Detection of Ectopic B-cell Follicles with Germinal Centers in the Meninges of Patients with Secondary Progressive Multiple Sclerosis

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Multiple sclerosis (MS) is characterized by synthesis of oligoclonal immunoglobulins and the presence of B-cell clonal expansions in the central nervous system (CNS). Because ectopic lymphoid tissue generated at sites of chronic inflammation is thought to be important in sustaining immunopathological processes, we have investigated whether structures resembling lymphoid follicles could be identified in the CNS of MS patients. Sections from post-mortem MS brains and spinal cords were screened using immunohistochemistry for the presence of CD20$^+$ B-cells, CD3$^+$ T-cells, CD138$^+$ plasma cells and CD21$^+$, CD35$^+$ follicular dendritic cells, and for the expression of lymphoid chemokines (CXCL13, CCL21) and peripheral node addressin (PNAd). Lymphoid follicle-like structures containing B-cells, T-cells and plasma cells, and a network of follicular dendritic cells producing CXCL13 were observed in the cerebral meninges of 2 out of 3 patients with secondary progressive MS, but not in relapsing remitting and primary progressive MS. We also show that proliferating B-cells are present in intrameningeal follicles, a finding which is suggestive of germinal center formation. No follicle-like structures were detected in parenchymal lesions. The formation of ectopic lymphoid follicles in the meninges of patients with MS could represent a critical step in maintaining humoral autoimmunity and in disease exacerbation.

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INTRODUCTION

Multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (CNS), is characterized by immune cell infiltration in the white matter, resulting in myelin destruction, axonal damage and neuronal loss, the main cause of permanent neurological deficits. Current concepts on the pathogenesis of MS indicate that a T-cell response directed against viral or CNS antigens is responsible for the inflammatory component and that lesion development requires additional immune mechanisms, which may involve demyelinating antibodies (17, 61). B-cell activation is a consistent immune abnormality in MS, in which oligoclonal IgG of unknown specificity are produced intrathecally (53) and plasma cells accumulate in lesions and CSF (3, 12, 35, 40). The involvement of antibody-mediated effector mechanisms in demyelination is supported by capping of surface IgG on microglia/macrophages engaged in myelin breakdown (38) and by codeposition of IgG and activated complement fragments and complexes at the borders of actively demyelinating lesions (38, 48). Moreover, antibodies specific for a number of oligodendroglial/myelin antigens have been detected in the CSF and peripheral blood of MS patients (31, 57, 62) and at sites of myelin breakdown (14, 43).

During the past few years, there have been considerable advances in defining the characteristics of the humoral immune response developing in the CNS of MS patients. Analyses of the V gene repertoire of B-cells isolated from MS lesions and CSF showed a high frequency of clonally expanded memory B-cells and of extensive somatic mutations, a pattern which is indicative of antigen-driven B-cell activation and not of a random bystander response (5, 8, 33, 34, 41, 42). The demonstration of unique oligoclonal and highly mutated Ig heavy chain sequences confined to the MS brain is strongly suggestive of an antigen-driven and CNS-targeted humoral immune response (8, 33). Whether peripherally activated B-cells are selectively recruited to the MS brain where they differentiate into antibody producing plasma cells, or expand and mature locally mimicking a germinal center reaction, remains to be determined.

The germinal center of lymphoid follicles is the microenvironment where antigen-activated B-cells undergo clonal expansion and selection to differentiate into memory B-cells or into plasma cells secreting high affinity antibodies (23). These events require interactions of B-cells with T-cells and follicular dendritic cells (FDCs). The latter cells have a critical role in presenting intact antigen to B-cells and in providing B-cell survival and proliferation signals (21, 52). Moreover, FDCs produce the lymphoid chemokine CXCL13 which regulates the migration of B-cells and a subset of T helper cells expressing CXCR5, the CXCL13 receptor, thus playing a key role in organizing cells within follicles and in their development (10).

Tissues affected by autoimmune diseases, such as rheumatoid arthritis, Sjogren syndrome, Crohn disease and Hashimoto thyroiditis, often contain ectopic lymphoid aggregates comprising interfollicular T-cell zones and B-cell follicles with germinal centers, which are thought to be important for the production of autoreactive antibodies and the maintenance of the autoimmune response (for review, see 18, 60). Despite extensive neuropathological investigations, to date there is no indication that ectopic follicles form in the autoimmune CNS and might contribute to intrathecal B-cell activation. In a morphological study performed in the late 70s, Prineas described the presence of immune cell infiltrates reminiscent of lymphoid structures in chronic MS lesions (37). Thin-walled channels resembling lymphatic capillaries and containing lymphocytes, macrophages and plasma cells interacting with reticular cells

Patient	Age/ sex	MS type	Disease duration (yrs)	Post mortem delay (hours)	Cause of death	Number of tissue blocks	Number of lesions	Lesional stage		Presence of meningeal infiltrate
								Ch. Active	Ch. Inactive	
MS 79	49/F	SP	21	7	Bronchopneumonia	4	8	2	6	Yes
MS 80	71/F	SP	34	24	Post-operative complications	3	2		2	No
MS 85	59/F	SP	35	34	Cerebrovascular disease	4	5		5	Yes
MS 83	54/M	PP	16	13	Bronchopneumonia	4	6	3	3	Yes
MSG1	43/F	PP	10	24	Cerebrovascular disease	9	15	11	4	Yes
MSG2	28/F	RR	10	26	D.I.C.	2	8	5	3	No
C16	92/M			13	Cardiac failure	1				

Table 1. Clinical and autopsy details. SP = secondary progressive; PP = primary progressive; RR = relapsing remitting; F = female; M= male; D.I.C.= disseminated intravascular coagulopathy.

(presumably FDCs or stromal cells) were localized in the perivascular space of parenchymal blood vessels. Since no proliferating lymphoblasts were observed in chronic MS lesions (40), it is likely that the perivascular compartment represents a niche for B-cell differentiation and plasma cell survival, but not for germinal center formation.

Recently, we have shown that follicles containing B-cells and FDCs expressing CXCL13 form in the inflamed meninges of mice with relapsing EAE (26). The identification of lymphoid structures in the less immunoprivileged meningeal compartment prompted us to investigate their existence in post-mortem brain and spinal cord tissue from MS patients with different disease courses. The immunohistochemical data presented in this study demonstrate, for the first time, the presence of follicles comprising proliferating B-cells and a network of FDCs in the meninges of patients with secondary progressive (SP) MS and suggest their possible involvement in sustaining intrathecal B-cell responses during disease exacerbation.

MATERIALS AND METHODS

Patients and tissues. This study was performed on post-mortem CNS tissue from three patients with SP MS, two patients with primary progressive (PP) MS, one patient with relapsing remitting (RR) MS and one control subject who died for cardiac failure without evidence of neurological disease or neuropathological alterations. Tissues were provided by the UK Multiple Sclerosis Tissue Bank, the Institute of Pathological Anatomy, Policlinico A. Gemelli, Rome, and the Department of Neurosciences, Opthamology and geriatrics, University of Genova, Genova, Italy. The present study was approved by the Ethics Committee of the Istituto Superiore di Sanità.

On the basis of the available clinical documentation, all patients with PP and SP MS were in the progressive phase of the disease. Patients affected by PP MS had severe disabilities and all patients with SP MS were bedbound at the time of death. No treatment is reported for these patients in the six months before death and no Expanded Disability Status Scale assessments are available. The patient with RR MS was treated with steroid pulses at the time of death. The clinical and autopsy data are summarized in Table 1. No data are available about IgG levels in the CSF of the patients examined, with the exception of one patient with PP MS (MS83), in which markedly elevated IgG and IgG to albumin index were detected in the CSF.

Twenty brain (4 cm^3 each) and 6 spinal cord (one cm^3 each) tissue blocks from MS patients and one block of cerebral tissue from a control patient were studied. A total of 44 lesions (27 subcortical, 8 periventricular, and 9 in the spinal cord) and 3 areas of normal appearing white matter were analysed. Normal appearing white matter was defined as an area, far away from the lesion, which showed no sign of demyelination by histology. With one exception, all the blocks examined in this study showed at least one lesion in the white matter. Positive control tissues (axillary and tracheoesophageal lymph nodes) were autoptic samples from a female patient who died from heart failure.

Classification of lesions and neuropathological findings. The lesional activity and the extent of the inflammatory infiltrates in MS brain tissues were quantified by histopathological evaluation, using hematoxylin-eosin staining and the combined Luxol fast blue-periodic acid-Schiff (LFB-PAS) reaction to evaluate areas of myelin breakdown and the presence of PAS-positive material in phagocytic cells, and by immunohistochemical detection of infiltrating immune cells (T- and B-cells, plasma cells, macrophages), MHC class II molecule expression and astrocyte reactivity. Lesions were classified using the staging system described by De Groot et al (11). To evaluate the extent of demyelination, LFB/PAS-stained sections from each tissue block were acquired using a laser scanning microscope (LSM 5 Pascal, Carl Zeiss, Jena, Germany) and the morphometric analysis of the areas of demyelination with respect to the total area of white matter was performed using KS300 Imaging System.

In tissue blocks from the 3 patients with SP MS, 12 chronic lesions were identified in the brain hemispheres (3 periventricular and 9 subcortical) and 3 in the spinal cords by LFB staining. Among these, 13 lesions were classified as chronic inactive, as they were hypocellular, had well demarcated borders, and comprised rare $CD68^+$, PAS^+ macrophages and perivascular lymphocytic infiltrates of variable size, essentially at the lesion borders. Two partially demyelinated subcortical plaques with hypercellular edges, centrally located inflamed blood vessels and signs of active demyelination, were classified as chronic active. These lesions contained numerous PAS^+ and/or LFB^+ macrophages and MHC class II-expressing cells. Spinal cord sections from patients MS79 and MS85 showed massive and partial demyelination, respectively, with reactive gliosis, modest perivascular cuffing and scattered intraparenchymal lymphocytes.

Antigen	Cell specificity	Clone	Dilution	Source
CD68	Macrophages/ microglia	KP1	1:50	Dako, Carpinteria, Calif
CD3	T cells	PS1	Pre-diluted	Immunotech, Marseille, France
CD20	B cells	L26	Pre-diluted	Immunotech
CD138 (Syndecan-1)	Plasma cells	B-B4	1:100	Serotec, Oxford, United Kingdom
CD21 (C3d receptor, CR2)	FDC	1F8	1:25	Dako
CD35 (C3b/C4b receptor, CR1)	FDC	Ber/MAC/DRC	1:25	Dako
CXCL13 (B cell-attracting chemokine 1)	FDC		1:15	R&D Systems, Minneapolis, Minn
CCL21 (secondary lymphoid tissue chemokine)	HEV, stromal cells, dendritic cells		1:30	R&D Systems
PNAd carbohydrate epitope (CD62L ligand)	HEV	MECA79	1:10	BD Biosciences Pharmingen, San Diego, Calif
Ki67 nuclear antigen	Proliferating cells	MIB 1	1:20	Immunotech
Ki67 nuclear antigen	Proliferating cells		1:400	Novocastra Labs, Newcastle, United Kingdom

Table 2. Primary antibodies used for immunohistochemistry. Antigen retrieval procedures utilized microwave of sections in citrate buffer (0.01M, pH 6.0), except for CD21 and CD35 stainings, in which heat treatment with Dako target retrieval solution was performed.

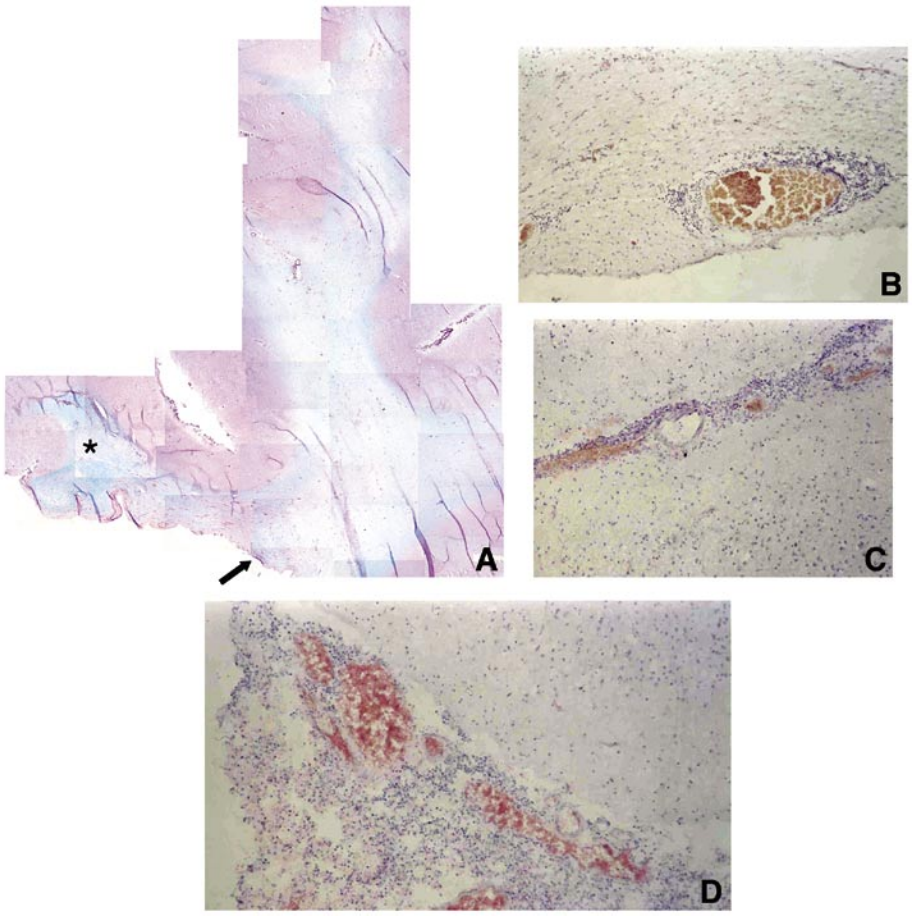

Figure 1. Localization of demyelinated lesions and inflammatory cell infiltrates in a patient with SP MS (MS79). Cryostat sections from occipital (**A-C**) and parietal (**D**) lobes are shown. Panel **A** is a montage of overlapping images acquired from a LFB/PAS-stained section showing a large inactive demyelinated lesion extending from the subependymal region (arrow) to the subcortical white matter, and a subcortical chronic active lesion (asterisk). Hematoxylin-eosin stainings in panels **B-D** show perivascular leukocyte infiltrates in the demyelinated parenchyma close to the ependymal layer (**B**) and in the meninges, both in the depth of a cerebral sulcus (**C**) and in the subarachnoid space (**D**). Original magnifications: **A** = 100×; **B-D** = 250×.

Of 21 lesions examined in tissue blocks from two patients with PP MS (3 periventricular, 12 subcortical, 2 in the medulla oblongata and 4 in the spinal cord), 7 were classified as chronic inactive and 14 were actively demyelinating, with a massive presence of PAS^+ foamy macrophages, strong microglial activation, elevated MHC class II expression and moderate lymphocytic infiltration. The periplaque tissues often showed a reduction in myelin density. The only patient with RR MS examined in this study showed one subcortical and 2 large periventricular inactive lesions, and 5 subcortical chronic active lesions.

Immunohistochemistry. Most CNS tissues used in this study were fixed in 4% paraformaldehyde for several days, cryoprotected in sucrose, frozen by immersion in isopentane precooled on a bed of dry ice and stored a -80°C until use. CNS tissue from the patient with RR MS and from one of the 2 patients with PP MS (MSG1) were fixed in buffered formalin and embedded in paraffin wax. Both paraffin embedded and fixed frozen control tissues were used.

Five-µm-thick paraffin and 10-µm-thick cryostat sections were cut from the tissue blocks, mounted on Super Frost plus slides and stored at room temperature and at -20°C, respectively, until use. For immunohistochemical stainings, deparaffinized or air dried, acetone fixed cryostat sections, were rehydrated with PBS, subjected to the most appropriate antigen unmasking procedure (see Table 2), and incubated for 20 minutes with 0.3% H_2O_2 in PBS to eliminate endogenous peroxidase activity. Sections were then pre-incubated with 10% normal sera and immunostained with the polyclonal and monoclonal antibodies (Abs) listed in Table 2. Except for anti-CXCL13 and anti-CCL21 polyclonal Abs which were diluted in PBS, all the primary Abs used in this study were diluted in PBS + 1% BSA and incubated overnight at 4°C. After extensive washing with PBS, sections were incubated with the corresponding biotinylated secondary Abs (rabbit anti-mouse IgG and donkey anti-goat IgG, from Jackson Immunoresearch

Laboratories) and avidin-biotin horseradish peroxidase complex (ABC), using the ABC Vectastain Elite kit (Vector Laboratories, Burlingame, Calif), according to the manufacturer's instructions. Staining reactions were performed with 3,3 diaminobenzidine (DAB, Sigma) as substrate. Negative controls included the use of IgG isotype controls or omission of the primary Ab. Sections were counterstained with hematoxylin and viewed under an Axiophot microscope (Carl Zeiss). Some images were acquired using a digital camera (JVC TK-C1381 color video camera).

Confocal microscopy. Cryosections of MS brain and control lymph node were stained using double indirect immunofluorescence techniques and images were analysed and acquired using a laser scanning confocal microscope (LSM 5 Pascal, Carl Zeiss). Briefly, sections were air dried and post-fixed as described above. After an initial blockade with 10% normal goat serum in PBS, sections were incubated overnight at 4°C with anti-Ki67 polyclonal Ab and anti-CD20 or anti-CD3 mAb. The bindings were visualized using a mixture of rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG (Jackson Immunoresearch) diluted in PBS. The incubation with the secondary Abs was carried out for one hour at room temperature. Negative controls were performed by replacing primary antibodies with preimmune serum and IgG isotype control.

RESULTS

Distribution of B-cells and plasma cells in the lesions and inflamed meninges of MS patients. As the main aim of this study was to investigate whether ectopic B-cell follicles form in the CNS of MS patients, we first screened brain and spinal cord sections from patients with different MS disease courses (see Table 1) for the presence of B-cells and plasma cells using immunohistochemical techniques.

Abundant B-cell infiltration was detected only in the CNS of 2 patients with SP MS. Patients MS79 and MS85 had large, confluent demyelinated lesions in the periventricular white matter and smaller subcortical lesions (Figure 1A shows Luxol-PAS staining of a cerebral section from patient MS79). Mean percentage of demyelination in cerebral and spinal cord white matter was 51.5 and 58.0 in patient MS79, and 33.8 and 10 in patient MS 85, respectively. With the exception of 2 chronic active subcortical lesions identified in patient MS79, all lesions examined were of the chronic inactive type (n = 11). Perivascular immune infiltrates were present in all lesions, the largest ones being detected in the subependymal region of patient MS79 (Figure 1B). In these 2 patients, abundant inflammatory infiltrates were also common around blood vessels of the cerebral leptomeninges (Figure 1C, D). Numerous B-cells accumulated perivascularly in chronic inactive lesions and in the subarachnoid space (Figure 2A, B), whereas no B-cells were found in chronic active lesions or inside the demyelinated parenchyma. Within the leptomeninges, B-cells often formed large, compact aggregates (Figure 3C). All perivascular cuffs in chronic active and inactive lesions and in the meninges contained $CD3^+$ T-cells and fewer $CD68^+$/MHC class II^+ macrophages. Immunostainings performed on consecutive sections showed that $CD3^+$ T-cells were a regular component of intrameningeal B-cell aggregates (Figure 2D).

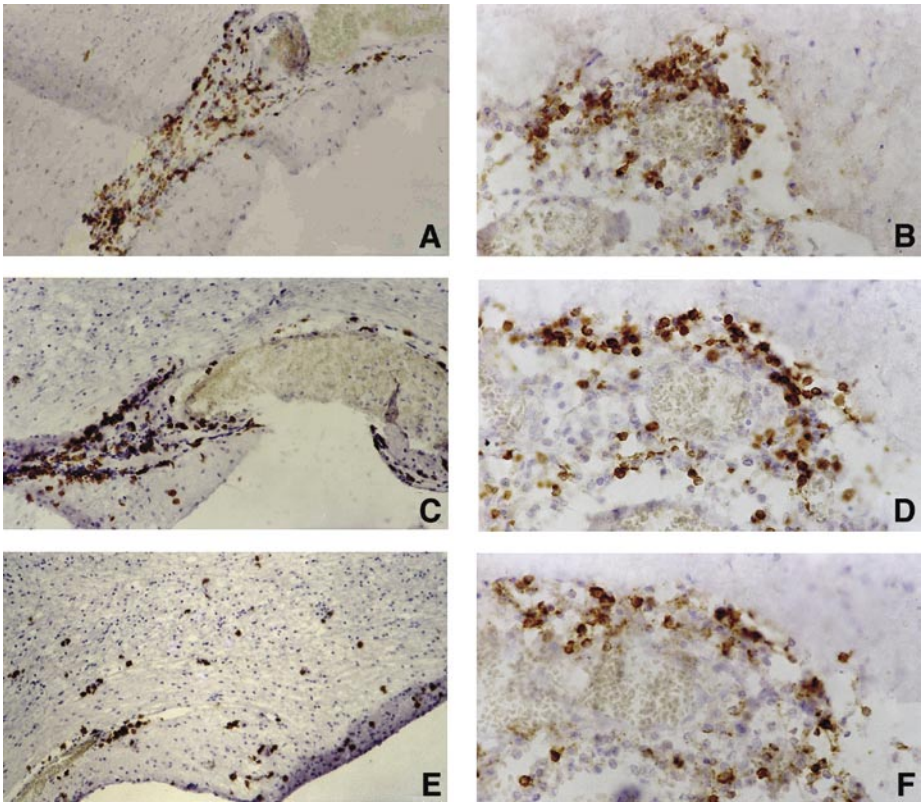

Figure 2. Detection of B-cells and plasma cells in the parenchymal and meningeal inflammatory cell infiltrates in patient MS79. Serial sections from the occipital lobe (panels **A, C, E**) and superior frontal gyrus (panels **B, D, F**) are shown. Numerous $CD20^+$ B-cells (**A**) and $CD138^+$ plasma cells (**C**) accumulate in a perivascular location in the chronic inactive periventricular lesion. Plasma cells also infiltrate the demyelinated parenchyma (**E**). Inflammatory cell infiltrates containing $CD20^+$ B-cells (**B**), $CD3^+$ T lymphocytes (**D**) and $CD138^+$ plasma cells (**F**) are detected around large venules in the subarachnoid space. Original magnifications: **A** and **C** = 500×; **E** = 250×; **B, D, F** = 1000×.

In the brain of patient MS79, but not MS85, numerous $CD138^+$ plasma cells were present around most parenchymal and meningeal blood vessels (Figure 2C and F, respectively), as well as scattered inside the parenchyma of chronic inactive lesions (Figure 2E). Stainings performed in serial sections showed that plasma cells were always found in association with intrameningeal B-cell clusters, in which they had a more peripheral distribution compared to B-cells (Figures 2F, 3H). In the spinal cord of patients MS79 and MS85, only a few $CD20^+$ cells and no plasma cells were present around parenchymal blood vessels, but none was detected in the meninges. Conversely, numerous $CD3^+$ T lymphocytes accumulated perivascularly and infiltrated the spinal cord white matter as well as the leptomeninges (data not shown).

Compared to patients MS79 and MS85 described above, the third patient with SP MS examined in this study (MS 80)

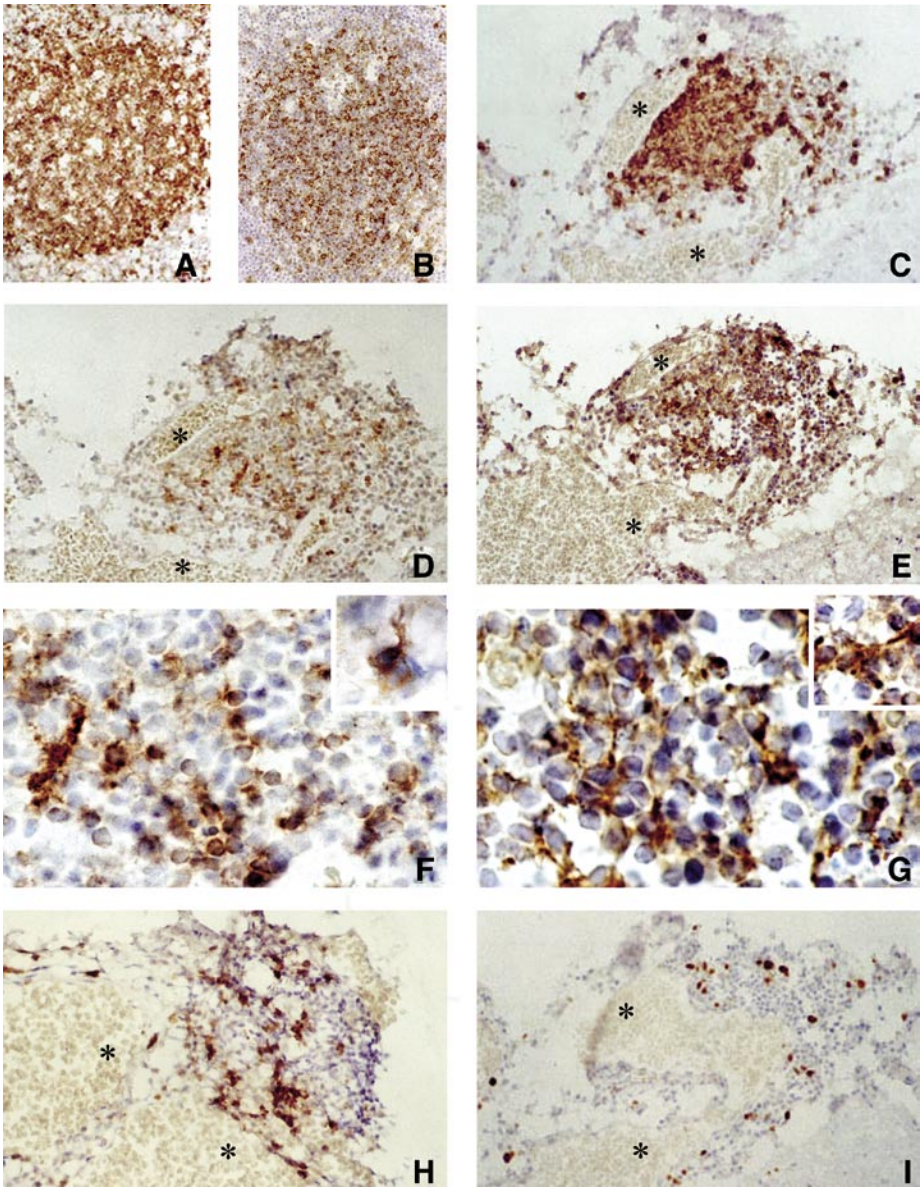

Figure 3. Detection of B-cell follicles containing a network of CXCL13-expressing FDCs and Ki67$^+$ cells in the inflamed meninges of patient MS79. Immunostainings for CD20 (**A**) and CD35 (**B**) performed in cryosections of control human lymph node show a B-cell follicle containing a network of CD35$^+$ FDCs. Stainings performed on serial sections from the occipital lobe (**C-E**) show a follicle-like structure in the subarachnoid space which is located between 2 blood vessels (asterisks) and comprises a large aggregate of CD20$^+$ cells (**C**) and a reticulum of FDCs expressing CD35 (**D**) and the lymphoid chemokine CXCL13 (**E**). Panels **F** and **G** are high power magnifications of fields selected from **D** and **E**, respectively. Note that CD35$^+$ (**F**) and CXCL13$^+$ (**G**) cells display a ramified morphology, with oval nuclei and cytoplasmic processes intermingled with the surrounding lymphocytes (insets in **F** and **G**). CD138$^+$ plasma cells distribute predominantly at the periphery of the B-cell follicle (**H**). Immunostaining for the proliferation antigen Ki67 (**I**) shows the presence of several proliferating cells in the same intrameningeal follicle shown in **C-E**. Note that some proliferating cells appear in small clusters. Original magnifications: **A-E, H, I** = 500×; **F** = 1000×; **G** and insets = 1575×.

displayed more limited demyelination (mean percentage of demyelination in cerebral white matter was 11.9) and modest perivascular cuffing in the periventricular white matter (data not shown). Only rare, isolated B-cells were found associated with some inflamed blood vessels in chronic inactive lesions. A scarce degree of immune cell infiltration, consisting of scattered CD3$^+$ T-cells and CD68$^+$ macrophages, but no CD20$^+$ B-cells or plasma cells, was observed in the cerebral leptomeninges.

Neither B-cells nor plasma cells were detected in brain and spinal cord sections from 2 patients (MS83 and MSG1) with PP MS, in which demyelinated lesions with prominent macrophage/microglia activation were observed. Mean percentage of demyelination in cerebral and spinal cord white matter was 27.2 and 61.5 in patient MS83, and 17.2 and 32.8 in patient MSG1, respectively. As previously described (45), lesions of patients with PP MS contained fewer and smaller perivascular infiltrates as compared to lesions of patients with SP MS, which were composed mainly of CD3$^+$ T-cells. T-cells were also found scattered throughout the damaged white matter and in the inflamed meninges. However, no perivascular cuffing was observed around leptomeningeal venules.

In the patient with RR MS, perivascular infiltrates comprising numerous CD3$^+$ T-cells and fewer CD68$^+$ macrophages, but neither B-cells nor plasma cells, were detected in both chronic active and inactive lesions (mean percentage of demyelination in cerebral white matter was 18.1). The meninges, which were well preserved only in one of the 2 brain tissue blocks examined, contained scattered T-cells, but no B-cells or plasma cells.

The above observations confirm previous findings that extensive B-cell and plasma cell accumulation occurs in the brain of patients with SP MS (39, 40), and show that large B-cell aggregates comprising T-cells and plasma cells can form in the inflamed cerebral meninges. Because these aggregates are reminiscent of ectopic lymphoid follicles described in the thyroid, joint synovial tissue and salivary glands during autoimmune diseases (4, 49, 51, 59), we explored further their cellular and molecular organization by using Abs specific for FDCs and for chemokines and adhesion molecules that are important in cell trafficking to secondary lymphoid tissues.

Detection of FDCs within intrameningeal B-cell aggregates in SP MS. FDCs are an essential component of B-cell follicles in secondary lymphoid tissues and have a key role in presenting intact antigen and providing survival and differentiation signals to B-cells (21, 56). To assess whether FDCs accumulate in the MS brain, immunohistochemical stainings were performed using two mAbs recognizing the FDC markers CD35 (CR1) and CD21 (CR2). For comparison, panels A and B of Figure 3 show the network of CD35$^+$ FDCs in a B-cell follicle of control human lymph node. Immunostainings performed in serial cerebral sections from patients MS79 (Fig-

ure 3C, D, F) and MS85 (Figure 4A, B) revealed that CD35$^+$ cells with slender dendritic protrusions and oval nuclei typical of FDCs were present within the intrameningeal B-cell aggregates. In some of the B-cell clusters identified in patient MS79, CD35$^+$ cells formed a complex reticular network in intimate contact with the surrounding lymphocytes (Figure 3F), which closely resembles that observed in lymph nodes (Figure 3B). In patient MS79, CD35$^+$ FDCs were found in the meninges of all three cerebral blocks examined (from the frontal, parietal and occipital lobes), whereas in patient MS85 FDCs were present in only one of 2 cortical blocks. Interestingly, despite the presence of numerous perivascular B-cells, no CD35$^+$ were detected around inflamed parenchymal blood vessels.

In both patients, the localization and morphology of cells stained with anti-CD21 mAb was similar to that observed with anti-CD35 mAb, although reticular networks of CD21$^+$ cells were not detected (Figure 4D). Occasionally, isolated CD21$^+$ cells with a round, lymphocytic morphology were identified in the perivascular cuffs of meningeal and parenchymal vessels (data not shown). This is consistent with the reported finding that, in addition to FDCs, CD21 is expressed on subsets of B-cells (7). Neither CD21$^+$ nor CD35$^+$ cells were detected in areas of the cerebral meninges that did not contain B-cell aggregates or in the spinal cord meninges (data not shown).

No CD21 or CD35 immunopositive cells were detected in the parenchyma and meninges of patient MS80 with SP MS and of patients with PP and RR MS, all of which had no or minimal B-cell infiltration in the CNS.

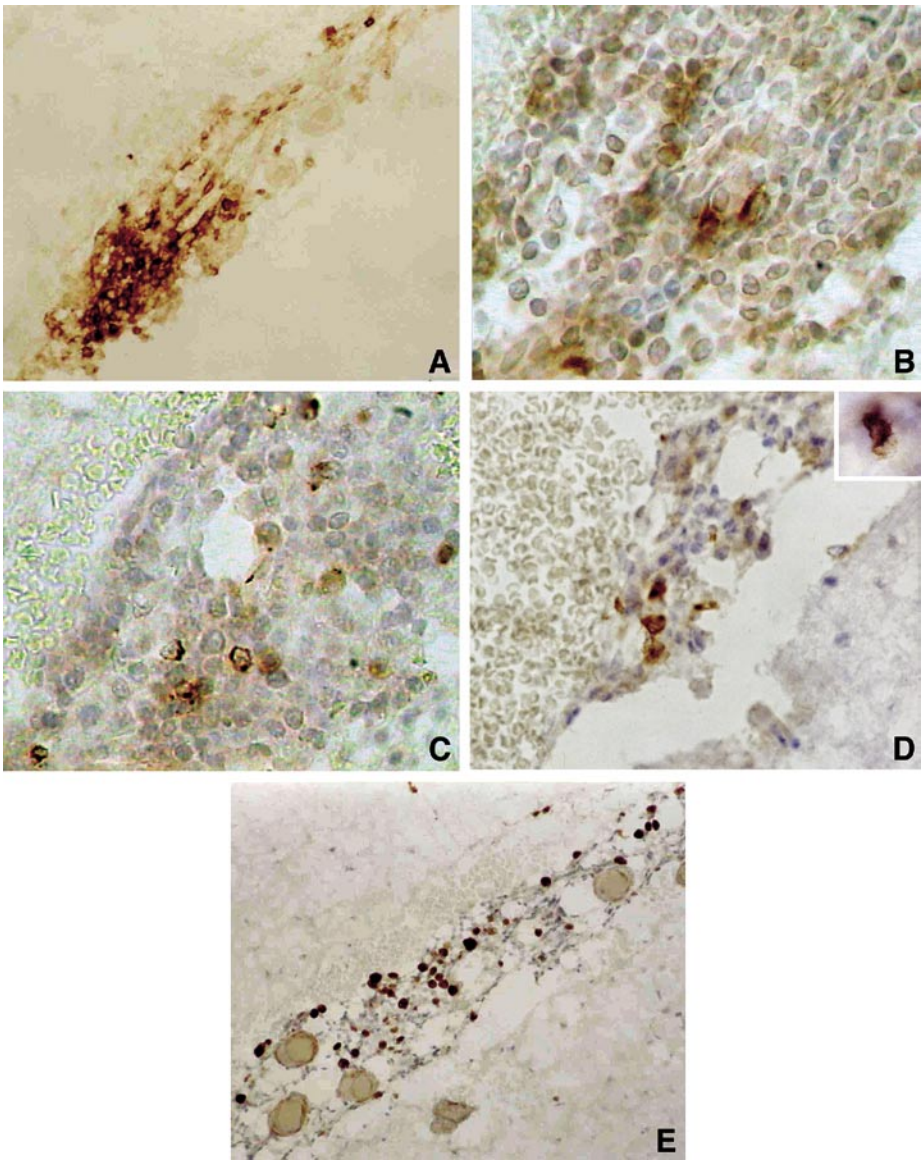

Figure 4. Detection of B-cell follicles containing CXCL13-expressing FDCs and Ki67$^+$ cells in the inflamed meninges of patient MS85 with SP MS. A follicle-like structure containing numerous CD20+ B-cells (**A**) and some CD35$^+$ (**B**) and CXCL13$^+$ (**C**) FDCs is present in the meninges in the depth of a cerebral sulcus (panels **A-C** show serial sections). FDCs appear as scattered cells and do not form an organized network comparable to that observed in patient MS79 (see Figure 3). Panel **D** shows CD21+ cells, some with a process-bearing morphology (inset in **D**), within an inflammatory infiltrate close to a meningeal vessel. Immunostaining for Ki67 antigen (**E**) shows an intense proliferative activity within the same B-cell cluster shown in **A-C**. The section shown in panel **A** is not counterstained, whereas those in panels **B-E** are counterstained with hematoxylin. Original magnifications: **A, D, E** = 500×; **B, C** = 1000×; inset in **D** = 1575×.

Expression of lymphoid homing chemokines and adhesion molecules in the MS brain. The expression of lymphoid homing chemokines, which are known to be involved in the formation and maintenance of follicles (1) and have been detected in ectopic lymphoid tissue (4, 47, 51, 58), was also examined in the CNS of MS patients. Cerebral and spinal cord sections were stained with a polyclonal Ab specific for CXCL13, also called B-lymphocyte chemoattractant. CXCL13 is constitutively produced by some FDCs in secondary lymphoid tissues and acts on naive B-cells and subsets of CD4$^+$ T-cells (called follicular T helper cells) expressing CXCR5, allowing their migration into B-cell follicles (2, 10).

Stainings performed in serial brain sections from patients MS79 and MS85 revealed that, similarly to CD21 and CD35, CXCL13 immunoreactivity was confined to dendritiform cells inside intrameningeal B-cell follicles (Figure 3E and Figure 4C, respectively), whereas no CXCL13$^+$ cells were ever detected around inflamed parenchymal blood vessels. In some B-cell clusters, particularly in those detected in patient MS79, CXCL13$^+$ cells formed a reticular network comparable to that of intrameningeal CD35$^+$ FDCs (Figure 3G), suggesting that most FDCs identified in these structures are functionally similar to those present in lymphoid follicles. No CXCL13$^+$ cells were identified in any of the tissue blocks from the other MS patients examined.

CNS tissue sections from all MS patients were also stained for CCL21, also known as 6Ckine, secondary lymphoid tissue

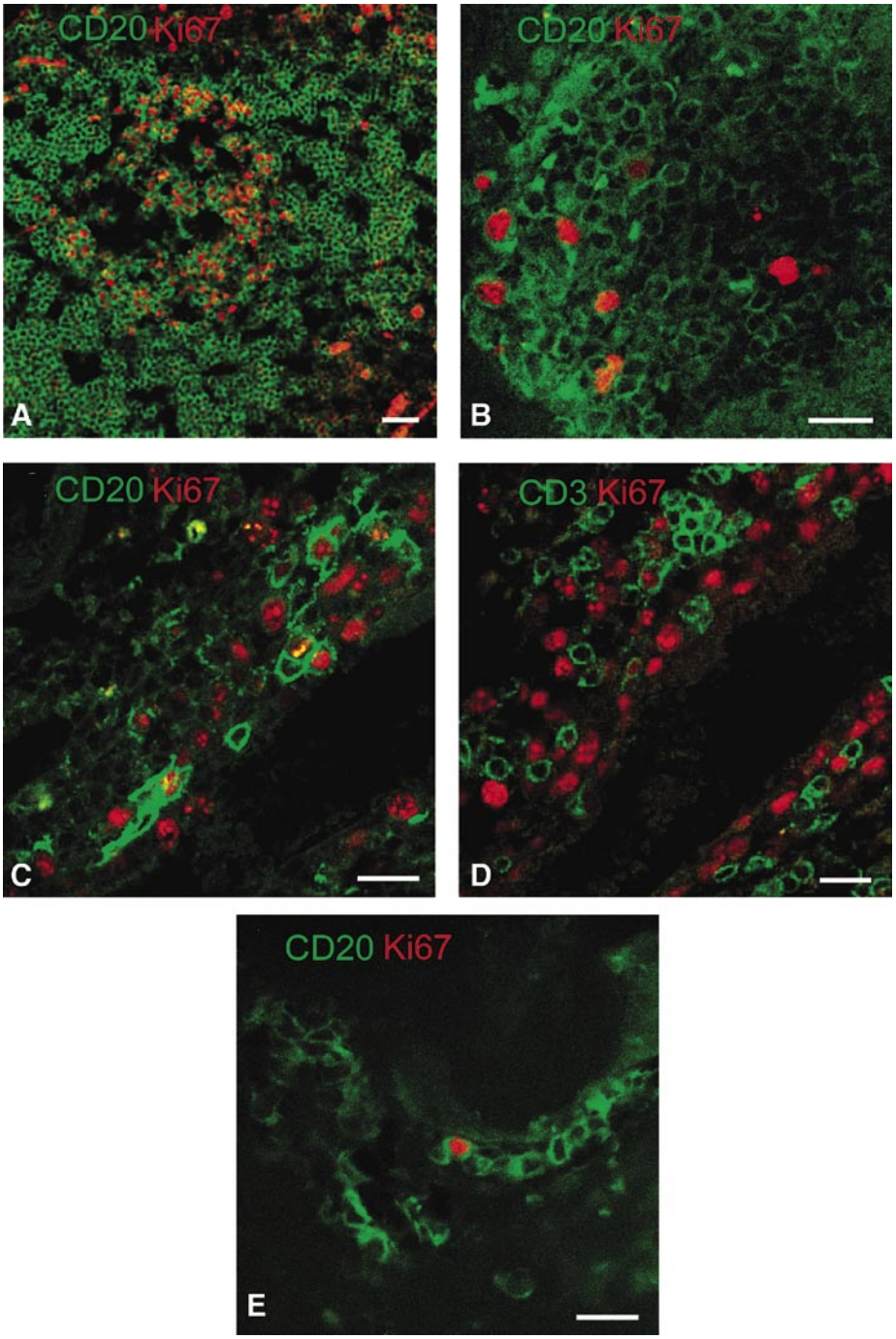

Figure 5. Presence of proliferating B-cells in intrameningeal follicles of patients with SP MS. Confocal micrographs of cryosections from control lymph node (**A**) and brain tissue from patients MS79 (**B, E**) and MS85 (**C, D**), double stained with anti-CD20 (green in **A-C** and **E**) or anti-CD3 mAb (green in **D**) and anti-Ki67 polyclonal Ab (red in **A-E**), are shown. Double staining for CD20 and Ki67 in a lymph node section shows that proliferating B-cells accumulate in the germinal center of a secondary follicle (**A**). In patients MS79 (**B**) and MS85 (**C**), the majority of the proliferating cells in the intrameningeal follicles are $CD20^+$ B-cells. Note that in patient MS79 (**B**), $Ki67^+$ nuclei form an eccentric cluster within the follicle, which is reminiscent of a small germinal center. This micrograph is from a section adjacent to those shown in Figure 3C-I. The majority of $CD3^+$ T-cells infiltrating the meninges of patient MS85 do not stain for Ki67 (**D**). Only one $Ki67^+$ proliferating B-cell is detected among numerous $CD20^+$ B-cells accumulating around a parenchymal blood vessel located in a chronic inactive periventricular lesion from patient MS79 (**E**). Bars = 40 μm in **A**; 20 μm in **B-E**.

chemokine and Exodus-2, a key molecule for the migration of naive T- and B-cells into interfollicular T-cell zones and for the development of lymphoid tissue compartments (9). CCL21 was previously reported to be expressed in lymphatic vessels and high endothelial venules (HEV), and in stromal and dendritic cells present in T-cell zones of mouse and human lymph nodes (9, 29). CCL21 expression was also described in ectopic lymphoid tissue at sites of chronic inflammation (4, 58, 59). Although anti-CCL21 polyclonal Ab selectively stained numerous process-bearing cells (bona fide stromal and dendritic cells) in T-cell zones of normal human lymph nodes, no $CCL21^+$ cells were detected in brain and spinal cord sections from any of the MS patients examined in this study. Vascular endothelial cells were negative for CCL21, both in control lymph nodes and MS brains (data not shown).

To investigate further the cerebral microenvironment that could favour the formation of ectopic lymphoid tissue, we analysed the expression of the adhesion molecule peripheral node addressin (PNAd) using the mAb MECA 79. This mAb recognizes a carbohydrate epitope that is specifically found on glycoproteins expressed on the surface of HEV (50). PNAd binds L-selectin which is highly expressed on naive T and B lymphocytes and allows their selective homing to secondary lymphoid organs (50). Although anti-PNAd mAb strongly stained HEV in fixed-frozen lymph node sections, no PNAd-positive endothelia were detected in the parenchyma and meninges of any of the MS patients examined (data not shown).

To summarize, while the identification of CXCL13-producing FDCs in the intrameningeal follicles of some patients with SP MS suggests that CXCL13 is involved in attracting B-cells and possibly T helper cells into such structures, the lack of CCL21 and PNAd immunopositivity in the meninges indicates that interfollicular T-cell zones with HEV do not develop at these sites. Thus, different sets of chemokines and adhesion molecules are likely to control the extravasation of B-cells, presumably memory B-cells, through meningeal vessels, allowing the subsequent organization of ectopic lymphoid follicles.

Presence of proliferating B lymphoblasts within intrameningeal follicles is indicative of germinal center formation. We next evaluated the possibility that ectopic lymphoid follicles forming in the meninges of MS patients contain germinal centers. The germinal centers are the specialized sites of the secondary lymphoid follicles where antigen-stimulated B-cells undergo clonal ex-

pansion and selection before differentiation into memory B-cells or into plasma cells (23). By performing immunostainings with anti-Ki67 mAb, as proliferation marker, and anti-CD20 mAb in serial cerebral sections, we investigated whether lymphocytic proliferation occurs in the intrameningeal follicles. Groups of $Ki67^+$ nuclei were detected inside the B-cell follicles of patients MS79 (Figure 3I) and MS85 (Figure 4E). Numerous proliferating cells were present even in those follicles that apparently did not contain a well developed FDC network. Scattered proliferating cells were also present throughout the meninges of these patients, whereas no or only rare $Ki67^+$ nuclei were observed in the perivascular compartment inside demyelinated lesions (data not shown).

We next used double immunofluorescence techniques with anti-Ki67 polyclonal Ab and anti-CD20 or anti-CD3 mAb and confocal microscopy to evaluate the identity of the proliferating cells in the intrameningeal follicles identified in patients MS79 and MS85. We found that most proliferating cells in the intrameningeal follicles were B-cells (Figure 5B, C), whereas only rare proliferating T-cells were identified in the inflamed meninges (Figure 5D). The percentage of proliferating cells in the B- and T-cell populations of different intrameningeal follicles ranged between 8% and 43% and between 0% and 2%, respectively. In patient MS79 which showed the largest B-cell aggregates with a well organized FDC network, Ki67+ nuclei were clustered on one side of the B-cell follicle (Figure 5B), just like a germinal center in a secondary follicle of lymph node (Figure 5A). No or only rare proliferating B-cells were present around blood vessels in demyelinated lesions (Figure 5E).

DISCUSSION

In this study, we provide the first immunohistochemical evidence that follicles containing B-cells, T-cells, plasma cells and FDCs expressing the lymphoid chemokine CXCL13 can form in close association with inflamed blood vessels in the cerebral leptomeninges of a subset of MS patients. The presence of proliferating B-cells in these structures is strongly suggestive of germinal center formation, possibly driven by (auto)antigen presented to B-cells on the surface of FDCs. Taken together with previous findings demonstrating the presence of B-cell clones with somatic hypermutations in the CNS of MS patients (5, 8, 33, 34, 41, 42), these results suggest that intrameningeal follicles can be regarded as ectopic lymphoid follicles where antigen-specific stimulation, expansion and maturation of B-cells takes place. This proposal is further sustained by a recent study showing that all stages of B-cell maturation, ranging from germinal center centroblasts and centrocytes to memory B-cells and plasma cells, can be identified in the CSF of MS patients (55). As many autoimmune diseases are accompanied by the formation of ectopic lymphoid structures in the target tissues (18, 60), it is conceivable that the local organization of the immune response improves the efficiency of autoreactive responses and might be implicated in disease exacerbation.

Despite the limited number of MS cases examined and the risk of underestimating the real frequency of intrameningeal follicles due to their scattered distribution and to the difficulty of maintaining the integrity of the meningeal compartment during tissue processing, our results suggest that the finding of ectopic lymphoid tissue might be associated with SP SM. Interestingly, among three patients with SP MS examined in this study, all with a long clinical history and very severe disability, the extent of demyelination was much greater in the 2 cases in which CNS infiltration by B-cells and intrameningeal follicles were demonstrated. A scenario can be envisaged in which following an initial destructive phase caused by CNS invasion by macrophages and T-cells, production of proinflammatory cytokines and synthesis of pathogenic autoantibodies, degradation of the neural tissue would induce a more complex and local immune process generating a lymphoid environment. Long-lived plasma cells persisting in MS chronic lesions and CSF have been indicated as the main source of intrathecally produced antibodies and responsible for lesion development in the absence of new inflammatory activity (40). Since different pathogenic pathways of demyelination have been proposed to occur in subgroups of MS patients (22), it would be important to determine if there is any relationship between formation of intrameningeal follicles and the lesional pattern characterized by deposition of activated complement and Ig.

The idea that intrameningeal follicles may represent a major source of B-cells and plasma cells accumulating in MS lesions is supported by the following observations. First, prominent B-cell infiltration was detected in the brains from the 2 cases with SP MS which showed intrameningeal follicle formation, whereas no or rare B-cells were present in the MS brains lacking intrameningeal follicles. Second, in one patient (MS79) in which accumulation of plasma cells was prominent in both lesions and meninges, numerous plasma cells were also detected in the periphery of the intrameningeal follicles, suggesting that they may have differentiated from B-cells activated within the FDC network. In this patient, the complexity of the FDC networks and the presence of small eccentric groups of proliferating B-cells within such networks point to a close similarity between intrameningeal follicles and secondary lymphoid follicles. The observation that smaller and less organized intramenigeal follicles were detected in the CNS of a second MS patient (MS85) showing prominent accumulation of B-cells, but no plasma cells in lesions and meninges, could indicate either an ongoing process or an abortive attempt at germinal center formation.

The close association between lymphoid follicles and leptomeningeal blood vessels in the MS brain suggests that the development of germinal centers might be initiated by circulating naïve or memory B-cells extravasating in the meningeal connective tissue and raises the question of which are the initial triggers for FDC differentiation and cellular aggregation. Recent studies have elucidated the molecular basis of lymphoid organogenesis and have shown that the tumor necrosis factor family member lymphotoxin $\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) and the lymphoid chemokines CCL19, CCL21 and CXCL13 have a critical role in the development, organization and function of secondary lymphoid organs and in the formation of ectopic lymphoid structures (13, 15, 19, 30, 46). $LT\alpha_1\beta_2$ is expressed on the surface of activated lymphocytes and a subset of resting B-cells, and regulates several aspects of T-cell and B-cell mediated immune responses, including maintenance of FDC networks and synthesis of lymphoid chemokines (15, 30). In the experimental

animal, ectopic expression of LT, CXCL13 or CCL21 is sufficient to induce formation of lymphoid structures (13, 19, 25). FDCs are a specialized population of fibroblastoid cells (7) whose differentiation and function, including CXCL13 production which is essential for B-cell recruitment and positioning within follicles, are regulated by $LT\alpha_1\beta_2$ (15). It is conceivable that $LT\alpha_1\beta_2$ expressed on activated T- and B-cells infiltrating the MS brain can induce fibroblast precursors residing in the leptomeningeal connective tissue to differentiate into FDCs and to produce CXCL13 which in turn would attract CXCR5-expressing B-cells and T helper cells, allowing the formation of functional lymphocytic aggregates. T helper cells provide the appropriate signals for B-cell activation and differentiation, whereas FDCs have a key role in presenting intact antigen to B-cells and in the affinity selection process (56).

Although in MS there is preference for accumulation of inflammatory cells and lesion formation around venules of the white matter, meningeal inflammation has been reported in a substantial number of MS cases (16). However, intrameningeal immune infiltrates have not been extensively characterized, presumably due to detachment of the meninges at autopsy. Prineas and Wright identified macrophages, lymphocytes and plasma cells around blood vessels in the pial connective tissue of patients with chronic MS (40). Despite the presence of blood-CSF barriers, the leptomeningeal and ventricular compartments behave as less immunoprivileged sites compared to the CNS parenchyma and are connected to cervical lymph nodes (24). Recent work by Ransohoff and colleagues in the normal human brain indicates that trafficking of T-cells through choroid plexus and meningeal vessels is important for CNS immune surveillance in physiological conditions (20). Following extravasation across postcapillary venules at the pial surface of the brain, leukocytes may enter the subarachnoid space and the Virchow-Robin perivascular spaces, thus patrolling the interfaces between the neural parenchyma and the periphery (44). In EAE, the meninges are the sites where inflammation develops earlier, residual immune infiltrates can persist for long periods of time after disappearance of clinical symptoms (28, 54, and our unpublished observations), and follicle-like structures form during chronic-relapsing disease (26). This study confirms that also in human disease the meninges represent a site of intense immune traffic and can provide a favourable microenvironment for ectopic lymphoid follicle formation. Synthesis of anti-myelin antibodies in this compartment could be involved in the subpial demyelination observed in a substantial proportion of MS patients (6).

The major question that arises from this study is whether meningeal follicles are involved in MS pathogenesis. The accumulation of somatic mutations in B-cells isolated from MS lesions and CSF indicates that extensive revision of the receptors occurs in these cells and may be important for the production of high affinity pathogenic antibodies (5, 8, 33, 34, 41, 42). However, the nature of the eliciting antigen (self or foreign) remains elusive. The proximity of intrameningeal follicles to the subarachnoid space and the increased levels of neural antigens in the CSF of MS patients (27, 32, 36) could favour exposure of B-cells to autoantigen and hence allow continuous B-cell activation and differentiation into autoantibody producing plasma cells. An additional mechanism through which locally activated B-cells could maintain an autoimmune response is through presentation of CNS antigen to autoreactive T-cells. However, it cannot be excluded that viral or bacterial antigens might induce an intrathecal immune response by directly activating B-cells within intrameningeal follicles. The scenarios depicted above suggest that the immunopathological process in MS would be more difficult to control once lymphoid follicles have developed in the inflamed meninges and could explain why current immunomodulatory drugs, like interferon-β and glatiramer acetate, fail to prevent MS progression despite their ability to reduce, at least in part, development of new lesions and relapse incidence.

Intrathecal B-cell clonal expansion is a common and early feature in MS (5, 8, 33, 34, 41, 42), which can even precede the appearance of oligoclonal Ig bands and disseminated magnetic resonance imaging lesions (41). Whether ectopic secondary lymphoid follicles develop early in disease, possibly within restricted areas in the ventricular/meningeal compartment, remains to be determined. In this respect, it is worthwhile mentioning that in mice with relapsing EAE follicle-like structures were detected exclusively in the meninges lining the brain stem, often close to the fourth ventricle and its lateral recesses (26). Further investigations are needed to establish whether similar "niches" exist in the meninges of MS patients with relapsing disease and short disease duration.

In conclusion, by showing that ectopic lymphoid follicles can develop within the meninges of the immune privileged CNS, this study supports the concept that events taking place in the target tissue are important in the pathogenesis of autoimmune diseases. The possible involvement of intrameningeal lymphoid tissue in maintaining humoral autoimmunity in MS suggests that local targeting of molecules involved in B-cell immunity or lymphoid tissue development should be considered as a possible therapeutic strategy to control the disease.

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