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A new model for multiple sclerosis: Chronic experimental allergic encephalomyelitis induced by immunization with cerebral endothelial cell membrane*

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Summary. Multiple sclerosis is considered to be an autoimmune demyelinating disease of the central nervous system. Damage to the blood-brain barrier, of which endothelial cells are the main constituent, occurs in multiple sclerosis, probably due to immunological mechanisms. We report here the results of immune-mediated damage to these cells, produced by immunizing guinea pigs with an endothelial cell membrane fraction. The fraction was obtained from cerebral endothelial cells grown in vitro and was free from myelin basic protein. The immunized animals developed a chronic neurological illness with evidence of delayed hypersensitivity to the cell membrane fraction but not to myelin antigens. Histological examination of the brain in the acute stage showed mononuclear cell infiltrates around blood vessels, while in the chronic phase large areas of demyelination, especially in the periventricular region, were present. This bore a striking similarity to the brain in multiple sclerosis. This may prove to be a useful new animal model for the investigation of the human demyelinating disease.

Key words: Multiple sclerosis – Cerebral endothelial cells – Experimental allergie encephalomyelitis

The blood-brain barrier (BBB) is mainly composed of brain endothelial cells that line the capillary of the blood vessels. These cells possess a significantly high metabolic capability. It has been suggested that a cell membrane of cultured endothelial cells, derived from human umbilical cord, may possess a cell-specific antigenicity which reacts with monoclonal antibodies and that the brain endothelial cell reacts with the membrane impermeable reagent (Drewes and Lidinsky 1980; Park et al. 1985).

Under normal conditions, brain endothelial cells have only a few pinocytic vesicles. In multiple sclerosis (MS), the BBB is often disrupted, leading to an increased vascular permeability and the appearance on electron microscopy of large numbers of pinocytic vesicles in cerebral venules (Broman 1964; Brown 1978). A variety of immunological mechanisms, directed at the endothelial and smooth muscle cells of the blood vessels, or at antigens in the surrounding parenchyma, could cause this disruption. The incidence of antibodies to smooth muscle has indeed already been reported to be increased in patients with MS (McMillan et al. 1980).

Our own studies and that of others on the brain lesions associated with the Forssman carotid artery syndrome led us to investigate the effect of injury to the BBB caused by immunizing guinea pigs with an endothelial cell membrane fraction (Tsukada et al. 1986a, b).

Materials and methods

Cultured brain endothelial cells

Capillaries were separated from the homogenized brain of 7-day-old Osborne-Mendel rats by homogenization and centrifugation using a discontinuous sucrose gradient (1.0/1.5 M) under sterile conditions. They were carefully collected, and washed with Simm's balanced salt solution. Endothelial cells were derived from the isolated capillaries by trypsin-collagenase digestion, using an established method (Mrsülja et al. 1976; Spatz et al. 1980), and grown in medium 199 (Bibco, Long Island, NY) supplemented with 30% heat-inactivated fetal bovine serum, penicillin-streptomycin-fungizone and endothelial cell supplement (Collaborative Research, Inc., Lexington, Mass) as monolayers. This procedure is more strigent in respect to contamination by non-endothelial cells than the other methods for isolation

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of rat endothelial cells. Monolayer cultures of endothelial cells were washed three times with cold Hank's balanced salt solution (HBSS). The antigen used for this study was the plasma membrane prepared by ultracentrifugation according to the method of Schimmel et al. (1973). The cell pellet was suspended in 0.25 M sucrose at pH 7.4, and then homogenized in a Dounce homogenizer. The homogenate was centrifuged at 1,700 *g* for 10 min: the supernate was removed and centrifuged at 33,000 *g* for min before use in the experiment.

Confirmation of endothelial cells and plasma membrane fractions

The presence of endothelial cells in vitro was confirmed by staining cells attached to coverslips for the enzymes of alkaline phosphatase and γ-glutamyl transpeptidase characteristic of endothelial cells (DeBault and Cancilla 1980). Enzyme activity for alkaline phosphatase or γ-glutamyl transpeptidase was measured using the previously described method for assessment of enzyme activity in brain homogenate, capillary, and membrane fractions (Orlowski et al. 1974). To confirm the presence of cell membranes from the cultured brain endothelial cells, the pellet was observed by electron microscopy.

Finally, possible contamination by myelin basic protein (MBP) was sought using an enzyme-linked immunosorbent assay (ELISA). A high titer rat anti-guinea pig MBP (GPMBP) was used, capable of reacting with 25 ng of GPMBP.

Electrophoresis of cell membrane products

Slab polyacrylamide gel electrophoresis of cell membrane products and standard samples in sodium dodecyl sulfate (SDS) was carried out by the method of Laemmli (1970). Proteins were fixed with water-ethanol-acetic acid (9:9:2, v/v/v) and stained with Coomassie brilliant blue-R 250 in water-ethanol-acetic acid (83:10:7, v/v/v).

Animals immunized with cell membrane products

Eighty Hartley guinea pigs, each of approximately 300 g were used. They were divided into two groups of 40 animals and immunized with the endothelial cell membranes. In each group, the inoculum consisted of 250 μg and 500 μg cell membrane in 0.25 ml physiological saline plus 0.25 ml complete Freund's adjuvant (CFA) and 2.5 mg *Mycobacterium tuberculosis*, as presented in Table 2, injected subcutaneously into the flank as a single dose. In the first (acute) group, the 40 guinea pigs were dead or killed soon after first attack at intervals from 7 to 21 days after immunization while in the other (chronic) group, the animals were sacrificed from 4 to 20 weeks after inoculation.

Histological and ultrastructural studies

Animals in which ultrastructural studies were carried out were perfused with 3% glutaraldehyde, in phosphate-buffered saline (PBS) (pH 7.2), injected into the heart at 100 mg Hg using a micrometering pump. After perfusion with glutaraldehyde, the brains were carefully collected and sectioned at the levels of the thalamus, midbrain, pons and medulla oblongata. For routine histology, specimens from animals perfused with 3% glutaraldehyde were fixed in 10% formalin solution and the sections were stained with hematoxylin and eosin (H & E), Luxol fast blue and H & E and Woelke stains. For electron microscopic evaluation, blocks of guinea pig brain were fixed in 3% glutaraldehyde for 60 min after perfusion. They were washed three times with PBS (pH 7.2) and postfixed with 1% osmium tetroxide. After dehydration with graded alcohols, they were embedded in Epon and sections were cut with an ultramicrotome. They were stained with uranyl acetate and lead citrate and examined under a HU electron microscope (Hitachi, Japan).

Table 1. Enzyme activities in rat brain capillary fractions

Fraction	γ-Glutamyl transpeptidase	Alkaline phosphatase
Homogenate	1.08	0.45
Capillary	67.9	4.0
Plasma membrane	56.2	2.9

Values are expressed as units of enzyme activity/mg protein. 1 unit is the amount of enzyme that catalyse the formation of 1 μ mole of the products per 30 min

Delayed hypersensitivity

To detect cell immunity to endothelial cell membrane, MBP and proteolipid apoprotein (PLP)[1], delayed hypersensitivity was assessed by a skin test, and proliferative response using lymph node cells of guinea pigs immunized with endothelial cell membrane.

Skin test. Each solution (0.05 ml) containing 50 μg of antigen was injected intradermally in the flanks of the guinea pigs immunized 14 days before with 250 μg of the brain endothelial cell preparation in CFA. The injected sides were inspected after 24, 48 and 72 h.

In vitro lymphocyte proliferative assay. The method we used was same as that described by Richert et al. (1981). Three weeks after sensitization with brain endothelial cell membrane, draining lymph nodes were removed and trimmed of excess fat. Single cell suspensions were obtained by teasing the tissue through a stainless steel wire mesh. Cells were then washed three times in HBSS and adjusted to a concentration of 1×10^6 cells/ml in a total of 0.2 ml complete medium consisting of RMPI (Gibco) supplemented with 5% fetal bovine serum (Gibco), 2 mM glutamine (Sigma, St. Louis, Mo), penicillin 100 U/ml (Gibco), 25 mM Hepes buffer (Gibco) and 5×10^{-5} 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY). The cells (1×10^6/ml) were cultured in RPMI medium prepared as above with addition of 50 μg/ml of GPMBP or PLP in the wells of flat-bottomed 96-well microplates (Nunc, Kamstrup, Denmark). The microplates were incubated for 56 h at 37°C in 5% CO_2, at which time the cells in each well were pulsed with 1.0 μCi per well of [^{3}H]thymidine (2 Ci/ml, Amersham Arlington Heights, Ill) and incubated for an additional 18 h. Cultures were harvested on fiberglass filters and the incorporation of [^{3}H]thymidine was measured via liquid scintillation counting. The proliferative response of lymph node cells to MBP or PLP stimulation was recorded as the stimulation index (SI), calculated for each proliferative assay as the mean cpm of triplicated wells containing GPMBP or PLP.

Results

Confirmation of endothelial cell membrane fractions

The capillary and membrane fractions were enriched five times over the starting homogenate in the enzyme

[1] PLP: we received PLP from Dr. T. Kunishita (National Nervous, Mental and Muscular Disorders, Kodaira, Tokyo)

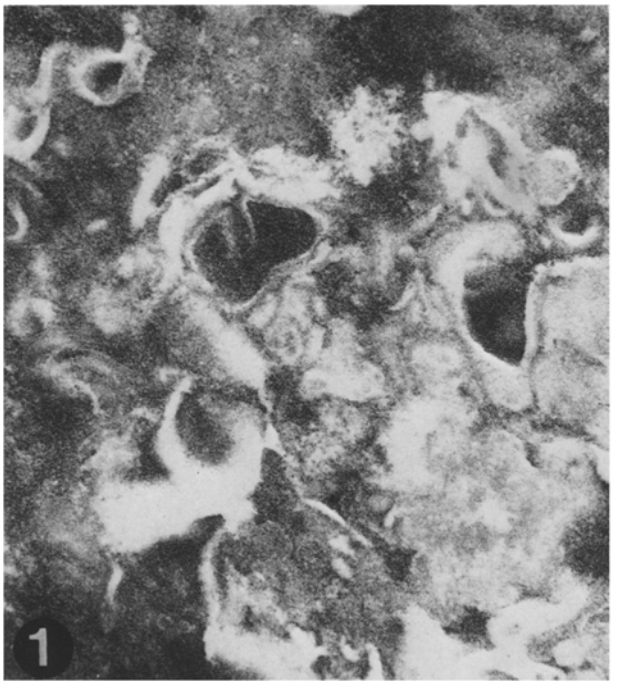

Fig. 1. On the electron microscopy, cell membrane products showed membrane structures distributed in amorphous homogeneous materials. × 30,000

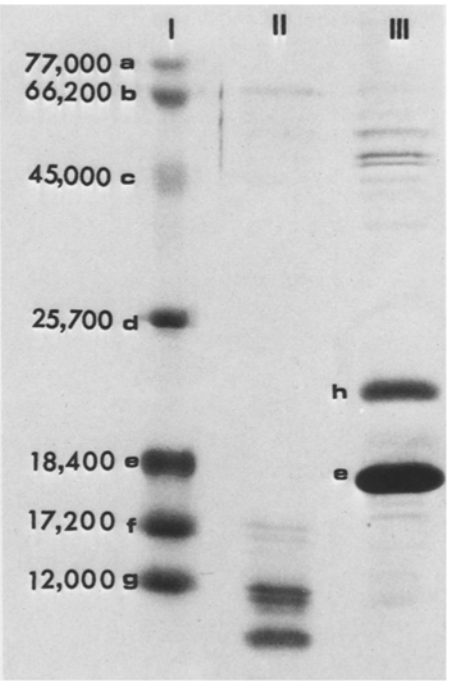

Fig. 2. Polyacrylamide electrophoresis (13%) of membrane antigen in sodium dodecyle sulphate. *I* Standard molecular weight, *a* ovotransferrin (77 kDa), *b* bovine serum albumin (66.2 kDa), *c* ovalbumin (45 kDa), *d* chymotrypsinogen A (25.7 kDa), *e* MBP (18.4 kDa), *f* myoglobin (17.2 kDa), *g* cytochrom (12 kDa); *II* membrane sample; *III* Purified myelin of Hartley guinea pig, *h* proteolipid apoprotein (PLP). Endothelial cell membrane proteins were compared to molecular weight standard. Two major protein bands with a molecular weight less than 12.3 kDa were detected by electrophoresis

markers of endothelial cell alkaline phosphatase and γ-glutamyl transpeptidase (Table 1). On electron microscopy, cell membrane products administered to the animals showed membrane structures distributed in amorphous homogeneous materials (Fig. 1). Electrophoresis of cell membrane products revealed two major protein bands with a molecular weight less than 12,300 dalton. No protein band for MBP or PLP was detected by electrophoresis of brain endothelial cell membrane fractions (Fig. 2).

No MBP was found in a 250 μg sample of endothelial cell membrane products by the ELISA method so that, if any was present, it formed less than 0.01% of the materials. Similar studies were done at The Multiple Sclerosis Laboratory (Dr. M. L. Cuzner), National Hospital, Queen Square, and confirmed the absence of MBP.

Central nervous system (CNS) involvement

Clinical signs. The first signs of illness were loss of hair and body weight. This was followed, in each group, at days 14–21, by the development of ataxia, flaccid paralysis of the hind legs and occasional myoclonus, 76 of 80 (Table 2). The mortality rate during the first attack was higher in the 20 guinea pigs inoculated with 500 μg cell membrane, than in the 20 animals inoculated with 250 μg cell membrane. In the second (chronic) group of the guinea pigs inoculated with 250 μg cell membrane, where the animals were allowed to survive, ten animals had a chronic progressive course, while four recovered from the first attack and then remained well. Six guinea pigs, however, showed recurrent attacks of weakness and paralysis, which lasted on average for 15 days (Table 2). The incidence of the chronic progressive course in animals immunized with 500 μg cell membrane was also higher than in animals immunized with 250 μg cell membrane.

Histological features. The earliest pathological changes in the acute phase noticed in the cerebrum were infiltrates of mononuclear cells attached to the endothelial cells and around the post-capillary venules (Fig. 3). Slight pallor of myelin staining was observed in the white matter of the cerebrum and cerebellum in the late acute phase. Occasional tiny hemorrhages and evidence of oedema were present around the small vessels. At 40 days and thereafter, severe loss of myelin was recognizable in the white matter regions of the cerebrum, pons and cerebellum (Figs. 4, 5). The areas of demyelination were always centred on blood

Table 2. Clinical course and signs in animals immunized with endothelial cell membranes

Groups	Killed at 1st attack or (dead)	Paralysis	Myoclonus	Ataxia	Chronic progressive or (dead)	Relapse
Cell membrane (250 μg/gp)						
Acute 20	16 (4)	18	6	10		
Chronic 20		16	8	9	10 (4)	6
Cell membrane (500 μg/gp)						
Acute 20	13 (7)	19	11	10		
Chronic 20		20	10	12	12 (6)	2

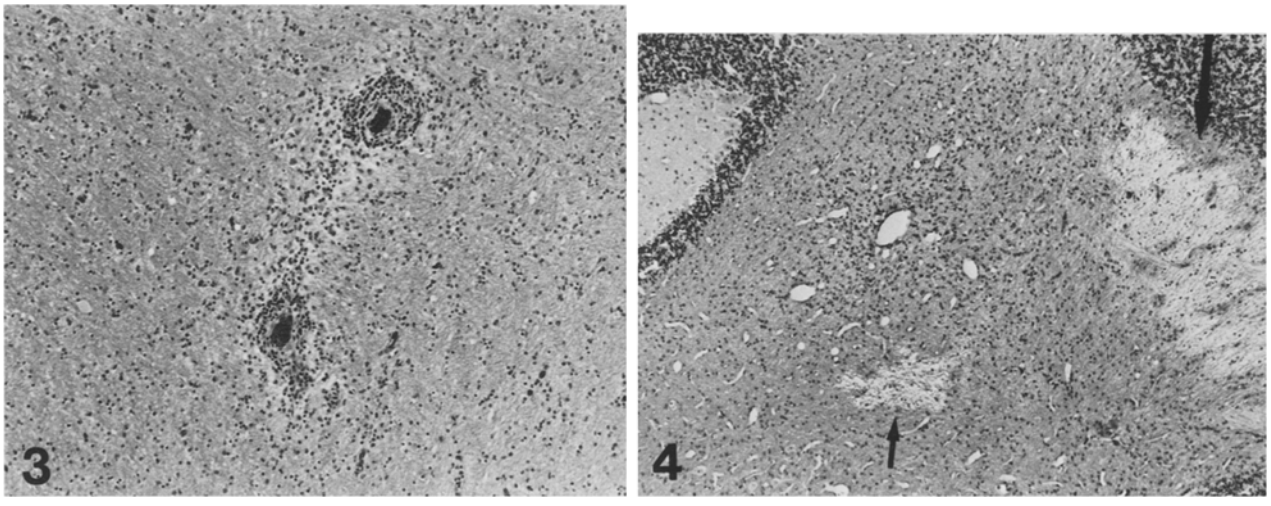

Fig. 3. Infiltrates of mononuclear cells attached to the cerebral capillaries in the acute phase (7 days after immunization). H & E stain. ×300

Fig. 4. Perivascular mononuclear cell infiltrates accompanied by astrocytic proliferation were conspicuous and demyelination could be seen in the adjacent regions (*arrows*) (30 days after immunization). H & E stain. ×200

vessels, although some were large and irregular. All animals had conspicuous periventricular myelin loss (Fig. 6). The spinal cord showed very few demyelination (Fig. 7).

Electron microscopic findings. Ultrastructural examination of the lesions revealed widespread demyelination and reactive gliosis in the cerebrum, pons, cerebellum and spinal cord (Figs. 8, 9). In the acute phase, mononuclear cells and astrocytes were seen, but these were absent in chronic lesions. Polymorphonuclear and mononuclear cells were found around the small blood vessels attached to the endothelial cells (Fig. 10). Electron-dense deposits were demonstrated between the endothelial cell and the basement membrane (Fig. 10).

Renal and respiratory findings

Mononuclear cell infiltrates with hyperplasia of mesangial cells were seen in the glomeruli. Interstitial infiltrates of mononuclear cells were noted in all animals. Pulmonary hemorrhages were accompanied histologically by interstitial infiltrates of mononuclear cells.

Delayed hypersensitivity

A positive skin test was found when the animal was injected with the brain endothelial cell membrane fraction. Skin test to MBP or PLP was negative in all animals immunized with cell membrane products (Table 3). No significant effect on proliferative response was observed from the addition of MBP or PLP (Table 3).

Discussion

MBP has an inflammatory encephalitogenic activity with little or no demyelination, whereas demyelination is the major characteristic of the mild form of experimental allergic encephalomyelitis (EAE) (Paterson 1977). There are, however, major differences which prevent EAE from being accepted as the animal

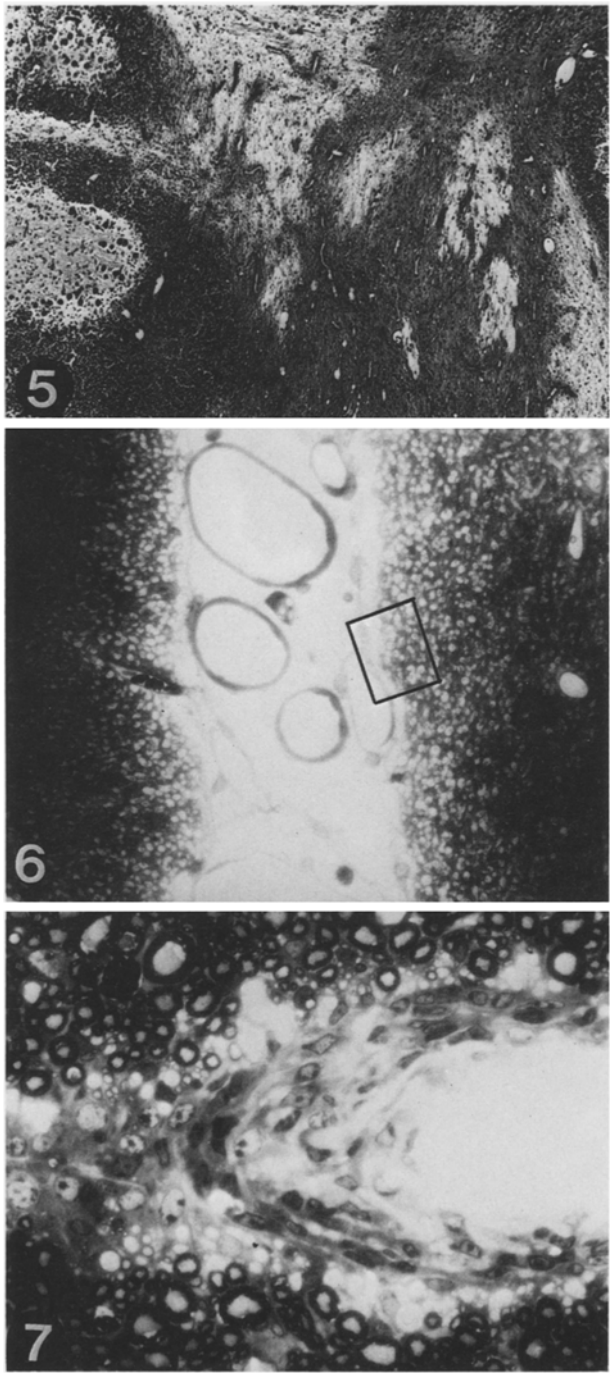

Fig. 5. Severe loss of myelin staining in the white matter of the cerebellum (66 days after) immunization). Luxol fast blue and H & E stain. ×200

Fig. 6. Periventricular foci of demyelination seen in the white matter of the cerebrum (30 days after immunization). Toluidine blue stain. ×40

Fig. 7. Demyelinating lesions around the small blood vessel in the white matter of the spinal cord in the chronic phase (60 days after immunization). Toluidine blue stain. ×800

counterpart of the demyelinating disease, MS. The histological lesions are different between EAE and MS with many more inflammatory cell being present in EAE, while the large centrifugal demyelinating plaques characteristic of MS are not present. Therefore it is difficult to compare the lesions in the two disorders (Field and Raine 1966; Poser and Behan 1982). Indeed, some investigators consider that EAE is primarily a vasculopathy, with secondary demyelination (Poser and Behan 1982).

More recently chronic relapsing EAE (CREAE) has been induced in Strain 13 guinea pigs with a clinical course characterized by cycles of exacerbations and remissions accompanied histologically by widespread demyelination mainly in the spinal cord (Wiśniewski and Keith 1977; Raine and Stone 1977; Keith 1978; Lassmann and Wisńiewski 1978; Traugott et al. 1982). Although, at present, CREAE may be the best experimental animal model for the pathogenetic study of MS (Lassmann and Wiśniewski 1979), the demyelinating factors of CREAE induced by immunization with emulsion of whole spinal cord remain uncertain. In this study, it has been shown that the brain endothelial cell membrane, which includes neither MBP nor PLP, has a severe demyelinating encephalitogenic activity. Immunization with cell membrane products from cultured brain endothelial cell induced a chronic course of the disease accompanied histologically by widespread demyelination in guinea pigs, as in MS. The pathological feature in the early lesion induced in the experiment was characterized by infiltrates of mononuclear cells attached to the cerebral endothelial cells, differing from EAE lesions in which severe infiltrates of mononuclear cells could be detected. The main pathological feature in the chronic phase is the presence of diffuse demyelination plaques, as seen in MS. It is very interesting to observe that few demyelinated axons surrounded by macrophages were found in these demyelinating regions. In this respect, the demyelinating lesions induced by brain endothelial cell membrane products are quite different from those of EAE in which many more inflammatory cells accompanied by a few demyelinated axons invaded by macrophages are often observed around blood vessels.

The identity of cell membrane fraction with a demyelinating encephalitogenic activity is unknown. Delayed hypersensitivity was observed prior to the appearence of clinical signs by skin testing. This result suggests a pathogenetic role for a cell-mediated response to endothelial cell membrane in demyelination. It is not possible to state that the demyelinating lesions in the CNS are induced by dysfunction of the endothelial cells produced during the immunization procedure, but it is suggested that the small amount

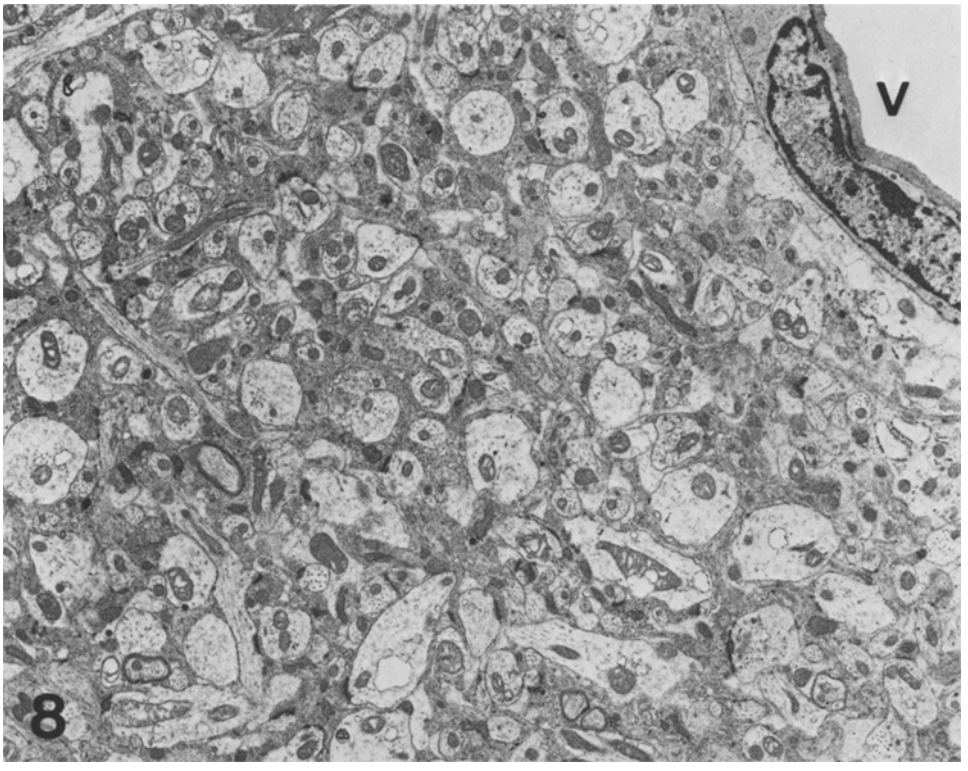

Fig. 8. High magnification of rectangle in Fig. 6 showed well-preserved axons separated by glia scar tissue but complete demyelination around the periventricular blood vessel (*V*) in the white matter of cerebrum. ×4,000

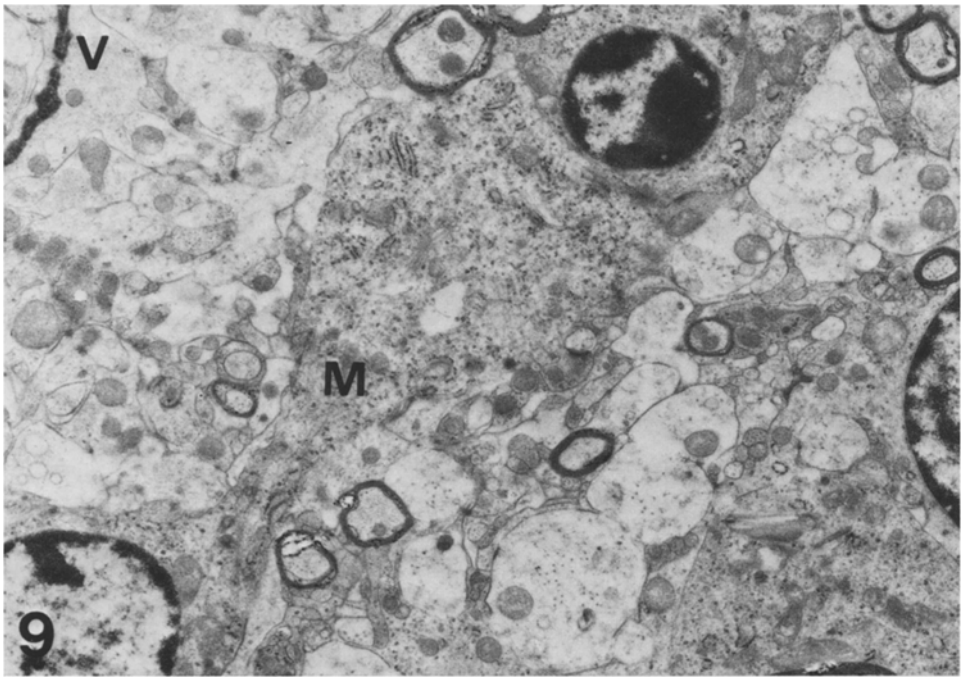

Fig. 9. High magnification of Fig. 7 showed demyelination around the blood vessel (*V*). Macrophage (*M*) seen in the demyelinating lesion. ×6,000

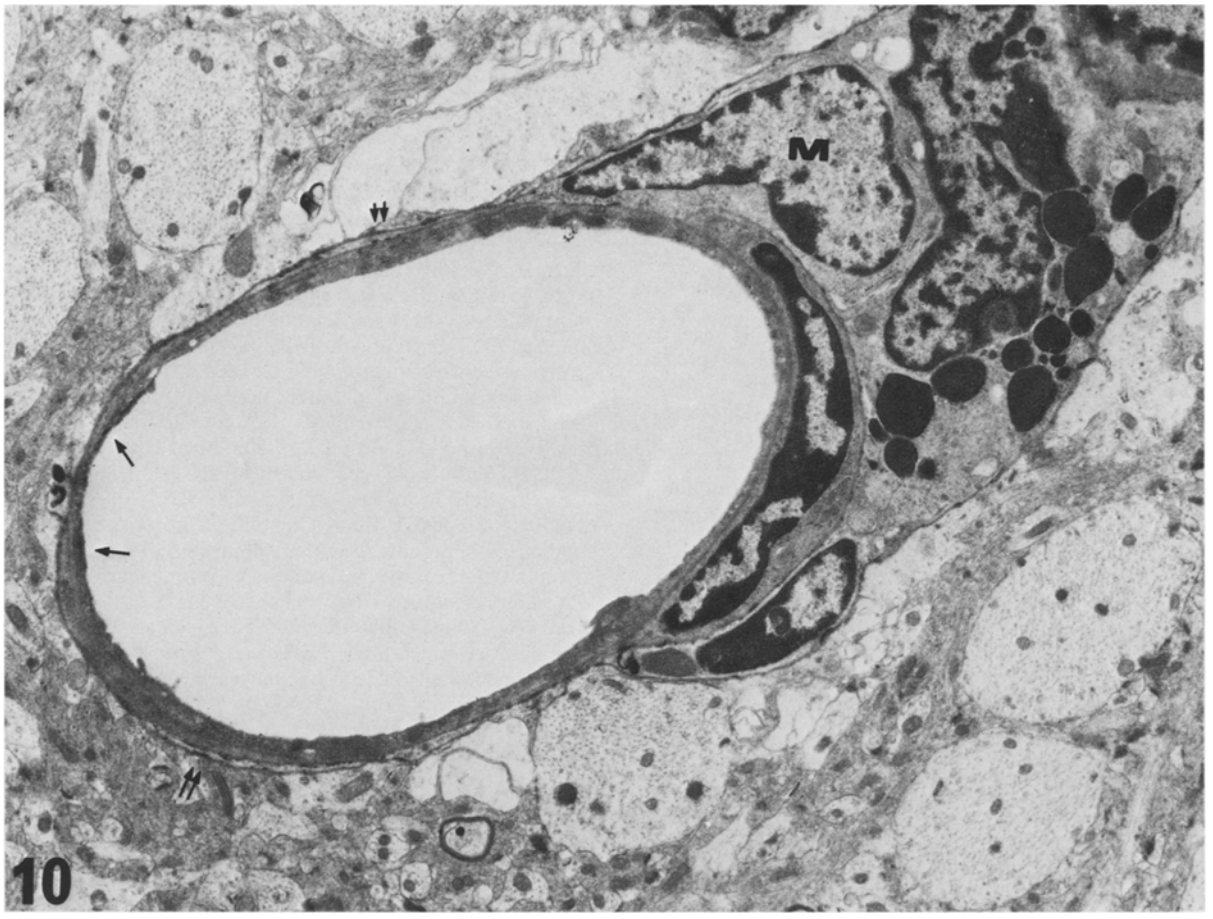

Fig. 10. Infiltrates of polymorphonuclear and mononuclear (*M*) cells attached to the endothelial cells around a small blood vessel in the chronic demyelinating lesion of the cerebral white matter. Electron-dense deposits are present within the endothelial cell (*arrows*) and in the wall of the capillary (*double arrows*) (30 days after immunization). × 5,500

Table 3. Delayed hypersensitity in guinea pigs immunized with endothelial cell membrane

Antigen	Positive skin test	Proliferative response ^{3}H-TdR		
		Expt 1	Expt 2	Expt 3
NIL		3,015	759	1,355
MBP	–	3,769	881	2,078
PLP	–	3,971	944	1,791
ECM	+			

Positive skin test was judged by diameter of erythema and degree of induration. Lymph node cells (1×10^6/well) were cultured with each antigen. Data of proliferative response present the mean cpm of quadruplicate determinations of tritiated thymidine (^{3}H-TdR) incorporation
MBP = Myelin basic protein; PLP = proteolipid apoprotein; ECM = endothelial cell membrane

of the antigen, the encephalitogenic cell membrane fraction of endothelial cells, may be a stronger inducer of cell-mediated immune reaction than serum antibody production. The studies reported here indicate that there are obvious similarities between chronic autoimmune demyelinating disease induced by cultured brain endothelial cell membrane, in which extensive demyelination is seen in the white matter of cerebrum, cerebellum and spinal cord, and the human demyelinating disorder, MS. Although the cause of MS is unknown, the sequence of events leading to widespread demyelination of MS appears to be similar to that seen in the experiment. Pathogenetic examination of cell membrane with a antigenicity producing the demyelinating lesions may, therefore, be of importance in the study of human and experimental demyelinating diseases.

Further biochemical and immunological analysis of endothelial cell membrane products with a demyelinating encephalitogenic activity may, therefore, provide a clue in resolving the pathogenesis of MS.

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