 26 inflammatory plaques, 19 areas of normal appearing white matter and cerebral spinal fluid. Analysis of 1833 B cell receptor heavy chain variable region sequences demonstrated that antigen-experienced clones were consistently shared among these distinct compartments. This study establishes a relationship between extraparenchymal lymphoid tissue and parenchymal infiltrates and defines the arrangement of B cell clones that populate the central nervous system of patients with multiple sclerosis.

Keywords: multiple sclerosis; B cells; clonal expansion; antigen experience; central nervous system

Abbreviations: Ig = immunoglobulin; VH = variable region heavy chain

Introduction

Multiple sclerosis is an inflammatory disease of the CNS characterized by demyelination of the brain and the spinal cord (Lucchinetti et al., 1998; Noseworthy et al., 2000; O’Connor et al., 2001; Filippi and Rocca, 2005; Hafler et al., 2005). Although most of the tissue damage is found within the parenchyma, several recent studies have implied that extraparenchymal structures, such as the meninges, may play a role in the disease pathology. The first of these seminal studies demonstrated that organized B cell structures, reminiscent of germinal centres, are present in the cerebral meninges of patients with multiple sclerosis (Serafini et al., 2004; Magliozzi et al., 2007). These tertiary lymph node-like structures include proliferating B cells with a network of follicular dendritic
cells and appear to be primarily present in the meninges and sporadically in the parenchyma (Prinezas, 1979). The second major finding showed that the choroid plexus, a distinct meningeal structure, is the port through which Th17 lymphocytes enter the CNS where they then initiate experimental autoimmune encephalomyelitis (Reboldi et al., 2009). While these studies point towards a role for the meninges in the inflammatory response, it is not known whether the B cells present at these extraparenchymal sites are related to those that populate the CNS and potentially contribute to the pathophysiology of multiple sclerosis. Through analysis of the B cell repertoires derived from meningeal B cell aggregates and the corresponding parenchymal infiltrates from brain tissue derived primarily from patients with progressive multiple sclerosis, we defined the organization of antigen experienced B cell populations present in distinct locations of the multiple sclerosis CNS. This study provides new evidence for a correlation between the extraparenchymal sites and the clonal B cell population that infiltrates multiple sclerosis CNS tissue.

Materials and methods

Specimens

Plaque, adjacent normal appearing white matter or distant normal appearing white matter, and adjacent grey matter or distant grey matter were dissected and bisected at autopsy from 11 subjects with clinically defined multiple sclerosis. Seven of the 11 subjects had a secondary progressive clinical course, three had a chronic progressive course and one had a relapsing clinical course. Half of the bisected samples were placed in 10% buffered formalin then stored at room temperature and half were immediately snap-frozen then stored at −80°C. The CSF of Patient MS7 was removed post-mortem, centrifuged, then the isolated cell pellet and supernatant were stored at −80°C. The clinical features of each patient with multiple sclerosis and the respective brain specimens are summarized in Supplementary Table 1. Non-multiple sclerosis controls included two white matter and grey matter samples derived from an individual (autopsy) without history of any neurological disease and the peripheral blood from three subjects without neurological diseases. Local human research internal review boards approved all human subject related work.

Histology and immunohistochemistry

Immunohistochemical analysis was performed on either paraffin-embedded formalin-fixed or frozen tissue as previously described (Willis et al., 2009). Primary anti-human CD3, CD20, CD138 and CD68 (all from Dako) were applied for 1 h to the specimens MS4–11. Slides were washed in Tris–HCl (50 mM, pH 7.4) and incubated with Dako Cytomation Envision kit according to the manufacturer’s instructions. After further washing, immunoperoxidase staining was developed using diaminobenzidine chromogen and counterstained with haematoxylin. Luxol Fast Blue was used to identify the areas of demyelination. Due to the limited size of the meningeal B cell aggregates, on MS1, MS2 and MS3 brains it was possible to perform only the CD20 staining.

Immunoglobulin variable region cloning and B cell repertoire analysis

Immunoglobulin (Ig) variable region heavy chain (VH) libraries were constructed from two non-consecutive sections of each specimen (15 × 20 mm area; 14 μm thickness). RNA was extracted from tissue sections using the RNeasy® Kit (QIAGEN) according to the manufacturer’s instructions. From the total RNA, complementary DNA was synthesized and human Ig variable region genes were amplified as previously described (Wang and Stollar, 1999), with minor modifications described by our group (Willis et al., 2009).

The VH libraries were examined for evidence of clonal expansion, somatic mutation and isotype distribution. The variable region cloning procedure captures the 5’-end of the Ig constant region, allowing the Ig isotype to be determined. VH sequences were analysed using software available from the human variable region database (IMGT®; http://www.imgt.org) (Ford et al., 1994). This analysis afforded identification of the most homologous germline segments (VH, heavy chain diversity gene segment (DH) and heavy chain joining gene segment (JH)) and allowed determination of the extent of somatic mutation relative to germline and the presence of insertions or deletions. The first 10 codons of framework 1, being primer coded, were excluded from this analysis. Allelic polymorphism was not considered in the assessment of somatic mutation, because the Ig variable gene alleles have very few such nucleotide substitutions (Cook and Tomlinson, 1995) and the IMGT® database includes various alleles for alignment (Lefranc, 2001). Chimeric molecules arising from polymerase chain reaction amplification artefacts were not included in any analysis (Ford et al., 1994).

Clonal expansion

Clones were identified through their invariably unique CDR3 sequences. Identical sequences derived from separate (non-consecutive) tissue sections defined clonal expansion, whereas identical sequences within one tissue section library were considered to be the product of polymerase chain reaction amplification rather than expanded clones, as the two cannot be reliably distinguished. Two or more sequences were considered to be derived from clonally related B cells, which we term clonal variants, if they had identical CDR3 and differed by at least two different somatic mutations in the VH region.

Variable region heavy chain gene family usage

Perturbations of VH gene family usage are indicative of antigen-driven stimulation. Accordingly, we compared the usage of the different VH gene families in meninges, plaques, normal appearing white matter and CSF of the multiple sclerosis cases with that of the B cells in the peripheral blood of three control subjects. Differences in VH gene family usage were measured using Fisher’s exact test with P < 0.005 considered significant.

Results

To examine the role of the extraparenchymal meningeal lymph-like structures in multiple sclerosis, we selected meningeal specimens containing B cell aggregates and corresponding parenchymal white matter areas isolated from distinct locations of multiple
sclerosis brain tissues (Fig. 1). In order to identify the B cell clones present in each specimen, we generated B cell receptor VH libraries from two non-consecutive sections of each multiple sclerosis specimen and of a control brain from a subject without neurological disease (Fig. 2A). By examining the repertoires derived from the meningeal aggregates and the corresponding parenchymal infiltrates, we observed similar features of antigen experience for the B cell clones populating each area (Fig. 2B). The relative clonal expansion of the B cells in the meningeal aggregates and in the parenchymal infiltrates was 24 and 28%, respectively. The mutation frequency in the VH sequences (number of nucleotide and amino acid mutations accumulated) was similar in the two areas. The isotype distribution showed that ~90% of the clones in both locations used the IgG isotype and the remainder the IgM isotype (Fig. 2B). This ratio was substantially different from that found in peripheral blood of healthy subjects where the IgG/IgM ratio is typically 15:85 (Klein et al., 1998) further highlighting that these B cells were antigen experienced.

Through analyzing the distribution of B cell clones in the meningeal aggregates and the corresponding parenchymal infiltrates, we found that the majority of B cells were exclusive to each location (Fig. 2C). However, several clones were shared between the meningeal aggregates and parenchymal infiltrates in Patients MS1 and MS2, but not in Patient MS3. When our analysis was restricted to only expanded, antigen-experienced clones, 71% and 89% of the expanded B cell clones present in the meningeal aggregates of Patients MS1 and MS2 (respectively) were present in the parenchymal infiltrates. Seven representative VH sequences

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**Figure 1** The multiple sclerosis brain contains a prominent B cell infiltrate within the meninges and parenchyma. Immunohistochemistry identified meningeal B cell aggregates (CD20 staining), with follicle-like appearance, in a subset of multiple sclerosis brain specimens (A, x 200; B, x 400). CD20 staining also identified B cell infiltration in multiple sclerosis plaques (C) and scattered B cells adjacent to vessels in normal appearing white matter (D). Grey matter from a multiple sclerosis brain contained a sparse B cell infiltrate within the meninges but not in the parenchyma of these specimens (E, meninges; F parenchyma).
Figure 2 Subsets of meningeal B cells are clonally related to those present in the parenchyma of the multiple sclerosis brain. The B cell receptor VH region was amplified from two non-consecutive sections (first and third sections) from matched meningeal (containing B cell aggregates) and parenchymal (containing infiltrates) specimens derived from three multiple sclerosis brains (MS1, 2, 3; A). No B cell receptor VH region was amplified from healthy control brain (CO) containing no detectable B cell infiltrate by immunohistochemistry (A). Sequence analysis of the B cell receptor VH region demonstrated that the B cells populating both compartments appeared antigen experienced as indicated by clonal expansion, significant somatic mutation and isotype distribution (B). Sequence comparison between meningeal and parenchymal B cell clones demonstrated that while most B cells localized exclusively to one area (unique), a small but significant proportion of B cells were present in both locations in MS1 and MS2 brains (shared; C). The distance between the meningeal and parenchymal specimens in each brain is indicated on the top of the columns. Analysis of the subset of B cells that were clonally expanded revealed that 70 and 90% of the expanded clones populated both the meningeal aggregates and parenchymal plaques of MS1 and MS2, respectively (C). Variable region sequence alignments of seven representative B cell clones shared between meningeal aggregates and parenchymal infiltrates in MS1 and MS2 are shown (D). Asterisk indicates absence of sequences. The CDR regions are indicated by vertical bars.
with their relative clonal variants shared between meningeal B cell aggregates and parenchyma in MS1 and MS2 subjects are represented in Fig. 2D.

These data then led us to investigate how B cells populate additional anatomical locations of the multiple sclerosis CNS including the meninges, plaques, normal appearing white matter and CSF. To this end, we built B cell receptor VH libraries from 20 grey matter samples containing meninges, 26 plaques, 19 normal appearing white matter sections and one CSF from 11 different multiple sclerosis brains (Supplementary Tables 1 and 2, Supplementary Fig. 1A) totalling 1833 individual sequences. The cases studied were derived from secondary and chronic progressive multiple sclerosis and a single case representing the relapsing remitting multiple sclerosis course. Consistent with the immunohistochemical analysis, the amplification of B cell receptor VH regions highlighted the presence of B cells not only in plaques, but also in all normal appearing white matter and in the grey matter specimens that showed sparse meningeal CD20+ and CD138+ cells by immunohistochemistry (Supplementary Fig. 1B, Supplementary Table 2). The B cell clones present in these different areas possessed the characteristics of antigen experience in terms of clonal expansion, number of mutations in the B cell receptor VH, and isotype distribution (Fig. 3A). Among the 1833 sequences (421 clones) analysed, only four clones (one in each of four cases MS3, MS7, MS10, MS11) carried germline sequences without mutations in the B cell receptor VH sequence. All the other clones carried nucleotide mutations in the VH sequences. Perturbations of VH gene family usage, also indicative of antigen-driven stimulation, were present not only in plaques and CSF as previously described (Owens et al., 1998, 2001; Qin et al., 1998; Baranzini et al., 1999; Smith-Jensen et al., 2000), but also in meninges and normal appearing white matter. The VH gene family usage in the peripheral blood B lymphocytes of control subjects reflected the relative distribution of the VH gene germline in the different families (Supplementary Table 3). These data also independently confirmed that the reverse transcriptase-polymerase chain reaction protocol used in this study did not artificially skew the repertoire.

While the majority of B cells were exclusive to a single region (Fig. 3B), a number of clones were shared among different anatomical areas, including a clone that populated the meninges, plaque and CSF (Fig. 3C). When our analysis was restricted to only expanded, antigen-experienced clones, the fraction of B cells that were shared among distinct locations ranged from 39 to 62% in the 11 multiple sclerosis brains examined (Fig. 3B, Supplementary Table 4). No clones were shared between different subjects, which confirmed the absence of cross contamination in the cloning procedures.

Discussion

The presence of Ig in the CNS of patients with multiple sclerosis is a hallmark of the disease. Accordingly, humoral immunity is thought to play an important role in the autoimmune response and the development of demyelinated plaques. Several recent studies imply that extraparenchymal structures, such as the meninges, also play a role in multiple sclerosis immunopathology. The presence of B cell follicle-like structures in the cerebral meninges of some patients with multiple sclerosis supports such a role. It is not clear whether these meningeal aggregates harbour antigen experienced B cells and their relationship to the B cells known to partially comprise parenchymal infiltrates is also not understood. Identifying a relationship between the immune cells that populate these distinct compartments would further define the role that the cerebral meninges may play in multiple sclerosis pathophysiology. In this study, the detailed molecular analysis of the B cell receptor variable region demonstrates that antigen experienced B cell clones are shared between the meningeal aggregates and the corresponding parenchyma. Notably, a high percentage of the expanded, antigen-experienced B cell clones present in the meningeal aggregates of two of the three brains examined were also present in the corresponding parenchymal infiltrates. Interestingly, the distance between the meningeal and parenchymal specimens in the third brain that showed no overlap was considerably higher than that in the others, possibly indicating that B cell trafficking within the CNS may be constrained by distance.

It has been recently described in the experimental autoimmune encephalomyelitis model that peripheral CCR6+ Th17 lymphocytes, critical for initiating parenchymal damage, can use meningeal structures such as the choroid plexus to enter the CNS (Reboldi et al., 2009). Because the CCR6 ligand CCL20 is constitutively expressed in the epithelial cells of choroid plexus in mice and humans, these structures are also suggested to control the immune surveillance of the human CNS. The meningeal B cell aggregates that we identified are likely to be the same structures previously identified as germinal centre-like tertiary lymph nodes (Serafini et al., 2004). Following negative selection, proliferation and maturation in these structures, expanded antigen experienced B cells may then migrate to the parenchymal sites and possibly contribute to tissue damage. Although we could not determine the direction in which B cells traffic in the multiple sclerosis brain, this scenario represents one of the possible schemes of B cell maturation and infiltration in the multiple sclerosis meninges and parenchyma.

We also defined the arrangement of antigen experienced B cells present in distinct locations of the multiple sclerosis CNS providing insight into the manner by which the B cells populate the tissue. The analysis of the B cell repertoire in different anatomical locations showed the presence of antigen experienced B cells in all areas of the CNS tissue from patients with multiple sclerosis. Interestingly, even though the normal appearing white matter showed a more restricted number of clones in comparison to the plaques and meninges, the B cells present in this area nevertheless showed features of antigen experience similar to the B cells present in plaques and meninges. These features included clonal expansion, somatic mutations in the B cell receptor VH region, isotype switching and a skewed VH gene family repertoire. The antigen experienced B cells present in normal appearing white matter might represent an early inflammatory event that occurs prior to parenchymal tissue damage. It follows that such B cells may gain antigen experience within extra-parenchymal compartments, such as the meninges or the periphery, and then subsequently populate sites in the multiple sclerosis brain.
Figure 3 Clonally related B cells populate the meninges, plaques, normal appearing white matter and CSF in the multiple sclerosis CNS. Analysis of the B cell repertoires derived from the meninges, plaques, normal appearing white matter and CSF of 11 multiple sclerosis brains demonstrated the characteristic features of antigen experience for the B cells present in all locations (A). Sequence comparison between the B cell receptor repertoires derived from different locations determined that the majority of the B cells resided exclusively in one area, however a small proportion of clones were shared among different locations (B). Only the MS3 case did not show any shared clones. Analysis of the subset of B cells that were clonally expanded revealed that 39–62% of these clones populated different locations within the multiple sclerosis CNS (B). Representative examples of sequence alignments from seven B cell clones shared among CNS compartments, including meninges, plaque, normal appearing white matter and CSF (C). Distinct B cell clones, activated in extra-parenchymal sites such as peripheral lymph nodes or meningeal lymphoid structures, might populate different anatomical locations of the multiple sclerosis brain and potentially contribute to tissue damage. Further diffusion and seeding of a subset of B cells to multiple locations of the CNS may occur through the CSF circulation (D). Asterisk indicates absence of sequences. NAWM = normal appearing white matter.
While the majority of B cell clones present in multiple sclerosis CNS tissue resided in a single exclusive location, a number of antigen-experienced B cell clones were shared among different locations. We observed shared B cell clones in multiple locations of each multiple sclerosis brain, including multiple meningeal areas, plaques, normal appearing white matter and CSF. The meningeal B cell aggregates were not dissected from the surrounding tissue. Consequently, these B cell libraries may have included sequences from those very few B cells (confirmed through immunohistochemistry) found in the grey matter. Still, these libraries accurately represented the meningeal infiltrate because the majority of the sequences were most likely derived from the B cell follicles that contain large numbers of cells. As B cell clones were shared between the CNS tissue and the CSF, these data indicate that sampling of CSF B cells can provide insight into parenchymal B cell populations that are often proximal to tissue damage.

Our data complement the work of Junker et al. (2007) who demonstrated the presence of a pervasive T cell response in distinct regions of multiple sclerosis brain comprised of ‘private’ T cell clones unique for each brain region and ‘public’ T cell clones shared in multiple sites of the brain. These data collectively indicate that both CNS B and T cells may be targeting a CNS antigen or antigens. Furthermore, our data define a relationship between meningeal structures and multiple sclerosis parenchymal tissue. We propose that the B cell distribution in the multiple sclerosis brain may be due to a hierarchy of the B cell clones involved in the disease, some of them dominant and possibly involved in the lesion formation in multiple sites, and others exclusively involved in a single site of inflammation. One possible scenario concerning B and T cell migration is that at the initial phases of the disease, distinct B and T clones from extra-parenchymal sites such as peripheral lymph nodes or meningeal lymphoid structures populate different anatomical locations of the multiple sclerosis brain. A subpopulation of these immune cells may subsequently diffuse to multiple locations of CNS through CSF circulation and contribute to the tissue damage (Fig. 3D). In conclusion, following the recent evidence that supports a primary role for the meningeal structures in T cell infiltration and B cell proliferation and maturation, this study provides new evidence for a direct correlation of extraparenchymal sites to the clonal B cell population that infiltrates multiple sclerosis CNS tissue.

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Supplementary material

Supplementary material is available at Brain online.

References


