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Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis

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ABSTRACT **Nitric oxide (NO) has been implicated as a pathogenic mediator in a variety of central nervous system (CNS) disease states, including the animal model of multiple sclerosis (MS) and experimental allergic encephalomyelitis. We have examined post-mortem brain tissues collected from patients previously diagnosed with MS, as well as tissues collected from the brains of patients dying without neuropathies. Both Northern blot analysis and reverse transcriptase (RT)-driven *in situ* PCR (RT-*in situ* PCR) studies demonstrated that inducible NO synthase (iNOS) mRNA was present in the brain tissues from MS patients but was absent in equivalent tissues from normal controls. We have also performed experiments identifying the cell type responsible for iNOS expression by RT-*in situ* PCR in combination with immunohistochemistry. Concomitantly, we analyzed the tissues for the presence of the NO reaction product nitrotyrosine to demonstrate the presence of a protein nitrosylation adduct. We report here that iNOS mRNA was detectable in the brains of 100% of the CNS tissues from seven MS patients examined but in none of the three normal brains. RT-*in situ* PCR experiments also demonstrated the presence of iNOS mRNA in the cytoplasm of cells that also expressed the ligand recognized by the *Ricinus communis* agglutinin 1 (RCA-1), a monocyte/macrophage lineage marker. Additionally, specific labeling of cells was observed when brain tissues from MS patients were exposed to antisera reactive with nitrotyrosine residues but was significantly less plentiful in brain tissue from patients without CNS disease. These results demonstrate that iNOS, one of the enzymes responsible for the production of NO, is expressed at significant levels in the brains of patients with MS and may contribute to the pathology associated with the disease.**

We have reported (1) that Borna disease virus, rabies virus, and herpes simplex virus induce the increased expression of inducible nitric oxide synthase (iNOS) mRNA in the brains of intrathecally infected mice and rats. We and others (2) also found that the induction of experimental allergic encephalomyelitis resulted in a similar increase in iNOS mRNA expression, suggesting that a similar phenomenon may occur in the brains of patients with multiple sclerosis (MS). We investigated this hypothesis by detecting iNOS mRNA in extracts of brains from patients who died with MS and from extracts of brain tissues from patients who died from nonneurological diseases. We have also colocalized the iNOS expressing cells by using immunocytochemical techniques combined with reverse transcriptase (RT) driven-*in situ* PCR and demonstrated the presence of iNOS protein by the same double labeling procedures. Finally, as a surrogate marker for nitric oxide (NO) presence, we have immunocytochemically localized nitrotyrosine adducts in histological sections of brain from MS patients.

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MATERIALS AND METHODS

Sources and Selection of Specimens. The samples used in these studies were collected from patients with an antemortem diagnosis of MS based on clinical presentation and reconfirmed by histopathology after death. All MS samples were found to contain pathognomonic MS plaques; control brains were examined and found to be pathologically unremarkable. In most cases, autopsy samples were of frontal lobes, which provided mixed white and gray matter.

In two of the MS cases, special efforts were made to preserve the tissue morphology. Small portions of the brain specimens were frozen in liquid nitrogen immediately after sectioning and then stored at −70°C. This procedure preserved the architecture of the brain significantly better than direct freezing of fresh brain specimens at −80°C, which results in ice crystal formation in the tissue and distorts its architecture.

Cell Culture and Cytokine Activation. A549 human pulmonary epithelial cells were purchased from the American Type Culture Collection. Cells were grown in Ham's F-12 medium supplemented with 10% (vol/vol) fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and 5 μM L-glutamine. Cells were seeded into 6-well tissue culture clusters (Falcon) for Northern blot analysis or seeded into 8-well Lab-Tek culture slides (Nunc) for RT-*in situ* PCR. For those cultures stimulated for the expression of iNOS, interleukin 1β (IL-1β at 100 units/ml), tumor necrosis factor α (TNF-α at 10 ng/ml), and interferon γ (IFN-γ at 500 units/ml) were added for 8 h. For use in Northern blot analysis, the cells were scraped from the culture substrate, washed three times in PBS, pelleted, and then frozen at −20°C until analyzed.

Northern Blot Analysis. Total RNA was extracted from samples of human brains by the method of Chomczynski and Sacchi (3). After denaturation with 10 mM sodium phosphate, pH 7.4/50% (vol/vol) formamide at 65°C for 15 min, the extracts were electrophoresed in a 1.2% agarose gel containing 1.1 M formaldehyde and 10 mM sodium phosphate buffer. RNA was transferred and covalently fixed to nylon membrane. Hybridization probes were prepared from human hepatocyte iNOS cDNA (4) or from glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA (5) and labeled with ^{32}P by using a nick-translation kit (Stratagene).

Immunohistochemistry. Identification of cells in the central nervous system (CNS) was carried out by immunohistochemical staining. Fluorescein isothiocyanate (FITC) and rhoda-

Abbreviations: NO, nitric oxide; MS, multiple sclerosis; RT, reverse transcriptase; iNOS, inducible NO synthase; CNS, central nervous system; RCA-1, *Ricinus communis* agglutinin 1; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α; IFN-γ, interferon γ; FITC, fluorescein isothiocyanate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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mine-conjugated *Ricinus communis* agglutinin 1 (RCA-1; Sigma) were utilized to identify microglia/macrophages. The antibodies to nitrotyrosine were purchased from Upstate Biotechnology (Lake Placid, NY) and the antibodies reactive with human iNOS were from Timothy R. Billiar (University of Pittsburgh Medical Center). All the antibodies used in these studies were unconjugated. Appropriate secondary antibodies were labeled with FITC or rhodamine and were from Sigma.

RT–*in Situ* PCR. Tissue sections of the brains were transferred to specially designed silanated slides, containing a 20-mm single well (Erie Scientific, Portsmouth, NH) and processed for RT–*in situ* PCR, as described (6), by using the iNOS-specific primer pair (sense primer, 5′-CACCTGACCTTGTGCTTGAGGTGGCCATGG-3′; antisense primer, 5′-CTCTTCTCTTGGGTCTCCGCTTCTCGTCCT-3′). Human A549 cells unstimulated or cytokine-stimulated were used as internal negative and positive controls, respectively. For additional positive internal controls, the conserved region of HLA-DQA1, using primers HLA-DQ-GH26-27 (Synthetic Genetics, San Diego), was amplified in DNase-untreated slides. After reverse transcription, a DNA *in situ* PCR amplification was performed by placing the slides in a specifically designed heat block of a thermocycler (MJ Research, Watertown Cambridge, MA; MS-16). Thirty cycles of amplification in this automatic thermal cycler set at 94°C, 55°C, and 72°C with cycling times of 30 sec, 1 min, and 1 min, respectively, were employed. Hybridizations were performed with either FITC or biotinylated oligonucleotide probe for MS-amplified sequence (25-mer oligonucleotide 5′-AACATTGCTGTGATCCATAGTTTTC-3′) and HLA-DQ-A1 was identified by the biotinylated probe GH64 (5′-TGGACCTGGAGAGGAAGGAGACTG-3′). Negative controls for each assay were performed on brain sections from individuals without neurological disease. Hybridizations were performed as described (6). These tissue sections were washed to remove unbound probe and incubated with avidin-peroxidase for 1 h at 37°C. Color was developed with 0.03% H_2O_2 and 3′-amino-9′-ethylcarbazole (AEC) dissolved in 50 mM sodium acetate (pH 5.0), or for fluorescein-tagged probes, no development of color was necessary, as the cells were viewed under UV epifluorescence microscopy after the cells were washed. The tissues were counterstained with 2% (vol/vol) Gill's hemotoxylin for 30 sec.

RESULTS

Northern Blot Detection of iNOS mRNA. The data presented in Fig. 1 demonstrate that iNOS mRNA was present in the extracts of brains from MS patients but was absent in the brain extracts collected from patients who died without neurological disease. The specificity of the hybridization with human iNOS was demonstrated by the presence of a band of the appropriate molecular weight in extracts of cytokine activated A549 human lung epithelial cells (a cell type that expresses iNOS after exposure to IL-1β, TNF-α, and IFN-γ).

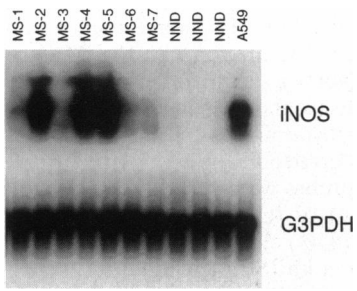

FIG. 1. Northern blot analysis of brain samples from normal or MS brains or cytokine-activated A549 human lung epithelial cells. RNA from samples of brain tissue from patients who died with MS (MS-1 through MS-7) or with a nonneurological disease (NND) or A549 cells activated with TNF-α, IFN-γ, and IL-1β for 8 h. After electrophoresis, the RNA was electroblotted to a nylon membrane and hybridized with a ^{32}P-labeled probe for human iNOS (*Upper*) or stripped and reprobed with a ^{32}P-labeled probe for G3PDH (*Lower*). Samples MS-3 and MS-7 had detectable bands of the appropriate molecular weight after longer autoradiographic exposures and PhosphorImager analysis.

The expression of iNOS mRNA was highly variable between MS brains, as indicated by the intensities of the hybridization bands. Equivalent amounts of mRNA were loaded on the gel as indicated by essentially equal band intensities after hybridization with a probe specific for human G3PDH. Two of four samples shown in Fig. 1 (MS-3 and MS-7) did not show a visually specific band at the autoradiographic exposure used but contained detectable hybridization products of the appropriate molecular weight after prolonged exposure or PhosphorImager analysis (data not shown).

RT–*in Situ* PCR for iNOS mRNA. Because the Northern blot analysis demonstrated iNOS mRNA activity in human A549 lung epithelial cells, we also chose to use these cells as positive control cells for RT–*in situ* PCR amplification of iNOS mRNA. *In situ* labeling shows that specific iNOS reactivity was present only in A549 cells after cytokine activation (Fig. 2 *B* and *C*) and was absent if the cells were not induced (Fig. 2*A*). Only a minority of the cells were positive by RT–*in situ* PCR.

Identification of iNOS Expression by Microglial Cells. Because by Northern blot analysis only a fraction of the specimens showed positive bands, we decided to utilize a more sensitive method to detect a low abundance of iNOS mRNA and to identify the histological cell type expressing the iNOS mRNA (6, 7). RT–*in situ* PCR was utilized to demonstrate iNOS expression in the brain tissues of MS patients as shown in Fig. 3. We utilized the dual-straining method, where we simultaneously stained the brain cells containing iNOS mRNA and also identified the cell type with fluoresceinated RCA-1, a lectin specific for cells of monocyte/microglial lineage. As shown in Fig. 3, iNOS-positive reactivity after RT–*in situ* PCR was only rarely seen in histological sections from control brains (Fig. 3*A*). In brain tissues from MS patients, the iNOS-positive cells were much more frequent in, but not limited to, the areas of plaques (Fig. 3 *B–D Left*). Lectin immunohistochemistry in these experiments also demonstrated that iNOS-expressing cells were predominantly, but not exclusively, RCA-1-positive microglia/macrophages (Fig. 3 *B–D Right*). In addition, iNOS-positive RCA-1-positive cells were more concentrated around and within proximal areas of demyelination. In all seven MS brain specimens, the majority of the iNOS-expressing cells were RCA-1-positive, although occasional RCA-1-negative iNOS-positive cells were observed (Fig. 3).

Concomitant Cellular Expression of Both iNOS mRNA and iNOS Protein. To assure that the cells found to express iNOS mRNA by RT–*in situ* PCR were also producing the iNOS protein, we carried out dual staining with FITC-conjugated iNOS probe and rhodamine-conjugated anti-iNOS antibodies. Both MS and control brain specimens were examined and

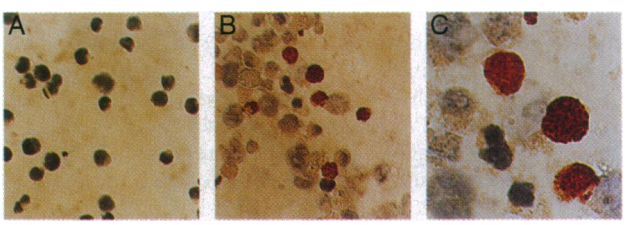

FIG. 2. RT–*in situ* PCR analysis of A549 human lung epithelial cells. Cells were cultured in the absence (*A*) or presence (*B* and *C*) of TNF-α, IFN-γ, and IL-1β for 8 h. After fixation, the cells were processed for RT–*in situ* PCR with an iNOS-specific biotinylated probe. The color was developed with 3′-amino-9-ethylcarbazole, which gives a red-brown color in positive cells. Cells were lightly counterstained with Gill's hematoxylin. (*A* and *B*, ×70; *C*, ×270.)

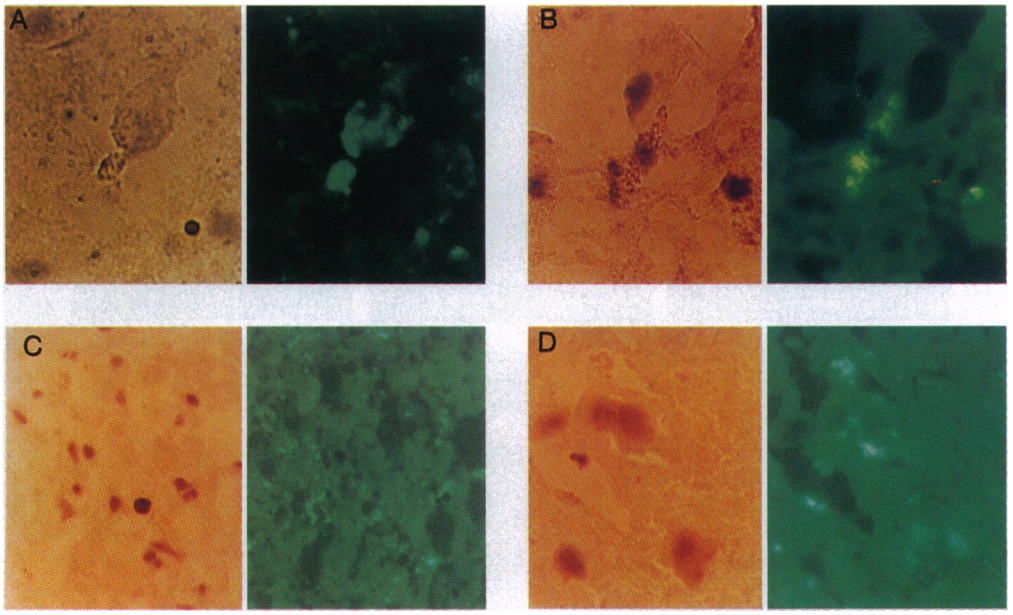

FIG. 3. RT–*in situ* PCR and immunohistochemistry were performed to evaluate the histological type of iNOS expressing cells in brain tissue sections. After RT–*in situ* PCR, biotinylated iNOS probe was utilized to identify iNOS mRNA expressing cells (*Left*). In the identical field, immunohistochemistry was performed with rhodamine-labeled ricin lectin (RCA-1) to identify macrophage/microglia cells (*Right*). (*A*) Brain specimen from a normal individual shows no signals for iNOS mRNA in the RCA-1-positive cells. (*B–D*) Three MS patients' brain sections show multiple iNOS-positive cells identified as macrophage/microglia cells (RCA-1), suggesting the expression of iNOS in these cell types.

representative data are presented in Fig. 4. The vast majority of cells exhibiting mRNA for iNOS, as determined by RT–*in situ* PCR, also exhibited the presence of iNOS protein, as detected by rabbit anti-human iNOS antibodies. The brain sections from the non-MS brain sections were negative for iNOS mRNA and reactivity with anti-iNOS antibodies.

Immunohistochemical Detection of Nitrotyrosine Residues. As shown in Fig. 5, only MS-positive brains exhibited significant concentrations of nitrotyrosine. Normal brains showed little or no reactivity with anti-nitrotyrosine antibodies. It was also noted that although most of the immunoreactive foci were in close proximity to cells that also showed RT–*in situ* PCR-specific iNOS labeling, some of the nitrotyrosine-specific antibody localization was distinct from the iNOS-expressing cells (data not shown). Nitrotyrosine-specific staining was significantly more dense in areas in and around MS plaques and appeared to be more intense within areas of demyelination but less dominant in areas immediately delimiting plaques. Morphologically, the nitrotyrosine-positive cells resembled microglia.

DISCUSSION

The experiments reported here demonstrate that tissue extracts from MS brains contained increased levels of iNOS mRNA. We found no detectable signals in extracts of brain tissue from patients without neurological disease. The mRNA detected was specific for iNOS as illustrated by the presence of a reaction product of identical molecular weight in an extract of A549 human lung epithelial cells after cytokine activation (Fig. 1). This cell line has been shown (8) to express iNOS after exposure to TNF-α, IFN-γ, and IL-1β. These results complement and extend the previous findings of Bo *et al.* (9) who reported the presence of iNOS associated with the plaques present in the brains of MS patients. These investigators used semiquantitative RT-coupled PCR amplification to demonstrate the presence of iNOS-specific mRNA associated with MS lesions. The data presented here were derived by employing the Northern blot technique show that the expression of iNOS mRNA varied across at least two orders of magnitude between patients (Fig. 1). Because we did not select tissue samples for Northern blot analysis based on the presence of

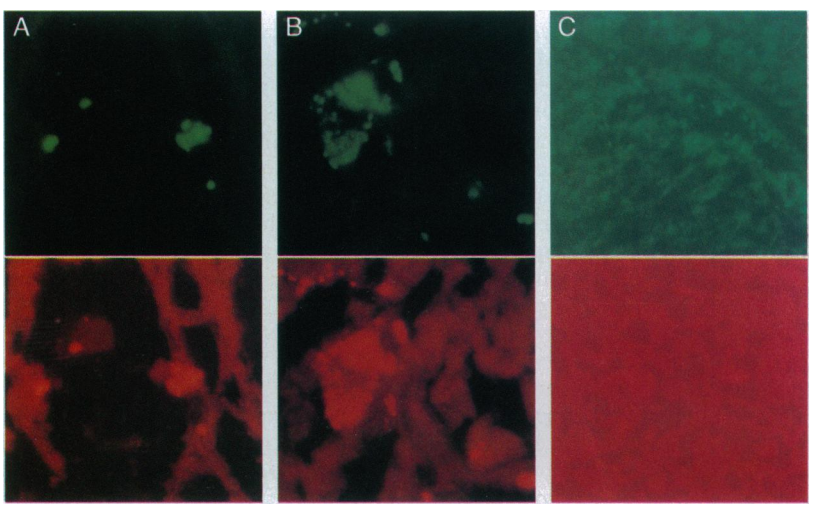

FIG. 4. To further evaluate the association of iNOS-expressing cells with the protein products, the brain sections were dually stained. After RT–*in situ* PCR for iNOS, cells were hybridized with FITC-labeled iNOS probe (*Upper*), and immunohistochemistry was performed with a murine monoclonal antibody against human iNOS. The iNOS antigen was visualized with rhodamine-labeled anti-mouse secondary antibody (*Lower*). As shown in *A* and *B Upper*, two MS patients' brain cells stained positive for activated iNOS mRNA and in the identical field, these cells were also positive for iNOS protein. The control brain sections from one of the non-MS brains show absence of iNOS mRNA and protein.

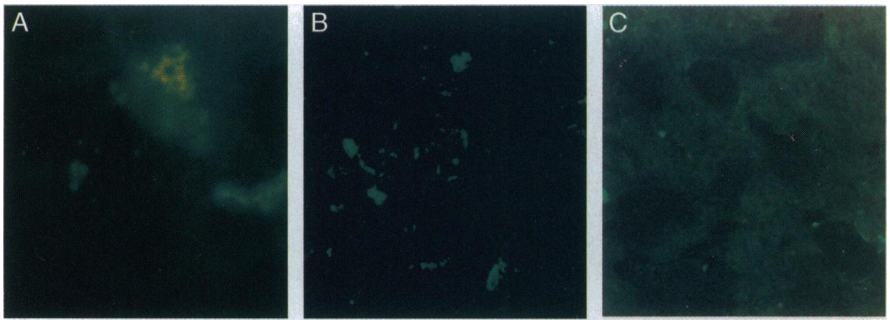

FIG. 5. Presence of nitrotyrosine in brain samples from two MS patients (*A* and *B*) or from a patient without neurological disease (*C*). The presence of nitrotyrosine was shown by immunolabeling with a mouse anti-nitrotyrosine monoclonal antibody. Specific labeling was detected after treatment of the sections with FITC-conjugated goat anti-mouse antibody.

MS plaques, we cannot assign the higher concentrations of iNOS mRNA to sites of active disease. However, our results employing an extremely sensitive detection method, RT–*in situ* PCR, where a single copy of a gene can be amplified *in situ* and then detected by a specific probe (7), support this hypothesis.

Again employing cytokine-activated A549 cells, we found that a significant number of the cells expressed iNOS mRNA detectable by RT–*in situ* PCR (Fig. 2). Cells not exposed to TNF-α, IL-1β, and IFN-γ were universally devoid of detectable iNOS mRNA. By utilizing the same technique, we were able to demonstrate the presence of specifically reactive cells in the brain samples of seven out of seven MS samples tested. Only rare reactive cells were found in the samples of three brains tested that had been collected from patients who died without primary CNS disorders. The density of the iNOS-specific signal was greatest in areas immediately adjacent to MS plaques and appeared to concentrate within plaques with histological indications of demyelination. These observations are similar to those contained in a recent report in which a direct correlation between iNOS activity and the perilesional density of cells with NADPH diaphorase activity was found (9). NADPH diaphorase activity has been shown to closely correlate with the presence of NOS activity (10), suggesting, but not proving, that iNOS activity is quantitatively increased in active demyelinating lesions, and to a lesser extent in chronic active MS plaques. The data presented here, employing a specific iNOS detection method, fully substantiates this conclusion.

We further attempted to identify the cell types responsible for the expression of iNOS within the brains of patients with MS. Significant colocalization of iNOS mRNA occurred with cells that bound FITC-conjugated RCA-1. This lectin has been shown to selectively adhere to human brain microglial cells and to microvascular endothelial cells (11, 12), cells that have also been shown to express NO (13). Although the capacity of human monocytes to produce NO has been difficult to establish (14), recent publications have reported the expression of NO *in vitro* (15, 16). Rat microglial cultures have also been shown to express iNOS activity after cytokine activation (17). In light of the precedents cited, it would be surprising if human microglial cells were completely refractory to NO expression after the application of appropriate stimuli.

The cellular source of iNOS expression reported in these studies is different from that identified by Bo *et al.* (9) who found that most NADPH diaphorase activity colocalized with immune reactivity for glial fibrillary acidic protein, a selective marker of astrocytes, but not with cells staining with anti-CD45 (leukocyte common antigen) used in their studies as a monocyte/microglial marker. Our experiments employed RCA-1, a selective marker of endothelial cells and microglia in human nervous tissue (11, 12), to identify cells expressing iNOS mRNA. Microglia and endothelial cells are easily differentiable on morphological criteria. We cannot readily resolve the differences between the two studies but suggest that the results are not mutually exclusive.

NADPH diaphorase activity does not distinguish between the type I and type II NOS enzymes (18) and, under the conditions used by Bo *et al.* (9), would be expected to detect both forms of NOS equally well. It has been shown that both rat (17) and human (19) astrocytes can be induced to elaborate iNOS after exposure to specific cytokines, many of which have also been shown to be present in increased concentrations in MS lesions (20–22). Additionally, several reports have demonstrated that astrocytes are also capable of expressing type I NOS activity (23) in response to stimuli distinct from those required for the type II enzyme. The type I NOS isoforms cannot be distinguished from the type II enzyme based solely on NADPH diaphorase reactivity (18), suggesting that the NOS activity represented by astrocyte-associated NADPH diaphorase staining might have been representative of either or both the type I and type II NOS enzymes. Our results, derived from reactivity specific for type II NOS mRNA combined with colocalization with microglia detected by RCA-1 binding, indicate that the majority of cells elaborating the type II mRNA were not of astrocyte origin but, rather, were microglial cells. We suggest that the astrocyte predominance of NADPH diaphorase activity observed by Bo *et al.* (9) may represent predominantly type I NOS.

To assure that the microglia found to express iNOS mRNA were indeed synthesizing the iNOS enzyme, we sequentially processed brain sections for iNOS mRNA by using RT–*in situ* PCR and then immunohistochemistry to detect the presence of iNOS enzyme. Again, we found simultaneous presence of the two signals, indicating that transcription of the gene was followed by translation to the protein product providing further evidence that NO is indeed elaborated within the CNS of patients with MS.

We have shown the presence of nitrotyrosine in CNS cells of MS patients. The distribution of the reactivity was greatest in central plaque areas but was more widely dispersed than was iNOS mRNA or immunoreactivity with iNOS protein as described. The presence of nitrotyrosine in MS brains may indicate that brain cells may contain peroxynitrite, a reaction product of superoxide and nitric oxide (24). It has been shown (25) that NO reacting through peroxynitrite and superoxide dismutase can add a nitrate moiety to phenolic ring structures including a protein-associated tyrosine. The presence of nitrotyrosine residues detected by a specific monoclonal antibody was found in tissues collected from several disease states such as atherosclerosis (26), adult respiratory distress syndrome (27), and rheumatoid arthritis (28). If the nitrotyrosine indicates the presence of peroxynitrites, one should stress that these substances are highly toxic agents causing dysfunction of mitochondria (29) and may contribute to the cytotoxicity for oligodendrocytes in MS brains.

The results presented here indicate that both iNOS mRNA and the iNOS enzyme are present in the brain tissues of patients who died with MS but absent in equivalent tissues collected from patients without MS. These data further demonstrate that cells of monocyte/macrophage lineage, probably microglial cells, are the source of the majority of the detected iNOS activity. We have also provided evidence that NO production appears to mediate the formation of nitrotyrosine residues that are concentrated within and immediately surrounding MS plaques and suggest that the peroxynitrite adduct may play a pivotal role in the neurotoxicity of iNOS-induced pathway. In addition, nitrotyrosine may serve as a relatively stable marker of previous NO elaboration by microglial cells. We do not know how long nitrotyrosine residues persist in tissues after their formation or whether the adducts contribute directly to the diseased state.

The administration of iNOS antagonists can significantly ameliorate the clinical manifestations of experimental allergic encephalomyelitis in experimental animals (30) and certain virally induced encephalitides that result in significantly increased intrathecal NO concentrations. These observations suggest that MS patients may benefit from the administration of iNOS inhibitors once selective and nontoxic compounds become available.

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