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RESEARCH ARTICLE

Elevated Activity and Microglial Expression of Myeloperoxidase in Demyelinated Cerebral Cortex in Multiple Sclerosis

Elizabeth Gray, BSc, PhD; Taya L. Thomas, BSc; Samar Betmouni, MRCPath, DPhil; Neil Scolding, FRCP, PhD; Seth Love, FRCP, FRCPath, PhD

Glial Cell Biology Laboratories, University of Bristol Institute of Clinical Neuroscience, Frenchay Hospital, Bristol, UK

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Corresponding author:
Seth Love, PhD, Department of Neuropathology, University of Bristol Institute of Clinical Neuroscience, Frenchay Hospital, Bristol BS16 1LE, UK (E-mail: *seth.love@bris.ac.uk*)

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Abstract

Recent studies have revealed extensive cortical demyelination in patients with progressive multiple sclerosis (MS). Demyelination in gray matter lesions is associated with activation of microglia. Macrophages and microglia are known to express myeloperoxidase (MPO) and generate reactive oxygen species during myelin phagocytosis in the white matter. In the present study we examined the extent of microglial activation in the cerebral cortex and the relationship of microglial activation and MPO activity to cortical demyelination. Twenty-one cases of neuropathologically confirmed multiple sclerosis, with 34 cortical lesions, were used to assess microglial activation. HLA-DR immunolabeling of activated microglia was significantly higher in demyelinated MS cortex than control cortex and, within the MS cohort, was significantly greater within cortical lesions than in matched non-demyelinated areas of cortex. In homogenates of MS cortex, cortical demyelination was associated with significantly elevated MPO activity. Immunohistochemistry revealed MPO in CD68-positive microglia within cortical plaques, particularly toward the edge of the plaques, but not in microglia in adjacent non-demyelinated cortex. Cortical demyelination in MS is associated with increased activity of MPO, which is expressed by a CD68-positive subset of activated microglia, suggesting that microglial production of reactive oxygen species is likely to be involved in cortical demyelination.

INTRODUCTION

Although multiple sclerosis (MS) has long been regarded as a disease that mainly affects white matter, lesions can occur in any part of the central nervous system. Recent studies have demonstrated extensive demyelination in the cerebral cortex in MS (4, 10, 21). Several types of cortical demyelinating lesion have been delineated: intracortical perivenous plaques, leukocortical plaques (at the border between cortex and white matter and affecting both) and subpial plaques (4, 8, 17). The last of these tend to be the most numerous (4, 17).

In cortical plaques the inflammatory cell content is much lower than in white matter plaques (3, 17). Most of the inflammatory cells are activated microglia, rather than infiltrating lymphocytes and macrophages. In active gray matter lesions, activated microglia were reported to appose and ensheath apical dendrites, neurites and neuronal perikarya (17). Activated microglia are a major source of cytokines and oxidizing radicals such as superoxide, hydroxyl radicals, hydrogen peroxide and nitric oxide (1, 6, 9, 13). One source of oxidizing species in activated microglia (18) and macrophages (16) is myeloperoxidase (MPO), which catalyzes the formation of hypochlorous acid, a highly cytotoxic oxidant (15).

In the present study we have investigated the extent of microglial activation in the cerebral cortex in MS and the relationship of microglial activation to cortical demyelination. Our findings demonstrate that MS is associated with widespread microglial activation in the cerebral cortex, even in cortex which is not demyelinated. We have also shown that cortical demyelination is accompanied by elevated myeloperoxidase activity and that the expression of this enzyme in MS is largely restricted to cells that have morphological and immunohistochemical features of macrophages and are not present in non-demyelinated cortex.

MATERIALS AND METHODS

Brain tissue

Tissue for this study was obtained from the UK Multiple Sclerosis Tissue Bank, Imperial College, London and from Frenchay Neuropathology Department archives. The research was approved by Frenchay Research Ethics Committee. Frozen tissue from multiple regions of cerebral cortex and white matter was available from nine cases of neuropathologically confirmed MS and seven controls (see Table 1 for clinical details). The tissue had been snap-frozen by

Table 1. Clinical details of cases studied.

Case number	Age (years)	Sex	Cause of death	Duration of MS (years)	Course of MS	β interferon	Other diseases	Tissue
Multiple sclerosis (MS) cases								
MS1	60	F	Bronchopneumonia caused by MS	25	Progressive	No		P*
MS2	51	M	Pseudomembranous colitis caused by MS	23	Secondary progressive	No		P
MS3	38	M	Acute MS	<1	Fulminant	No		P
MS4	46	M	Bronchopneumonia caused by MS	6	Secondary progressive	No		P
MS5	74	F	Chronic obstructive airways disease and MS	9	Secondary progressive	No		P
MS6	67	F	Bronchial carcinoma	13	Primary progressive	No		P
MS7	63	M	Not known	24	Progressive	No		P
MS8	61	M	Massive haemoptysis (source not specified)	35	Not known	No		P
MS9	53	M	Myocarditis with heart failure	Not known	Not known	No		P
MS10	61	F	Bronchopneumonia caused by MS	19	Primary progressive	No		P
MS11	44	F	Bronchopneumonia caused by MS	7	Secondary progressive	No		P
MS12	27	F	Bronchopneumonia caused by MS	1.5	Secondary progressive	No		P
MS13	43	F	Bronchopneumonia caused by MS	Not known	Not known	No		P
MS14	50	M	Bronchopneumonia caused by MS	4	Primary progressive	No		P
MS15	63	F	Old myocardial infarct and probable terminal arrhythmia	24	Secondary progressive	No		P
MS16	27	M	Acute MS	<1	Fulminant	No		P
MS17	53	F	MS	Not known	Not known	Not known		P
MS18	44	F	MS	Not known	Not known	Not known		P
MS19	44	M	Bronchopneumonia caused by MS	>10	Secondary progressive	Not known		P
MS20	51	M	Bronchopneumonia caused by MS	10	Secondary progressive	Yes		P
MS21	40	M	Respiratory failure and sepsis caused by MS	12	Secondary progressive	Yes		P
MS22	49	F	Bronchopneumonia caused by MS	23	Secondary progressive	No	Hypothyroid	F
MS23	63	M	Pneumonia and MS	39	Secondary progressive	No		F
MS24	66	F	Carcinoma of the lung and MS	25	Primary progressive	No		F
MS25	80	F	Cerebrovascular aneurysm	Not known	Not known	No		F
MS26	49	F	Respiratory infection	19	Secondary progressive	No		F
MS27	78	F	Metastatic carcinoma of bronchus	42	Secondary progressive	No		F
MS28	64	F	Aspiration pneumonia and gastrointestinal bleed	36	Secondary progressive	No		F
MS29	77	F	Lung infection	>25	Progressive	No		F
MS30	46	M	Pneumonia	8	Secondary progressive	No		F
Controls								
C1	92	M	Heart failure, old age				Multiple CVAs	F
C2	82	M	Not known				Chronic schizoprenia, skin cancer	F
C3	64	F	Cardiac failure				Myocardial infarct	F
C4	90	F	Old age				Skin cancer, breast cancer, hypothyroidism	F/P
C5	84	F	Ischaemic heart disease, atrial fibrillation, heart failure				Thyroid adenoma, myelodysplasia, atrial fibrillation, cerebral infarcts	F/P
C6	35	M	Carcinoma of the tongue					F/P
C7	60	F	Ovarian cancer					F/P
C8	78	F	Myeloid leukaemia					P
C9	60	F	Acute pancreatitis					P
C10	60	M	Carcinoma of the lung					P

*F = snap-frozen tissue; P = paraffin embedded tissue.

Table 2. Antibodies used for immunoperoxidase and immunofluorescent labeling. Abbreviations: IP = immunoperoxidase; IF = immunofluorescence; MBP = myelin basic protein; NF = neurofilament; MPO = myeloperoxidase; GFAP = glial fibrillary acidic protein

Antigen	Clone	Species	Pre-treatment	Dilution		Source
				IP	IF	
MBP	12	Rat	EDTA	1:3200 (P)*1:100 (F)	–	Serotec, Oxford, UK
MBP	–	Rabbit	Citrate	–	1:100 (P)	Abcam, Cambridge, UK
NF	–	Rabbit	Citrate	–	1:100 (P)	Abcam, Cambridge, UK
MPO	2C7	Mouse	Citrate	–	1:100 (P)	Abcam, Cambridge, UK
HLA-DR	CR3/43	Mouse	Citrate	1:800(P)	1:100 (P)	Dako, Ely, UK
CD68	KP1	Mouse	Citrate	1:400(P)	1:200 (P)	Dako, Ely, UK
CD68	C-18	Goat	Citrate	–	1:100 (P)	Santa Cruz, Calne, UK
GFAP	–	Rabbit	Citrate	–	1:200 (P)	Dako, Ely, UK

*F = snap-frozen tissue; P = paraffin-embedded tissue.

immersion in isopentane and stored at −80°C. Paraffin blocks of formalin-fixed tissue from multiple regions of cerebral cortex were available from 21 cases of neuropathologically confirmed multiple sclerosis MS and seven controls (Table 1).

Immunoperoxidase labeling of paraffin sections

Sections 7 μm in thickness were cut from blocks of the frontal and temporal lobes. Sections were immunostained with antibodies to myelin basic protein (MBP) and HLA-DR and sections from selected blocks also with antibody to CD68 (Table 2). Sections were dewaxed, hydrated, and immersed in 3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity, rinsed and microwaved in sodium citrate buffer (0.01 M, pH 6.0, 5 minutes) or EDTA buffer (1 mM, pH 8, 10 minutes) as appropriate and rinsed in phosphate-buffered saline (PBS). Non-specific binding was blocked with Vectastain blocking serum (20 minutes). After addition of the primary antibody, sections were incubated overnight at 4°C. The sections were then rinsed in PBS before incubation for 20 minutes with secondary antibody (Vectastain Biotinylated Universal antibody) and 20 minutes with VectaElite ABC Complex (PK-6200, Vector Laboratories, Peterborough, UK) followed by a 10-minute incubation with 3,3′-diaminobenzidine (DAB) and 0.01% H_2O_2. Sections were washed in water, immersed in copper sulphate DAB enhancer (4 minutes), counterstained with hematoxylin, dehydrated, cleared and mounted. Controls in each run included sections known to contain cortical plaques and sections of normal brain, as well as sections incubated overnight in PBS instead of primary antibody.

Further sections were stained for myelin with solochrome cyanin. In general there was close correspondence between the areas of demyelination demonstrated by immunostaining for MBP and staining with solochrome cyanin. However, immunostaining for MBP provided clearer demarcation of cortical plaques and proved more sensitive at demonstrating smaller plaques, particularly in the subpial region and was therefore used to assess whether demyelination was or was not present and to classify cortical plaques as subpial, intracortical, leukocortical or transcortical (ie, extending across the full thickness of the cortex from the pial surface to the white matter) according to their location within the cortex.

Quantitative assessment of activated microglia in paraffin sections

Histometrix software (Kinetic Imaging, Nottingham, UK), driving a Leica DM microscope (Milton Keynes, UK) with a motorized stage, was used to quantify HLA-DR immunolabeling. Areas of demyelination or of non-demyelinated cortex were outlined interactively and the software was programed to make an automated unbiased selection of 10 random ×20 objective fields (covering a total of 4 mm^2) within the outlined area. Every non-demyelinated area was outlined in a region of cortex that was of approximately the same size, shape and intracortical location as an outlined demyelinated area in the same section. All of the demyelinated (and corresponding non-demyelinated) areas that we analyzed were >4 mm^2 (ie, exceeded the area sampled by the software). The captured images were interactively edited to exclude blood vessels. The mean percentage area of brain parenchyma that was immunopositive for HLA-DR was determined for each area of demyelinated cortex and each paired area of non-demyelinated cortex.

Immunfluorescent labeling of paraffin sections

Sections were dewaxed, hydrated and rinsed as above. To reduce auto-fluorescence, sections were incubated in 5 mM copper sulphate and 50 mM ammonium acetate for 1 h at room temperature prior to microwaving of the sections in sodium citrate buffer (0.01 M, pH 6.0, 5 minutes). The distribution of MPO was assessed by single and double immunofluorescence using rabbit anti-MPO antiserum alone or in combination with antibodies to MBP, phosphorylated neurofilament (NF), glial fibrillary acidic protein (GFAP) or CD68 (see Table 2). Non-specific binding was blocked with 10% normal goat or donkey serum diluted in PBS containing 0.1% triton. Sections were incubated at 4°C overnight with primary antibodies. Sections were then washed in PBS and incubated for 30 minutes in the dark with the appropriate secondary antibodies (1:1000): donkey anti-mouse Alexa Fluor 555, donkey anti-goat Alexa Fluor 488, donkey anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 546 or goat anti-rabbit Alexa Fluor 488 (all from Invitrogen, Paisley, UK), before being washed in PBS and mounted in Vectashield medium containing the nuclear dye 4′6′-diamidino-2-phenylindole (DAPI)

(H-1200, Vector Laboratories). For all of the immunofluorescence, images were acquired using an inverted Leica CTR 6000 fluorescence microscope and merged with Leica Application Suite Advanced Fluorescence software.

Assessment of demyelination in blocks of frozen tissue

Sections 15 μm in thickness were cut from the blocks of frozen tissue (each measuring approximately 2 × 2 cm in cross-section and 1 cm in thickness) and immunostained for MBP. The sections were fixed in acetone, pre-treated in ice-cold methanol, rinsed, immersed in 3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity and rinsed in PBS. Non-specific binding was blocked by incubation for 20 minutes in Vectastain blocking serum (Vector Laboratories). Sections were incubated for 1 h at room temperature with myelin basic protein antibody (Table 2). The sections were then rinsed in PBS before incubation for 20 minutes with secondary antibody (Vectastain Biotinylated Universal antibody) and 20 minutes with VectaElite ABC Complex (PK-6200, Vector Laboratories) followed by a 10-minute incubation with 3,3′-diaminobenzidine and 0.01% H_2O_2. Sections were washed in water, immersed in copper sulphate DAB enhancer (4 minutes), counterstained with hematoxylin, dehydrated, cleared and mounted. Cortical plaques were classified as subpial, intracortical, leukocortical or transcortical. However, many of the cryostat sections included multiple plaques of different types and sizes, and to provide an indication of the extent of demyelination in each block, we also made the following semiquantitative 3-tier assessment of the proportion of cortex that was demyelinated: mild (<10%), moderate (10%-50%) or severe (>50%).

Measurement of MPO activity in cerebral cortex

After the sections had been cut and used to assess the distribution of cortical demyelination and proportion of cortex involved, the frozen tissue blocks were thawed on ice and the cortex carefully dissected away from underlying white matter. The cortex was homogenized on ice in a ratio of 300 mg cortex to 1 mL of lysis buffer (0.1 mM NaCl, 10 mM Tris (pH 7.6), 100 μM phenylmethylsulfonylfluoride, 1 μg/mL aprotinin and 1% sodium dodecyl sulphate in distilled water). The supernatants were removed and stored at −80°C until required. MPO activity was subsequently assayed in the supernatants (Innozyme Myeloperoxidase Activity kits, Calbiochem, Merck Chemicals, Nottingham, UK). The assay uses immobilized polyclonal antibody to human MPO immobilized to capture the enzyme on a 96-well plate. One hundred microliters aliquots of homogenate containing 3 mg/mL of protein were added in duplicate and incubated at room temperature for 1 h. Each plate included a 0–100-ng/mL range of MPO standards that was used to construct a standard curve and blank wells that contained sample buffer alone. The reaction was started by adding 100 μL of tetramethylbenzidine (TMB) and the plates incubated at 37°C for 30 minutes. The reaction was stopped by addition of sulphuric acid, the absorbance of the oxidized TMB read at 450 nm and the concentration of active MPO (ng/mL) determined by interpolation from the standard curve.

Statistical analysis

Paired *t*-tests were used for comparison of HLA-DR immunolabeling of cortical plaques and matched areas of non-demyelinated cortex in the same sections and for comparison of post-mortem delays in samples used to measure MPO activity. The relationship between MPO activity and post-mortem delay was assessed by Pearson regression analysis. Kruskal–Wallis test was used to compare HLA-DR immunolabeling in cortical plaques of different type in the MS brains and Dunn's (non-parametric) multiple comparison test to compare the values in both demyelinated and non-demyelinated cortex with those in control brains. Because some of the MPO measurements were on different samples from the same brains, we could not treat all of the values independently and for statistical analysis therefore fitted a maximum restricted likelihood mixed-effect regression model that assumed both random effects between and within subjects and a fixed effect between subjects, based on presence/absence of MS and, within the MS cases, presence/absence of cortical demyelination in the sample. The model was chosen to take account of variable repeated measurements coming from different specimens of the same brain. The analysis performed with the help of STATA v9.0 (Timberlake Consultants, London, UK). For all tests, values of $P < 0.05$ were considered statistically significant.

RESULTS

Topography of cortical lesions in multiple sclerosis

Areas of cortical demyelination were readily identified in the sections immunostained for MBP (Figure 1A–F). Of the four types of cortical plaque detected, the commonest was transcortical (n = 19), extending from the pial surface to the junction of cortex and white matter. Next, most numerous were subpial plaques (n = 10), some of which affected more than one adjacent gyrus. Leucocortical plaques were relatively uncommon (n = 5) and only a single intracortical plaque was included. No demyelination or other cortical lesions were seen in the sections of control brain.

Distribution of HLA-DR and CD68 in cerebral cortex

In control brains immunolabeled for HLA-DR (Figure 2A), few microglia were demonstrable in the cerebral cortex. Occasional microglia were present adjacent to blood vessels; others were very sparsely distributed within the cortex. They typically had a ramified morphology with small perikarya and thin processes. In MS cases (Figure 2B,C), HLA-DR-positive microglia were scattered throughout the cortex but were abundant in areas of demyelination and particularly prominent toward the plaque edge, where they had enlarged perikarya with thicker processes. In non-demyelinated cortex, microglia were less numerous and more evenly distributed.

Immunolabeling for CD68 revealed very little antigen in cortex outside of plaques (Figure 3). Like HLA-DR-positive cells, those containing CD68 were most numerous toward the edge of the plaques. However, comparison of CD68 and HLA-DR labeling of serial sections through the same plaques showed that many more cells were immunopositive for HLA-DR than CD68 (Figure 3).

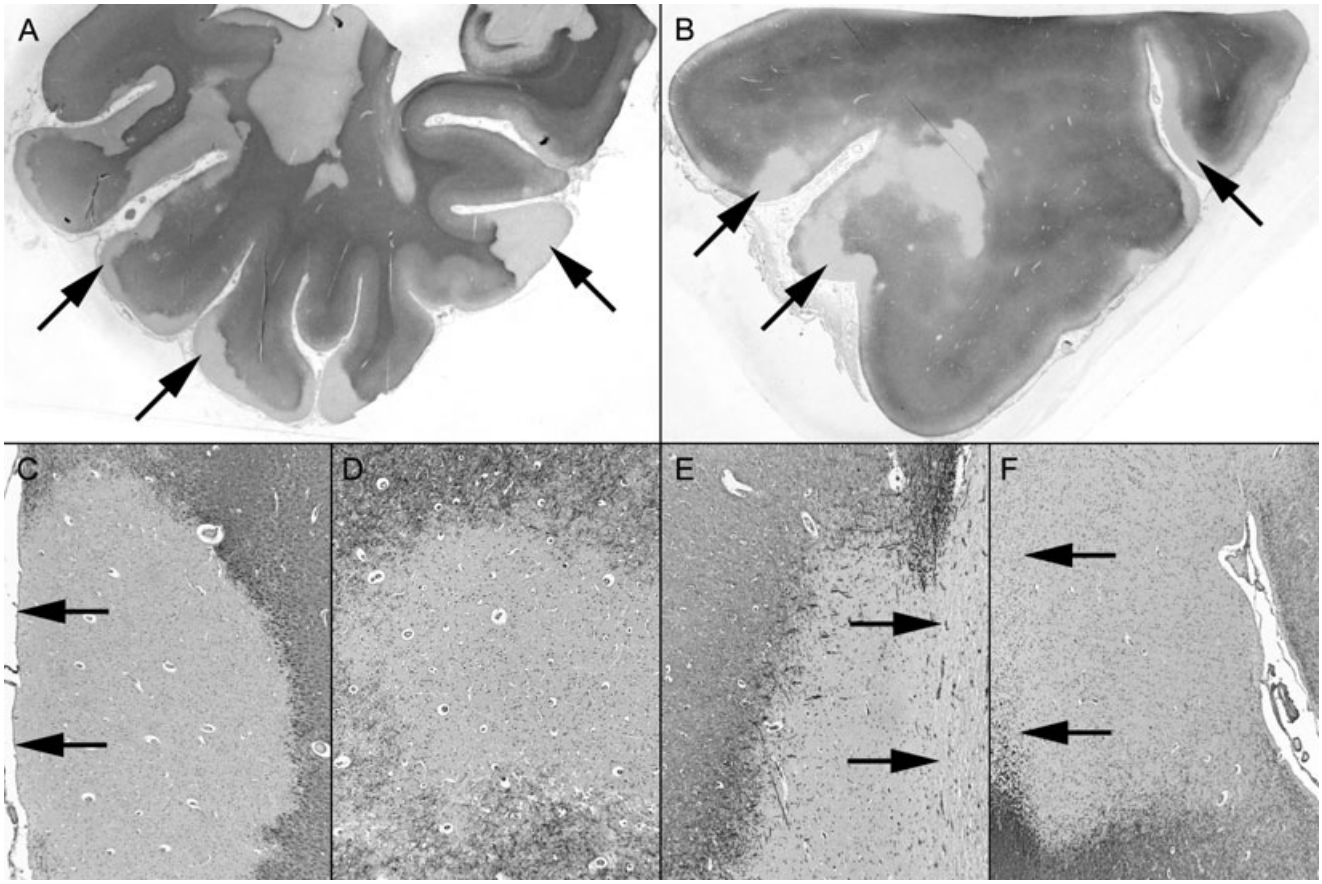

Figure 1. *Cortical demyelination in multiple sclerosis, demonstrated by labeling of paraffin sections for myelin basic protein.* **A,B.** Multiple well-defined areas of subpial and transcortical demyelination (arrows) in sections of temporal (**A**) and frontal (**B**) cortex. **C.** Subpial plaque. The arrows indicate the pial surface. **D.** Intracortical plaque. **E.** Leucocortical plaque. The arrows indicate the junction between the cerebral cortex and underlying white matter. **F.** Transcortical plaque. The arrows indicatie the junction between cortex and white matter.

Quantitation of HLA-DR immunolabeling

No significant differences were detected in the percentage area of HLA-DR immunolabeling in subpial, leucocortical and transcortical plaques (data not shown) in MS brains and the measurements were therefore pooled for further analysis. HLA-DR immunolabeling was significantly greater within 34 plaques (mean = 4.619, SE = 0.767, median = 3.366) than in matched non-demyelinated areas of cortex in the same sections (mean = 2.890, SE = 0.420, median = 2.355; $P = 0.0016$, paired *t*-test on data transformed logarithmically for normalization). Comparison of both sets of values (from demyelinated and non-demyelinated MS cortex) with measurements on sections from control brains (n = 12) (Figure 4) indicated that HLA-DR immunolabeling was higher in all regions of MS than control cortex but the difference was statistically significant only for the comparison of demyelinated cortex with control cortex ($P < 0.05$, Dunn's test).

MPO activity

MPO activity was measured in homogenates prepared from MS (32 demyelinated and 14 non-demyelinated) and control (n = 20) cortex. Post-mortem delay was significantly greater in the control (mean = 18 h, SE = 1.690, median = 18.0, range 13–24) than the MS group (mean = 7.944 h, SE = 0.604, median = 7.5, range 5–11; $P < 0.001$). However, within none of the three groups assessed below (MS demyelinated, MS non-demyelinated and control) was there a significant relationship between MPO activity and post-mortem delay.

MBP immunolabeling was used for semiquantitative assessment of the overall extent of cortical demyelination in cryostat sections from the blocks used to prepare these homogenates. Demyelination was classified as mild (<10% of cortex) in nine, moderate (10–50% of cortex) in a further nine and severe (>50% of cortex) in 14. The MPO measurements were right-skewed but were normalized by logarithmic transformation. MPO activity did not differ significantly between the corresponding homogenates (Figure 5) and the data from MS cases were therefore pooled for subsequent analysis.

Regression analysis of the contributions to MPO activity of presence/absence of MS and, within the MS cases, presence/absence of cortical demyelination indicated a strong positive association between presence of cortical demyelination in MS tissue samples and levels of MPO (linear coefficient 0.79, 95%

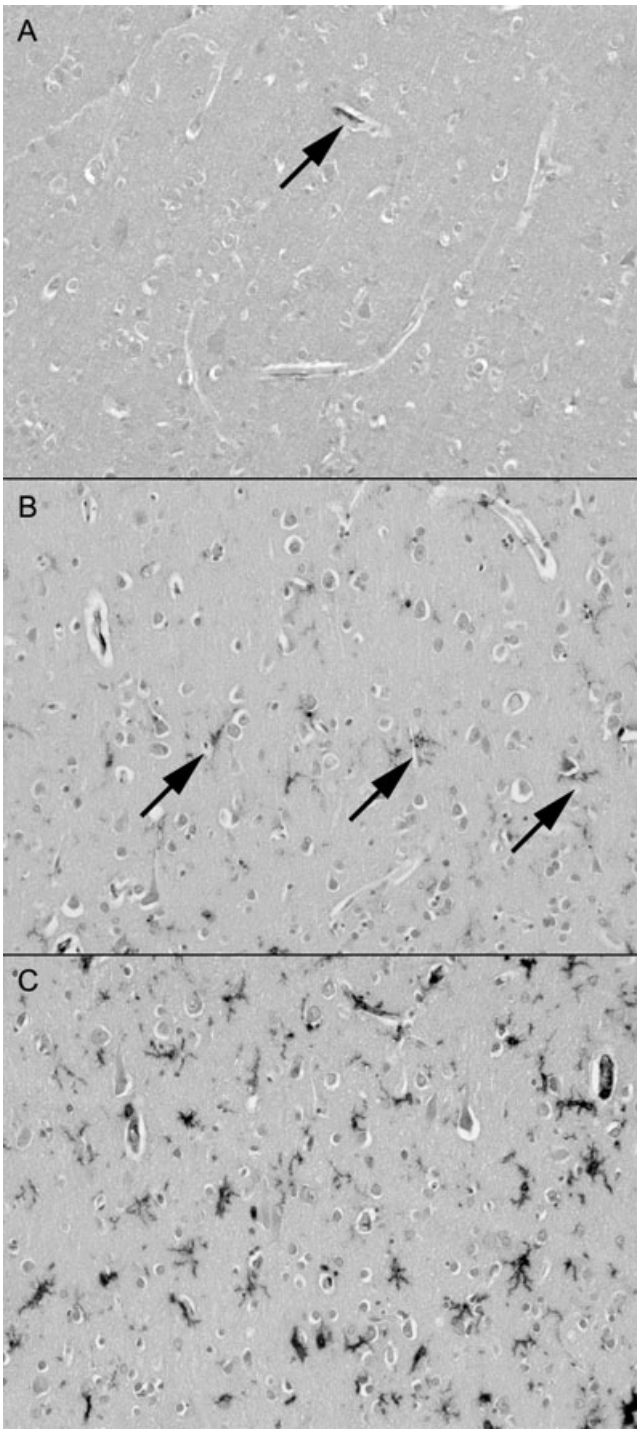

Figure 2. *Distribution of HLA-DR-positive cells in cerebral cortex.* **A.** This section of cortex from a control brain includes only a single HLA-DR-positive cell (arrow), adjacent to a capillary. **B.** Non-demyelinated cortex from a patient with multiple sclerosis includes sparsely distributed HLA-DR-positive microglia (arrows). **C.** Multiple activated microglia are labeled in a region of cortical demyelination.

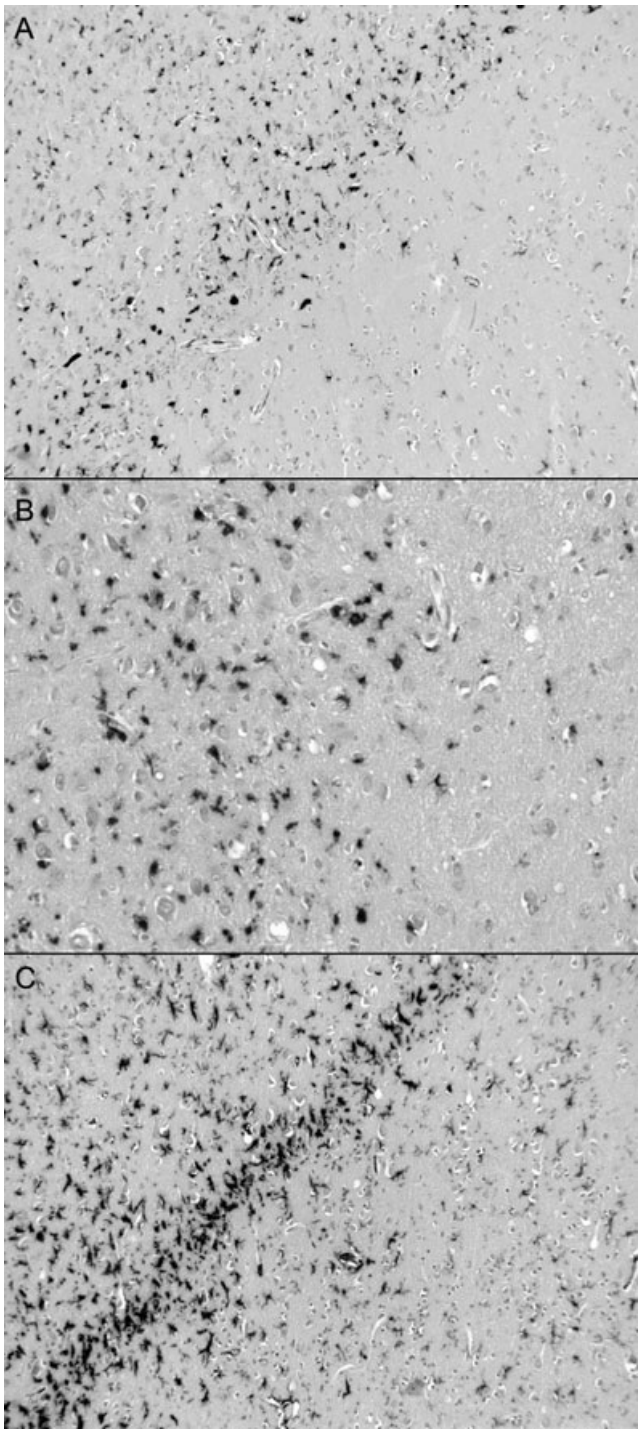

Figure 3. *Comparison of HLA-DR- and CD68-labeling in cerebral cortex.* **A.** CD68-positive cells are present within a cortical plaque (top left part of figure) but not in the adjacent, non-demyelinated cortex. The labeled cells are most numerous along the edge of the plaque. **B.** At higher magnification, CD68 is seen to be present in rounded cell bodies with little labeling of finer cell processes. **C.** In a serial section through the edge of the plaque illustrated in **A**, antibody to HLA-DR labels more cells than that are positive for CD68 both within the central part of the plaque and along its edge. The labeling extends along the cytoplasmic processes of many of the cells. HLA-DR-positive cells are also present, albeit at lower density, in the adjacent, non-demyelinated cortex.

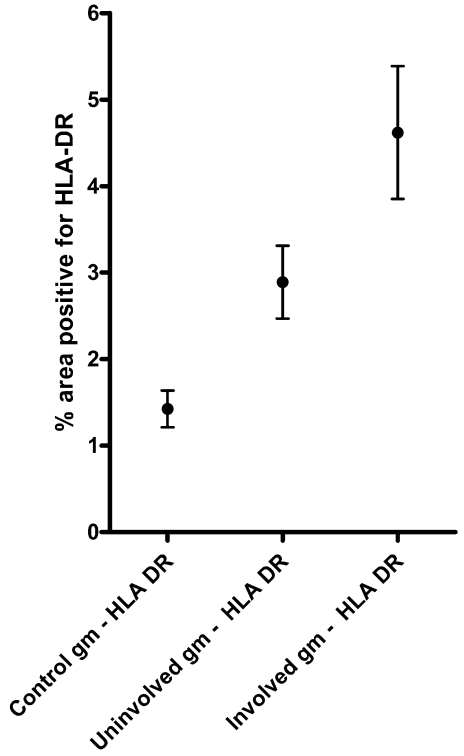

Figure 4. HLA-DR-labeling of plaques, non-demyelinated multiple sclerosis (MS) cortex and control cortex. The mean values ± SE are shown for 32 demyelinated and 14 non-demyelinated samples of MS cortex and 20 control samples. The labeling is significantly greater within plaques than that in matched non-demyelinated areas of cortex in the same sections ($P = 0.0016$). HLA-DR immunolabeling is higher in all regions of MS than control cortex but the difference is statistically significant only for the comparison of demyelinated with control cortex ($P < 0.05$). Abbreviation: gm = grey matter.

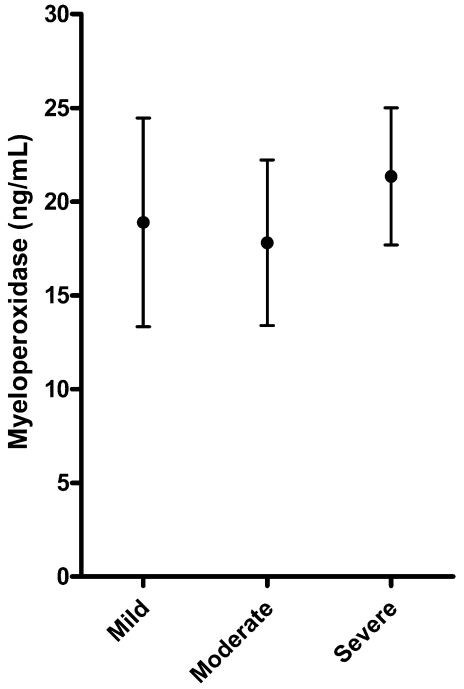

Figure 5. Levels of active myeloperoxidase in relation to extent of demyelination in frozen tissue blocks. The mean values ± SE are shown for nine blocks with mild, nine with moderate and 14 with severe demyelination. There is no significant difference between the groups.

confidence intervals 0.20–1.38, $P = 0.0008$) (Figure 6). MPO activity in non-demyelinated MS cortex was similar to that in control cortex.

Distribution of MPO in MS cortex

To identify the cells responsible for MPO production in MS cortex, we performed immunofluorescent examination of serial sections through cortical plaques. MPO immunoreactivity was located solely within plaques and particularly toward the edge of plaques. MPO staining was cytoplasmic and granular, in keeping with the lysosomal location of this enzyme. In sections through the same plaque, labeled with mouse monoclonal antibodies to MPO, CD68 and HLA-DR (Figure 7), the distribution and proportion of cells showing MPO immunoreactivity corresponded closely to those expressing CD68. Double immunofluorescent labeling (Figure 8) with mouse anti-MPO and rabbit anti-MBP confirmed that MPO-labeled cells were most numerous at the lesion edge and that almost all were immunopositive for CD68. Some of the MPO-positive cells also contained cytoplasmic MBP. Double immunofluorescent labeling of the plaques for MPO and GFAP showed the MPO-labeled cells to be interspersed between the GFAP-positive astrocyte processes. The MPO-positive cells were also interspersed between NF-positive processes and neuronal cell bodies.

DISCUSSION

In this study, we have demonstrated that regions of cortical demyelination in MS brains are associated with significantly elevated levels of myeloperoxidase activity. Immunofluorescent labeling of this enzyme has shown its expression to be restricted to a subset of microglia that are predominantly located near the margins of cortical lesions and that have morphological and immunohistochemical features in keeping with a phagocytic role for these cells.

Previous neuropathological studies demonstrated that cortical demyelination is extensive in MS (4, 11, 10, 21). This was confirmed in the present study. Our approach to categorizing the cortical plaques was similar to that used in previous studies (17), supplemented for the analysis of MPO activity in frozen tissue by semiquantitative assessment of the total extent of cortical demyelination. The methods had some limitations. Although the individual blocks of tissue were relatively small, we cannot exclude the possibility that some of those from MS brains may have included plaques that were not sampled in the superficial histological sections. However, the difference in MPO activity between samples assessed as being demyelinated and those thought not to be demyelinated suggests that the superficial cryostat sections provided a good representation of the presence or absence of demyelination in most of the blocks of frozen tissue. In addition, our study did not take account of possible remyelination within the cortex (2). Particularly in cryostat sections, cortex showing substantial remy-

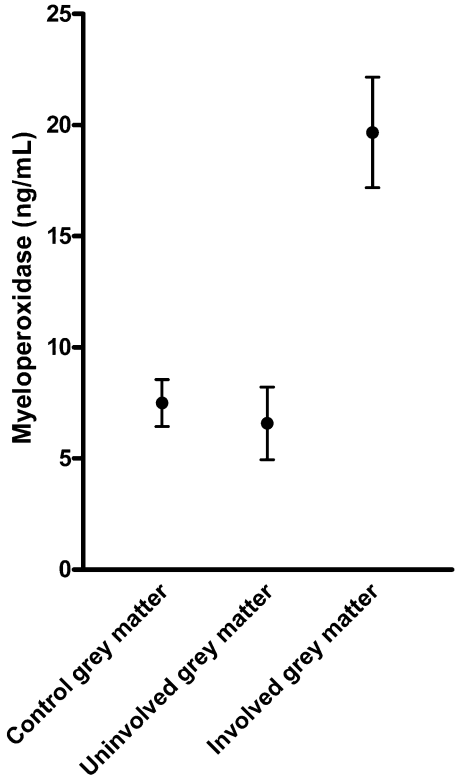

Figure 6. Levels of active myeloperoxidase in control, non-demyelinated and demyelinated multiple sclerosis (MS) cortex. The mean values ± SE are shown for 20 control samples, 14 of non-demyelinated and 32 of demyelinated MS cortex. Myeloperoxidase levels are significantly associated with cortical demyelination ($P = 0.0008$) but not with MS in the absence of demyelination.

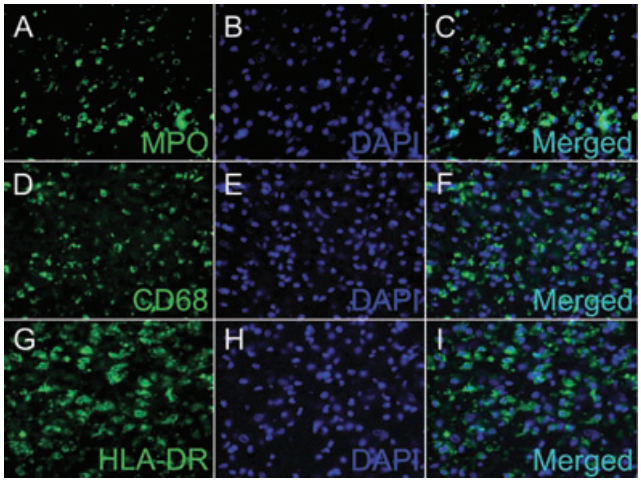

Figure 7. Immunofluorescent labeling (green) of myeloperoxidase (MPO) (**A**), CD68 (**D**) and HLA-DR (**E**) in a cortical plaque with dye 4′6′-diamidino-2-phenylindole nuclear counterstain (blue in **B**, **E**, **F**). The merged images are shown in **C**, **F** and **I**. The MPO labeling is cytoplasmic and granular, in keeping with the lysosomal location of this enzyme and appears similar in distribution to that for CD68. HLA-DR labeling is more abundant than either MPO or CD68.

elination may have appeared indistinguishable from other non-demyelinated cortex. This is unlikely to have influenced our findings in relation to microglial activation on assessment of paraffin sections, where the fine detail of MBP labeling was much more clearly resolved; we took care to perform measurements only on plaques that were clearly lacking entirely in MBP or on areas of cortex in which MBP labeling appeared normal. Finally, although all of the samples used to measure MPO activity were from brains removed within 24 h of death, the possibility should be considered that differences in post-mortem delay between the control and MS cases could have influenced our findings. Arguing against this are (i) the absence of correlation between MPO activity and post-mortem delay in any of the groups analyzed; and (ii) the very similar MPO activity in normal control cortex and uninvolved MS cortex, despite the difference in mean post-mortem delay in these two groups and the marked increase in MPO activity in demyelinated cortex from the same patients.

Previous studies have indicated that the degree of inflammation in MS cortex may be very mild (3, 8), but that active cortical demyelination is associated with microglial activation (10). These findings were confirmed in the present study, in which we found a significant increase in HLA-DR immunolabeling in demyelinated MS cortex. Microglial activation was also increased in non-demyelinated cortex in MS patients compared with controls, but not significantly so. The possibility should be considered that systemic infection, present terminally in 17 of the 30 patients with MS, may contribute to microglial activation in MS patients. Lemstra et al (12) recently demonstrated an increase in the number of CD68-immunopositive microglia in the gray matter of patients who had died of sepsis. However, unlike in our study, they found that Major histocompatibility complex class II antigen labeling of microglia was not significantly increased. Furthermore, the predominantly plaque-associated distribution of activated microglia in MS differs from the mild generalized increase in CD68-immunopositive microglia described by Lemstra et al (12).

The pathogenesis of the cortical lesions in multiple sclerosis is largely unknown (14). It has been suggested that in the chronic, progressive phase of the disease, inflammation occurs throughout the brain, T and B lymphocytes being found within the meninges (3, 10). The leptomeningeal inflammation may contribute to the subpial demyelination in the cerebral cortex (10). It has been proposed that soluble factors produced within the meningeal infiltrates diffuse into the cortex and initiate myelin destruction either directly or indirectly through microglial activation (10). The microglial inflammation is probably important not only for cortical demyelination but also axonal degeneration and neuronal loss (17, 22). In addition, Wegner et al observed a decrease of glial cells in leukocortical lesions relative to normal-appearing MS neocortex; however, these authors did not determine the specific type(s) of glial cells lost (22).

MPO is not expressed by quiescent microglia in normal brain parenchyma but is elevated in a variety of neurodegenerative diseases such as Alzheimer's disease (AD) (7, 18), Parkinson's disease (5) and MS (15, 16). Until recently, phagocytic white blood cells were generally believed to be the only cellular sources of MPO (5). However, neuronal MPO expression was reported to be increased in AD, in which a by-product of MPO activity, 3-chlorotyrosine, is

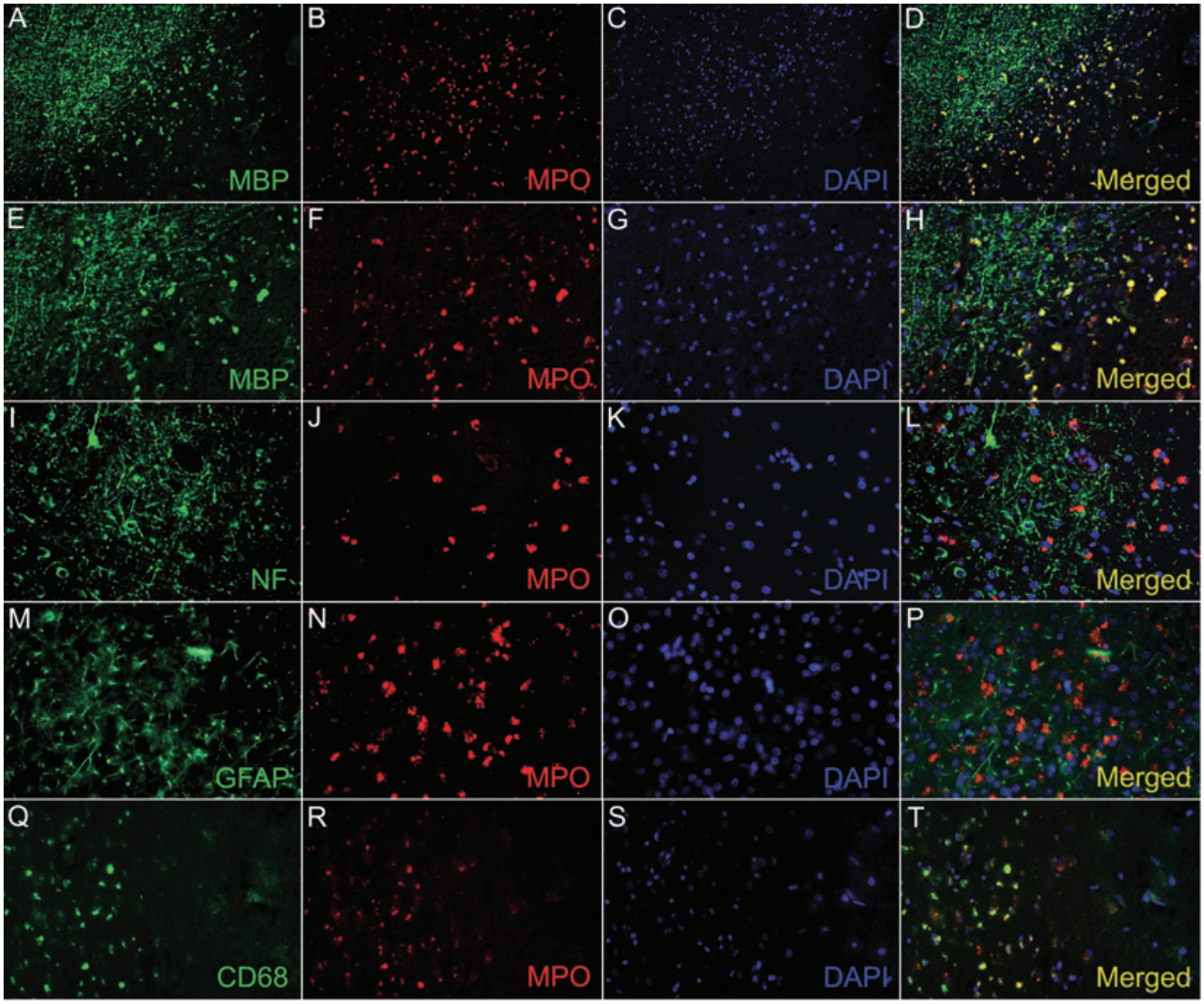

Figure 8. Double immunofluorescent labeling of myelin basic protein (MBP) (green in **A**,**E**), neurofilament (NF) (green in **I**), glial fibrillary acidic protein (GFAP) (green in **M**), CD68 (green in **Q**) and myeloperoxidase (MPO) (red in **B**, **F**, **J**, **N**,**R**) in a cortical plaques, with dye 4′6′-diamidino-2-phenylindole (DAPI) nuclear counterstain (blue in **C**, **G**, **K**, **O**, **S**). **D**, **H**, **L**, **P** and **T** are merged images. Examination of **A–D** shows MPO-positive cells to be concentrated near the edge of a cortical plaque, demarcated by the labeling of MBP in adjacent non-demyelinated cortex. At higher magnification (**E–H**), MBP is clearly visible within some of the MPO-positive cells, which appear yellow in the merged image. The MPO-positive cells are separate from the neurofilament-positive neuronal somata and processes in the cortical plaque illustrated in **I–M**. MPO-positive cells are also distinct from the GFAP-positive astrocytes in the plaque shown in **M–P**. In contrast, most of the MPO-positive cells near the edge of a cortical plaque co-localize with CD68 (**Q–T**).

demonstrable (7). MPO in the brain can also be derived from astrocytes (5, 24). However, these seem to express MPO only under certain pathological conditions (5, 24). Our immunohistochemical findings suggest that neither neurons nor glia contribute significantly to MPO activity in the cerebral cortex in MS.

In 1997, Nagra et al (16) reported that myeloperoxidase was present in macrophages/microglia in MS plaques in the white matter. The present study, MPO expression was shown to be present in the cerebral cortex as well, in macrophage-like cells within areas of demyelination. These findings were supported by measurement of MPO activity, which was significantly elevated in demyelinated but not non-demyelinated MS cortex—in keeping with the distribution of MPO-positive cells on immunohistochemistry. Phagocytosis of myelin by activated microglia/macrophages in the white matter is very well documented in MS [eg, Tanaka et al. (19), Williams et al. (23)] and has been shown to involve the generation of reactive oxygen species (ROS) (20, 23). The increase in myeloperoxidase activity in demyelinated MS cortex suggests that ROS are likely to be involved in cortical demyelination as well.

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