Immunocytochemical Characterization of the Expression of Inducible and Constitutive Isoforms of Nitric Oxide Synthase in Demyelinating Multiple Sclerosis Lesions

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Abstract. The cellular localization and distribution of inducible and constitutive nitric oxide synthase (iNOS/cNOS) was determined in tissue sections from multiple sclerosis (MS) and control brain and spinal cord. Immunocytochemical techniques were applied using specific iNOS- and cNOS-directed antibodies. In addition, NADPH-diaphorase histochemistry was performed. To establish the identity of iNOS-, cNOS- and NADPH-diaphorase-positive cells single and double staining was performed on tissue sections with the macrophage marker KP1 (CD68) and with the astrocyte marker glial fibrillary acidic protein (GFAP). Areas of myelin breakdown and demyelination were determined using a staining for neutral lipids, Oil Red O (ORO). Furthermore, macrophages isolated from active demyelinating MS lesions were stained for iNOS, cNOS, KP1 and ORO. In active MS lesions strong iNOS immunoreactivity was found exclusively in perivascular and parenchymal macrophages distributed within regions of active demyelination.

In these active MS lesions immunoreactivity for cNOS was also found in macrophages. Macrophages isolated from active MS lesions also showed immunoreactivity for iNOS and cNOS. Moreover, these isolated macrophages produced nitric oxide (NO; >30 μM) in vitro. NADPH-diaphorase activity was detected in KP1-positive perivascular and parenchymal macrophages and in GFAP-positive reactive astrocytes in active MS lesions and in reactive astrocytes located in the hypercellular rims of chronic active MS lesions. cNOS-positive reactive astrocytes were detected in both active and chronic active MS lesions. Inside chronic active lesions some residual macrophages were weakly iNOS-positive. In control brain and spinal cord no iNOS immunoreactivity could be detected. These results suggests an important role for human macrophages capable of producing the free radical nitric oxide (NO), which may contribute to the cytotoxicity of oligodendrocytes and destruction of myelin in MS brain and spinal cord.

Key Words: Astrocytes; Brain; Human macrophages; Immunocytochemistry; Macrophage isolation; Nitric oxide synthase; Spinal cord.

INTRODUCTION

In multiple sclerosis (MS), a demyelinating disease of the central nervous system (CNS), the major pathological features are edema, perivascular inflammation, demyelination, oligodendrocyte loss, and astrogliosis. The perivascular cuffs of the inflammatory lesion contain T cells and macrophages filled with degraded myelin (1–4). The mechanisms triggering myelin destruction, oligodendrocyte loss, and the ensuing astrogliosis in MS are poorly understood, but T cells, macrophages, microglia and astrocytes are clearly implicated in MS as effector cells (5–9). It is thought that myelin breakdown in MS is caused by inflammatory mediators, among which is the free radical nitric oxide (NO). The release of NO by inflammatory cells is increased by several cytokines, including

IFN-γ and, synergistically, TNF-α, lymphotoxin, and IL-1 (10). Nitric oxide has a variety of activities, including vasodilation, neurotransmission, anti-microbial activity, and cytotoxicity. Constitutive and inducible isoforms of NO synthase (constitutive and inducible nitric oxide synthase [cNOS and iNOS]) have been identified (11, 12). Several studies have demonstrated a role for NO in the pathogenesis of experimental animal models of CNS autoimmune diseases (13–16). Recently, significant levels of iNOS mRNA have also been demonstrated in MS brains (9, 17). However, the cell types that express iNOS in MS are a matter of debate. Though the expression of iNOS has been described extensively in rodent macrophages (14), most studies have failed to detect iNOS in human monocytes/macrophages. Bagasra et al (17) demonstrated for the first time that iNOS mRNA expression can be detected in the cytoplasm of cells identified as macrophages/microglial cells within the brains of MS patients. From this study, however, the type of lesion in which iNOS expression is found could not be determined. To our knowledge, no studies have been reported demonstrating both cNOS and iNOS at the protein level in MS brain and spinal cord lesions with different stages of cellular activities. Given its potential role in tissue damage, a careful and exhaustive study of iNOS and cNOS expression in MS-affected tissue is warranted. Therefore,

in the present study the cellular localization and distribution of iNOS- and cNOS-positive cells were investigated in active demyelinating and chronic active demyelinating MS CNS lesions. Immunocytochemical techniques were applied using specific iNOS- and cNOS-directed antibodies.

Furthermore, we have applied NADPH-diaphorase histochemistry to compare our staining results with earlier descriptions made by Brosnan et al (18) and Bö et al (9), and in the brain it has been demonstrated that NADPH-diaphorase in neuronal cells, endothelial cells, and macrophages represents NOS (19–21). In addition, NADPH-diaphorase histochemistry was also combined with immunocytochemistry using the astrocytic marker GFAP or the macrophage marker KP1 (CD68).

Postmortem brains and spinal cords obtained from patients with and without neurological disease were used as control tissue.

Furthermore, we have isolated perivascular and parenchymal macrophages from actively demyelinating MS CNS lesions. The cells were characterized with monoclonal antibodies (mAbs) KP1 (CD68) and LeuM5 (CD11c). Their iNOS and cNOS immunoreactivity was determined and their ability to produce NO in vitro, as measured by the accumulation of nitrite in culture supernatants, was investigated.

MATERIALS AND METHODS

Tissue

Human brain and spinal cord tissue was obtained at autopsy (with short postmortem intervals; see Table 1 for details) from 5 MS patients, from 3 patients without neurological complications, and from 1 patient with amyotrophic lateral sclerosis (ALS). The autopsies were performed under the management of the Netherlands Brain Bank (coordinator, Dr R. Ravid). In all MS cases, tissue samples were taken from lesions located in the brain and/or spinal cord. From every control case tissue samples were taken from the subcortical white matter or corpus callosum and/or spinal cord. The clinical diagnosis of MS was confirmed neuropathologically.

CNS tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. Hematoxylin and eosin (H&E)/-stained sections were always prepared from the obtained CNS tissue. Tissue sections from MS lesions were stained with the neutral lipid marker Oil Red O (ORO) to delineate areas of myelin breakdown and demyelination.

In order to isolate perivascular and parenchymal macrophages from active demyelinating MS lesions, tissue samples were collected in DMEM/HAMF10 (1:1) medium (Gibco, Breda, The Netherlands) containing penicillin (100 IU/ml) and streptomycin (50 μg/ml).

Cell Isolation

Human brain macrophages were obtained using a modification of the method reported previously (22, 23) for the isolation

TABLE 1
Autopsy Data

Case no:	Age	Sex	Post-mortem delay	Cause of death/ neuropathology
Multiple sclerosis:				
S350	81	F	5 h 30 min	Cerebral vascular accident/ definitive MS
S009	78	F	6 h 00 min	Aspiration pneumonia/ definitive MS
S136	46	M	8 h 00 min	Cachexia/definitive MS
S234	81	F	3 h 00 min	Respiratory insufficiency/ definitive MS
S276	56	M	5 h 30 min	Respiratory insufficiency/ definitive MS
Controls:				
S64	51	F	4 h 00 min	Aspiration pneumonia/ALS
S110	75	M	7 h 30 min	Adeno carcinoma
S202	75	M	7 h 00 min	Myocardial infarction
S281	89	F	6 h 00 min	Aspiration pneumonia

MS = multiple sclerosis; ALS = Amyotrophic lateral sclerosis; h = hours; min = minutes.

of brain macrophages of rats with experimental allergic encephalomyelitis (EAE). If present, meninges were aseptically removed from the collected CNS samples with MS lesions. Tissues were minced into small fragments (approximately 2 mm^3). The tissue fragments were incubated at 37°C for 20 minutes (min) in a Hank's Balanced Salt Solution (HBSS) containing 2.5 mg/ml trypsin (Sigma, St. Louis, USA), 0.2 mg/ml EDTA, 1 mg/ml glucose and 0.1 mg/ml bovine pancreatic DNase I (Boehringer, Mannheim, FRG). After digestion, the cell suspensions were gently triturated using a 10 ml pipet and washed with DMEM/HAMF10 medium containing 10% (w/v) Fetal Calf Serum (FCS, Gibco). Single cell suspensions were plated into 80 cm^2 tissue culture flasks (Polystyrene; Greiner, Alphen a/d Rijn, The Netherlands) to isolate brain macrophages (by adherence to the plastic). The flasks were incubated for 2 hours (h) at 37°C in an humidified, 5% CO_2 atmosphere. Adherent cells were harvested by trypsinization and resuspended in DMEM/HAMF10/10% FCS medium. Cytocentrifuge preparations from the harvested cells were made and used for immunocytochemical characterization. Cell yield and viability, using Trypan blue exclusion, was determined in a hemocytometer. From one MS lesion sample (S234), macrophages were isolated and used both for immunocytochemical characterization and the measurement of spontaneous NO production (see below).

Measurement of Nitrite

Macrophages isolated from an MS lesion sample, as described above, were plated in triplicate for 48 h in a 96-well plate without further stimulation (seeded at a density of 5 × 10^4 cells/well). The total cell yield from the tissue sample was 4 × 10^5 cells/ml; remaining cells were used for immunocytochemical characterization. The production of NO, as measured by formation of the stable decomposition product nitrite, was determined in cell-free culture supernatant using a microplate assay described by Ding et al (39). Nitrite was measured by mixing 100 μl of culture supernatant with 100 μl of Griess
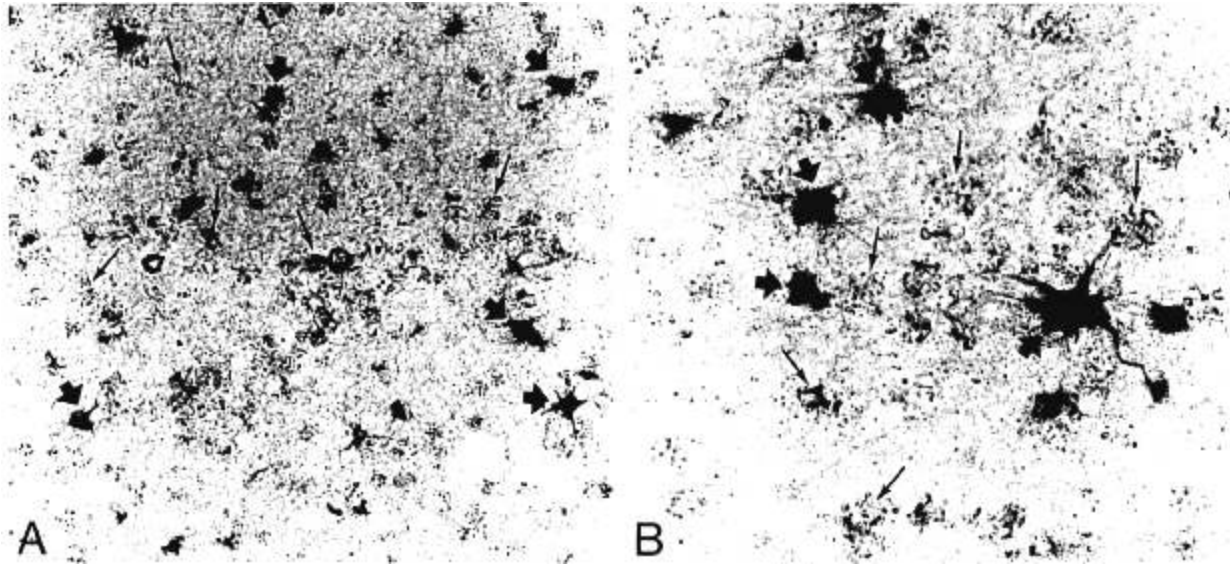
Fig. 1. NADPH-diaphorase staining in cryostat sections (14-μm-thick) from an active demyelinating MS brain lesion (case no. S136). NADPH-diaphorase-positive cells having the morphology of reactive astrocytes are located in the lesion center (A, arrows). Numerous NADPH-diaphorase-positive cells resembling parenchymal macrophages are randomly distributed throughout the demyelinated area (A, small arrows). ×100. At higher magnification it is found that NADPH-diaphorase activity is distributed diffusely throughout the reactive astrocytes (B, arrows), whereas a granular staining of NADPH-diaphorase is detected in parenchymal macrophages (B, small arrows). ×200.
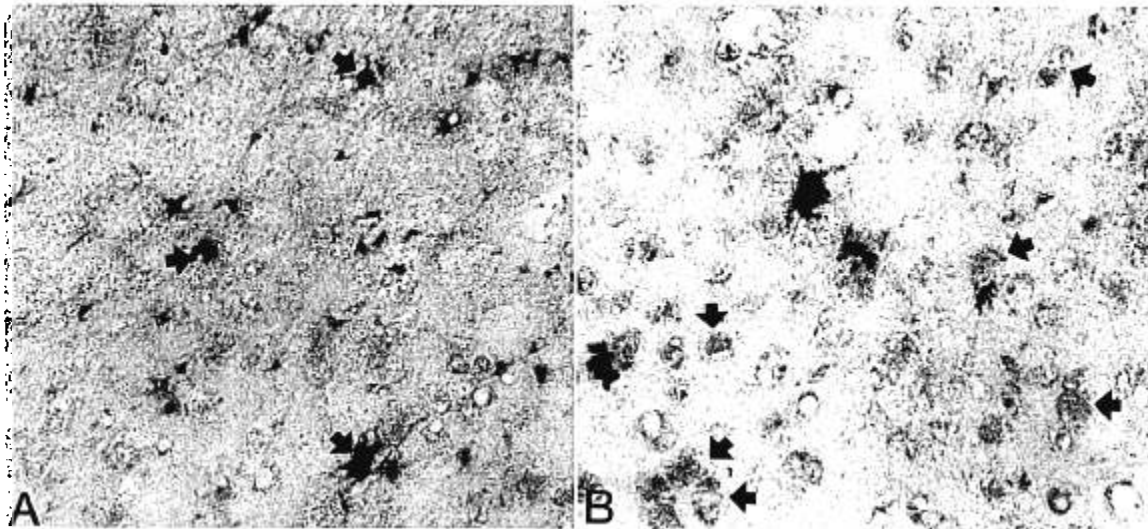

Fig. 2. Cryostat sections (14-μm-thick) from an active demyelinating MS spinal cord lesion (case no. S136) double-labeled with NADPH-diaphorase and glial fibrillary acidic protein (GFAP) antibodies (A), and KP1 antibodies (B). Dark-blue NADPH-diaphorase-positive cells are double-labeled by GFAP (brown staining) antibodies (A, arrows). A blue granular staining of NADPH-diaphorase activity is detected in cells that are double-labeled by KP1 (brown staining) antibodies (B, arrows). ×100.

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Fig. 3. Cryostat sections (7-μm-thick) from an active demyelinating MS spinal cord lesion (case no. S136) stained with a mAb specific for inducible nitric oxide synthase (anti-iNOS) (A, B), isotype control mAb L26 (D), anti-iNOS mAb applied on a tissue section from human tonsil (C), the neutral lipid marker Oil Red O (ORO) (E), and the macrophage marker KP1 (F). Parenchymal macrophages are strongly stained with anti-iNOS mAb (brown staining) antibodies (A, arrows). ×200. At higher magnification their characteristic foamy appearance can be detected (arrows), displaying a strong immunoreactivity for iNOS mAb. ×400. No immunoreactivity for iNOS mAb can be detected on human tonsil (C). ×200. B cells located in a perivascular
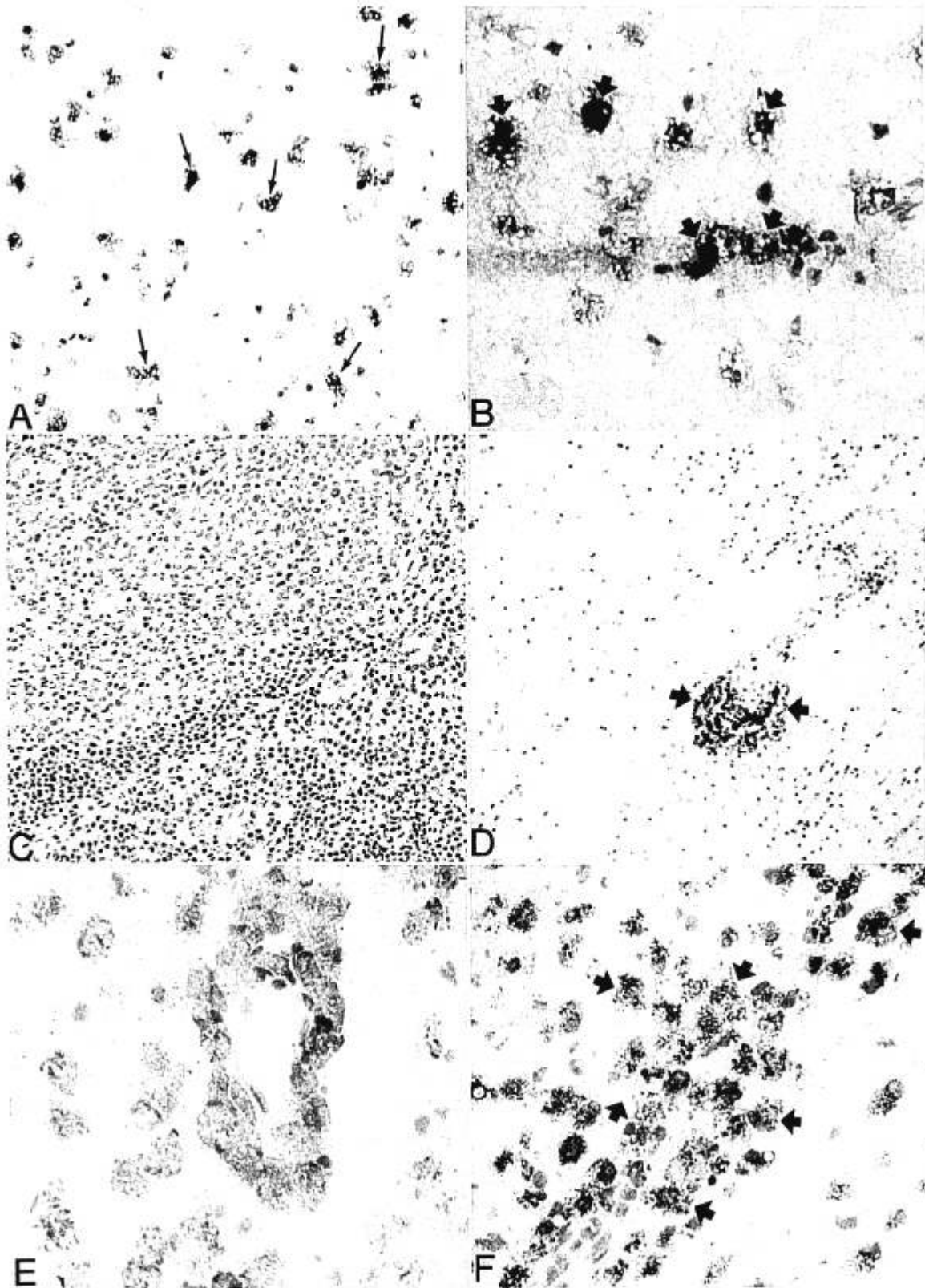
cuff are positively stained with L26 mAb (arrows). No aspecific binding is detected on parenchymal macrophages (D). ×100. Abundant lipid-filled ORO-positive (red staining) perivascular and parenchymal macrophages are distributed throughout the demyelinated lesion (E). ×200. On an adjacent section numerous KP1-positive perivascular and parenchymal macrophages (brown cells) can be detected (F, arrows). ×200.

reagent (1% sulfanilamide, 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride, 2.5% H₃PO₄). After 5 min incubation at room temperature, the optical density at 540 nm was measured in a microtiter plate reader, against culture medium as blank. In parallel, a sodium nitrite standard curve (1–200 μM) was generated. Also, nitrite content in wells containing medium without cells was measured and subtracted.

Monoclonal and Polyclonal Antibodies

Antibodies used in the present study were mouse anti-mouse iNOS (macNOS; IgG2a; 24) obtained from Transduction Laboratories (Lexington, KY, USA). Monoclonal antibodies (mAbs) raised in mice against the peptide sequence 941–1144 of murine iNOS are affinity-purified and have been tested by Western blotting and are considered to be specific for the specific antigen. Immunoreactivity with the iNOS mAb was found in postmortem human tissue (25). Rabbit anti-rat cNOS (cross-reacting in human tissue [26]) was purchased from The Wellcome Research Laboratories (Beckenham, UK) and was raised against the peptide sequence 1409–1429 of rat cNOS. The pAb have been tested by Western blotting and immunoabsorption and are considered to be specific for the antigen (26). Mouse anti-human KP1 (CD68; IgG1), rabbit anti-cow glial fibrillary acidic protein (GFAP), mouse anti-human leukocyte common antigen (LCA; IgG1) and an isotype-match mouse anti-human L26 (B cel marker, CD20-Cy; IgG2a) were all obtained from Dakopatts (Copenhagen, Denmark). Mouse anti-human HLA-DR/DQ (OKIa, class II, IgG1) was obtained from Ortho (Beerse, Belgium). Mouse anti-human Leu-M5 (CD11c; IgG2b) mAb was obtained from Becton & Dickinson (Aalst, Belgium).

Immunocytochemical Staining

Serial 7-μm-thick cryostat sections of MS lesions and control CNS and cytocentrifuge preparations of isolated macrophages derived from active demyelinating MS lesions were fixed in acetone for 10 min and air dried for at least 10 min. Sections were immunostained by the streptavidin-biotin complex (sABC) procedure described previously (23). Briefly, the slides were washed three times with 0.01 M phosphate buffered saline (PBS, pH 7.4) and subsequently preincubated with 10% (mAbs) normal rabbit (mAbs) or 2% normal swine serum (pAbs) for 20 min at room temperature (RT). Recently, it has been shown that macrophages/microglia in MS lesions strongly express receptors for the Fc portion of IgG (FcR) (27). Such expression may give false positive binding of IgG antibodies. To investigate the specificity of the iNOS expression, control experiments were therefore performed with FcR blocking. We have applied the anti-iNOS mAb and an isotype control mAb L26 (both of the IgG2a subclass) on sections preincubated with 10% normal rabbit serum for 20 min at RT. Primary antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and incubated for 1 h at RT in biotinylated rabbit anti-mouse-IgG F(ab′)₂ or swine anti-rabbit-IgG F(ab′)₂ containing 1% BSA and 2% normal human-pooled serum (Vector Laboratories, Burlingame, CA), diluted 1:500 for 30 min at RT in peroxidase (HRP)-linked biotin (1:200) and streptavidin (1:200) (Vector Laboratories), and immersed in PBS solution for 1 h, and in 0.5 mg/ml diaminobenzidine solution (DAB; Sigma, St. Louis, MO) in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.03%

H₂O₂ for 5 min. Cells were lightly counterstained with hematoxylin, and slides and cytocentrifuge preparations were mounted with coverslips using DePeX (BDH, Poole, UK).

NADPH-Diaphorase Histochemistry

Fourteen-μm-thick cryostat sections of MS lesions and (normal) control CNS were fixed in 4% paraformaldehyde/PBS for 10 min. Whereas several other enzymes besides NOS demonstrate NADPH-diaphorase, they are inactivated by paraformaldehyde fixation (28). NADPH-diaphorase activity was detected by incubating the sections with 0.5 mM nitroblue tetrazolium (Sigma) and 1 mM β-NADPH (Sigma) in 50 mM Tris-HCl/pH 7.6 at 37°C in a humified chamber for approximately 30 min, until a visible dark blue reaction product was formed. Cryostat sections of human skeletal muscle were used as the positive control; slides incubated with reaction buffer without βNADPH comprised the negative control. Sections to be double-labeled with NADPH-diaphorase and GFAP or KP1 antibodies were first stained with NADPH-diaphorase. Slides were mounted using Aquamount (BDH, Poole, UK).

RESULTS
Neuropathological Evaluation

In order to evaluate MS lesion activity and histological characteristics of the investigated tissue samples, cryostat sections from all different MS brains and spinal cords were histochemically stained with H&E and ORO, and immunocytochemically stained with KP1, HLADR/DQ, LCA, and GFAP antibodies. Conforming to the classification described by Bö et al (8, 9), 5 MS lesions from the brain and 3 from the spinal cord were identified that contained abundant phagocytic macrophages and were classified as active demyelinating lesions (case no. S136/S234/S350), 3 MS lesions from the brain and 2 from the spinal cord had a hypocellular center and hypercellular rims and were classified as chronic active demyelinating lesions (case no. S009/S276). In normal control brain and spinal cord and in the brain from the ALS case no inflammatory cells were detected.

Single and Double NADPH-Diaphorase Histochemical and Immunocytochemical Staining

Cryostat sections (14-μm-thick) from MS and control CNS were cut adjacent to the 7-μm-thick sections (used for histological and single immunocytochemical stainings) and stained either for NADPH-diaphorase activity alone or for NADPH-diaphorase followed by immunocytochemistry using GFAP or KP1 antibodies.

In tissue sections from (normal) control subcortical white matter, corpus callosum, and spinal cord, NADPH-diaphorase activity was associated with blood vessels and neurons and not with glial cells such as astrocytes and microglia. In tissue sections of the white matter from the ALS case no changes in NADPH-diaphorase activity

were detected as compared to the normal control CNS tissues.

In active MS lesions derived from brain and spinal cord, NADPH-diaphorase activity was distributed diffusely in cell bodies having the morphology of reactive astrocytes inside and at the edge of the demyelination regions (Fig. 1A, B). In the center of the active MS lesions a granular NADPH-diaphorase activity was detected throughout the cytoplasm of perivascular and parenchymal macrophages (Fig. 1A, B). To identify the phenotypes of the cells involved, double staining with NADPH-diaphorase and GFAP or KP1 was performed. The intensely dark-blue stained NADPH-diaphorase cells resembling reactive astrocytes associated with active demyelinating MS lesions were also immunoreactive for GFAP antibodies (Fig. 2A). The NADPH-diaphorase-positive cells, morphologically resembling perivascular and parenchymal macrophages, were double-labeled with the macrophage marker KP1 (Fig. 2B).

In chronic active lesions, cells with strong NADPH-diaphorase activity were double-labeled with GFAP and predominantly located in the hypercellular rims surrounding the hypocellular center of the demyelinated region. In the center of these chronic active lesions a few parenchymal macrophages were detected that still contained residual ORO reactivity. NADPH-diaphorase activity was not detected in these macrophages.

Immunocytochemical Staining for iNOS and cNOS on CNS Tissue Sections

iNOS-immunoreactive cells could not be detected in any of the examined tissue sections of control brain or spinal cord tissue.

In tissue sections of active demyelinating MS lesions from both brain and spinal cord virtually all cells (as could be judged by light-microscopical inspection) were strongly immunoreactive for iNOS mAb. The iNOS-positive cells were found accumulated in perivascular cuffs and randomly distributed within regions of active demyelination and at the outer edge of the demyelinated region. The iNOS-positive cells were large, round, and without processes (Fig. 3A, B). No iNOS immunoreactivity could be detected on human tonsil or on human spleen tissue sections (Fig. 3C). The isotype-specific control mAb L26 only stained B cells present in perivascular cuffs of active demyelinating MS lesions (Fig. 3D). In adjacent sections, abundant neutral lipid could be detected in these phagocytic cells (Fig. 3E). Immunocytochemical staining for the macrophage marker KP1 confirmed their macrophage phenotype (Fig. 3F). A strong immunoreactivity for cNOS was detected in virtually all perivascular and parenchymal macrophages within regions of active demyelination (Fig. 4A). Moderately cNOS-positive reactive astrocytes were found in the center of

the demyelinated lesion and at the edge of the lesion site (Fig. 4A).

In chronic active lesions, a few weakly iNOS- and cNOS-positive parenchymal macrophages were detected in the center of the lesion (not shown). Cells resembling reactive astrocytes located in the center and at the outer edge of the demyelinated region were not immunoreactive for iNOS. However, immunocytochemical staining for cNOS showed strongly positive stained cells that morphologically resembled reactive astrocytes, located mainly in the hypercellular rim of the demyelinated region (Fig. 4B). Immunocytochemical staining for GFAP on adjacent sections confirmed their astrocytic nature (not shown). Furthermore, a weak endothelial staining could be observed throughout the examined sections (Fig. 4B). In normal controls, CNS tissue cNOS immunoreactivity was only detected in neurons and blood vessels.

Table 2 summarizes the results of the immunocytochemical stainings performed on cryostat sections from MS and normal control CNS.

Immunocytochemical Staining for iNOS and cNOS and In Vitro Production of NO by Isolated Macrophages from Active Demyelinating MS Lesions

To exclude the possibility that the isolated macrophage populations obtained from MS lesions are due to any contamination with resident microglial cells and/or macrophages from the CNS vasculature/meninges, brain cell suspensions obtained from normal control white matter tissue were incubated in uncoated tissue culture flasks for 2 h at 37°C. No adherent cells could be detected.

From MS lesion samples of the brain and spinal cord (S136/S234/S350), macrophages could be isolated by means of adherence, and the majority of cells isolated from these active demyelinating MS lesions contained degraded neutral lipids stained with ORO, and >95% of the cells were immunoreactive for KP1 and LeuM5. The immunoreactive cells had a round or oval morphology with irregularly shaped nuclei (Fig. 5A, B). All KP1/LeuM5-positive macrophages were also immunoreactive for iNOS (Fig. 5C) and cNOS (Fig. 5D). From one MS lesion brain sample (S234), macrophages were isolated by means of adherence and the cells were cultured for 48 h without any further stimulation. The total nitrite content of the supernatants (triplicate) measured by the Griess reaction revealed spontaneous NO production by the cultured macrophages. A mean level of 30.7 ± 1.5 μM (triplo) nitrate was measured in the culture supernatants of untreated macrophages derived from an active demyelinating MS lesion. Immunocytochemical staining performed on cytocentrifuge preparations made from the isolated macrophages obtained from the same brain sample revealed that all cells were immunoreactive for iNOS (see Fig. 5C).
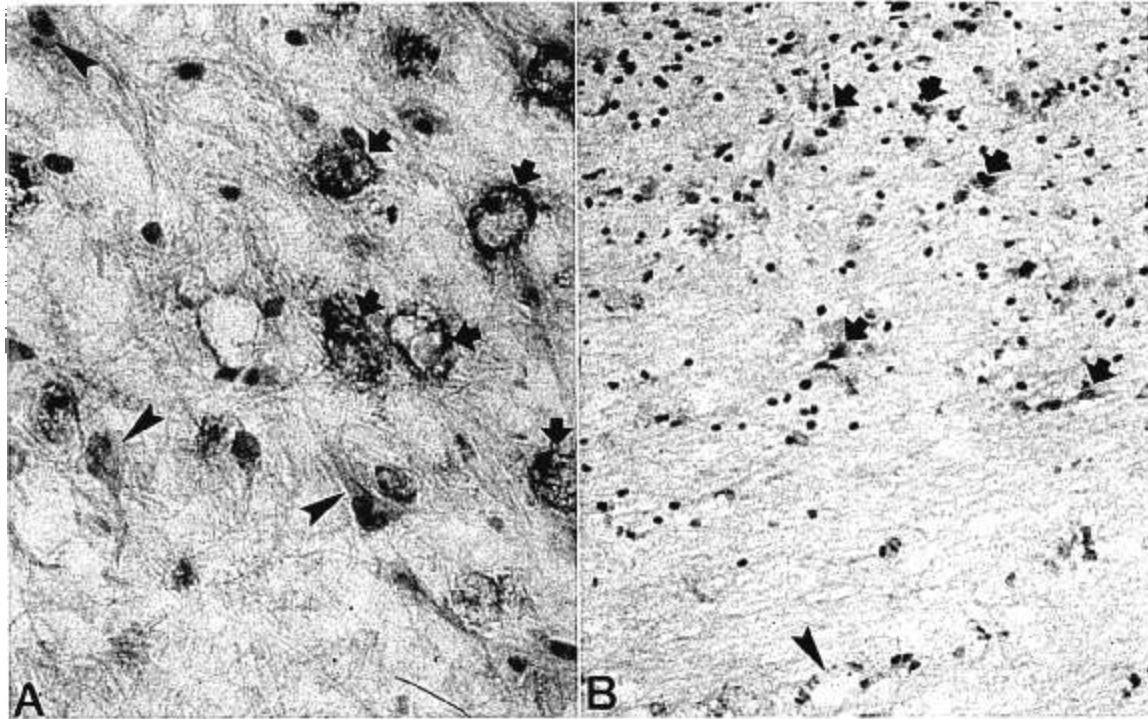
Fig. 4. Cryostat sections (7-μm-thick) from an active (case no. S136) and a chronic active demyelinating MS brain lesion (case no. S276) stained with an antiserum specific for cNOS. Immunoreactivity for cNOS was found both in parenchymal macrophages (arrows) and in reactive astrocytes (arrowheads) located in an active demyelinating MS lesion (A) ×400. In a chronic active MS lesion strongly cNOS-positive reactive astrocytes were present in the hypercellular rim of the demyelinated lesion (B, arrows). A weak staining of the blood vessels in the demyelinated regions is visible (arrowhead). ×200.

TABLE 2
Summary of NADPH-diaphorase and
Immunocytochemical Stainings on CNS Tissue of MS
and Control Cases

Cell type/staining	Active demyelinating lesions	Chronic active demyelinating MS lesions	(Normal) control CNS
Phagocytic macrophages:			
NADPH-diaphorase	+	−	−
iNOS	+ +	±	−
cNOS	+ +	±	−
Reactive astrocytes:			
NADPH-diaphorase	+ + +	+ + +	−
iNOS	−	---	−
cNOS	+	+ +	−

−, no staining; ±, weak staining; +, moderate staining; + +, strong staining; + + +, intense staining.

DISCUSSION

In this study we clearly demonstrated the presence of both inducible and constitutive isoforms of NOS at the protein level in different tissue samples obtained from

MS brain and spinal cord undergoing active demyelination. Our results, derived from specific immunocytochemical staining, using iNOS- and cNOS- directed antibodies, demonstrated iNOS- and cNOS-positive perivascular and parenchymal macrophages, whereas reactive astrocytes in the lesioned CNS regions only showed immunoreactivity for cNOS.

It has already been shown using various molecular biological techniques that tissue extracts and tissue sections from MS brains contain increased levels of iNOS mRNA (9, 17). However, identification of the cell type(s) responsible for the expression of protein iNOS in MS has led to conflicting results. Bö et al (9) and Brosnan (18) found most of the NADPH-diaphorase activity in reactive astrocytes in active demyelinating MS lesions and at the edge of chronic active demyelinating lesions, whereas cells staining with the macrophage/microglial markers leukocyte common antigen (LCA; CD45) or EBM11 (CD68) did not show NADPH-diaphorase activity. They suggested that the strong NADPH-diaphorase activity found in reactive astrocytes in active and chronic active demyelinating MS lesions is representative for both iNOS and cNOS. However, NADPH-diaphorase activity does not distinguish between the constitutive and inducible forms of the enzyme NOS (18), suggesting that the NOS
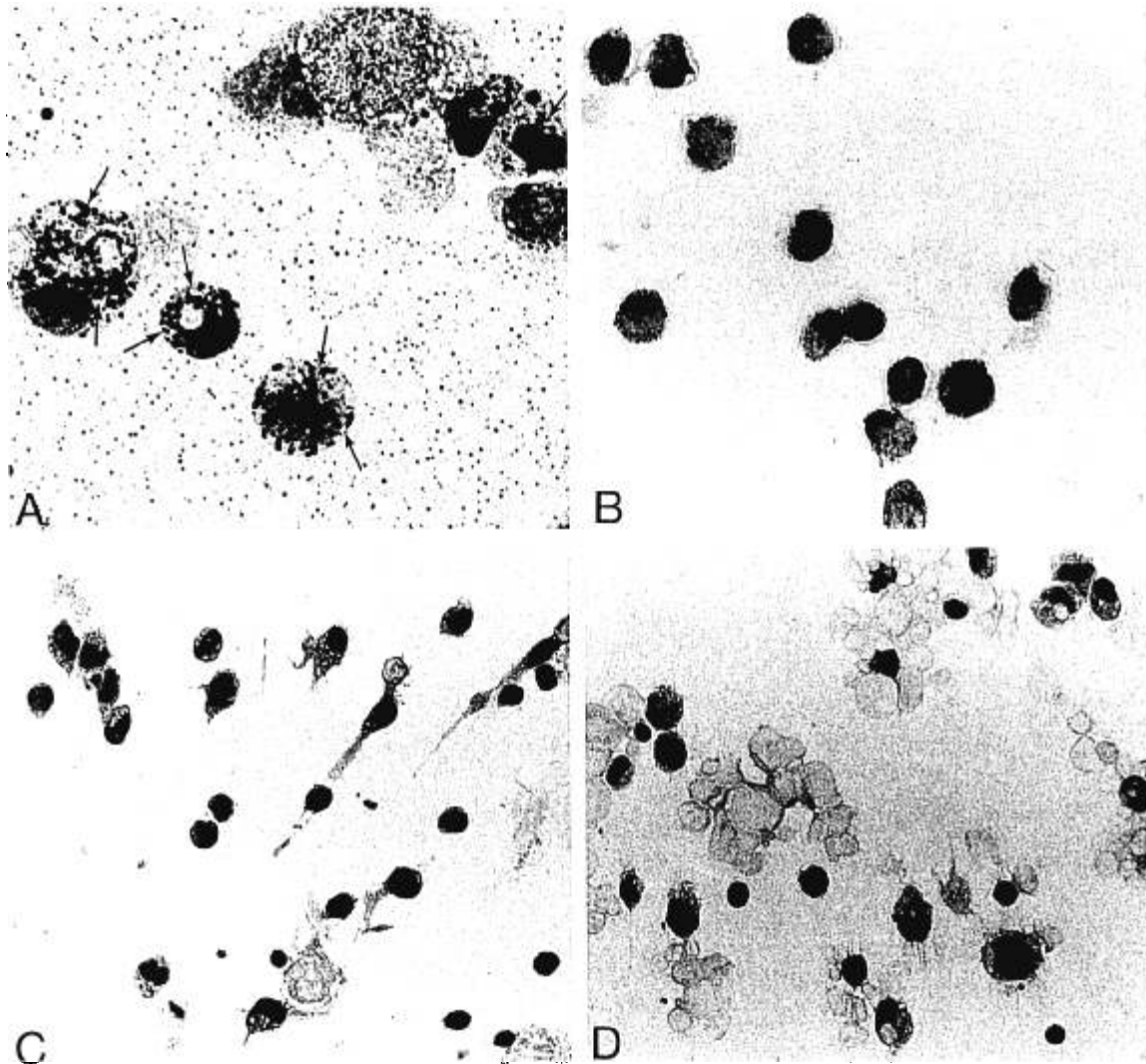
Fig. 5. Oil Red O staining and immunocytochemical staining for KP1, iNOS, and cNOS of isolated perivascular and parenchymal macrophages from acute demyelinating MS brain lesions (case numbers S136 and S234). Oil Red O (ORO)-stained neutral lipids are detectable in isolated perivascular and parenchymal macrophages (A, arrows). Strongly stained KP1-positive macrophages (granular staining pattern) with a round or oval morphology and irregular shaped nuclei (B). These isolated cells showed strong immunoreactivity for iNOS (C) and cNOS (D). ×200.

activity in astrocyte-associated NADPH-diaphorase staining could be representative of either one or both of the enzyme isoforms. Recently, Bagasra et al (17) reported the presence of iNOS mRNA expression, using RT-in situ PCR combined with immunohistochemistry, in macrophages/microglial cells in MS brain tissue of unknown cellular activity. Furthermore, they also demonstrated the presence of the iNOS protein using immunohistochemistry. However, the cell morphology of these iNOS-positive cells was difficult to interpret, probably due to the immunofluorescence staining that was used.

In the present study, the cell types responsible for iNOS and cNOS protein expression were identified by immunostaining of cryostat sections of demyelinating MS brain and spinal cord lesions with varying cellular activities. Interestingly, in active demyelinating MS lesions a strong immunoreactivity was detected for iNOS in perivascular and parenchymal macrophages, and not in reactive astrocytes. Moreover, immunoreactivity for cNOS and NADPH-diaphorase activity was also found in virtually all perivascular and parenchymal macrophages. This finding suggests that both iNOS and cNOS activity can coexist in an individual cell. Furthermore, a moderate cNOS immunoreactivity and a high level of NADPH-diaphorase was found in reactive astrocytes in active and chronic active MS lesions. Thus, these results indicate that the predominance of astrocytes in NADPH-diaphorase activity predominantly represented cNOS enzyme activity.

Furthermore, we have isolated macrophages from active demyelinating MS lesions that showed strong immunoreactivity for both iNOS and cNOS. Moreover, we present evidence for the spontaneous production of NO

by macrophages isolated from an active MS lesion, as measured by the accumulation of nitrite in the culture supernatant. In agreement with this finding, Ruuls et al (16) have demonstrated the spontaneous production of NO in vitro by brain macrophages isolated from the brains of rats with EAE during the clinical period. Our data show that macrophages derived from MS-lesioned CNS have the capacity to produce NO in vitro; whether this NO production actually occurs in vivo remains to be investigated. Using anti-nitrotyrosine antibodies, Bagasra et al (17) reported that MS brains with detectable amounts of iNOS mRNA exhibited significant concentrations of nitrotyrosine in microglia/macrophages. The presence of nitrotyrosine in MS brains may indicate that brain cells contain peroxynitrite, a reaction product of superoxide and NO. Whether the peroxynitrite adducts contribute directly to the cytotoxicity for oligodendrocytes in MS is unknown (17).

In chronic active demyelinating MS lesions, however, iNOS immunoreactivity was considerably decreased. A few iNOS-positive parenchymal macrophages could be detected in the center of the lesion, whereas again no iNOS-positive reactive astrocytes could be detected. Reactive astrocytes located at the border of the lesion were strongly immunoreactive for cNOS. Thus, in spite of change in cellular activity in developing MS lesions, no expression of iNOS protein can be detected in reactive/hypertrophic astrocytes. Preliminary data we have obtained from in vitro experiments in which cultured human adult astrocytes obtained from MS and control CNS tissue were treated with LPS, IL-1β, IFN-γ, or TNF-α, single or in combinations, showed only low levels of NO (29). This suggests that astrocytes are not efficiently induced by inflammatory cytokines to produce NO. Our finding that reactive astrocytes present in both active and chronic active MS lesions are cNOS and not iNOS-positive suggests that they do not contribute to the actual cell and tissue damage, because NO production by cNOS is supposed to be low. Whether cNOS is involved in the formation of the gliotic scar tissue is yet to be determined.

In rodents, iNOS activity can be found in vitro in cells of the monocyte/macrophage lineage after stimulation with lipopolysaccharide (LPS), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (11, 24, 30). Also, in neonatal rat and mouse microglial cultures, synthesis of NO has been demonstrated following exposure to various combinations of LPS and cytokines (31, 32). In EAE, a known animal model for MS, an immunohistochemical examination of the CNS using iNOS-specific antibodies showed that the iNOS-positive cells were mainly inflammatory macrophages (14, 15). A correlation between the severity of EAE and the frequency of iNOS-positive cells that were present in the areas with necrotic foci and destruction of the CNS tissue was also found (14). In mice

infected with the neurothrophic JHM strain of mouse hepatitis virus (MHV-JHM), iNOS was expressed by astrocytes in chronic demyelinated spinal cords (33), whereas in MHV-induced acute encephalitis, iNOS-positive cells had the morpology of macrophages and very few appeared to be astrocytes. This latter observation is in line with our results which show iNOS expression only in macrophages in active demyelinating MS lesions and a weak iNOS immunoreactivity in a few residual macrophages present in chronic active lesions.

Considering the presence and localization of large numbers of iNOS-positive perivascular and parenchymal macrophages in MS lesions classified as active (containing abundant lipid-filled foamy macrophages), we suggest that iNOS-positive macrophages contribute to the destruction of the blood-brain barrier and to the destruction of myelin and oligodendrocytes in the ongoing pathological events. Merrill et al (34) demonstrated that activated microglia can kill oligodendrocytes in a TNF- and NO-dependent manner. The cytotoxic effect of TNF-α and lymphotoxin on oligodendrocytes in vitro has been reported (35, 36). TNF-α has been detected by immunocytochemical investigation in MS lesions (37, 38). Interestingly, it has been shown that TNF-α and lymphotoxin may enhance the effect of NO-mediated cytotoxicity (34, 39). Thus, in early MS lesions the production of cytokines such as TNF may play a crucial role in lesion formation, not only through their own cytotoxicity but also via induction of iNOS and enhancement of NO-mediated cytotoxicity. The decreased expression of iNOS found in chronic active demyelinating lesions may be caused by shifts in cytokine production. Immunoregulatory cytokines such as transforming growth factor (TGF)-β, IL-10, and IL-4, reported to suppress NO production in vitro, might be involved (40–43). It has been suggested that an insufficient supply of these cytokines can cause prolonged inflammation and NO production in the CNS, and can therefore contribute to the development of EAE (14). In MS lesions, cytokine mRNA expression for IL-4, IL-10, and TGF-β1 & 2 has been detected predominantly in the perivascular inflammatory cuffs and not in parenchymal glial cells (44). Lack of the production of immunoregulatory cytokines such as TGF-β, IL-10, and IL-4 by parenchymal glial cells can explain why suppression of the ongoing demyelination in MS fails once inflammation has started.

The activation of cNOS results in the release of relatively low levels of NO for short periods of time and induction of iNOS results in the release of high levels of NO for extended periods; this is known to exert numerous toxic effects against a wide variety of mammalian cell targets (45). Our data demonstrate that iNOS-positive perivascular and parenchymal macrophages have the capacity to produce NO in vitro and therefore they may be

the effector cells primarily responsible for causing tissue damage occurring in MS.

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