Endothelial Cell Class II Major Histocompatibility Complex Molecule Expression in Stereotactic Brain Biopsies of Patients with Acute Inflammatory/Demyelinating Conditions

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Abstract. To determine if central nervous system (CNS) microvascular endothelial cells express class II major histocompatibility complex (MHC) molecules in early demyelinating lesions in humans, cerebral white matter (WM) biopsies from patients with acute inflammatory/demyelinating conditions, including 4 with multiple sclerosis (MS), were immunostained for class II MHC and other antigens. Eight of 9 biopsies showed focal MHC class II-positive endothelial cells; there were none in the CNS of 1 of the MS patients at autopsy. There were more vessels with class II-positive endothelial cells in areas with intact WM and gliosis than in areas with active demyelination or control WM; class II-positive endothelial cells in small vessels and capillaries were adjacent to transmigrating and perivascular CD4-positive cells. By immunoelectron microscopy, class II molecules were localized to vesicles in endothelial cell cytoplasm, suggesting the potential for antigen processing. Perivascular cells, parenchymal microglia, mononuclear cells and the perinuclear cytoplasm but not the processes of astrocytes were class II-positive. These data indicate that in acute CNS inflammatory/demyelinating lesions, endothelial cells focally and apparently transiently express class II MHC molecules. This expression implies potential antigen-specific interactions, immunoregulatory or signalling functions in endothelial cells, or it may render them susceptible to CD4-positive cell-mediated cytotoxicity. Thus, class II-positive endothelial cells may have pivotal immunologic roles in initial stages of T-cell responses in human CNS WM, particularly in acute MS lesions.

Key Words: Antigen presentation; Astrocytes; Endothelial cells; Immunoelectron microscopy; Major histocompatibility complex; Microglia; Multiple sclerosis.

INTRODUCTION

Expression of class II major histocompatibility complex (MHC) molecules on the surfaces of antigen-presenting cells (APCs) is essential for recognition of antigenic peptides and activation of CD4-positive T cells (1). To understand mechanisms of cellular immune reactions in the central nervous system (CNS), potential resident and infiltrating APCs need to be identified and the stages of the response during which they express MHC molecules determined. In the early 1980s, HLA-DR antigen was first demonstrated on normal human brain white matter microvascular endothelial cells (2). At that time we found that in guinea pigs sensitized for acute experimental allergic encephalomyelitis (EAE), an animal model of MS, CNS microvascular endothelial cells exhibited enhanced class II MHC molecule expression prior to the accumulation of detectable T cell infiltrates or the onset of clinical disease (3). Later, we demonstrated by immunoelectron microscopy (IEM) that this expression was on endothelial cell luminal surfaces as well as on perivascular and inflammatory cells (4). In a study of cerebral cortical biopsies of patients with acute herpes simplex encephalitis in whom clinical signs evolve over several days, we also found MHC class II expression on microvessels in association with T cell infiltrates in affected tissues (5). These observations suggested that enhanced class II MHC molecule expression by endothelial cells is among the numerous alterations that characterize their cytokine-mediated activation in initial phases of immune responses in the CNS (6). In particular, they raised the possibility of antigen presentation by endothelial cells to CD4-positive lymphocytes in acute human CNS inflammatory diseases.

In the major human CNS inflammatory/demyelinating disease multiple sclerosis (MS), this issue has been controversial. Traugott et al reported class II MHC expression on CNS endothelial cells and astrocytes in MS lesions (7, 8). However, in those studies endothelial cell and astrocyte class II MHC expression was not distinguished from perivascular and parenchymal microglial cell expression. Endothelial cell class II MHC expression in MS lesions has more recently been discounted (9-11). With few exceptions (e.g. 12-14), investigations addressing class II MHC expression in MS lesions have been performed on cryostat sections of postmortem tissue samples (7-11, 15, 16). In such samples, the disease is usually of years in duration, the majority of lesions are chronic and noninflammatory, and even in inflammatory lesions, correlations with clinical disease expression and radiographic abnormalities often cannot be made. Determinations of lesion ages based on pathologic criteria alone are, therefore, imprecise, and cell identification and immunohistochemical reaction product localization can be difficult in cryostat sections. Most recent reviews emphasize perivascular cell and parenchymal microglial
class II MHC molecule expression and do not consider CNS endothelial cells or astrocytes either to express class II MHC molecules or to be likely candidates for primary antigen-presenting cells in MS lesions (17–22).

Based on our earlier observations, we hypothesized that CNS white matter (WM) microvascular endothelial cells also express class II MHC molecules in initial stages of immune responses in inflammatory/demyelinating conditions in humans. To address this question we analyzed archival CNS samples from patients who had had a recent onset or exacerbation of neurological disease and who had undergone diagnostic stereotactic biopsies of MRI-localized cerebral WM lesions. All of the samples showed acute inflammatory/demyelinating WM lesions by routine methods and none showed unequivocal evidence of a viral infection or other disease. The use of reagents that immunostain class II MHC molecules and CD4 in paraffin sections following microwave antigen retrieval permitted definitive identification of endothelial cell MHC class II expression in association with CD4+ cell infiltrates in all but 1 of the WM biopsies. IEM supported this localization in endothelial cell cytoplasm. Furthermore, 1 of the patients with MS died 5 months after his biopsy and, consistent with the numerous studies of MS autopsies, brain and spinal cord lesions in his autopsy samples did not show class II MHC molecule-positive endothelial cells. These results indicate that although class II MHC molecule expression is abundant in microglia, macrophages, and inflammatory cells in inflammatory and actively demyelinating CNS WM lesions, microvascular endothelial cells show focal class II MHC molecule expression only in early phases of these lesions in humans, particularly in patients with clinically active MS.

Follow-up information for periods of 4 months to 7 years after the hospitalizations was obtained from the patients’ primary physicians and 1 autopsy record. Based on this information, the clinical diagnoses were as follows: apparently solitary demyelinating lesion (3 cases); acute MS (2 cases); probable MS (1 case); autopsy-confirmed MS (1 case); and relapsing acute disseminated encephalomyelitis (ADEM) (1 case). One patient with multiple bilateral lesions had AIDS, but his biopsy showed no viral inclusions and immunohistochemistry for papovavirus on paraffin sections was equivocal (as was the case in the patient with autopsy-confirmed MS). Viral particles were not demonstrated by EM. Therefore, although it is most likely that this AIDS patient had progressive multifocal leuкоencephalopathy (PML) with inflammatory/demyelinating lesions, that diagnosis could not be made. An alternative possibility includes an inflammatory-demyelinating leuкоencephalopathy associated with HIV-1 infection (24). Control samples included archival paraffin blocks of histologically normal WM in temporal lobectomy specimens from 2 patients and samples of surgically removed normal spleen from 3 patients.

Monoclonal Antibodies (mAbs) and Immunohistochemistry

Six-μm-thick paraffin sections were baked for 2 h at 60°C, deparaffinized, rehydrated to 70% ethanol and rinsed in phosphate-buffered saline (PBS) pH 7.4, 3 times for 10 minutes (min). Slides were washed twice with distilled water, then placed in alternate slots of microwave staining dishes (Shandon-Lipshaw, Pittsburgh, PA), and immersed in either of 2 buffers determined in preliminary studies to be optimal for each mAb. For mAb 1F6 (see below), 1 mM ethylenediaminetetraacetic acid, pH 8.0 was used and for the other mAbs 10 mM citric acid monohydrate, pH 6.0, was used. The dishes were covered with a loose fitting lid and heated in a microwave for 15 min (initial 2–3 min at 100% power level, remaining minutes at 80% power level, microwave wattage 1.25 kW) (23). The slides were then cooled in the uncovered dish at room temperature (RT) for at least 30 min and rinsed (2 × 10 min each) in distilled water and PBS.

Slides were incubated in 10% normal horse serum for 10 min at RT and then in mAb diluted in PBS at 2–8°C overnight. The mouse mAbs and dilutions used were TAL.1BS (anti-HLA-DR, α chain, Dako A/S, Denmark, 1:50), IQU9 (anti-HLA- [DR, +DP+DQ], β chain, Vector Lab., Newcastle, UK, 1:50), FMC14 (anti-HLA-DR, Harlan Sera-Lab, Ltd., Sussex, UK 1:2), and 1F6 (anti-human CD4, Vector, 1:20). On the following day slides were incubated sequentially in 0.3% H2O2 in PBS, biotinylated horse anti-mouse immunoglobulin (lg), and avidin-biotin horseradish peroxidase complex reagents (Vectastain Elite kit, Vector Laboratories, Burlingame, CA) with washes of PBS (3 × 10 min) between each step. Immunoperoxidase reaction product was visualized with 3,3'diaminobenzidine (DAB) (Sigma, St. Louis, MO) and fixed in neutral buffered formalin and the sections were counterstained with hematoxylin. Normal spleen samples were used as positive staining controls and for determinations of optimal dilutions for each mAb. In experiments in which samples were stained for glial fibrillary acidic protein (GFAP), a rabbit polyclonal antibody (Dako, 1:1

MATERIALS AND METHODS

Case Material

Seventeen archival paraffin blocks containing sufficient amounts of diagnostic tissue from cerebral WM biopsies of 9 patients with acute inflammatory/demyelinating conditions diagnosed at Stanford University Medical Center, Stanford, CA from 1988 to 1997 were studied (Table 1). Clinical histories were obtained from review of patient hospital records. The known neurological syndromes of these patients prior to biopsy were from 9 days to 2.5 years in duration (median = approximately 3 months). Four had single cerebral hemisphere lesions and 5 had multiple WM lesions on MRI examination. All had acute inflammatory/demyelinating lesions diagnosed by routine histology, including Luxol fast blue stains and Bielschowsky preparations. The diagnoses were confirmed using 1-μm sections and electron microscopy (EM) of Epon-embedded samples in 5 cases and using 1-μm sections only in 1 case. All cases showed relative axon preservation but in the majority some evidence of acute axonal injury was also present.

J Neuropathol Exp Neurol, Vol 58, April, 1999
### TABLE 1

**Case Material**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/sex</th>
<th>Clinical History</th>
<th>MRI Findings</th>
<th>Additional Pathologic Data</th>
<th>Clinical/Final Diagnosis</th>
<th>Follow-up (duration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35 M</td>
<td>IV drug use, bipolar disorder, hepatitis B, progressive ataxia, abnormal gait, visual dysfunction (\times 3) mo, nystagmus, dysmetria of UE, hyperreflexia, HIV</td>
<td>Multiple WM lesions in both hemispheres</td>
<td>No viral inclusions, IH for papovavirus equivocal</td>
<td>MS (autopsy confirmed)</td>
<td>Died 5 mo after biopsy</td>
</tr>
<tr>
<td>2</td>
<td>24 M</td>
<td>AIDS, verigo, blurred vision, nystagmus, photophobia (\times 1) mo</td>
<td>Multiple enhancing 5.0 mm–2.0 cm lesions in both hemispheres</td>
<td>EM: myelin disruption, foamy macrophages, reactive astrocytes, axonal spheroids, no viral particles. IH for papovavirus equivocal</td>
<td>Multiple lesions, possible PML</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>65 F</td>
<td>Ataxia (\times 1) yr, blurred vision, emotional lability for a few mo, staggering gait</td>
<td>L parietal enhancing lesion with surrounding edema</td>
<td>EM: demyelination, no viral particles</td>
<td>Probable MS</td>
<td>Follow-up MRI: resolution of large lesion, multiple additional lesions compatible with MS, &quot;severely compromised&quot; (7 yr)</td>
</tr>
<tr>
<td>4</td>
<td>30 F</td>
<td>Diagnosed with MS 1 yr prior with episodes of R side numbness, dysarthria. 9 da prior to biopsy progressed to bilateral spasticity and aphasia over 24 h</td>
<td>Bilateral large periventricular lesions</td>
<td>EM: several nerve fibers with preserved myelin; many axonal spheroids</td>
<td>Fulminant MS</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>37 F</td>
<td>Homeless. Disorientation, mutism, global apathy, seizures</td>
<td>Multifocal enhancing WM lesions</td>
<td>EM: demyelinated axons at lesion perimeter</td>
<td>Probable acute MS</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>8 M</td>
<td>HA, eye pain, dizziness, photophobia, fever (\times 3) mo, fluctuating course with steroid Rx</td>
<td>Multiple WM abnormalities in both hemispheres, cerebellum and brain stem</td>
<td>EM: demyelinating process</td>
<td>Relapsing acute disseminated encephalomyelitis</td>
<td>Symptom free for 1 yr (15 mo)</td>
</tr>
<tr>
<td>7</td>
<td>34 F</td>
<td>Episodes of diplopia, dizziness, confusion, sensory symptoms (\times 2.5) yr; migraine HA, gait imbalance, slurred speech. Hypertension, probable Hashimoto’s thyroiditis, normal cerebral angiogram</td>
<td>R occipital WM high intensity lesion on T2, not noticeable on T1, with some enhancement</td>
<td>CSF cytology negative for malignant cells</td>
<td>Demyelinating lesion, solitary</td>
<td>No MRI progression. Workup for MS negative (2 yr)</td>
</tr>
<tr>
<td>8</td>
<td>35 F</td>
<td>Alcohol abuse; progressive L hemiparesis (\times 5) wks</td>
<td>2 cm posterior R parietal mass lesion</td>
<td>One micron sections: foamy macrophages, reactive astrocytes, relative axon preservation</td>
<td>Demyelinating lesion, solitary</td>
<td>No neurological disease (2 yr)</td>
</tr>
<tr>
<td>9</td>
<td>65 F</td>
<td>Hemiparesis and aphasia (\times) several wks</td>
<td>Single hemisphere lesion, suggestive of tumor</td>
<td>None</td>
<td>Demyelinating lesion, solitary</td>
<td>Clinical and MRI response to steroid Rx (4 mo)</td>
</tr>
</tbody>
</table>

*Abbreviations: CSF: cerebrospinal fluid; da: days; EM: electron microscopy findings; h, hours; HA, headache; IH, immunohistochemistry; L, left; mo, months; MS, multiple sclerosis; PML, progressive multifocal leukoencephalopathy; R, right; Rx, treatment; UE, upper extremities; wks, weeks; WM, cerebral white matter; yr, year.
Fig. 1. Endothelial cell HLA-DR and CD4+ cell infiltrates. (A). Case 5. Endothelial cell staining is predominantly on the side of the vessel with an adjacent, eccentric accumulation of inflammatory cells. The inflammatory cell cuff is also diffusely HLA DR-positive. Myelin in this field is intact. mAb FMC14. (B) and (C). Case 9. Two portions of a vessel in which there are individual HLA-DR-positive endothelial cells (arrows). Intraluminal leukocytes and perivascular foamy macrophages are also HLA DR-positive. mAb TAL.1B5. (D). Adjacent section to B and C stained for CD4. Intensely stained cells are present in the perivascular space. Macrophages show faint, more diffuse staining. Endothelial cells are negative. mAb 1F6. All are \( \times 543 \).

500) was used with an anti-rabbit Ig kit (Vector) and appropriate reagent substitutions. For double staining, a second overnight incubation with a different primary antibody was followed by the use of either an alkaline phosphatase or avidin-biotin staining kit. The second reaction product was visualized with a purple substrate (Vector VIP, SK-4600). Double stained slides were not counterstained.

Semiquantitative Analysis and Histologic Correlations

To correlate the presence of staining for MHC class II on endothelial cells with the stage of tissue injury, 3 histologic patterns were identified in the disease biopsy samples (Table 2). The classifications were based on patterns in the class II MHC- and anti-GFAP-immunostained and routine sections. “Normal-appearing white matter” (NAWM) consisted of normal or minimally hypercellular areas with intact myelin and axons and only scattered reactive astrocytes. Those areas were indistinguishable from the WM in normal control samples (“Control WM”). “Gliotic WM” areas were those in the disease biopsy samples that showed intact myelin and axons but had numerous immunostained perivascular cells and parenchymal microglia (in stains for class II MHC) and astrocytes (anti-GFAP stain). Some of these areas had small numbers of perivascular CD4+ cells. “Demyelinated WM” areas were those that contained hypercellular regions with marked myelin loss, sheets of foamy macrophages, astrocytosis, axonal spheroids, and some loss of axons. All of these areas had perivascular and parenchymal CD4+ cell infiltrates in adjacent serial sections. The majority of the slides contained 1 section from each paraffin block, but for some blocks with very little tissue, up to 3 serial sections were put on each slide. Total WM areas and areas within each slide that contained each of these histologic patterns were measured and the numbers of vessels in each area with positively stained endothelial cells were counted by 2 observers.

Immunoelectron Microscopy (IEM)

Several protocols were tested and the following method was developed to maximize antigen retrieval, tissue preservation, and specific immunogold particle visualization by EM. After completion of the light microscopy analysis, tissue fragments in biopsies with positive endothelial cell immunostaining for HLA-DR and with sufficient remaining tissue for further studies were identified in 4 paraffin blocks (1 each from cases 2 and 8; 2 from case 7). Fifteen-\( \mu \)m-thick sections were cut from these blocks using a Leitz model 1512 microtome with a steel blade. The sections were placed on Formvar-covered microscope slides and baked 2 h at 60°C. The sections were then
TABLE 2
Histologic Correlates of Endothelial Cell Class II MHC Expression*  

<table>
<thead>
<tr>
<th>Histologic features</th>
<th>Control WM</th>
<th>NAWM</th>
<th>Gliotic WM</th>
<th>Demyelinated WM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelin</td>
<td>Intact</td>
<td>Intact</td>
<td>Intact</td>
<td>Demyelinated</td>
</tr>
<tr>
<td>Gliosis</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>CD4+ cell infiltrates</td>
<td>Absent</td>
<td>Absent</td>
<td>Variable</td>
<td>Present</td>
</tr>
<tr>
<td>Mean vessels with positively stained endothelial cells/Slides ± SE</td>
<td>0 ± 0‡</td>
<td>0.45 ± 0.19</td>
<td>0.93 ± 0.25‡</td>
<td>0.43 ± 0.13</td>
</tr>
<tr>
<td>(No. of slides with histologic pattern)</td>
<td>(6)</td>
<td>(10)</td>
<td>(15)</td>
<td>(13)</td>
</tr>
</tbody>
</table>

* Data are from 17 inflammatory/demyelinating disease biopsy slides in which there was endothelial cell class II staining and from 6 control slides. Each slide was stained with one of the 3 anti-class II MHC mAbs.
† Criteria for histologic patterns are described in Materials and Methods.
‡ P < 0.03, by Student's t-test.

Deparaffinized in xylene and graded alcohols, followed by 3 10 min washes in 10mM PBS and 2 brief rinses in deionized H2O (DI H2O). They were then placed into the citrate buffer and microwaved as described above. After cooling, they were rinsed briefly in DIH2O and washed in 10 mM PBS 3 times for 10 min each.

The following steps were carried out at RT, unless otherwise noted. The slides were washed in 20mM Tris buffer, pH 7.4 (TBS) x 2, incubated with 20mM glycine in TBS for 10 min at 37°C, and washed in TBS x 3. A wash buffer (TBS-AG) consisted of 0.8% bovine serum albumin, 0.1% ImmunoGold-Silver Stain Quality Gelatin (Amersham, Arlington Heights, IL), and 2mM NaN3 in TBS. The slides were treated with blocking solution (5% goat serum in TBS-AG) for 30 min, washed in TBS-AG x 3, and incubated in mouse mAb FMC14 diluted 1:5 in TBS-AG with 1% normal goat serum for 1 h at RT and then overnight at 2–8°C. The slides were then washed in TBS-AG x 2, TBS x 2 and in PBS x 3 over 1 h, fixed in 2% glutaraldehyde in PBS for 10 min, washed in PBS x 3, dehydrated by immersion in 50% and 70% ethanol for 10 min each and in 96% ethanol x 3 for 10 min each. The tissue was then infiltrated with 100% LR-White (Sigma), first briefly and then with fresh 100% LR-White for 60 min at RT, followed by 100% LR-White overnight at 2–9°C, and the next day in fresh 100% LR-White for 6 h at RT. A size 1 gelatin capsule filled with LR White was then inverted over the tissue fragment of interest and polymerization was allowed to take place at 30°C ± 1°C for 72 h. The tissue was then retained within the LR White block when the blocks were removed from the slides.

Ultrathin (80-nm-thick) sections were cut on an Ultracut S (Leica) ultramicrotome with glass knives and placed on formvar covered carbon-coated Ni grids. The grids were washed in TBS x 2, incubated for 10 min at 37°C in 20mM glycine in TBS, washed in TBS x 3, washed in TBS with 0.8% bovine serum albumin for 30 min, washed in TBS-AG x 3 and incubated in 20 nm gold particle-conjugated goat anti-mouse IgG Reagent (E-Y Laboratories, Inc., San Mateo, CA) diluted 1:25 in TBS-AG with 1% normal goat serum for 1 h. The grids were then washed in TBS-AG, TBS x 2 and PBS x 3, fixed in 2% glutaraldehyde in PBS for 10 min, washed in PBS, DIH2O x 3. They were then stained with 4% uranyl acetate overnight for 15 min in the dark, washed with boiled DIH2O x 2, washed with 20 mM NaOH and further stained with 0.2% lead citrate in a Petri dish with NaOH granules. The grids were then washed once in NaOH and in boiled DIH2O x 4, air dried, and examined by EM. Fields for photographs were selected on the basis of tissue preservation and the presence of multiple gold particles on microvessels.

RESULTS

In control brain samples, there were scattered class II MHC+ perivascular cells and parenchymal microglia to the extent previously reported in comparable cryosections and isolated brain microvessels (25, 26). Microvessels in these samples showed no endothelial cell staining. By contrast, all but 1 biopsy case (Case 6) had positive MHC class II staining of endothelial cells in at least 1 slide with 1 or more of the anti-class II MHC mAbs (17/48 single-stained slides, 35.4%). Most of the vessels with stained endothelial cells were in small venules and capillaries. There were no clear differences in staining patterns among the 3 anti-class II MHC mAbs evident, except for some nonspecific nuclear staining with mAb IQU9. Of the 17 slides with vessels with positive endothelial cells, 8 were stained for anti-HLA-DR α chain, 7 for anti-HLA-DR, and 2 for anti-[DR,DP,DQ] β chain. In vessels observed in cross-section, the staining was generally not circumferential. Rather, individual stained endothelial cells were often on the portions of the vessel walls that had accumulations of inflammatory cells on their abluminal sides (Fig. 1A, B, C). In adjacent sections stained for CD4, these infiltrates contained strongly CD4-positive lymphocytes and macrophages that had weaker and more diffuse, apparently cytoplasmic staining but no
staining of endothelial or other CNS resident cells (Fig. 1D).

To determine if the extent of endothelial cell staining correlated with specific stages of the pathologic processes, 3 histologic patterns were identified in the disease biopsies and the extent of microvessel class II MHC expression in each determined. Vessels with positive endothelial cells were identified both in gliotic WM with intact myelin and minimal CD4+ cell infiltrates (Fig. 2A, C, E, G) and in NAWM. In demyelinated WM with numerous macrophages and CD4+ cells and marked astrogliosis, class II-MHC-positive endothelial cells were also identified (Fig. 2B, D, F, H). Overall there tended to be fewer vessels that had positive endothelial cells in these areas, suggesting that as inflammation and demyelination progressed, fewer endothelial cells express class II MHC molecules. In the 17 slides that contained microvessels with stained endothelial cells, positive vessels were most numerous in WM with intact myelin and gliosis ("Gliotic WM", Table 2). The number of vessels with positive endothelial cells was significantly greater in those areas than in control WM (Table 2).

In agreement with many previous studies, HLA-DR was prominently expressed on perivascular cells and parenchymal microglia (Fig. 3A, B), in perivascular mononuclear inflammatory cell cuffs and on foamy macrophages in demyelinated foci (Figs. 1A–C, 2F, H). Some HLA-DR-positive parenchymal glia had abundant perinuclear cytoplasm (Fig. 3B), suggesting that they might be astrocytes. To assess this possibility, double staining with anti-GFAP was performed. In double stained sections of control samples and NAWM in disease biopsies, 2 distinct populations, i.e. HLA-DR-positive microglia and GFAP-positive astrocytes, were identifiable (Fig. 3C). In areas in disease samples with more prominent gliosis and demyelination, some astrocytes exhibited HLA-DR staining in perinuclear cytoplasm, but not in GFAP-positive processes (Fig. 3D).

Patient no. 1 came to autopsy 5 months after her brain biopsy and his CNS tissues were obtained from a medical examiner. He had numerous MS plaques and, despite the presence of possible risk factors, there was no evidence of HIV-1 infection or PML in the CNS. Three samples of brain and spinal cord plaques that contained prominent perivascular mononuclear cell cuffs and sheets of parenchymal foamy macrophages were immunostained using the FMC14 and TAL1B5 mAbs with positive and negative controls. Staining for class II MHC molecules was present on perivascular mononuclear cells, parenchymal microglia, and macrophages in these lesions, but not on endothelial cells or astrocytes (data not shown).

Neurons in gray matter and oligodendrocytes appeared to be uniformly class II-negative in all brain samples. In control spleens class II staining was observed on dendritic cells, lymphocytes and endothelial cells. No consistent staining for HLA-DR or CD4 was detected in spleen or CNS samples that had not undergone the microwave antigen retrieval procedure.

By IEM, small numbers of immunogold particles labelling HLA-DR were found in CNS microvascular endothelial cell cytoplasm in samples from all 4 paraffin blocks (from Cases 2, 7 and 8) analyzed (Fig. 4). The specificity of the immunolabelling was indicated by absence of gold particle labeling on adjacent red blood vessels, vessel lumens, perivascular inflammatory cells, basement membranes or neural structures. More numerous gold particles labelling perivascular cell processes served as an internal positive control in some fields (Fig. 4A, B), whereas in other fields endothelial cells were the only cells labelled (Fig. 4C). In endothelial cells, the particles were most frequently found in intracytoplasmic vacuoles, whether vacuoles were numerous (as in Fig. 4A), or whether they were sparse (Fig. 4B, C). The labelled vacuoles were predominantly found near the luminal surfaces of the endothelial cells (Fig. 4B, C); labelling on the luminal surface of an intact endothelial cell was observed once. Similar endothelial cell HLA-DR subcellular localization patterns were demonstrated in an additional diagnostic frozen section sample from Case 8 that had been preserved in OCT, but tissue preservation in that sample was less optimal (not shown). Thus, the IEM data indicate specific immunogold particle localization using the methods developed for this analysis, confirm localization of HLA-DR molecules in endothelial cells in the biopsy fragments that were positive using the immunoperoxidase procedure, and suggest specific localization of the MHC molecules within endothelial cells.

Discussion

Breakdown of the blood-brain barrier can be detected by MRI in MS patients and it may precede the onset of symptoms (27). Brain tissue samples obtained when there is a recent onset of disease activity indicate that inflammation occurs early and prior to widespread myelin breakdown (28–30). Thus, the pathophysiology of acute clinical disease activity in MS patients involves immunological as well as barrier alterations of the CNS microvasculature. In certain animal models of MS and in some acute human encephalitides, endothelial cells, the major components of the blood-brain barrier, exhibit immunologic activation, i.e. increased class II MHC molecule expression. Therefore, the present study was undertaken to determine whether similar alterations are found in CNS microvascular endothelial cells in acute human CNS WM inflammatory/demyelinating conditions.

In addition to the expected widespread expression on microglia and macrophages, several factors may have contributed to the detection of class II MHC expression.
Fig. 2. Correlation of microvascular DR expression with histologic patterns. A, C, E, G. Case 5. Adjacent serial sections of NAWM with intact myelin (A) and minimal astrocytosis (C). Focal endothelial cell surface staining (arrow) is present on this vessel (E) and there is staining of perivascular cells and parenchymal microglia. In a double-stained section (G), there are no CD4+ (brown) cells in the field, but DR staining (purple) of the endothelium (white arrow) and of microglia is evident. B, D, F, H. Case 9. Adjacent serial sections of demyelinated WM (B) with marked astrocytosis (D). The vessel in the upper left corner of (B) and (D) is seen at higher power in (F) with focal luminal surface staining for HLA-DR (arrow). Stained foamy macrophages in the Virchow-Robin space and in the parenchyma are also evident. Double staining of an adjacent vessel demonstrates intraluminal CD4+ cells (brown) and a cell that appears to be migrating under the surface of a (purple) HLA-DR-positive endothelial
Fig. 3. Microglial and astrocyte staining for HLA-DR. (A). Case 5. Perivascular cells and parenchymal microglia with delicate processes in NAWM are HLA-DR-positive but endothelial cells in this field are negative. (B). Case 5. Most parenchymal microglia have delicate cytoplasmic processes but other glia (e.g. cell in field center) have more abundant perinuclear cytoplasm with coarser processes. (C). Case 9. Double stain for DR (brown) and GFAP (purple) in a field of NAWM with minimal glial reaction, intact myelin and no inflammation. GFAP-positive astrocyte processes extend to the outer vessel wall and delicate parenchymal microglia are DR-positive. A small amount of HLA-DR staining is also present on the vessel luminal surface (arrow). (D). Case 5. Double stain demonstrating DR-positive astrocyte cytoplasm (brown), but not GFAP-positive (purple) processes in a field with marked astrocytosis and demyelination. A, B, mAb FMC14. C, D, double stain with anti-GFAP (purple chromogen) and anti-HLA-DR mAb TAL.1B5 (brown chromogen). All are ×543.

on endothelial cells and astrocytes in the present study. Our use of archival biopsy samples rather than cryostat sections of autopsy samples eliminated potential peri- and postmortem effects such as proteolysis of MHC molecules. Furthermore, the biopsies were performed at the time of relatively acute clinical events and were directed to WM regions with identified MRI abnormalities. Thus, in contrast to the majority of tissue samples from autopsies, the biopsy samples analyzed represent the earliest clinical and radiographic lesions obtainable from patients with these conditions.

The pathological processes in the patients studied, however, may have diverse etiologies. Since brain biopsy is only used for the diagnosis of demyelinating disease under extraordinary circumstances, even the 4 patients in this series diagnosed with MS may not be completely representative of patients with that disease. Three patients had apparently solitary demyelinating lesions but the periods of follow-up after their biopsies were brief and their ultimate diagnoses are uncertain. In a large series of biopsies of similar lesions, only a small proportion of the patients documented subsequently developed MS in follow-up periods of up to 12 years (31). One patient in the present series may have had PML, a disease with a known etiologic agent that could not be demonstrated, and another had ADEM. Nevertheless, all of the biopsy samples exhibited inflammatory demyelination indistinguishable by histologic and ultrastructural criteria from acute MS lesions. Therefore, the present observations are likely highly relevant to the acute inflammatory events.
associated with active clinical disease in MS patients, as well as to other conditions.

It is unlikely that technical factors contributed to results different from those in other studies of MS lesions. Microwave antigen retrieval was necessary for the demonstration not only of endothelial cell and astrocyte class II expression but also of rare perivascular cell staining in control WM (25) and of microglial and macrophage expression in demyelinated foci (7–11). We did not, however, observe any unexpected cell staining patterns, e.g. of neurons. Therefore, there is no evidence to suggest that the use of microwaving resulted in any nonspecific staining. Immunogold labelling of endothelial cell cytoplasm was also detected by IEM using an entirely different
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detection method, indicating that the endothelial cell staining observed by light microscopy cannot be attributed to the edge artifact of conventional immunoperoxidase-stained sections. Furthermore, endothelial cell class II staining was occasionally observed in NAWM (Fig. 2E, G) and associated with accumulations of inflammatory cells in Virchow-Robin spaces (Fig. 1A–C), but was seen to a lesser degree in vessels in areas with frank demyelination and perivascular accumulations of foamy macrophages (Fig. 2F; H; Table 2). This suggests that the endothelial cell staining was related to earlier inflammatory cell migration and not to class II expression on cells beyond the blood vessel wall and within the parenchyma. The trend towards less staining in demyelinated foci is also consistent with the absence of endothelial cell and astrocyte class II MHC expression in MS lesions in autopsies reported by others and in those in the patient in this study who came to autopsy. Taken together, these data suggest that the endothelial cell class II expression identified does occur in situ but may be transient and only present in the earliest lesion stages.

Endothelial cell class II MHC molecule expression is consistent with other evidence of endothelial cell activation in inflammatory MS lesions, including their acquisition of a high endothelial venule-like morphology, accumulation of plasma proteins, and altered adhesion molecule, integrin and CD34 expression (15, 32–35). It is likely that the endothelial cells are induced to express class II MHC molecules by proinflammatory cytokines, particularly interferon-γ (6, 36–38). Furthermore, the trends towards less class II expression in demyelinated WM in the biopsies and its absence on endothelial cells in autopsies might be due to development of unresponsiveness to interferon-γ-induced class II induction, as reported in rat cerebrovascular endothelial cells in the recovery phase of acute EAE (39). The foci of the endothelial cell class II expression observed is also consistent with cytokine effects, since it implies localized effects on specific vascular beds. Secondary acquisition of class II molecules through direct contact with other class II-positive cells or by uptake of molecules or membrane fragments shed by other cells, e.g. by pinocytosis, is also possible. There was, however, no clear relationship of the extent of endothelial cell staining to the proximity of other stained cells.

The proximity of class II MHC molecule-expressing endothelial cells to CD4+ cells demonstrated implies the potential for antigen-specific recognition, i.e. antigen presentation, by endothelial cells to CD4+ T cells as they come into direct contact with endothelial cells, pass through the microvessel wall and enter the CNS. In recent years, evidence of rodent CNS microvascular endothelial cell antigen presentation to encephalitogenic T cells has accumulated and in some EAE models endothelial cells may be critical determinants of susceptibility (40–42). The present observations of immunogold localization of class II MHC molecules to vesicular cytoplasmic compartments in endothelial cells is also consistent with the endogenous class II MHC molecule synthesis and peptide loading that occur in APCs (43).

On the other hand, several in vitro studies of rodent CNS endothelial cells indicate that they are less efficient antigen-presenting cells than bone-marrow derived cells (44), that they may differentially activate specific T cell populations (45), and that they may inactivate T cells or induce anergy (46, 47). Recognition of antigen by T cells is dependent on the APC type (48) and a particular array of simultaneously expressed costimulatory molecules would be necessary for proinflammatory endothelial cell antigen presentation in situ (19, 49–52). Furthermore, multiple potential antigen presentation pathways have now been identified (53) and alternative pathways may be particularly important for presentation by "nonprofessional" APCs such as endothelial cells in the induction of responses to a wide range of T cell antigens (54).

The patient in whose biopsy class II MHC-positive endothelial cells were not demonstrated was the 8-year-old child with ADEM (Case 6). This might be related to his age or other factors. However, since ADEM has a favorable prognosis and does not generally progress to MS, there might be a relationship between the presence or absence of demonstrable endothelial cell class II MHC expression in acute lesions and disease progression. An analogous correlation can be made in a comparison of 2 classic acute EAE models, i.e. in the adult Strain 13 guinea pig and the Lewis rat. In the former, endothelial cell class II expression is readily detected in early EAE lesions by light microscopy and in a patchy distribution similar to that observed in the present study by IEM (3, 4). This pattern of expression is associated with a severe, progressive, and usually fatal disease course. In contrast, in rats, class II MHC molecules are not detected on endothelial cells in inflammatory lesions, clinical disease and inflammation resolve spontaneously and there is subsequent unresponsiveness to reinduction of EAE (55). Thus, similar differences in endothelial cell class II expression in lesions in both the human and animal diseases are associated with differences in the disease outcomes. Since class II MHC/CD4+ cell interactions may influence T cell memory (56), differences in CNS endothelial cell class II MHC expression between these 2 animal models and between the ADEM case and the other cases might relate to differences in endothelial cell activation of memory CD4+ T cell populations that influence the later disease courses.

It is now appreciated that class II MHC molecules may have functional roles in addition to antigen presentation to CD4+ cells. Encephalitogenicity of neuroantigen-specific T cells correlates with their capacity to lyse class II MHC-expressing cell targets (57) and recognition of class
II molecules on brain microvascular endothelial cells by myelin antigen-specific CD4+ T cells induces their lysis in vitro (58–61). Similar results have also been reported using peripheral blood T cells from MS patients in relapse (62). Thus, endothelial cell class II expression could trigger their recognition and lysis by cytotoxic CD4+ T cells thereby resulting in a breakdown of the blood-brain barrier that facilitates leukocyte transvascular migration. Class II molecules may also function as adhesion molecules for CD4+ cells (17, 63–65) and as signal transducing molecules that affect cytokine secretion (66, 67). The beneficial effects of interferon-β therapy in patients with relapsing-remitting MS might also in part be related to its modulation of endothelial cell class II MHC expression in vivo (68).

As in previous studies (13, 15), astrocyte labelling for class II was generally less conspicuous and less frequently seen than that of microglia and was only in perinuclear cytoplasm. Astrocytes may also be “partially competent” APCs (69, 70) whereas microglia and macrophages, which are known as “professional” APCs, are regarded by many authors as being the critical APCs in MS lesions (11, 18–21, 71). Expression of class II MHC molecules by microglia and macrophages, however, does not necessarily indicate the presence of a primary T cell response (72, 73) and their class II expression in MS lesions might, therefore, indicate reaction to injury or other functions rather than only antigen presentation (74).

In summary, endothelial cells in biopsies from patients with active clinical disease and CNS inflammatory/demyelinating lesions show focal expression of class II MHC molecules in areas with CD4+ cell infiltrates and gliosis. These results lend support to earlier concepts of endothelial cell activation and antigen-specific interactions with leukocytes in initial stages of immune responses in CNS WM (3). Since the extent of endothelial cell expression appears to be less in frankly demyelinating foci and is absent in MS lesions in autopsy material (7–11, 15), this illustrates possible differences in the stage of evolution between biopsied lesions and the vast majority of those in MS autopsy samples. Therefore, the present findings underscore the need for cautious interpretation of putatively acute immunopathologic events in autopsy samples. Although the subcellular localizations of the MHC molecules in endothelial cells implies the possibility that they could act as APCs in situ, the precise functional implications of the endothelial cell class II MHC expression and whether it is critical to T cell activation and migration into CNS inflammatory sites in acute MS lesions and to subsequent immunopathologic events remain to be established.

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Received October 2, 1998
Revision received January 4, 1999
Accepted January 4, 1999