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# Sequence of Tissue Responses in the Early Stages of Experimental Allergic Encephalomyelitis (EAE): Immunohistochemical, Light Microscopic, and Ultrastructural Observations in the Spinal Cord

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**ABSTRACT** Experimental allergic encephalomyelitis (EAE) was induced in adult Lewis rats with purified guinea pig CNS myelin and Freund's adjuvant. As soon as the very earliest clinical signs appeared the animals were perfused with fixatives and the spinal cord analyzed by electron microscopy, silver methods, and immunocytochemistry.

Our findings suggest that in the early stages of EAE a sequence of events can be traced, although these events frequently overlap. The earliest morphological change appears to be astrocytic edema in both the cell body and processes. Increased amounts of glycogen particles and dispersion of glial filaments are prominent. These changes seem to occur just prior to the time when inflammatory cells begin to penetrate the capillary walls. Invasion of the neuropil mainly by macrophages and lymphocytes closely follows. Both macrophages and microglia seem to participate in phagocytosis of oligodendrocytes and myelin. Demyelination, however, is not a prominent feature at this early stage.

## INTRODUCTION

In a previous publication we examined the alterations of glial fibrillary acidic protein (GFAP) in astrocytes and the accompanying ultrastructural modifications at the onset of EAE (Eng et al., 1989). Since previous investigations by Smith et al. (1983) confirmed later by Aquino et al. (1988a,b) have shown that GFAP content did not increase during the first 13–18 days of development of EAE, we suggested that the increase in GFAP immunoreactivity observed by us was due to the exposure of more antigenic sites against GFAP brought about by the dissociation and dispersion of glial filaments within the edematous astrocytic cytoplasm and processes. In this report we suggest a sequence of tissue responses that take place during the course of the early stages of EAE.

## MATERIAL AND METHODS

### Animals

Adult male Lewis rats, about 250–300 g (purchased from Charles River, Wilmington, MA), were employed in this study.

### Immunization Procedures

On day 0 the rats were injected in the flanks of both hind legs with a total of 1 mg lyophilized purified guinea

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Fig. 1. EAE, lumbar spinal cord. A: Hypertrophic and intensely GFAP-immunoreactive perivascular astrocyte (Andriezen type, open arrow) in the white matter abuts upon the basement membrane of a capillary (ca). An inflammatory infiltrate of "mononuclear" cells has dissected the basement membrane (arrow, compare with Fig. 5). Section immunostained for GFAP (Sternberger method) and counterstained with hematoxylin. B: Control animal. Very small, "star"-shaped microglial cell, typical of the white matter of the spinal cord. Silver method for microglial cells. C: Longitudinal section of a blood vessel surrounded by an inflammatory cuff of "mononuclear" cells and a few

multinucleated macrophages (arrow). The MBP-immunoreactive fibers have normal appearance. Section immunostained for MBP and counterstained with cresyl violet. D: En masse macrophagic invasion of the neuropil. The macrophages show various sizes and shapes; some of them are seen in juxtavascular location around a capillary (ca), suggesting monocytic and/or pericytic origin; and others, in the neuropil, are suggestive of reactive microglia (arrowhead). Silver method, appropriate for the impregnation of macrophages, microglial cells, and pericytes. A,  $\times 536$ ; B,  $\times 2,430$ ; C,  $\times 800$ ; D,  $\times 400$ .

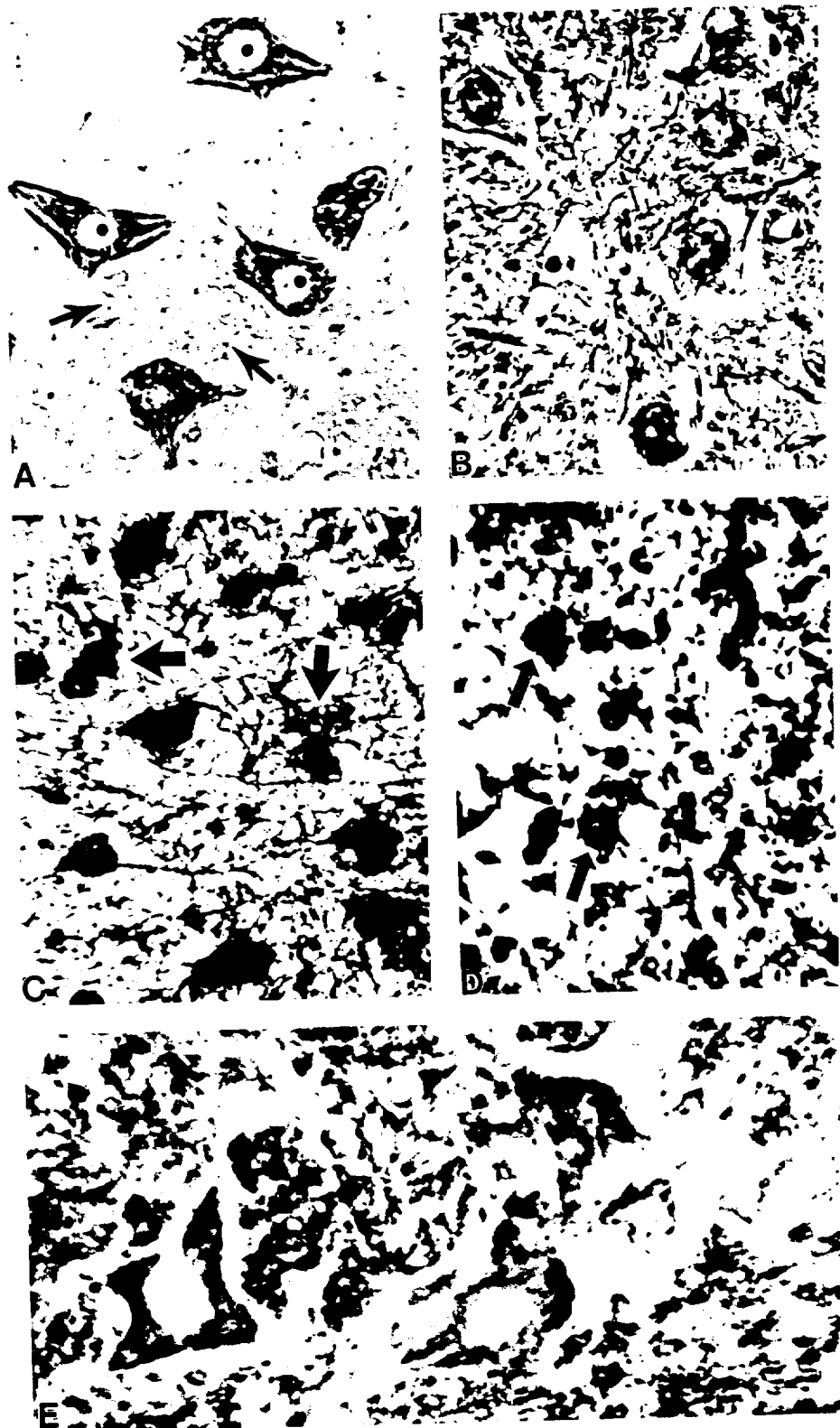


Fig. 2. EAE, lumbar spinal cord. A: Control animal. Motoneurons are surrounded by myelinated fibers (arrows) faintly stained with anti-MBP. B: Control animal. Motoneurons surrounded by a mesh of nerve fibers. C: Shrunken motoneurons, intensely argentophilic, in EAE animal. Some of the neurons reveal severe structural deterioration (arrows). In the center of the photograph a marked fibrogliosis is apparent. "Triple" silver impregnation method counterstained with gold chloride. D: Area of the gray matter invaded by phagocytic cells,

some of them multinucleated (arrows). Silver impregnation method for macrophages and microglia. E: Motoneurons with increased and diffuse basophilia and severe degenerative changes. The nerve cells are surrounded by nerve fibers in the process of desintegration of their myelin sheaths. Section immunostained for MBP and counterstained with cresyl violet. C-E: Photographs belong to sections taken from one animal in the same area of the spinal cord. A,  $\times 480$ ; B,  $\times 800$ ; C,  $\times 400$ ; D,  $\times 2,300$ ; E,  $\times 1,000$ .

pig CNS myelin in 0.25 ml Freund's complete adjuvant (Difco, Detroit, MI) to which was added 3 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco). When the first clinical signs of EAE became apparent (weight loss and tremor; 10–12 days) the rats were anesthetized with methoxyflurane and processed for immunohistochemical and structural studies as described below.

Control rats (FAC) were injected with Freund's adjuvant containing H37Ra *Mycobacterium tuberculosis* alone (Smith et al., 1983) and were processed together with the EAE rats.

### Light Microscopic Procedures

#### Immunohistochemistry

The rats were perfused with 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.4. Longitudinal samples of the spinal cord were infiltrated with paraffin according to standard procedures and sectioned at 10  $\mu$ m thickness. They were subsequently treated with antibodies to GFAP (Eng and Rubinstein, 1978) or myelin basic protein (MBP) and immunostained according to the peroxidase-antiperoxidase method developed by Sternberger (1986). Counterstaining with cresyl violet or hematoxylin was applied on some of the sections.

#### Silver impregnation techniques

The animals were perfused with 4% paraformaldehyde in distilled water containing 2% ammonium bromide. Samples of the spinal cord were cut with a freezing microtome at 20–30  $\mu$ m thickness and were processed for the visualization of macrophages/microglial cells, neuroglial components or neurons according to the methods originally developed by del Rio Hortega and described elsewhere (D'Amelio, 1981).

In some instances, for comparative purposes, the same sample of spinal cord of individual animals was used for both silver impregnation techniques and immunocytochemical procedures (MBP and GFAP).

### Electron Microscopic Procedures

The rats were perfused with 1% paraformaldehyde and 1.5% glutaraldehyde in 0.12 M phosphate buffer, pH 7.4. The samples were postfixed in 2% osmium tetroxide in phosphate buffer at pH 7.4, dehydrated by standard methods, and embedded in Quetol 651 epoxy resin. The blocks were sectioned at 600 Å, stained with uranyl acetate-lead citrate, mounted on copper grids, and observed in a Philips 300 electron microscope.

## RESULTS

The description of our observations reflect the sequence of events that we believe take place during the early stages of EAE.

### Astrocytic Alterations

The main changes found in the astrocytic population were described in a previous publication (Eng et al., 1989). Briefly, light microscopic preparations immunostained with anti-GFAP revealed hypertrophy of astrocytes and increased immunoreactivity for GFAP (Fig. 1A). These characteristics were also obvious in the gray matter that exhibited a marked proliferation of glial fibers visible in between the motoneurons (Fig. 2C). Distinctive ultrastructural features of astrocytes were a considerable "watery" swelling of cytoplasm and processes accompanied by widespread dispersion of the glial filaments and increased amounts of glycogen particles (Fig. 3A). These changes involved the most slender astrocytic extensions including the foot processes impinging upon the walls of blood vessels and capillaries. The latter appeared frequently without obvious morphological alterations (Fig. 4). In addition to these changes some areas showed what appeared to be glial filaments forming tight bundles, intensely osmiophilic, somewhat reminiscent of Rosenthal fibers (Fig. 3B,C).

### Inflammatory Infiltrates

The inflammatory reaction showed perivascular and diffuse patterns. The perivascular inflammatory cuffs were mainly composed of lymphocytes and macrophages that in both light microscopic and ultrastructural images appeared to dissect the vascular walls in between the endothelium and basement membrane (Fig. 1A,C, 5, 6). When stained with silver impregnation methods the macrophages, seen in perivascular situation or independent of vascular structures, showed a broad range of morphological variations such as large multinucleated elements, cells with ameboid features, rod-shaped components, and others with characteristics reminiscent of reactive microglial cells (Fig. 1D). These cells were also seen in some areas of the gray matter lying among neuronal elements, which showed profound alterations that included increased and diffuse cytoplasmic basophilia, irregularity of cell contours, retraction of soma and processes, increased metalophilia of the filamentous components, and complete loss of structure (Fig. 2C,D,E). On occasion, macrophages and cells identified as activated microglia were seen in the neuropil enclosing oligodendrocytes and myelin sheaths or containing cellular debris (Fig. 7). In other instances, perivascular microglial cells surrounded by astrocytic expansions appeared to become detached from the vascular walls as if derived from pericytic precursors (Barón and Gallego, 1972) (see Fig. 5).

### Myelin Alterations

Demyelination was not a prominent feature at this stage of development of EAE, although some myelin breakdown was revealed in light microscopic images by



Fig. 3. EAE, lumbar spinal cord. A: Markedly swollen astrocytic process (ap) containing dispersed and dissociated glial filaments (arrows). Particulate glycogen is also apparent. B: Astrocytic expansion adjacent to an oligodendrocyte (O) contains glial filaments (gf) and osmiophilic material that is interpreted as tight bundles of glial

filaments somewhat reminiscent of Rosenthal fibers (arrow). C: An astrocytic process that shows a gap junction (arrowhead) displays both glial filaments and osmiophilic material (arrow). The intracellular location is apparent. A,  $\times 25,650$ ; B, C,  $\times 31,250$ .



Fig. 4. EAE, lumbar spinal cord. Swollen astrocytic foot processes (fp) containing glycogen particles abutting upon the intact basement membrane of a capillary (arrows). E, cytoplasmic expansion of an endothelial cell; RBC, red blood cell.  $\times 14,175$ .

means of antiserum against myelin basic protein (MBP) and by electron microscopy. The areas of myelin disruption, as seen in light microscopic images, were frequently accompanied by macrophagic invasion and showed proliferation of glial fibers. This picture was particularly evident, for example, in the myelinated fibers traversing areas of the gray matter in which the neuronal lesions were more intense (Fig. 2E). In ultrastructural images it was possible to observe regions with denuded normal axons occasionally surrounded by densely osmiophilic material (Fig. 8).

## DISCUSSION

The immunohistochemical and morphological examination of the spinal cord in the early stages of EAE revealed lesions in various phases of development that overlap among and within the experimental animals. One of those phases involve the observations made in our first study (Eng et al., 1989). We then suggested that the increase in blood-brain barrier (BBB) permeability was responsible for the astrocytic edema with the resulting dissociation and dispersion of the glial filaments. The transport of fluid (mainly water and electrolytes) without visible damage to the walls of capillaries and blood vessels may be due to a "partial" breakdown of the BBB (see Lee, 1982; Miquel et al., 1982) elicited by

the antigenic aggression. Whether fluid extravasation precedes or is simultaneous with mononuclear cell invasion through the vascular walls cannot be thoroughly assessed in this study. However, the electron lucency and the lack of proteinaceous material in the astrocytic edema lead us to conclude that the first assumption is more tenable. Furthermore, quantitative determinations of BBB permeability and histological analysis during the course of EAE in Lewis rats showed that BBB permeability preceded the appearance of perivascular cellular infiltrates (Juhler et al., 1984). The finding of other morphological indicators of metabolic disturbances in the astroglial cytoplasm such as the presence of what appears to be the arrangement of glial filaments in tight, osmiophilic bundles, may represent a subsequent reactive response of astrocytes to the overloading with edema fluid. Although the first observations of these bodies suggested to us that they might represent fibrin, the fact that they are frequently seen occupying the same space as glial filaments or lying close to them speak in favor of their intracellular location (see Fig. 3B,C). Thus, it appears reasonable to correlate the structural changes of astroglia with the increased permeability of the vascular walls. Astroglial alterations would in turn play a significant role in the further disruption of the BBB and hence in the facilitation of cellular migration of inflammatory components toward the neuropil.



Fig. 5. EAE, lumbar spinal cord. Lymphocytes (arrowheads) and fragments of unidentified cells have broken through the basement membrane (arrows) of a capillary (ca). The swollen foot process (fp) of an astrocyte (A) containing glycogen particles impinges upon the disrupted basement membrane. A normal oligodendrocyte (O) is seen

in close apposition to normal axons. A perivascular microglial cell (M) is separated from the basement membrane of the capillary by an astrocytic expansion (a), suggesting a pericytic origin. E, endothelial cell nucleus.  $\times 5,600$ .



Fig. 6. EAE, lumbar spinal cord. A: A macrophage has "dissected" the basement membrane of a capillary (ca). B: Perivascular lymphocyte (Ly) and "mononuclear" cell (macrophage?) (Ma). The cells have broken through the basement membrane (arrows) of a capillary (ca) that is surrounded by some normal myelinated axons and some with irregular swelling of the myelin lamellae. A,  $\times 11,990$ ; B,  $\times 5,600$ .





Fig. 7. EAE, lumbar spinal cord. A: A phagocytic cell is seen completely enveloping another cell with a bilobed nucleus identified as an oligodendrocyte (O) and also closely apposed to myelinated axons (arrows). The process of the macrophage adjacent to an axon oriented longitudinally (star) appears to dissect the myelin sheath of the latter. A portion of the disrupted myelin lamellae is seen bound to the

cytoplasmic membrane of the oligodendrocyte (triangle, inset). B: Microglial cell (M) with a large cytoplasm occupied by a prominent phagosome, which is interpreted as a partially digested cell (oligodendrocyte?). Adjoining the phagosome there are lamellated myelin bodies (myelin debris?) (arrowheads and inset). A:  $\times 7,970$ ; inset,  $\times 18,500$ ; B:  $\times 7,970$ ; inset,  $\times 20,250$ .

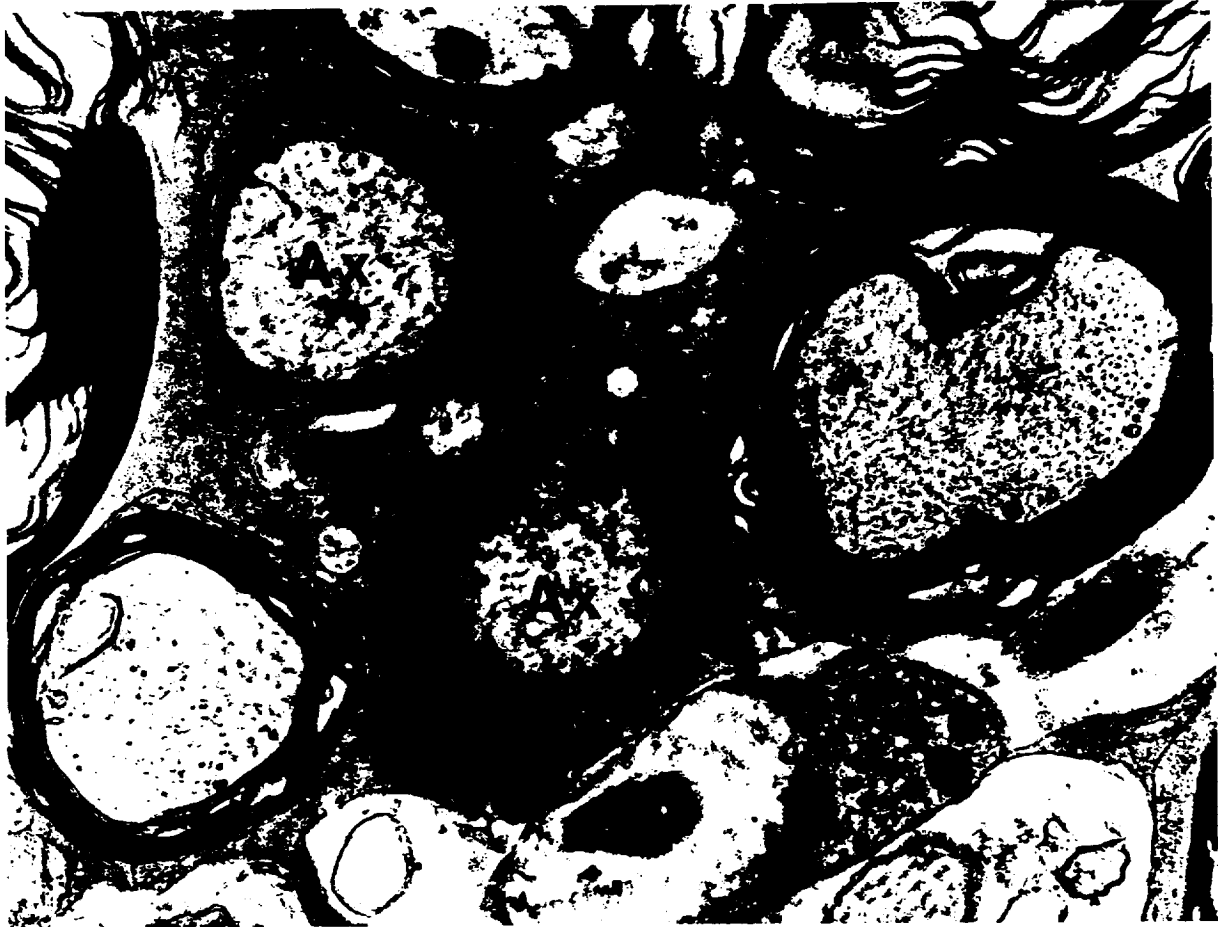


Fig. 8. EAE, lumbar spinal cord. Naked axons (Ax) are surrounded by osmiophilic material and by other axons with irregular swelling of the myelin lamellae. The axon in the upper left corner still retains a vestige of its myelin sheath.  $\times 21,875$ .

As brought out in our description, the dominant component of the cellular infiltrates are macrophages which can be found both in the perivascular regions and in the neuropil. The designation of "macrophage" refers to the morphological appearance of a cell endowed with phagocytic activity and does not assume any specific cell source. Different origins such as monocytes, pericytes, and microglial cells have been proposed in the past (Rio Hortega, 1932; Cancilla et al., 1972; Oehmichen, 1975; Imamoto and Leblond, 1978; Polak et al., 1982). In our opinion it is conceivable that these cell types might be complementary in function and band together in response to the antigenic insult. In some instances, however, it is possible to discriminate between a microglial cell with phagocytic activity and a macrophage of indeterminate origin. Microglial cells usually display some distinctive features such as an elongated nucleus with "patchy" peripheral condensation of the chromatin, sparse single and narrow strands of endoplasmic reticulum, and presence of osmiophilic dense bodies (Polak et al., 1982) (see Fig. 7). Recent studies have demonstrated the microglial nature of proliferating macro-

phages (Graeber et al., 1988; Streit and Kreutzberg, 1988; see also Streit et al., 1988). These modern investigations support the original contention by del Rio Hortega, who was the first to trace the evolution of changes sustained by microglial cells on their way to macrophage transformation (Rio Hortega, 1932). In an earlier radioautographic study of EAE in the rat (Kosunen et al., 1963), it has been suggested that the mononuclear cells of the perivascular infiltrates divide one or more times during the process of migration to the neuropil, where they take on the morphology of activated microglia. Moreover, microglial cell-derived macrophages can act as antigen presenting cells (APC) as they bear Ia antigens, which would enhance lymphocyte (T cells) recruitment (Fontana et al., 1987; Streit et al., 1988; see also Konno et al., 1989). In this way activated T cells would be able to secrete factors such as interferon- $\gamma$  (INF- $\gamma$ ) and contribute to the promotion of macrophagic activity involved in tissue injury (Fontana et al., 1987; Sobel et al., 1987). In this connection, Trotter and Smith (1984) have suggested that in cell-mediated demyelinating diseases myelin phagocytosis

may be initiated by an antibody bridge between the myelin sheath and the Fc receptor of the phagocytic cell (see also, Smith et al., 1989). A functional cooperation between microglial cells and astrocytes was suggested by Konno et al. (1989), who found clusters of Ia-immunoreactive microglial cells surrounded by astrocytes with increased GFAP-immunoreactivity in the gray matter of spinal cord of EAE Lewis rats.

Myelin breakdown or demyelination, although not dominant features in our observations at this early stage, were more easily visualized in the myelinated fibers running along some circumscribed regions of the gray matter than in the white matter. The difference in MBP immunoreactivity between the control animals (slight) and the EAE animals (very intense) is most likely due to the exposure of more epitopes at the onset of myelin breakdown. These regions of the gray matter contained abnormal neurons with alterations comparable to those described in the motoneurons of the mouse after axotomy (Lison, 1962; see also Cragg, 1970) and were spatially related to the invading phagocytic cells. In this connection it is interesting to note that in acute EAE Lewis rats, Konno et al. (1989) observed large numbers of Ia-immunoreactive microglial cells concomitant with extensive degeneration of axon terminals in the gray matter of the spinal cord where microglial cells are by far more numerous than in the white matter (Rio Hortega, 1921). We share the view that the appearance of demyelinated fields is directly related to phagocytic activity targeting both oligodendrocytes and myelin sheaths. Phagocytosis of oligodendrocytes has been observed at later stages (14–16 days) of EAE (Field and Raine, 1966). It has also been documented that the severity of demyelination differs according to the animal species (see Roizin et al., 1982, for review).

In conclusion, we believe that the pathogenesis of the early stages of EAE involve the following events: 1) edema induced by partial breakdown of BBB that affect predominantly astrocytes which exhibit increased GFAP immunoreactivity due to dissociation of glial filaments and consequent exposure of more antigenic sites; 2) as a consequence of further disruption of the BBB, "activated" lymphocytes and blood-borne macrophages penetrate the vascular endothelium "en route" to the neuropil; 3) myelin breakdown is initiated by macrophagic activity upon oligodendroglia and myelin sheaths. Sources of macrophages in the tissue are considered to be multiple (pericytes, blood-borne, microglia); 4) progression of the process would be enhanced by the ability of microglial cells/macrophages to express Ia-antigens induced by the secretion of factors (INF- $\gamma$ ) by "activated" lymphocytes.

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