Multiple Sclerosis: A Role for Astroglia in Active Demyelination Suggested by Class II MHC Expression and Ultrastructural Study

Sunhee C. Lee, M.D., G. R. Wayne Moore, M.D., FRCP (C), George Golenwsky, M.D., and Cedric S. Raine, Ph.D., D.Sc., FRCPATH

Abstract. Central nervous system (CNS) tissue was studied by immunocytochemistry and electron microscopy from three cases of multiple sclerosis (MS) in which evidence of ongoing myelin breakdown could be documented. The study focussed upon the role of glial cells in the pathogenesis of demyelination. In acute MS, demyelination involved the vesicular dissolution of myelin from intact axons and a paucity of fibrillary astrogliosis. Foamy macrophages, many of them probably derived from transformed and recently proliferated microglia, contained recognizable myelin debris and lipid droplets and were abundant throughout the lesions. These cells formed the major phagocytic population and stained positively for class II major histocompatibility complex antigens (HLA-DR; Ia). In acute MS lesions, rounded astrocytes were encountered which possessed membrane-bound compartments enclosing phagocytosed fragments of myelin basic protein-positive debris. Despite the superficial resemblance of these cells to foamy macrophages, the presence of intermediate filaments, glycogen granules and diffuse glial fibrillary acidic protein positivity supported an astroglial identity. Astrocyte processes were involved in myelin removal and invested recently demyelinated axons. Hypertrophic fibrous astrocytes were common in chronic active lesions, were capable of myelin degradation and on occasion, contained myelin debris attached to clathrin-coated pits. These astrocytes were sometimes Ia⁺. Oligodendrocytes were depleted from the center of active lesions but were numerous at the lesion margin, suggesting survival and proliferation. They stained positively for myelin-associated glycoprotein, a marker for immature oligodendrocytes. However, they were invariably Ia⁻. The findings confirm and further support a role for the astrocyte as both an antigen presenting cell and a phagocyte in the CNS during MS.

Key Words: Antigen presentation; Astrocyte; Ia; Major histocompatibility complex II; Microglia; Multiple sclerosis; Oligodendrocyte.

INTRODUCTION

In multiple sclerosis (MS), a demyelinating disease of the human central nervous system (CNS) of unknown etiology, there is abundant evidence that immunologic mechanisms might be involved in lesion pathogenesis since myelin damage is usually associated with CNS inflammation (1). With the aid of monoclonal antibodies, the phenotype of the infiltrating lymphocytes and the nature of certain immune system...
molecules expressed by CNS infiltrates and resident cells have been well documented (2–6). These investigations showed that a number of CNS cells (microglial cells, astrocytes and endothelial cells), expressed immune regulator molecules and were capable of interacting with T cells in vitro in a class I or class II major histocompatibility complex (MHC)-restricted manner (7, 8). The most extensively investigated MHC molecules are MHC II (HLA-DR; Ia) and their presence on cells usually denotes that the cells are capable of antigen presentation to CD4+ T cells (9). Although astrocytes do not normally express Ia, they can be induced to do so by viral infection or by cytokines including interferon gamma (IFNγ) and tumor necrosis factor (TNF) (10–13). The observation of Ia positivity on astrocytes in MS (4) brings into focus their possible functioning as accessory cells of the immune system within the immunologically-privileged CNS. This study on CNS tissue from three patients with acute and chronic progressive MS provides additional evidence that Ia+ astrocytes can function as phagocytes in MS. On the basis of our findings, the relative roles of astrocytes and oligodendrocytes in inflammatory demyelinating lesions are discussed.

MATERIALS AND METHODS

Case Histories

Case 1 (Acute MS): The patient was an 18-year-old woman with a three-month history of acutely developing right hemiplegia, sensory loss and spasticity associated with a white matter hypodensity in the left parieto-occipital region on CT scan. There was the development later of swelling of the ipsilateral cerebral hemisphere. A craniotomy and brain biopsy were done for histologic evaluation of the lesion.

Case 2 (Chronic Active MS): A 33-year-old woman presented seven years earlier had had an acute episode of double vision followed by progressive weakness and ataxia which confined her to a wheelchair within three years. Two years later, she developed incapacitating tremor in the arms, head and neck which became progressively worse. A left cryothalamotomy was performed for relief of the tremor, at which time a CNS biopsy was obtained from the subcortical parietal white matter.

Case 3 (Chronic Active MS): A 31-year-old white woman had an eight-year history of chronic neurologic illness characterized by progressive numbness and weakness of the limbs, gait disturbance and urinary incontinence. These symptoms were followed by tremor, nystagmus and blurred vision. She had been on intermittent steroid treatment and had also received total irradiation of the lymphoid system approximately three years before death. By the end of her life she was wheelchair-bound and developed episodes of seizures and aspiration pneumonia. Her entire CNS was examined postmortem.

Tissue Processing

For routine histology, CNS tissue was fixed in 10% formaldehyde, embedded in paraffin and stained with hematoxylin and eosin (H&E), Bodian’s silver protargol stain for axons and Luxol-fast blue for myelin. Other portions of the specimens were fixed in 2.5% or 5% glutaraldehyde, postfixed in osmic acid, dehydrated through a graded series of ethyl alcohol and embedded in Epon. One micrometer (µm) epoxy sections were obtained and stained with 1% toluidine blue for light microscopy (IM). For electron microscopy (EM), ultrathin sections were made, placed on uncoated grids, contrasted with uranyl acetate and lead citrate and examined in a Siemens 101 electron microscope.

Immunocytochemical staining was performed on 1 µm epoxy sections etched with sodium ethoxide: absolute alcohol (1:3) for 30 minutes (min) to one hour (h). These were then rinsed in absolute alcohol, distilled water and Tris-saline (pH 7.6) for 15 min, twice each. This was followed by incubation in 0.3% H2O2 for five min to block endogenous peroxidatic activity. The rest of the staining was performed according to the peroxidase-anti-peroxidase (PAP) method of Sternberger et al (14). Incubations with primary antibodies were performed over-
Fig. 1. Case 1. Acute MS. The demyelinated white matter contains infiltrating foamy macrophages (large arrows) with recognizable myelin debris (center) and lipid droplets. Myelin debris is scattered throughout the background in the extracellular space. A hypertrophic astrocyte (a) is seen in the upper left portion of the field. Note the surviving oligodendrocytes (asterisks), one of which (upper right) displays vacuolated cytoplasm—perhaps suggestive of incipient degeneration. Demyelinated axons (small arrows) are also evident. Epoxy, 1 μm. ×1,000. Toluidine blue.

night at 4°C. The dilutions used for primary antibodies were 1:25 or 1:50 for anti-gial fibrillary acidic protein (GFAP) and anti-myelin basic protein (MBP); 1:50 for anti-HLA-DR (Ia); and 1:100 for anti-myelin associated glycoprotein (MAG). Anti-GFAP antibody (rabbit) was kindly provided by Dr. J. E. Goldman, Columbia University, NYC, and anti-MAG (GEN S3) by Dr. N. Latov, Columbia University, NYC (15). Anti-MBP antibody (rabbit) was produced in this laboratory. The monoclonal mouse anti-human HLA-DR is an IgG1 which reacts with the alpha chain of the monomorphic HLA class II DR antigen and was purchased from Dako, Denmark. Unlike other anti-Ia antibodies, this particular monoclonal is marketed for use on conventionally-prepared tissue, i.e. formalin-fixed and paraffin-embedded. The secondary (bridge) antibodies were either rabbit anti-mouse (Dako) or swine anti-rabbit (Dako) immunoglobulin, applied at a 1:20 dilution for 30 min. This was followed by incubation with rabbit polyclonal PAP complex (Dako), 1:50, or mouse monoclonal PAP (Dako) 1:100, for one h. Three percent normal serum from the same species as the secondary antibody was administered for 15 min preceding incubation with primary antibody, secondary antibody and PAP to reduce non-specific binding. Diaminobenzidine (DAB) was used as a substrate. For double-labelling, 4 chloro-1-naphthol was used as a second chromagen.

As positive controls for Ia, lymph node and spleen tissue were obtained from an MS patient, fixed in 2.5% glutaraldehyde and post-fixed in osmium tetroxide. One μm epoxy sections of this tissue, as well as four μm sections of formalin-fixed paraffin-embedded lymph node tissue from other autopsy cases, were tested for Ia reactivity. For MBP, GFAP and MAG localization, sections of normal white matter fixed in a similar manner were used. Parallel sections with
the omission of the primary antibody or its replacement by normal serum were used as negative controls.

RESULTS

Light Microscopy

Routine histology on paraffin- and epoxy-embedded tissue confirmed the diagnosis of MS. Active lesions comprised hypercellular demyelinated areas of white matter, the hypercellularity due to foamy macrophages and reactive hypertrophic astrocytes. Oligodendrocytes were common and were sometimes swollen (Fig. 1). Some vessels showed cuffing by small lymphocytes (Fig. 2a). Many macrophages and some astrocytes contained recognizable myelin debris. Oligodendroglia were absent from the center of lesions but occurred at the lesion edge in clusters containing up to five to seven cells per high power field. Overall, axons were relatively well-preserved.

Electron Microscopy

The major cells responsible for the hypercellularity in active lesions were foamy macrophages, the cytoplasm of which was filled with myelin fragments, lamellar inclusions and lipid droplets. The cell outline was rounded with numerous finger-like processes radiating from the surface (Fig. 3). These cells frequently possessed a prominent perinuclear region containing a Golgi complex and associated endoplasmic vesicles. Equally common were centrioles and accompanying microtubules (mitotic spindles). Along the cell surface and filopodia, there were many coated pits and vesicles. Other ultrastructural features consisted of focal membrane densities along the plasmalemma, abundant haphazardly-arranged filaments and unusual cucumber-shaped inclusion bodies with periodic striations, features common to both monocyctic macrophages and microglial cells.

In the case of acute MS (Case 1), the acuteness of the disease process was represented by myelin vesiculation occurring around intact axons and abundant extracellular myelin (Fig. 4). This type of myelin degeneration, sometimes attributed to humoral mediators (16), was invariably associated with astrocytic processes interposed between the axon and its degenerating myelin (Figs. 4–8). In chronic active lesions, widespread myelin vesiculation was not encountered but instead, ongoing demyelination involved the phagocytosis of myelin droplets or tubular myelin arrays into phagolysosomes. Occasionally, myelin droplets were attached to clathrin-coated pits on the cell surface (Fig. 7), appearances described previously in MS (17, 18) and in its experimental model, experimental autoimmune encephalomyelitis (EAE) (19).

A subpopulation of astrocytes within the center of the acute MS lesion exhibited rounded contours with few cytoplasmic processes, undigested myelin debris and some lipid granules (Fig. 8a). While these cells superficially resembled foamy macrophages, they contained bundles of GFAP+ glial filaments and glycogen granules, thereby allowing us to identify them as astrocytes (Fig. 8a, b). Abutting these rounded astrocytes were numerous myelin fragments, apparently in the process of being internalized into phagolysosomes (Fig. 8a).

In all lesions studied, the most frequently encountered type of astrocyte was a hypertrophic cell with a characteristic pale cytoplasm containing vacuoles (mostly dilated endoplasmic reticulum) and dilated perinuclear cisternae. These cells had numerous filament-filled processes emanating from the cell soma. The nuclei (sometimes the cells were multinucleated) often showed granular and filamentous inclusion material described previously in MS and other conditions (20, 21). Viral particles were not seen. Rarely, small round astrocytes (resembling oligodendroglia at low
Fig. 2. a. Case 1. There is a uniform morphology of perivascular infiltrating cells. Foamy macrophages are present within the surrounding parenchyma which is almost devoid of myelin but still contains a few myelin droplets. Epoxy, 1μm. ×750. Toluidine blue. b. Parenchymal
Fig. 3. Case 1. Two foamy macrophages (microglial cells) in an acute MS lesion are filled with recognizable myelin debris. Demyelinated axons (A) are nearby. ×5,100.

magnification) containing centrioles and dense glial filaments were seen. The extracellular space of the acute MS lesion showed edema and lacked the dense glial meshwork seen in chronic active lesions. Occasional axonal spheroids were present in all lesions and sometimes reached 40–50 μm in diameter.

Oligodendroglial cells were infrequent within the lesion but were more numerous towards the plaque margin where demyelination was incomplete or where remyelination was in progress. Oligodendrocytes exhibited the usual cytoplasmic components and configuration although the cytoplasm was somewhat more condensed due to increased numbers of organelles including microtubules, ribosomes, rough endoplasmic reticulum and Golgi complex. Some cytoplasmic vacuolization occurred and partial investment of the oligodendroglial cell body by one to several layers of myelin was common. The morphologic features of oligodendrocytes in these areas have been discussed elsewhere (22).

A few small lymphocytes occurred within the parenchyma, especially in the acute MS lesion. Lymphocytes were seen most consistently within the Virchow-Robin space where they formed a row of morphologically homogeneous cells (Fig. 2c).

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macrophages stain positively, but small lymphocytes around the vessel stand out as unstained ghosts (arrows). Since B cells and activated T cells also bear surface Ia, the unstained lymphocytes are presumably unactivated T cells. Serial 1 μm epoxy section reacted with anti-Ia (HLA-DR). ×750. c. The uniform cytology of small lymphocytes within the Virchow-Robin space is apparent. The vessel lumen is above. ×4,700.
Fig. 4. a. Case 1. A demyelinated lesion from the case of acute MS shows a cross-sectioned axon (A) surrounded by a myelin sheath (large arrows) which has undergone vesicular dissolution. An astrocytic process lies in the periaxonal space closely applied to the axolemma (small arrows). Other demyelinated axons are present (asterisks). Microglial cells contain myelin debris and lipid. Note the abundant vesicular myelin debris (m) and the lack of dense gliosis in the background. ×3,400. b. Detail from a similar fiber to show a small astroglial cell process (note glial filaments) closely applied to a demyelinated axon (below) with vesicular myelin debris above. ×50,000.

Immunocytochemistry

Within the lesion, the cytoplasmic membrane of foamy macrophages stained strongly for Ia (Fig. 9a). The density of the staining varied, probably due to the presence of overlapping cell processes. Profiles identical to Ia− small lymphocytes (probably T cells) in close contact with Ia+ macrophages were frequently found,
perhaps the morphologic equivalent of antigen presentation (Fig. 9b). Occasional Ia⁺ astrocytes were seen with linear Ia positivity along the plasmalemma or with diffuse cytoplasmic staining (Fig. 8c, d). The staining was observed both on the rounded astrocytes resembling foamy macrophages (vide supra) and on typical fibrous astrocytes. The former cell type was also positive for GFAP in serial sections (Fig. 8b). On the other hand, oligodendroglial cells never stained positively for Ia (Fig. 9a). These cells, which were often present in clusters, stained intensely with anti-MAG antibody and in such preparations, macrophages were MAG⁻⁻ (Fig. 9c). Intact
myelin, myelin debris in the extracellular space, as well as myelin debris within microphages and astrocytes, stained positively with anti-MBP (Fig. 8b).

The cells in the perivascular cuffs consisted of a few Ia$^+$ foamy macrophages and Ia$^-$ lymphocytes (Fig. 2b). The latter were presumably T cells since hematogenous cells of monocyte and B cell lineage normally express surface Ia. Other T cell activation molecules, such as anti-Tac (anti-interleukin-2 receptor), were tested but the results were inconclusive. Endothelial cells failed to stain for Ia. In case 3, there was some linear Ia-positivity along some vessel walls which at higher magnification was found to be due to Ia$^+$ pericytes and perivascular macrophages.

Within the lymph node tissue (studied as a positive control for Ia), Ia positivity was observed as anticipated on sinusoidal macrophages and dendritic reticulum cells in paracortical areas. With omission of the primary antibody, there was complete lack of Ia staining.

DISCUSSION

While it is generally accepted that multiple sclerosis (MS) is a disorder with an immunologic background, its etiology and pathogenesis remain largely unresolved. Most hypotheses implicate mechanisms by which central myelin is seen by cells of the immune system due to a breach in the blood-brain barrier, perhaps the result of a viral infection occurring during childhood (18). The proposed initial stage of CNS disease is claimed to involve the entry of myelin-sensitized immune cells from the circulation into the CNS. Considerations of autoimmunity in this process have emanated largely from work on animals with experimental allergic encephalomyelitis (EAE) in which T cells sensitized to MBP produce a similar disease after adoptive transfer (23, 24). Interest in the nature and pathogenetic implications of the inflammatory infiltrates in MS burgeoned in the early 1980's when monoclonal markers for lymphocyte subsets and immune system molecules became available. The ensuing studies on MS were performed mostly on frozen material where tissue morphology was relatively poorly preserved. Observations emerging from these studies from a number of laboratories suggested that in the MS lesion, microglia, astrocytes and endothelial cells were chief among the resident cells of the CNS expressing Ia and that these cells might play a role in developing and perpetuating the disease process.
Fig. 7. a. Case 2. A large process of a fibrous astrocyte is shown from an actively demyelinating lesion. Some myelin fragments are being internalized following their attachment to a coated pit (arrows) to the upper right. Note the glial filaments and glycogen granules in the cytoplasm. Tubular myelin fragments are being drawn into membrane-bound compartments within the cell (arrowheads). ×27,000. b. Detail to show myelin droplet attached to the clathrin-coated pit (arrows) and myelin debris being internalized into a phagosome (center) (arrowhead). ×35,000.
Fig. 9. Case 1. In a section reacted for Ia, microglial cells (foamy macrophages) display abundant surface reactivity (shown here in tangential section) throughout the lesion, whereas surviving oligodendrocytes are focally clustered (lower right) at the lesion margin and are negative for Ia (arrows). ×750. b. A 1 μm epoxy section reacted with anti-HLA-DR (Ia) shows an Ia+ microglial cell in close contact with a small lymphocyte. This appearance may be representative of antigen presentation in situ. The relatively light staining for Ia (compared to Fig. 9a) is due to the section being somewhat thinner and the cell membrane being sectioned perpendicularly, as opposed to tangentially. ×750. c. An adjacent section reacted with anti-MAG antibody shows the oligodendrocytes to be MAG+ (arrows) and the microglial cells, MAG− (asterisks). ×750.

(2, 4). The predominance of the MHC II-restricted T helper/inducer subset (CD4) in active MS lesions (2) is also in accord with this hypothesis. Studies on brain microvessel endothelia and astrocytes in vitro showing induction of Ia expression by cytokines and the ability of these cells to present antigen (MBP) to sensitized T helper cells, lend additional support to the thesis (7, 8, 12).

Fig. 8. a. Case 1. The periphery of a rounded fibrous astrocyte engaged in myelin phagocytosis is shown within an area of active demyelination. Note the densely-packed astroglial filaments (small arrows) throughout the cytoplasm and the coated pits (large arrows) and membrane densities along the cell surface. Phagosomes containing vesicular myelin debris and myelin droplets are common (asterisks). ×35,000. b. Occasionally, rounded astrocytes (a) resembling microglia were seen to be apparently engaged in myelin phagocytosis. Note the diffuse cytoplasmic GFAP positivity (brown) confirming the astroglial identity of the cell. Elsewhere, GFAP+ astroglial cell processes are apparent (arrows). MBP+ myelin debris (blue-gray) is scattered in the background but is also seen along the cell surface and within the cytoplasm. One micron epoxy section. Double immunostaining, GFAP (DAB as substrate) and MBP (4 chloro-1-naphthol as substrate). ×1,200. c. Two rounded astrocytes which showed GFAP positivity in serial sections, stain positively for cytoplasmic and membrane Ia. A microglial cell with dense surface staining lies to the right. One micron epoxy section. ×750. d. Occasionally, processes of fibrous astrocytes possessed linear surface staining for Ia (center). Note also the Ia+ microglial cells. One micron epoxy section. ×750.
The present application of high resolution immunocytochemistry and ultrastructure has provided further supportive evidence in vivo that astrocytes might actively participate in the process of demyelination by acting as myelin phagocytes and antigen presenting cells (APC) to T cells. The observed direct participation of astrocytes in the removal of intact myelin from axons confirms findings from previous cases of active MS (25). Another intriguing feature was the interposition of astroglial processes between the axon and its myelin sheath undergoing vesicular dissolution. This feature, with layers of myelin lying loose within the extracellular space, has been previously suggested to represent antibody-, complement-, or protease-mediated myelin injury (16, 26). That such astroglial investment represents a non-specific phenomenon related to imminent gliosis is unlikely since a similar investment of demyelinated axons was not seen in areas of advanced demyelination. Recent studies on the production by astrocytes of a tissue necrosis factor (TNF)-like substance cytotoxic for oligodendroglia in vitro (27), the ability of TNF to lyse oligodendrocytes and myelin in organotypic CNS cultures (28), and the documented increases in astrocytic lysosomal activity in MS (29) might be relevant in this regard.

The observation of rounded astrocytes morphologically resembling foamy macrophages (some of which were undoubtedly transformed microglial cells) engaged in myelin phagocytosis, might indicate that astrocytes are capable of undergoing transformation. A similar transformation of elements of astrocytic lineage into macrophages has been described in organotypic cultures of mouse spinal cord (30). Astrocytes have not only been demonstrated here to be involved in phagocytosis of myelin into large membrane-bound compartments but also as minute fragments attached to clathrin-coated pits. The latter process which has been dubbed "receptor-mediated phagocytosis of myelin" (19), has been suggested to represent the pathway by which myelin antigen might be processed and presented to lymphocytes, thus elevating the astrocyte to the level of an antigen-presenting cell (18). The finding of myelin undergoing internalization via attachment to coated pits is apparently relatively uncommon, perhaps related to the infrequency or the transience of the phenomenon. In the context of the described MHC II molecule expression on astrocytes in vitro and in vivo, our findings further implicate the astrocyte as an accessory immune cell within the CNS.

The significance of the Ia+ astrocytes in this study, especially in the presence of the much more frequent Ia+ microphages, is not clear. Whether resident brain cells can express class II MHC in situ remains controversial, particularly in regard to the double-labelling of cells with GFAP and Ia. While overlapping Ia-GFAP staining on MS tissue was obtained by Traugott el al (4), Hayashi el al reported a non-overlapping pattern for these markers (31). Another study on human CNS tissue revealed that unlike the situation in vitro, GFAP+ cells did not stain positively for MHC antigens (neither class I nor class II) in tissue sections (32). The latter study was on a human temporal lobectomy specimen and perhaps the absence of Ia expression by astrocytes in this case was due to the lack of an immunologic response within the tissue. While Ia+ microglia are seen in a variety of neurologic diseases (33), as well as in normal brain tissue (albeit in small numbers), and are claimed to be the major cell type expressing Ia in human brain tissue (34), Ia+ astrocytes have only been documented in MS. The recently reported correlation between Ia inducibility on astrocytes and susceptibility to EAE in rats and mice during autoimmune demyelination (35) may have some relevance to the present findings.

While oligodendroglial cells in these lesions apparently survived initially and displayed evidence of proliferation, their eventual demise was underscored by their
disappearance from the lesion center. At no time during this process did oligodendrocytes express Ia, although their ability to express MAG, a marker for immature oligodendrocytes (36) and their occurrence in numbers greater than those seen in normal white matter, would support their recent derivation at the lesion perimeter. Therefore, oligodendrocytes appeared to play no immunomodulatory role during inflammatory demyelination.

In summary, we have reported further evidence of astrocytic involvement during active demyelination in MS. Moreover, we have shown that conventionally-processed tissue fixed in glutaraldehyde and osmium (fixatives usually detrimental to most membrane antigens) for EM may prove useful in the demonstration of certain immune system molecules with the bonus of well-preserved morphology.

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