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Unmasking of an Unusual Myelin Basic Protein Epitope during the Process of Myelin Degeneration in Humans

A Potential Mechanism for the Generation of Autoantigens

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A rabbit antiserum (anti-EP), induced against a synthetic peptide corresponding to residues 68 to 86 of guinea pig myelin basic protein, powerfully immunostained abnormal-appearing oligodendrocytic processes and cell bodies in demyelinating areas associated with multiple sclerosis plaques. However, it failed to recognize any structures in normal human, rat, or guinea pig brain. The antiserum recognized the synthetic peptide QDENPVV, which corresponds to human myelin basic protein residues 82 to 88. Immunoabsorption with this peptide eliminated immunohistochemical staining. By contrast, several commercial antibodies recognizing nearby sequences of human myelin basic protein intensely stained all myelinated structures in both normal and multiple sclerosis tissue. The unusual epitope recognized by anti-EP appears to be accessible only in areas of myelin degeneration. If insults occur that repeatedly expose a region of MBP normally sheltered from immunosurveillance, a self-sustaining immune reaction might result. (Am J Pathol 1997, 150:1253–1266)

Multiple sclerosis (MS) is the most common of the demyelinating diseases.[1] Despite intensive research, a precise etiology of this well known disease remains to be established. However, an autoimmune mechanism is suspected, based in significant part on analogies with experimental allergic encephalomyelitis (EAE).[1–3]

EAE was originally produced by Rivers through injection of whole spinal cord homogenates into monkeys.[1,4] The phenomenon has since been reproduced in several species using not only spinal cord homogenates but also proteins associated with myelin as well as some of their small peptide sequences. Proteins that have been extensively studied in this regard include myelin basic protein (MBP), proteolipid protein, myelin-associated glycoprotein, and myelin oligodendrocyte glycoprotein.[2,3] Of these, MBP is the most thoroughly investigated because of its relative abundance in myelin and its high degree of encephalitogenicity. MBP-specific T-cell lines have been isolated from both animals with EAE and MS patients.[5–8] Increased numbers of cells producing antibodies against whole myelin and MBP have been found in the cerebrospinal fluid (CSF) of MS patients.[9–13] As far as EAE is concerned, whole guinea pig MBP (gMBP) is the most potent preparation for inducing EAE in rats, especially the Lewis strain. A sequence of gMBP that readily induces EAE in Lewis rats corresponds to residues 68 to 86 (Figure 1, HYGSLPQKSQRSQDENPVV).[17,18] This peptide is referred to as the encephalitogenic peptide (EP), although the exact antigenic stretch has been slightly varied in different sets of experiments.[18–22] The minimal peptide containing the pathogenic T-cell epitope lies in residues 73 to 83.[20]

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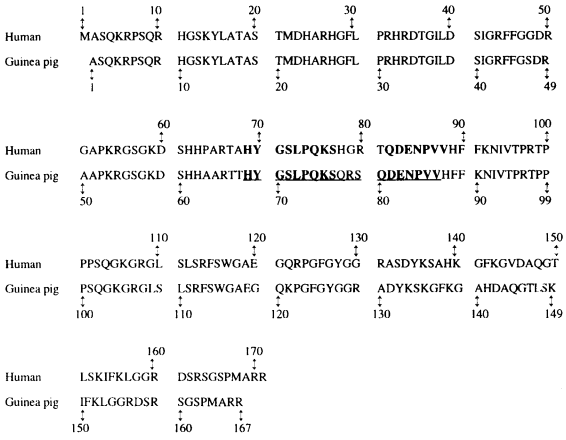

Figure 1. *Sequence comparison between human and guinea pig MBP. The oligopeptide used for the antigen of the EP antibody is underlined, and the homologous regions are indicated in bold type. Indicated numbering on top is for the human 18.5-kd form[17] and on the bottom for the guinea pig form.[18] Data were obtained from Genbank, accession number 307,160 for human and 126,797 for guinea pig. Note that the human sequence commences with methionine at position 1. The numbering is, therefore, one higher than some other published sequences (eg, 10 to 16 and 23 to 25).*

To determine whether comparable regions of human MBP (hMBP) are differentially exposed in normal and MS brain tissue, we studied the antigenic profile of hMBP by immunohistochemistry. We used a rabbit antiserum (anti-EP) raised against gMBP 68 to 86, as well as commercial antibodies recognizing several regions of hMBP. These included monoclonal antibodies recognizing probable hMBP sequences[17] in the region of 37 to 47 (clone 14),[23] 70 to 76 (clone 26),[23] 82 to 96 (clone 12),[18,24,25] 81 to 90 (clone 22),[23] and 119 to 131 (clone 2)[18,24,25] (Figure 1 and Table 1). In addition, we used a polyclonal rabbit antiserum raised against whole bovine MBP.

Materials and Methods

Production of EP Antiserum and Its Characterization

The immunogen was prepared by conjugating a synthetic oligopeptide (HYGSLPQKSQRSQDENPVV, EP), corresponding to residues 68 to 86 of gMBP,[18] to keyhole limpet hemocyanin (Figure 1). After immunization of a rabbit, the antiserum against EP-keyhole limpet hemocyanin was collected. The sensitivity of the antiserum was examined by enzyme-linked immunosorbent assay against EP conjugated to bovine serum albumin (BSA) and by detection of dot blots of oligopeptides corresponding to residues 69 to 88 of hMBP (HYGSLPQKSHGRTQDENPVV) and 82 to 88 of hMBP (QDENPVV; Figure 1).[17] The specificity of the antiserum was tested by absorption immunohistochemistry and by Western blotting.

Two absorbed EP antiserum solutions were prepared. In the first, the EP antiserum diluted 1:50,000 was incubated overnight at 4°C with 10 to 100 μg of EP conjugated to BSA. The antiserum was also incubated with the same amount of BSA alone. In the second, EP antiserum diluted 1:10,000 was incubated for 6 hours with 50 μg/ml of the synthetic peptide QDENPVV before standard immunohistochemical staining.

For companion immunoabsorption experiments with commercial clones reported to recognize sequences overlapping QDENPVV, solutions of clone 12 diluted 1:50,000 and clone 22 diluted 1:10,000 were also incubated for 6 hours with a solution of 50 μg/ml QDENPVV before standard immunohistochemical staining.

Table 1. *Primary Antibodies Used in This Study*

Antibody	Antigen	Probable sequence recognized*	Antibody type	Source	Preferred dilution
Anti-EP	gMBP 68–88	hMBP 82–88 QDENPVV	Rabbit polyclonal	Local	1 :50,000
clone 14	bMBP	hMBP 37–51[23] GILDSLGIFFG	Rat monoclonal IgG	Serotec	1 :100,000
MAb 384 (clone 26)	hMBP 65–79	hMBP 70–76[23] YGSLPQK	Mouse monoclonal IgG	Chemicon	1 :10,000
MAb 386 (clone 12)	bMBP	hMBP 82–95[24, 25] QDENPVVHFFKNIV	Rat monoclonal IgG	Chemicon	1 :100,000
clone 22	hMBP 76–90	hMBP 81–90[23] TQDENPVVHF	Mouse monoclonal IgG2b	Serotec	1 :10,000
clone 2	bMBP	hMBP 117–130[24, 25]	Mouse monoclonal IgG1	Serotec	1 :250
Anti-MBP	hMBP	–	Rabbit polyclonal	Zymed	1 :5,000
HB104	HLA-DR	–	Mouse monoclonal	ATCC	1 :1,000

bMBP, bovine MBP; ATCC, American Type Culture Collection.
*Numbering according to Figure 1.

Table 2. *Lowest Level of Synthetic Peptides Containing the QDEN Sequence Detectable by Anti-EP and Clones 26, 12, and 22*

	Micrograms of peptide per dot blot			
Antibody	hMBP 82–88 QDENPVV	hMBP 69–88 HYGSLPQKSHGTT-QDENPVV	gMBP 68–86 (EP) HYGSLPQKSQRS-QDENPVV	gMBP 73–83 (S55S) PQKSQRS-QDEN
Anti-EP	2	0.080	0.080	>10
Clone 26	>10	0.400	10	>10
Clone 12	>10	>10	>10	>10
Clone 22	>10	0.016	2	>10

For Western blotting, frozen tissues from white matter of neurologically normal cases were homogenized in 5 volumes of ice-cold 10 mmol/L Tris/HCl (pH 7.4) plus 150 mmol/L NaCl (TBS), containing 1 mmol/L EDTA, 1 mmol/L EGTA, phenylmethylsulfonyl fluoride (100 μg/ml), leupeptin (1 μg/ml), and aprotinin (1 μg/ml). The homogenates were centrifuged at 13,000 rpm for 20 minutes at 4°C. The pellets were suspended in TBS containing 1% sodium dodecyl sulfate (SDS). Aliquots containing approximately 100 μg of protein were electrophoresed on 15% SDS-polyacrylamide gels (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were blocked by incubating with TBS containing 0.5% Tween-20 and 5% skim milk for 30 minutes at room temperature before being incubated overnight at room temperature with clone 14 (1:50,000), clone 2 (1:250), anti-EP (1:10,000) or anti-whole hMBP (1:5000; Table 1). The rabbit polyclonal and mouse monoclonal antibodies were reacted with alkaline-phosphatase-labeled antibodies against IgG of the appropriate species (1:3000; Gibco BRL, Gaithersburg, MD) for 2 hours at room temperature. Alkaline phosphatase labeling was visualized by incubating with nitroblue tetazolium (0.33 mg/ml; Gibco BRL) and 5-bromo-4-chloro-3-indolyl-phosphate (0.165 mg/ml; Gibco BRL) in 100 mmol/L Tris/HCl (pH. 9.5) containing 100 mmol/L NaCl and 50 mmol/L $MgCl_2$. For rat monoclonal antibody detection, biotin-labeled anti-rat IgG antibody (1:1000; Vector Laboratories, Burlingame, CA) was used as the secondary antibody, followed by immunohistochemical treatment as described below.

For dot blots, anti-EP, and clones 26, 12, and 22, all of which are reported to recognize sequences in the 68 to 88 region of MBP, were tested against four synthetic peptides covering sequences in this region. The peptides were hMBP 82 to 88 (QDENPVV), hMBP 69 to 88, gMBP 68 to 86, and gMBP 73 to 83 (Table 2). The peptides were diluted in Tris/HCl, pH 7.6, at concentrations ranging from 20 to 0.002 μg/ml. A 1-μl aliquot of each solution was spotted on a nitrocellulose or polyvinylidene difluoride membrane, and the membrane was blocked for 30 minutes at room temperature with 5% skim milk in 10 mmol/L TBS, pH 7.4, containing 0.1% Tween. The membranes were then incubated for 2 hours with primary antibody in TBS/0.1% Tween containing 1% skim milk. The antibody dilutions were as follows: anti-EP, 1:5000; clone 26, 1:5000; clone 12, 1:50,000; and clone 22, 1:5000. Subsequent steps were the same as for Western blotting.

Immunohistochemical Procedures

Eight brains from MS cases were examined and compared with six brains from cases without neurological disease and six brains from cases where small cerebral infarcts of recent origin were detected postmortem. Details concerning age, sex, source of the MS brains, autolysis time, and method of preparation of the MS specimens before immunohistochemical staining are given in Table 3. For some of the brains, fresh tissue was directly fixed in 4% paraformaldehyde, pH 7.4, for 1 to 3 days before being transferred to a maintenance solution of 15% sucrose in 0.1 mol/L phosphate-buffered saline (PBS). Other brain tissues were formalin fixed. Some of the paraformaldehyde- or formalin-fixed tissue was paraffin embedded as in standard hospital pathological procedures. All methods of preparation yielded identical results with the various anti-MBP antibodies.

Male Wistar rats and male guinea pigs were used as the source of animal brains. They were sacrificed under sodium pentobarbital anesthesia. The rat brains were cut into 0.5-mm coronal slabs and fixed similarly to the human cases. The guinea pig brains were snap-frozen in liquid nitrogen and cut on a cryostat at 12 μm thickness. These sections were mounted on gelatin-coated glass slides, air dried, and then fixed in 4% paraformaldehyde for 2 hours at 4°C.

Sections of the human and rat brains that were not paraffin embedded were cut on a freezing microtome at 30 μm thickness. Some sections were directly stained by Klüver-Barrera's method to iden-

Table 3. *Multipe Scleorsis Cases Used in This Study*

Number	Age	Sex	Autolysis time (hours)	Fixation	Source
MS-1	33	F	22.5	Formalin	NNRSB*
MS-2	40	M	5.5	Formalin	NNRSB*
MS-3	69	M	72	Paraformaldehyde	University of B.C.
MS-4	54	M	8	Paraformaldehyde	NNRSB*.
MS-5	49	M	3	Formalin	University of B.C.
MS-6	26	F	>24	Formalin paraffin	University of B.C.
MS-7	59	M	2	Formalin paraffin	Canadian Brain Bank
MS-8	66	F	9	Paraformaldehyde paraffin	University of B.C.

F, female; M, male, NNRSB, National Neurological Research Specimen Bank, courtesy of Dr. Wallace Tourtellotte; B.C., British Columbia.

tify plaques. Others, used for immunohistochemical staining, were rinsed for several hours in 0.01 mol/L PBS (pH 7.4) containing 0.3% Triton X-100 (PBS-T). They were pretreated with 1.0% H_2O_2 for 1 hour to reduce endogenous peroxidase, washed in PBS-T, and blocked at room temperature for 2 hours with PBS-T containing 5% skim milk. They were then incubated for 48 to 72 hours at 4°C with one of the primary antibodies (Table 1). After incubation with primary antibody, the sections were washed and reacted with biotinylated antibodies against IgG of the appropriate species (1:1000; Vector Laboratories) for 2 hours at room temperature and for 1 hour with the avidin-biotin-peroxidase complex (diluted 1:2000; Vector Laboratories) at room temperature. They were rinsed and incubated in a staining mixture containing 0.001% 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO), 0.6% nickel ammonium sulfate (Fisher Scientific, Pittsburgh, PA), 0.05% imidazole, and 0.0003% H_2O_2 in 0.05 mol/L Tris/HCl buffer, pH 7.6, until a purple reaction product appeared. Where a brown reaction product was desired, the same mixture without nickel ammonium sulfate was used. The reaction was terminated by transfer of the sections to PBS-T. Sections were mounted on glass slides, dehydrated with graded alcohol, and coverslipped with Entellan (Merck, West Point, PA). In some cases, sections were counterstained with oil red O (ORO) without dehydration and then coverslipped with 80% glycerol buffered with 0.1 mol/L phosphate.

Some sections were double immunostained before slide mounting. After the first staining procedure, they were pretreated with 1.0% H_2O_2 for 1 hour to destroy excess peroxidase from the first cycle and then processed with the second primary antibody and appropriate secondary antibodies as described above. The final diaminobenzidine staining step was chosen to produce a counterstaining color different from the first step by addition or elimination of nickel ammonium sulfate. Control sections were stained without primary antibody or with a mouse monoclonal antibody indifferent to brain tissue. All such control sections showed negative staining.

For paraffin-embedded tissues, 15-μm-thick sections were cut on a microtome from the paraffin-embedded blocks. The sections were then deparaffinized by immersion in xylene for 20 minutes. They were then rehydrated through 100, 95, and 70% ethanol to H_2O and PBS-T. The sections were stained for EP and MBP by the free-floating technique described above.

Sources of the primary antibodies used and their dilutions are shown in Table 1. The immunohistochemical methods have previously been described in detail.[26]

Control and cerebral infarction brains were obtained 3 to 10 hours postmortem. They were treated by the same procedures as the MS brains. One control brain was paraffin embedded. The remainder were fresh fixed in paraformaldehyde.

Results

Characterization of the EP Antiserum

The titer by enzyme-linked immunosorbent assay of the antiserum against EP conjugated to BSA was 1:4500. As the antiserum was raised against EP conjugated to keyhole limpet hemocyanin, the titer was considered to be against the EP oligopeptide itself.

On Western blots using extracts of human brain homogenates, the EP antiserum recognized a major band at approximately 18.5 kd and a minor one at approximately 17.2 kd, corresponding to those reported for hMBP (Figure 2A, lane d).[17,27] Similar bands were recognized by the anti-whole hMBP polyclonal antibody (lane a), clone 14 (lane b), and clone 2 (lane c).

The antiserum to EP stained abnormal-appearing oligodendrocytic elements around MS plaques as shown in Figure 2, B and D. The antiserum absorbed

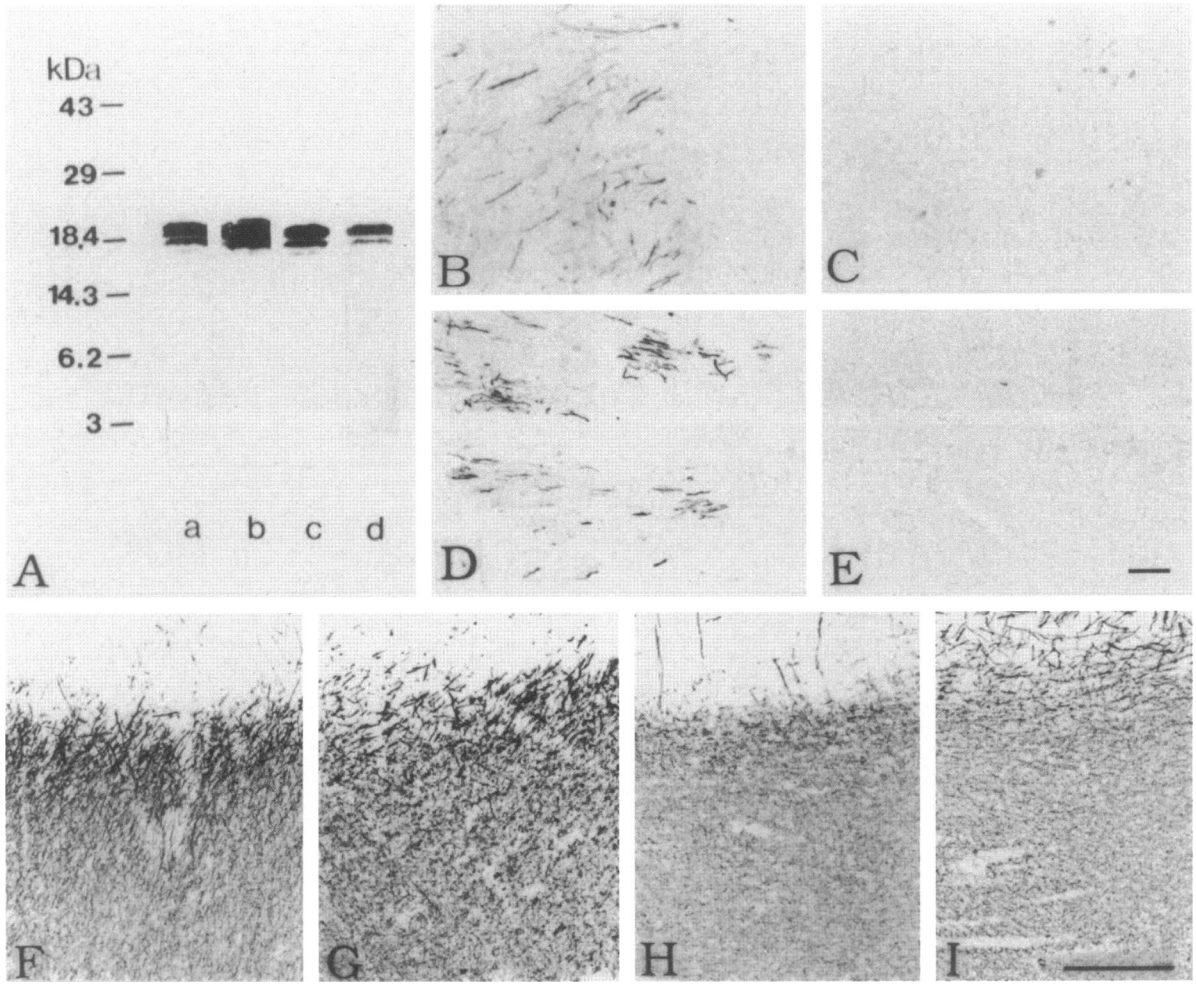

Figure 2. *The specificity of EP antiserum examined by Western blot* (A) *and immunoabsorption tests* (B *to* I). A: Lane a, *anti-whole hMBP antibody;* lane b, *clone 14;* lane c, *clone 2;* lane d, *EP antiserum. All antibodies detected a major 18.5-kd band and a weaker 17.2-kd band in extracts of normal human brain homogenates.* B: *EP immunoreactivity observed near a MS plaque in paraformaldehyde fixed tissue.* C: *Immunoreactivity disappeared in a serial section using EP antiserum that was preincubated with 100 μg/ml EP conjugated to BSA (see Materials and Methods).* D: *EP immunoreactivity observed near another MS plaque.* E: *Immunoreactivity disappeared in a serial section using EP antiserum preincubated with 50 μg/ml QDENPVV.* F *and* G: *Nearby areas of a plaque from a single paraformaldehyde-fixed MS section immunostained with clone 12 (1:50,000) without* (F) *or with* (G) *immunoabsorption with 50 μg/ml QDENPVV. No change in the intensity of immunostaining can be observed.* H *and* I: *Nearby areas of a plaque from a single paraformaldehyde-fixed MS section immunostained with clone 22 (1:10,000) without* (H) *and with* (I) *immunoabsorption with 50 μg/ml QDENPVV. Again, no change in the intensity of immunostaining can be observed. See Materials and Methods for experimental details.*

by 100 μg of EP conjugated to BSA did not stain any structures in any human sections (Figure 2C). Similarly, the EP antiserum absorbed by 50 μg of QDENPVV failed to stain any structures in any human section (Figure 2E). By contrast, clones 12 and 22, reported to recognize comparable or nearby sequences to QDENPVV, each strongly stained myelinated structures as well as the abnormal elements around MS plaques. Such staining was not absorbed by QDENPVV. This is shown for clone 12 in Figure 2 (F compared with G) and for clone 22 in Figure 2 (H compared with I).

Dot blot assays (Table 2) confirmed the differences between the antibodies in sequence recognition. The EP antiserum recognized a 2-μg blot of QDENPVV, whereas clones 12 and 22 were unable to recognize 10-μg blots. The EP antiserum more strongly recognized peptides corresponding to hMBP 69 to 88 and gMBP 68 to 86 than QDENPVV, which may have been due to better binding of the longer peptides to the membrane, but it failed to recognize 10 μg of gMBP 73 to 83, indicating that sequences carboxy-terminal to the asparagine (N) residue at position 83 were necessary for strong recognition. Clones 26, 12, and 22 also failed to recognize gMBP 73 to 83, also known as S55S,[20] which is the minimal peptide containing the pathogenic T-cell epitope for EAE in Lewis rats. Clone 22,

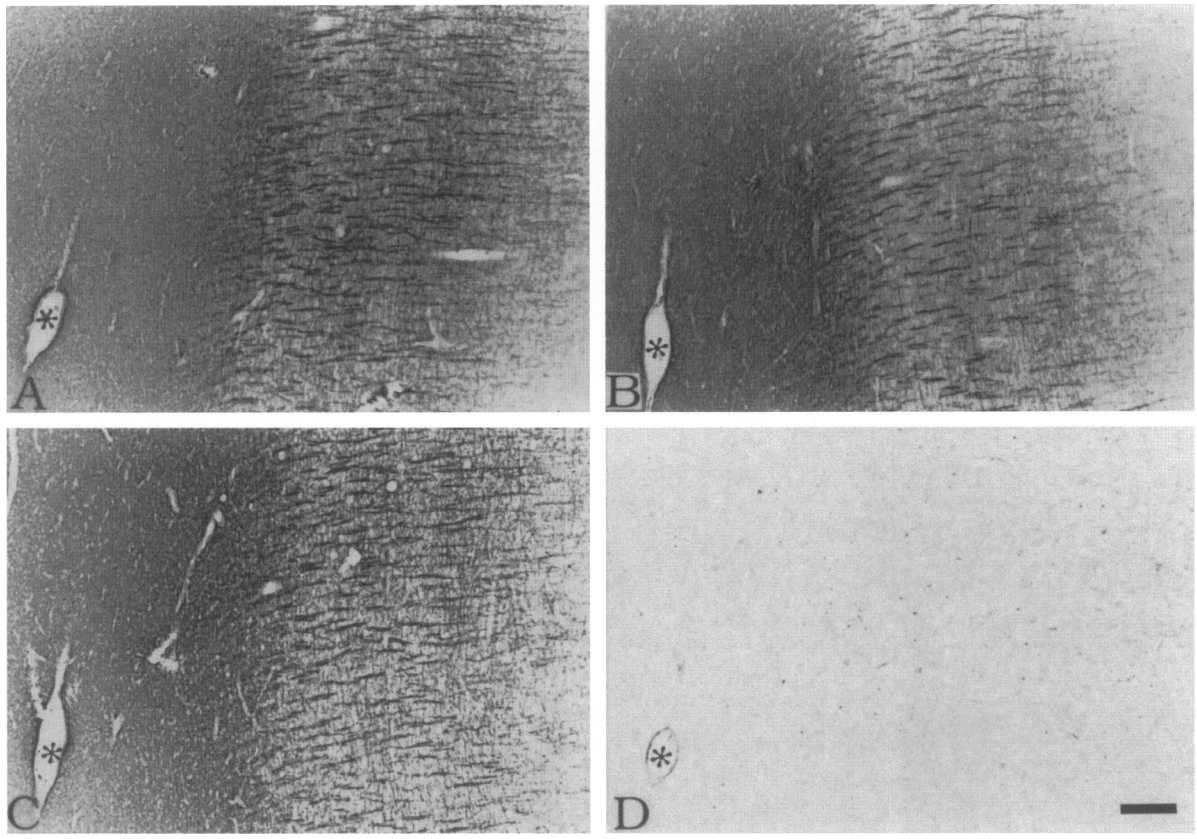

Figure 3. *Comparison between anti-MBP and anti-EP immunohistochemistry in a control brain. Antibodies for whole hMBP* (**A**), *clone 14* (**B**), *and clone 2* (**C**) *recognized all myelinated structures in control brains, whereas no immunoreactivity was detected by EP antiserum* (**D**). *The identical vessel in the four photomicrographs is indicated by the asterisks. Bar, 100 μm.*

prepared by immunizing with hMBP 76 to 90, recognized hMBP 69 to 88 more strongly than did anti-EP, indicating that residues carboxy-terminal to QDENPVV were necessary for strong recognition by this clone. By contrast, clone 12 failed to recognize any of the four peptides. It has been reported to recognize hMBP 82 to 95,[24] indicating that residues carboxy-terminal to QDENPVV are necessary for strong recognition by this clone.

Brain Immunohistochemistry

In all human brains, the polyclonal antibody to MBP, and each of the five monoclonal antibodies, strongly stained all myelinated structures. This is illustrated by immunostaining of serial sections of a normal paraformaldehyde-fixed brain with the polyclonal MBP antibody, clone 14, and clone 2 (Figure 3, A, B, and C, respectively). By contrast, the EP antiserum showed no positive staining of additional serial sections (Figure 3D). It similarly failed to stain any normal myelinated structure in any of the human brains.

In MS tissue, EP-like immunoreactivity was identified only in association with abnormal-appearing oligodendroglial elements in demyelinating areas in the vicinity of plaques. Fifty-six plaques were examined in the eight MS brains, and EP-positive structures were observed in association with every plaque. Surrounding normal myelinated structures were not stained. The same results were obtained irrespective of the postmortem interval or method of fixation or embedding in paraffin.

An example of EP-like immunoreactivity in paraformaldehyde-fixed tissue is shown in Figure 4A. Only structures in the immediate perimeter of the plaque are stained. Serial sections stained with anti-MBP (Figure 4B), clone 14 (Figure 4C), and clone 2 (Figure 4D) show that these antibodies, similarly to the other three monoclonals, intensely stained normal myelin in unaffected areas as well as the abnormal structures identified by anti-EP.

Identical results were obtained with formalin-fixed, paraffin-embedded tissue as illustrated in Figure 5. Serial sections of a brain stem area of a MS case with extensive plaque formation were stained. Figure 5A illustrates the results with anti-EP, whereas Figure 5, B–F shows the results with clones 26, 2, 14, 12, and 22, respectively. The anti-EP stained only abnormal

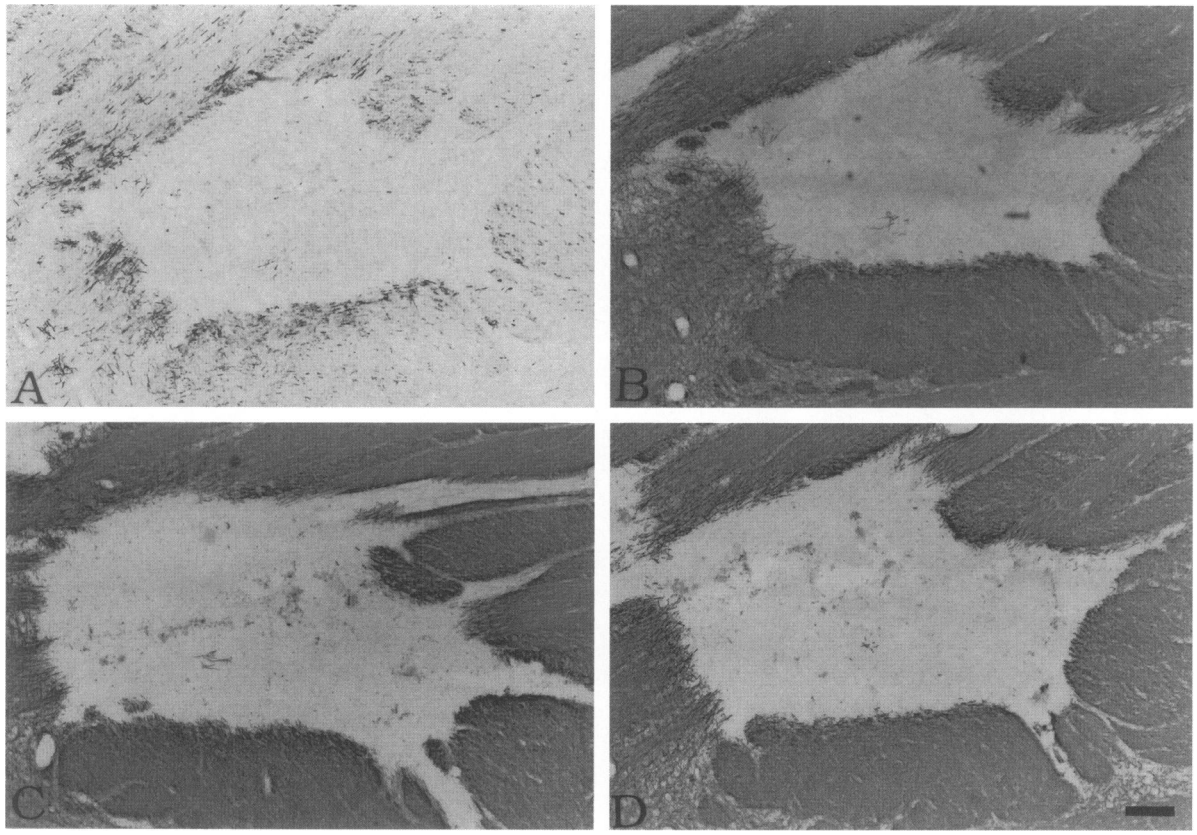

Figure 4. *Comparison of anti-EP immunohistochemistry with other antibodies in serial sections of a MS plaque from paraformaldehyde-fixed tissue.* **A:** *EP-positive structures were confined to abnormal appearing oligodendroglial elements within or around the MS plaque.* **B:** *Anti-MBP stained all myelinated structures.* **C:** *Clone 14 recognized all myelinated structures.* **D:** *Clone 2 also recognized all myelinated structures. See Materials and Methods for details. Bar, 200 μm.*

elements around the plaques, whereas each of the monoclonal antibodies strongly stained normal myelin in addition to the abnormal structures.

The EP-positive structures consisted mostly of long thickened fibers with no cell body visible (Figure 6A) or thickened, misshapen fibers extending from positively staining oligodendrocytes (Figure 6B). In some areas, densely staining cell bodies of irregular size were predominant, sometimes with thin, degenerate-appearing fibers extending from them (Figure 6C).

The plaques examined were in various stages as judged by inflammatory markers such as ORO and HLA-DR. HLA-DR is a marker of reactive microglia/macrophages and ORO turns a cherry red color when it is exposed to phagocytosed myelin in lipid-laden macrophages.[26,28] The criteria of Sanders et al[28] were used in judging the activity of the plaques. These criteria are as follows: type 1, pre-plaques consisting of a focal area of HLA-DR-positive reactive microglia; type 2, active plaques with a hypercellular demyelinating margin and many ORO-positive macrophages throughout the plaque; type 3, moderately active plaques with many ORO-positive macrophages at the demyelinating margin but not within the plaque; and type 4, least active plaques with scattered HLA-DR-positive reactive microglia at the plaque edge but few ORO-positive macrophages. Of the plaques observed, 2 were type 1, 12 were type 2, 25 were type 3, and 17 were type 4. Structures positive for EP were observed in association with each plaque of every type.

A typical example of a Sanders et al type 4 least active or inactive plaque is shown in Figure 7. A large demyelinated area is illustrated by Klüver-Barrera staining (Figure 7A). A nearby section stained for HLA-DR shows a rim of reactive microglia around the plaque (Figure 7B). Another nearby section stained for EP shows a few sparsely scattered oligodendroglial processes external to the rim of concentrated microglial cells (Figure 7C). A higher magnification of these processes reveals their swollen and irregular shape (Figure 7D).

Multiple staining gave greater insight into the interaction between EP-positive structures and reactive microglia/macrophages near active areas of my-

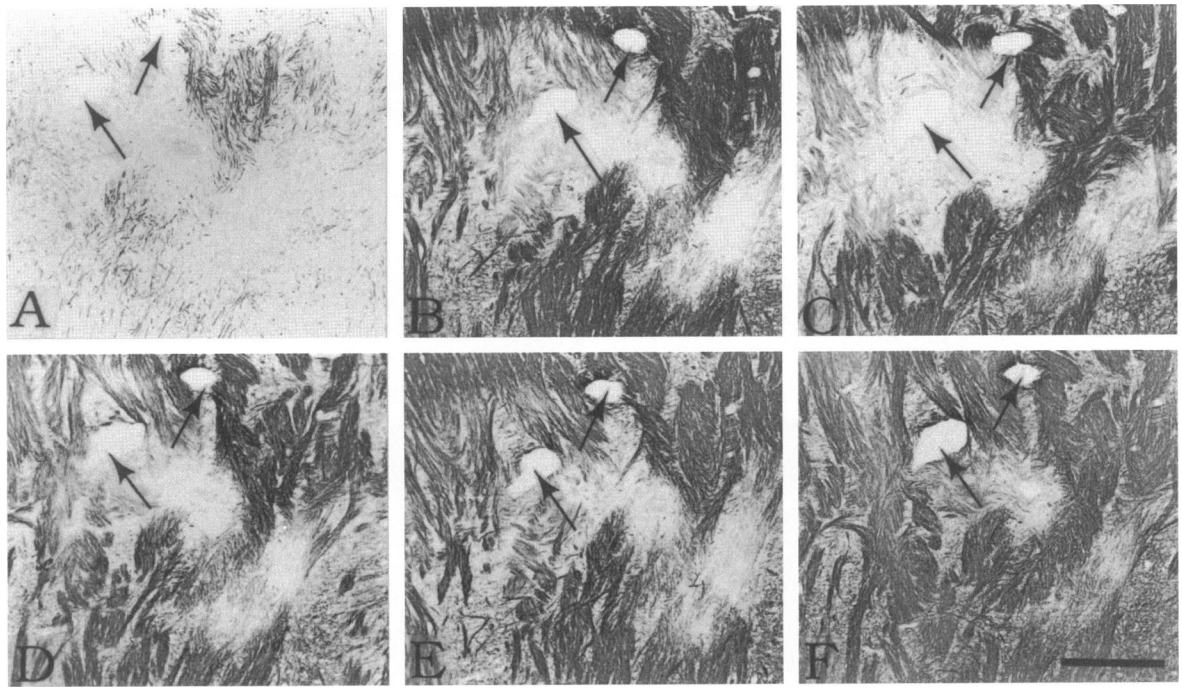

Figure 5. *Serial sections of paraffin-embedded MS tissue immunostained with anti-EP* (**A**), *clone 26* (**B**), *clone 2* (**C**), *clone 14* (**D**), *clone 12* (**E**), *or clone 22* (**F**). *Notice that only abnormal myelin tissue is strongly stained by anti-EP, whereas all other antibodies strongly stain the normal myelin surrounding the plaque area. See Materials and Methods for details.*

elin degeneration. Figure 8 illustrates double immunostaining for EP and HLA-DR as well as one example of ORO counterstaining to reveal those macrophages containing digested myelin. Figure 8A demonstrates double immunostaining around a hyperactive area near a brain stem vessel meeting the criteria of Sanders et al for a type 1 plaque. EP-positive structures appear next to the reactive microglia closest to the vessel. Figure 8B demonstrates double immunostaining for EP and HLA-DR in the brain stem near an early plaque meeting Sanders et al type 2 criteria. Degenerating EP-positive oligodendroglia and their processes can clearly be seen intermingling with HLA-DR-positive microglia gathered around the margin of the plaque. Figure 8C shows the same double immunostaining near a plaque meeting Sanders et al type 3 criteria. In this photomicrograph, many HLA-DR-positive lipid-laden macrophages are seen at the margin of the plaque. Degenerating EP-positive elements are seen external to this demyelinated region. Some macrophages contain EP-positive material at the margin, but the macrophages closer to the center of the plaque, where demyelination has already occurred, are EP negative. Figure 8D is a high-power photomicrograph of a type 3 plaque with double immunostaining plus ORO counterstaining. Again, EP-positive degenerating fibers can be seen at the margin of the plaque with some macrophages containing EP-positive material. In the demyelinated area, ORO-positive macrophages are seen that are not EP positive, suggesting that further myelin digestion results in disappearance of the epitope. Double immunostaining was also carried out for EP and T-4 as well as T-8 cells. As previously described,[26] T cells were overwhelmingly localized to perivascular cuffs and were not observed to be concentrated near EP-positive or ORO-positive structures.

To determine whether the EP-positive structures are specific for MS, we stained cerebral infarcts with demyelinating areas in six brains. The anti-MBP antibodies recognized all myelinated structures in the infarction brains, whereas the EP antiserum stained selectively abnormal-appearing fibers and cell bodies of oligodendrocytes near the infarcted areas. This is illustrated in Figure 9, which shows several sections of an infarcted area from a non-MS brain immunostained with EP antiserum and clone 22. As with MS plaque tissue, the EP antibody stained only abnormal oligodendroglial elements, whereas clone 22 stained all myelinated structures.

As with normal human tissue, the EP antiserum failed to stain any structures in normal rat or guinea pig brain, whereas clone 26 strongly stained the normal myelin (Figure 10).

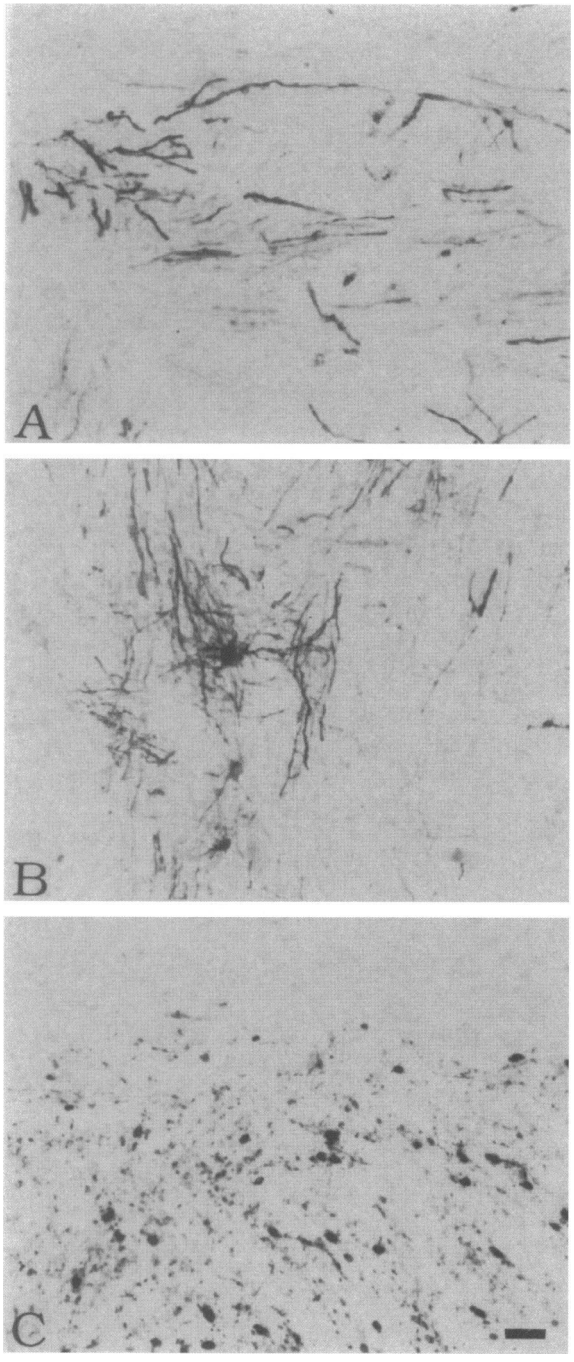

Figure 6. *Various patterns of EP-positive oligodendroglial elements observed in MS tissue.* A: *Long thickened fibers with no cell body visible.* B: *Thickened or long misshapen fibers extended from a positively staining oligodendrocyte.* C: *Densely stained cell bodies of irregular size and shape. Sometimes degenerate-appearing fibers extended from them. Bar, 25 μm.*

Discussion

The EP antiserum used in this study was raised in a rabbit against gMBP residues 68 to 86. The designation EP (encephalitogenic peptide) was given because it is the strongest encephalitogenic sequence for inducing EAE in Lewis rats.[18–22] The antiserum recognized hMBP 69 to 86, which has in common with gMBP the terminal sequence QDENPVV (glutamine-aspartate-glutamate-asparagine-proline-valine-valine), but it did not recognize the peptide S55S (gMBP 73 to 83).[19,20] This latter peptide is believed to contain the minimal sequence for T-cell epitope response in Lewis rats[20] but lacks the carboxyl-terminal sequence PVV, indicating that PVV is essential for recognition by the EP antiserum. The fact that anti-EP selectively stained damaged myelin and that this immunostaining was completely abolished by immunoabsorption with QDENPVV indicates that this sequence contains the key epitope exposed in areas of myelin damage.

Clones recognizing sequences very close to QDENPVV intensely stained normal myelin, as did antibodies recognizing sequences more remote from this area. Clone 26, for example, raised against hMBP 65 to 79,[23] recognized hMBP 69 to 88 and gMBP 68 to 86 but failed to recognize 10 μg/ml QDENPVV (Table 2). This slightly more amino-terminal-directed antibody strongly stained normal myelin.

Clone 22 was raised against hMBP 74 to 88.[23] This clone recognized hMBP 68 to 88 and gMBP 68 to 86, but it also failed to recognize 10 μg/ml QDENPVV. Therefore, clone 22, which strongly stains normal myelin, must also recognize sequences amino-terminal to QDENPVV.

Clone 12, raised against bovine MBP,[24,25] has been reported to recognize peptides corresponding to hMBP 81 to 98 but not 89 to 98 or 87 to 98.[24] The fact that this clone also failed to recognize QDENPVV indicates that the epitope recognized by the clone must require amino acids beyond valine 88, ie, possibly HFF. The supplier (Chemicon) reports that the clone only faintly recognizes peptides in which the FF bond has been cleaved, indicating the necessity of sequences carboxy-terminal to QDENPVV.

In a previous study, antibodies mapping to epitopes in the hMBP 80 to 89 region were highly sensitive to minor changes in peptide antigen sequences.[29] It is therefore reasonable to speculate that it would be relatively unexposed to the hydrophilic extracellular environment. The fact that the EP antiserum recognizes MBP that has been extracted in SDS and run on Western blots indicates that the epitope is accessible in conformationally altered MBP.

The EP antiserum stained the fibrils and cell bodies of damaged oligodendrocytes in regions of myelin removal after ischemic damage. As EP-positive structures appeared in damaged myelin regions of infarcted tissue, the exposure of EP epitopes on

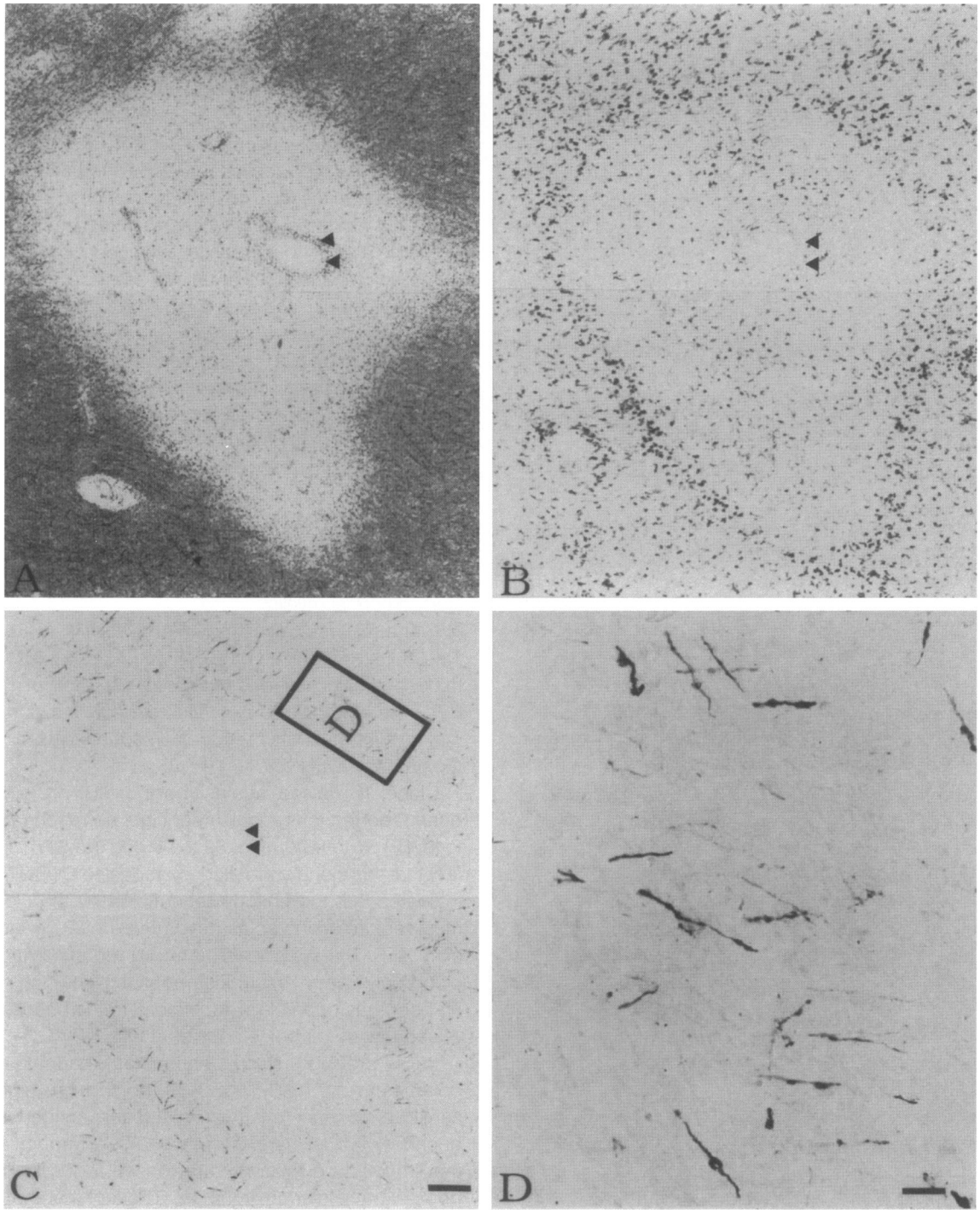

Figure 7. *EP-positive oligodendroglia remained around an inactive MS plaque.* A: *Klüver-Barrera's staining shows a demyelinated region.* B: *HLA-DR immunohistochemistry. Notice microglia were limited to the rim of this MS plaque.* C: *EP immunohistochemistry. EP-positive oligodendroglial elements were sparse although in a wider distribution than in active or moderately active stages.* D: *Higher-power magnification of the boxed area in* C. *Swollen and irregular processes are visible. The* arrowheads *indicate the identical vessel in* A *to* C. *Bar, 200 μm* (C) *and 25 μm* (D).

oligodendrocytes does not appear to be specific to MS pathology but rather to myelin digestion. The appearance of EP-positive material within fat-laden macrophages would suggest that the EP epitope survives initial phagocytosis. Further myelin catabolism may lead to this previously hidden but potentially pathogenic sequence being revealed to the immune system. Such exposure, which could follow

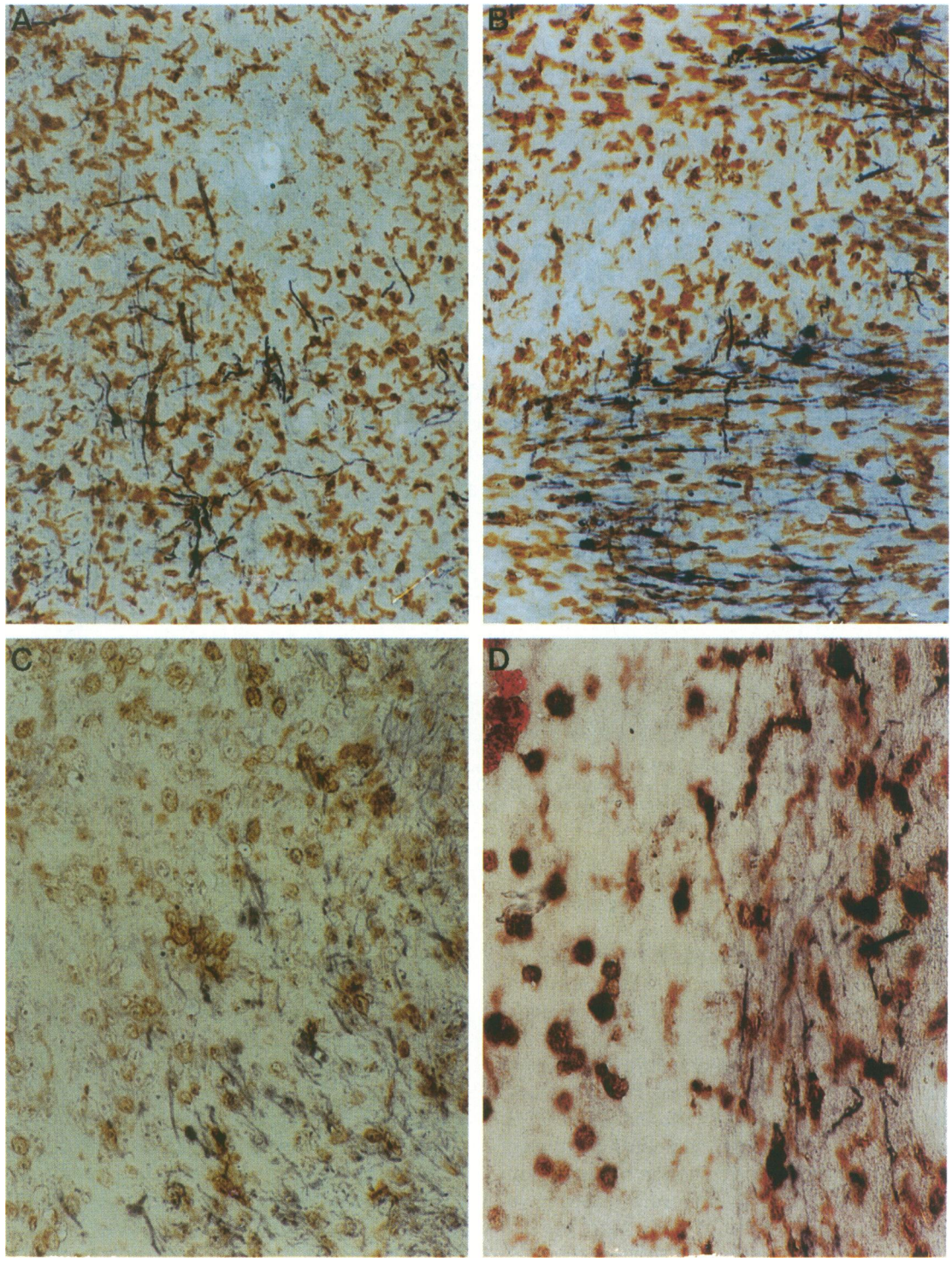

Figure 8. *Relationship of EP-positive structures to reactive microglia and lipid-laden macrophages in different MS plaque types as defined by the criteria of Sanders et al.*[21] **A:** *Double immunostaining of MS brain stem in a type 1 plaque. HLA-DR-positive reactive microglia (brown) surround a vessel, whereas EP-positive abnormal oligodendroglial fibers (purple) appear at the periphery.* **B:** *Double immunostaining of a type 2 plaque. HLA-DR-positive reactive microglia surround a demyelinated area with abnormal EP-positive oligodendroglia appearing just beyond the demyelinated area.* **C:** *Double immunostaining of a type 3 plaque. An external layer of brown HLA-DR-positive microglia lies just outside an internal layer of HLA-DR-positive, fat-laden macrophages at the plaque edge. EP-positive fibers (purple) are intermingled with the reactive microglia, but a few of the more externally located macrophages are EP positive.* **D:** *Double immunostaining with ORO counterstaining of a type 3 MS plaque. The same arrangement is visible as in* **C**, *except the ORO-positive macrophages (red) indicate that myelin digestion is occurring. Note also that some of the more externally located macrophages contain EP-positive material.*

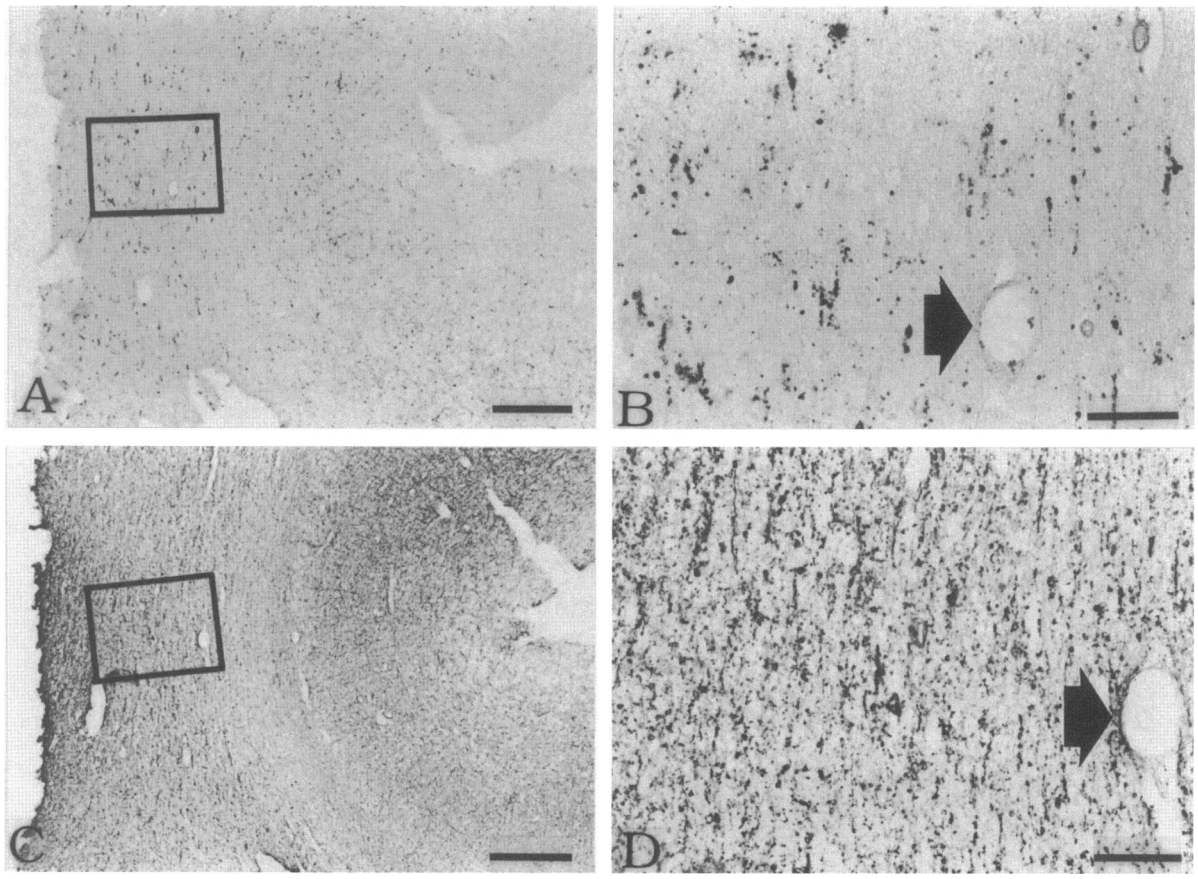

Figure 9. *Immunostaining with anti-EP* (A *and* B) *and clone 22* (C *and* D) *in nearby sections of an infarcted area in the occipital cortex of a non-MS case. Boxed areas in* A *and* C *are enlarged in* B *and* D. Arrows *in* B *and* D *identify the same vessel. Notice that the anti-EP antibody stains abnormal oligodendroglial structures, whereas clone 22 stains all myelinated structures. Bar, 500 μm* (A *and* C) *and 100 μm* (B *and* D).

vascular damage, trauma, infarction, or other injurious circumstances, could be the initiating event in an autoimmune cycle.

Human MBP is hypothesized to be the source of pathogenic peptides that might be responsible for a cell-mediated immune response in MS. Evidence supporting this general hypothesis is of two kinds: appearance of hMBP-immunoreactive material in CSF and urine of MS patients[2,9,30–32] and the appearance in MS brain tissue[14–16] and CSF[9–13] of immunoglobulins and immunoglobulin-producing cells that recognize selective peptide sequences of MBP. The sequences in all of these analyses were clustered in the hMBP region 75 to 95. Warren et al[15] found maximal IgG recognition of sequences in the MBP 85 to 95 region, which includes only the PVV sequence of the EP peptide. They interpreted this maximization as occurring for peptides corresponding to the minimal HLA-DR2b-restricted epitope previously identified for T cells reactive with MBP.[8] Marino et al[13] found that IgG-producing cells maximally recognized peptides in the MBP 70 to 89 region, which includes the complete QDENPVV sequence.

The epitope QDENPVV seems to be special in that it is not normally exposed to myelin-detecting antibodies. It appeared in actively degenerating areas at the margins of plaques and was largely restricted to a junctional zone between highly activated microglia and neutral-lipid-laden, ORO-positive macrophages. Clearly, this is a zone where these immunocompetent cells could pick up fragments of MBP for processing and later presentation to T cells in major histocompatibility complex class II restricted fashion. The peptides processed for this purpose might later appear in the CSF, blood, and urine of MS patients. In future studies, it will be important to determine the range of conformationally inaccessible epitopes in MBP that may be displayed when myelin degenerates in the fashion demonstrated in this study. It will also be of interest to learn whether the same epitopes also appear in a free form in body fluids and whether immunoglobulins and immunoglobulin-producing cells specific for these epitopes routinely

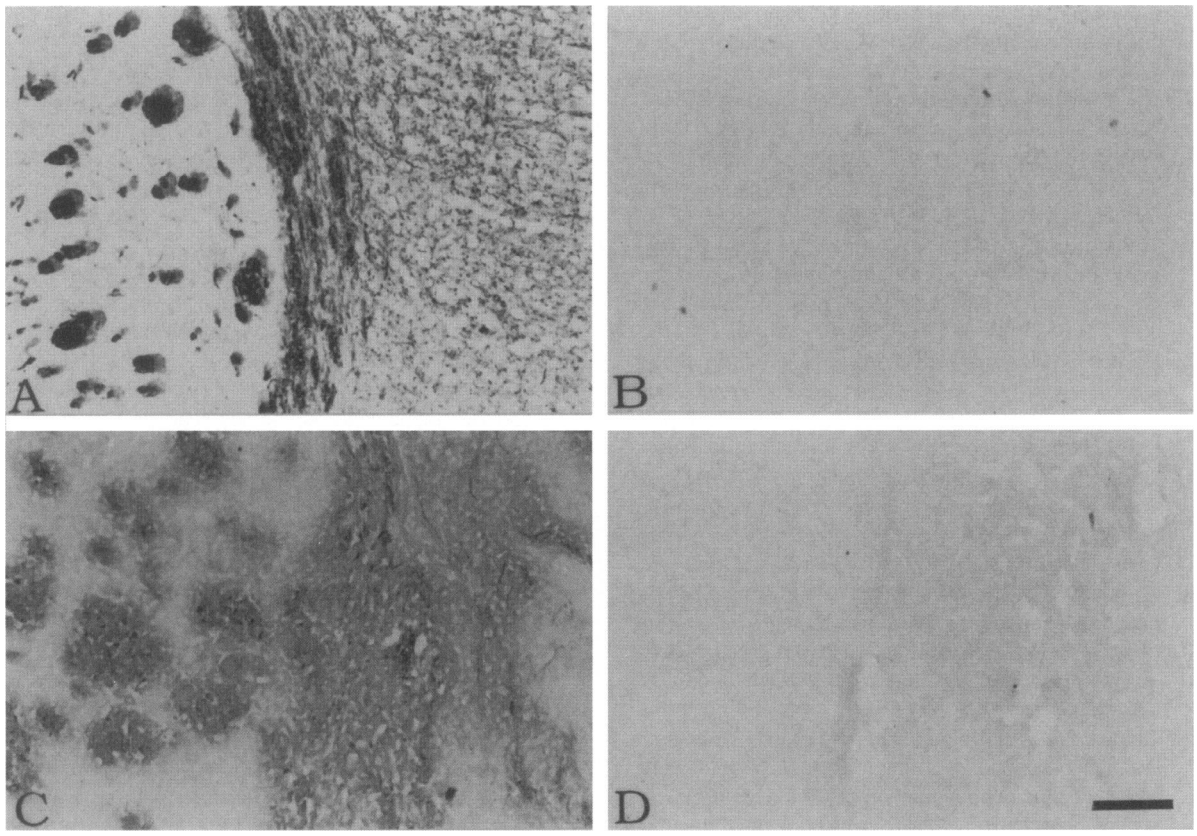

Figure 10. *Comparison between anti-hMBP and anti-EP immunohistochemistry in a rat and guinea pig brain. Clone 26 stained all myelinated structures in rat* (**A**) *and guinea pig* (**C**) *brain, whereas no immunoreactivity was detected by the EP antiserum (rat* (**B**) *and guinea pig* (**D**)). *Bar, 100 μm.*

appear in MS CSF and brain tissue. If precise epitopes can be identified as strongly antigenic in MS, therapeutic strategies might be designed to ameliorate immune responses to them.

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