Effects of TNF-α and IFN-γ on Nitric Oxide-Induced Neurotoxicity in the Mouse Brain

Véronique Blais and Serge Rivest

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Véronique Blais and Serge Rivest

The present study investigated the interaction between highly reactive gaseous-free radical NO and cytokines that are produced by activated Th-1 cells on the cerebral immune response and neuronal integrity. CD-1 mice received an intrastriatal infusion of different solutions containing the NO synthase inhibitor N(G)-nitro-l-arginine methylester, NO-releasing substance sodium nitroprusside (SNP), IFN-γ, and/or TNF-α. The solution containing both cytokines caused a profound and transient transcriptional activation of numerous genes encoding proinflammatory proteins in microglial/monocytic cells ipsilateral to infusion site. This increase in gene expression peaked 1 day after the cerebral bolus of cytokines and returned to basal levels from 3 to 7 days post activation of numerous genes encoding proinflammatory proteins in microglial/monocytic cells ipsilateral to infusion site. This phenomenon was greatly exacerbated by the coadministration of both cytokines, although TNF-α remained the most critical cytokine to enhance the damage of cerebral elements. These data provide evidence that NO has the ability to modulate the immune response, which is not by itself detrimental for the brain. However, SNP-induced NO production together with TNF-α in the cerebral environment are critical events leading to intense neurodegeneration and demyelination in vivo. The Journal of Immunology, 2004, 172: 7043–7052.

The detrimental effects of TNF-α in the CNS may also depend on the presence of other cytokines produced by either resident populations of cells or infiltrating leukocytes, such as T cells. Activated T cells have the ability to produce TNF-α along with IFN-γ that acts in both innate and specific cell-mediated immunities (2). Activated CD4+ Th1 cells and their secreted cytokines are believed to be crucial to the pathogenesis of multiple sclerosis, especially during the demyelinating episodes (3). The mixture of both TNF-α and IFN-γ may therefore be harmful to neuronal elements. In this regard, transgenic mice expressing IFN-γ in hippocampus exhibit a profound enhanced microglial reactivity to lesion-induced neuronal injury indicating that IFN-γ acts as an amplifier of the response (4). Four days of treatment with IFN-γ failed to alter cell survival or myelin basic protein gene expression in cultured human oligodendrocytes, but these cells are more susceptible to Fas-mediated apoptosis and this effect is augmented in presence of TNF-α (5).

NO is another important and versatile player in the immune system and accumulating evidence supports the hypothesis that it plays a critical role in chronic degenerative diseases (6). Activation of the inducible form of NO synthase (iNOS) and NO production take place during many neuropathological conditions (7). iNOS is responsible for the cytotoxic action of macrophages and neutrophils and NO has been implicated in neurodegeneration and chronic inflammation (8, 9). NO has also been found to participate in Fas-mediated apoptosis in Jurkat cells (10) and NO donor can induce apoptosis of murine thymocytes (11). Although induction of cell death by exogenous administration of NO donor has not yet been studied in the mouse brain, implication of this molecule in cellular toxicity has been suggested by the use of NO synthase inhibitors or knockout mice in different models of demyelinating and neurodegenerative diseases (12–15).

It is therefore possible that cytokines produced by the adaptive immune system together with a sharp increase in NO levels promote demyelination and neurological disorders. In the present study, we investigated the effects of TNF-α and IFN-γ in the brain of mice challenged with the NO donor sodium nitroprusside (SNP) or NO synthase inhibitor N(G)-nitro-l-arginine methylester (l-NAME). We evaluated the expression of genes encoding several proinflammatory molecules and investigated the consequences of such a response on the neuronal integrity. Although mice challenged with both cytokines exhibited a profound and transient cerebral immune reaction that was enhanced by l-NAME, their
brains did not show any signs of neurodegeneration. However, the neurotoxic effects of SNP were clearly exacerbated by the immune ligands.

Materials and Methods

Animals

Adult male CD-1 mice (30–35 g of body weight) were acclimated to standard laboratory conditions (14-h light and 10-h dark cycle, lights on at t = 0600 h and off at t = 2000 h) with free access to mouse chow and water. Animal breeding and experiments were conducted according to Canadian Council on Animal Care guidelines, as administered by the Laval University Animal Care Committee. A total of 130 mice were assigned to different protocols as described in Table I.

Experimental protocols

Mice were anesthetized with an i.p. injection (10 ml/kg of body weight) of a mixture of ketamine hydrochloride (15 mg/ml) and xylazine (1 mg/ml) and placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). The striatum was then reached (~2.0 mm lateral and ~3.0 mm dorsoventral to the bregma) with a 33-gauge stainless steel cannula (Plastics One, Roanoke, VA) that was connected to a 50-μl Hamilton syringe through an intramedic polyethylene tubing (PE-50; Caly Adams, Parsippany, NJ). A volume of 1 μl was infused over 2 min by means of a microinjection pump (model A-99; Razel Scientific Instruments, Stanford, CT). These coordinates were selected on the basis of preliminary data showing a robust hybridization signal and reliable pattern of cytokine gene expression over the ipsilateral cerebral cortex, hippocampus, corpus callosum, and basal forebrain (field, IL) and cut into 20-μm coronal sections from the olfactory bulb to the caudal medulla. The slices were collected in a cold cryoprotectant solution and stored at −20°C. Hybridization histochemical localization of Toll-like receptor (TLR)2, index of innate immune response; iNOS, index of phagocyte activation; and IL-12p40, a key cytokine involved in the transfer from the innate to adaptive immunity, mRNAs were performed on every 12th section of the entire rostrocaudal span of each brain as previously described (17–19). Plasmids were linearized and sense and antisense riboprobes synthesized as described in Table II.

Immunocytochemistry was combined with the in situ hybridization protocol to determine the type(s) of cells that express the different transcripts in the mouse brain. Double-labeling procedures were similar to those described in other studies published by our group (19–21).

Detection of demyelination, neuronal death, and apoptosis

Demyelination was determined via the Luxol fast blue (LFB) staining. Every sixth section of the whole rostrocaudal extent of each brain was mounted onto poly-L-lysine-coated slides, dried overnight under vacuum, dehydrated through graded concentrations of alcohol (50, 70, and 95%; 1 min each), and incubated at 60°C for 6 h in the LFB solution (solvent blue 38 at 1%; Sigma-Aldrich, St. Louis, MO) in ethanol 95% and 0.5% acetic acid. The sections were then rinsed in alcohol 95% (1 min), lithium carbonate 0.05% (Sigma-Aldrich, 1–5 min), and alcohol 70% (2 dips). Thereafter, the slides were incubated in eosine Y solution 1% (EM Diagnostic Systems, Gibbstown, NJ) for 40 s, rinsed in distilled water, incubated in cresyl violet 0.25% (Sigma-Aldrich) for 40 s, rinsed in distilled water, dehydrated through graded concentrations of alcohol (50, 70, 95, and 100%; 1 min each), cleared two times in xylene for 1 min, and coverslipped with distrene plasticizer xylene (Electron Microscopy Sciences, Fort Washington, PA).

Table I. Treatment, dose, and number of mice used in each condition

<table>
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<tr>
<th>Treatment*</th>
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<td>rmIFN-γ (100 ng)</td>
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<tr>
<td>rmTNF-α (100 ng)</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>SNP (1 μg) + rmIFN-γ (100 ng) + rmTNF-α (100 ng)</td>
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<tr>
<td>L-NAME (1 μg)</td>
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</tr>
<tr>
<td>L-NAME (1 μg) + rmIFN-γ (100 ng) + rmTNF-α (100 ng)</td>
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* Treatment using recombinant murine (rm) IFN-γ: catalog no. 485-MI (R&D Systems); rmTNF-α: catalog no. 410-MT (R&D Systems); SNP: catalog no. S-0501 (Sigma-Aldrich); L-NAME: catalog no. N-5751 (Sigma-Aldrich).

Table II. Plasmids and enzymes used for the synthesis of cRNA probes

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<td>Spe</td>
<td>V/7T</td>
</tr>
<tr>
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<td>Bluescript II*</td>
<td>1114</td>
<td>Hind</td>
<td>III/T3</td>
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<td>pGEMEX-1</td>
<td>578</td>
<td>Sac</td>
<td>I/I6</td>
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<td>1300</td>
<td>BamH</td>
<td>I/T7</td>
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<tr>
<td>iNOS</td>
<td>Bluescript SK II*</td>
<td>817</td>
<td>EcoR</td>
<td>V/7T</td>
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<tr>
<td>IL-12p40</td>
<td>pCL-Neo</td>
<td>1050</td>
<td>Not</td>
<td>I/T7</td>
</tr>
</tbody>
</table>

Enzymes

Sce I | Pst | I/3 |
BamH | I/T7 |
Kpn | I/T3 |
Xho | I/T3 |
A apoptotic cells were labeled by immunohistochemistry using a cleaved caspase-3 mAb. Brain sections were washed in sterile ethyl pyrocarbonate-treated 50 mM potassium PBS and incubated 48 h at 4°C with the cleaved caspase-3 Ab Asp175 (Cell Signaling Technology, Mississauga, Ontario, Canada), which was diluted in sterile potassium PBS (1/800) plus 0.4% Triton X-100 plus 1% BSA (fraction V; Sigma-Aldrich) plus 1% normal goat serum. After incubation with the primary Ab, brain slices were rinsed in sterile potassium PBS and incubated with a mixture of potassium PBS plus 0.02% Triton X-100 plus 1% BSA plus Cy2-conjugated anti-rabbit IgG Ab (1/500; Jackson ImmunoResearch Laboratory, West Grove, PA) for 2 h in a dark room at 20°C. Tissues were thereafter rinsed in sterile potassium PBS, mounted onto poly-L-lysine slides, and coverslipped with VectaShield mounting medium with propidium iodide (Vector Laboratories, Burlingame, CA). The phenotype of cleaved caspase-3 immunoreactive cells was determined via double immunofluorescence. Brain sections were incubated overnight at 4°C with an Ab directed against neuronal fibrillary acidic protein (Chemicon International), or Mac-1 α-chain (CD11b; BD Biosciences, San Jose, CA), which was diluted in sterile potassium PBS (1/1000, 1/100, or 1/500, respectively) plus 0.4% Triton X-100 plus 1% BSA plus 1% normal goat serum. After incubation with the primary Ab, brain slices were rinsed in potassium PBS and incubated with a mixture of potassium PBS plus 0.02% Triton X-100 plus 1% BSA plus Cy5-conjugated anti-mouse IgG Ab (1/500; Jackson ImmunoResearch Laboratory; for NeuN and glial fibrillary acidic protein) or Cy3-conjugated anti-rat IgG Ab (1/500; Jackson ImmunoResearch Laboratory; for mac-1) for 2 h in a dark room at 20°C. Tissues were thereafter rinsed in sterile potassium PBS and incubated overnight at 4°C with the cleaved caspase-3 Ab Asp175, which was diluted in sterile potassium PBS (1/800) plus 0.4% Triton X-100 plus 1% BSA plus 1% normal goat serum. After incubation with the primary Ab, brain slices were rinsed in sterile potassium PBS and incubated with a mixture of potassium PBS plus 0.02% Triton X-100 plus 1% BSA plus Alexa Fluor 488-conjugated anti-rabbit IgG Ab (1/500; Molecular Probes, Eugene, OR) for 2 h in a dark room at 20°C. Tissues were there after rinsed in sterile potassium PBS, mounted onto poly-L-lysine slides, and coverslipped with a polyvinyl alcohol (Sigma-Aldrich) mounting medium containing 2.5% 1,4-diazabicyclo(2,2,2)-octane (Sigma-Aldrich) in buffered glycerol.

Cell death by apoptosis was also detected using a TdT-FragEL DNA Fragmentation Detection kit (Oncogene Research Products, San Diego, CA). Positive controls were generated from brain sections of animals that received only sham treatments. These sections were mounted on the slides and covered with 1 µg/µl DNase I in 1× TBS/1 mM MgSO4 for 20 min at room temperature.

Confirmation of neuronal death was determined via the Fluoro-Jade B (FJB) method (22). Briefly, every sixth section of the whole rostrocaudal extent of each brain was mounted onto poly-L-lysine-coated slides, dried under vacuum 2 h, dehydrated through graded concentrations of alcohol (50, 70, and 100%; 1 min), rehydrated through graded concentrations of alcohol (100, 70, and 50%; 1 min each) and 1 min in distilled water. They were then dipped and shaken into potassium permanganate (0.06%) for 10 min, rinsed 1 min in distilled water, and dipped and shaken in a solution containing FJB 0.004% (Histochem, Jefferson, AR) plus acetic acid 0.1% (Sigma-Aldrich) plus DAPI 0.0002% (Molecular Probes) for 20 min. The slides were thereafter rinsed three times in distilled water (1 min each), dried, dipped in xylene three times (2 min each), and coverslipped with durethane plasticizer xylene.

Nissl stain was also used as a general index of cellular morphology that may be altered in response to the different treatments.

![FIGURE 1](http://www.jimmunol.org/) Expression pattern of genes encoding molecules of the innate immune system in response to an intrastriatal infusion of cytokines and the NO donor SNP. These darkfield photomicrographs were taken from nuclear emulsion-dipped coronal sections (20 µm) of mice killed 1 or 3 days after an intraparenchymal injection of saline solution (1 µl), IFN-γ (100 ng), TNF-α (100 ng), IFN-γ+TNF-α, SNP (1 µg), or SNP+IFN-γ+TNF-α. All photomicrographs were taken at a similar rostrocaudal level nearest the injection site. Please note the robust and widespread hybridization signal for TLR2 and MCP-1 transcripts all over the ipsilateral side. Cells expressing IL-12p40 were, conversely, less numerous and scattered across ipsilateral side of mice challenged with both cytokines either alone or combined with SNP and killed 1 day afterward. Magnification, ×3.25; scale bar, 1 mm.

<table>
<thead>
<tr>
<th></th>
<th>Saline 1 day</th>
<th>IFN-γ 1 day</th>
<th>TNF-α 1 day</th>
<th>IFN-γ+TNF-α 1 day</th>
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<tr>
<td>TLR2 mRNA</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1 mRNA</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IL-12 p40 mRNA</td>
<td></td>
<td></td>
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</tbody>
</table>

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**TLR2 mRNA**

- **Saline 1 day**
- **IFN-γ 1 day**
- **TNF-α 1 day**
- **IFN-γ+TNF-α 1 day**

---

**MCP-1 mRNA**

- **Saline 1 day**
- **IFN-γ 1 day**
- **TNF-α 1 day**
- **IFN-γ+TNF-α 1 day**

---

**IL-12 p40 mRNA**

- **Saline 1 day**
- **IFN-γ 1 day**
- **TNF-α 1 day**
- **IFN-γ+TNF-α 1 day**
FIGURE 2. Cellular distribution of TLR2, MCP-1, and IL-12p40 mRNA in cerebral tissue of mice that received IFN-γ, TNF-α, and SNP in the dorsal basal ganglia. Animals were killed 1 day after the intraparenchymal infusion of a solution (1 µl) containing IFN-γ (100 ng), TNF-α (100 ng), and SNP (1 µg). These darkfield and brightfield photomicrographs of dipped coronal sections (20 µm) into nuclear emulsion NTB2 depict positive signal within small-scattered microglial cells (TLR2 and MCP-1 mRNAs) and few larger monocytic cells (IL-12p40 mRNA) across the cerebral tissue. The dual-labeling procedure provided the anatomic evidence that although TLR2 and MCP-1 mRNAs were largely colocalized within parenchymal microglia (ibα1+), IL-12p40 transcript seemed associated with infiltrating monocytes/macrophages. Few microglial cells were nevertheless positive for the mRNA encoding the latter cytokine. TLR2, MCP-1, or IL-12p40 mRNA was hybridized on the same sections by means of a radioactive in situ hybridization technique using silver grains (right column). Arrowheads indicate dual-labeled cells (TLR2 mRNA/microglial cell (top row); MCP-1 mRNA/microglial cell (middle row); IL-12p40 mRNA/macrophage cell (bottom row)).

Data analysis

The relative intensity of mRNA signals throughout the brain of each animal was assessed on X-ray film images and graded according to the scale of undetectable (−), low (+), moderate (+ +), strong (+ + +), or very strong (+ + + +). Dipped emulsion slides were examined under microscopic evaluation to ascertain the subcellular localization of each transcript. Semi-quantitative analysis of the hybridization signal was conducted on X-ray films (Biomax; Kodak, Rochester, NY) over numerous brain sections ipsilaterally to injection site. Transmittance values (referred to in this study as OD) and extent of positive hybridization signal were measured under a Northern Light Desktop Illuminator (Imaging Research, St. Catherine’s, Ontario, Canada) using a Sony Camera Video System attached to a MicroNikkor 55-mm Vivitar extension tube set for a Nikon lens and coupled to a Macintosh computer (Power PC 7100/66) and Image software (version 1.61, non-FPU; a kind gift from W. Rasband, National Institutes of Health, Bethesda, MD). OD for each pixel was calculated using a known standard of intensity and distance measurements from a logarithmic specter adapted from BioImage Visage 110s (Millipore, Ann Arbor, MI). Sections from experimental and control animal were digitized and subjected to densitometric analysis, yielding measurements of integrated OD (area digitized × average OD). The OD for each section was corrected for the average background signal, determined on the contralateral section. Data are reported as mean ± SEM values for experimental and control mice. The number of FJB-positive neurons was quantified on brain sections that were digitized at ×100 of magnification with a RT-SPOT camera (Diagnostic Instruments, Sterling Heights, MI) mounted directly onto a BX-60 Olympus microscope and connected to a PowerMac G4 computer. All the FJB-positive cells were counted manually and the results were reported as the mean number of positive cells per area ± SEM values. The digitized areas were taken at the same rostrocaudal level ipsilateral to the injection site. Three different areas were delimited and quantified: surrounding the infarction in the caudate putamen (area a), dorsal to the infarction in the cortex (area b), and lateral to the infarction site in the cortical region (area c). Statistical analysis was performed by a two-way ANOVA (StatView 4.5, Macintosh computer) followed by a Bonferroni-Dunn test procedure as post hoc comparisons for each time postinjection (1 and 3 days). Quantification of the degenerated area was conducted on LFB-stained sections with an Olympus Optical System (BX-50, Bmax) coupled to a Macintosh computer (Power PC 7100/66) and Image software (version 1.61, non-FPU). The damaged area was digitized, and pixel values were measured under brightfield illumination at a magnification ×3.125. The percentage of the damaged area was calculated from the entire coronal section of each animal. Data are reported as mean ± SEM percentage values for experimental and control mice. Statistical analysis was performed by a two-way ANOVA (StatView 4.5, Macintosh computer) followed by a Bonferroni-Dunn test procedure as post hoc comparisons for each time postinjection.

Results

Effects of IFN-γ and TNF-α on expression of proinflammatory genes

Transcriptional activation of the gene encoding TLR2 is a very sensitive index of microglial activation in response to a large variety of immune stimuli and insults (1, 17, 23–25). However, only few cells were positive for TLR2 mRNA in the brain of mice that received a single intrastratial bolus of IFN-γ, and the hybridization signal remained localized near the injection site (Fig. 1, top). This was also the case for the mice that were infused with the vehicle solution into the striatum; the message was found quite specifically along the cannula track. In contrast to such localized induction, transcriptional activation took place in the infarced areas of animals challenged with the proinflammatory cytokine TNF-α. The signal diffused across the ipsilateral site 24 h after the single TNF-α infusion, but intensity and extent of hybridization signal were highest in mice challenged with both cytokines simultaneously (Fig. 1). Indeed, numerous small-scattered cells exhibited an intense hybridization signal across the cerebral tissue of mice killed 24 h after being infused with both cytokines (Fig. 2). Dual-labeling pro-
cEDURE provided the anatomical evidence that the gene encoding TLR2 was essentially expressed within microglial cells (see Fig. 2, which shows agglomerations of silver grains within iba1 immunoreactive cells). The signal for TLR2 transcript decreased 3 days following the cerebral challenge with IFN-γ and TNF-α and returned to background levels at time 7 days postinfusion (data not shown).

The mRNAs encoding numerous other genes were assessed in adjacent coronal sections. In this study again, saline-treated mice exhibited an increase in the expression levels of MCP-1 transcript in cells adjacent to the cannula track at time 24 h postinfusion. The signal essentially vanished 72 h after the single bolus of saline in the basal ganglia. A low but positive hybridization signal for MCP-1 was detected in the region ipsilateral to injection site of mice challenged with either IFN-γ or TNF-α, although the latter caused a more pronounced increase in MCP-1 gene expression (Fig. 1). However, the signal intensity was clearly highest in mice that received a single bolus with a mixture of both cytokines and killed 1 day afterward (Figs. 1 and 2). The pattern of MCP-1 gene expression was very similar to that of TLR2 mRNA, but the cells exhibited a lower number of silver grains (Fig. 2). Treatment with both cytokines also triggered transcriptional activation of genes encoding iκBα (index of NF-κB activity) and TNF-α, even if the signals for both iκBα and TNF transcripts were much lower than those of TLR2 and MCP-1 mRNAs (data not shown).

In contrast, a single intracerebral infusion of IFN-γ, TNF-α, or both cytokines together only provoked a very modest increase in iNOS gene expression within few isolated cells that were adjacent to the endothelium of the brain capillaries (Fig. 3). This was also the case for the gene encoding IL-12p40 that was induced only in few small-scattered cells 24 h after the intrastriatal infusion of both IFN-γ and TNF-α. The pattern of TLR2- and MCP-1-expressing cells was highly different from that of IL-12p40-expressing cells (Figs. 1 and 2). This suggests a different type of cells, although all transcripts were found within iba1-immunoreactive cells (Fig. 2).

It is nevertheless possible that the technique used in this study is not sensitive enough to detect IL-12p40 transcripts in all activated microglial cells or this cytokine is induced only in a selective subset of monocyteic cells. As depicted by Fig. 3, the innate immune response triggered by the intraparenchymal cytokine infusion essentially disappeared at time 3 days posttreatment.

Role of NO in mediating the inflammatory reaction in the CNS

Inhibition of NO pathway enhanced the effects of IFN-γ and TNF-α on the innate immune response in the CNS (Fig. 3). Indeed, the nonselective inhibitor of NO synthase l-NAME caused a profound transcriptional activation of genes encoding TLR2, MCP-1, iκBα, TNF-α, iNOS, and IL-12p40 24 h after the intracerebral infusion of IFN-γ and TNF-α. The hybridization signal for TNF-α, iNOS, and IL-12p40 transcripts was significantly stronger in the brain of mice that received a systemic injection of l-NAME into the intrastriatal area along with the mixture of both cytokines. All these transcripts returned to low or undetectable levels at time 3 days postinjection, and administration of l-NAME alone remained without notable effect on expression of the genes investigated in the present study.
To further assess the role of NO as mediator of the inflammatory response in the CNS, another group of mice was infused with the NO donor SNP either alone or in combination with IFN-\(\gamma\) and/or TNF-\(\alpha\). A low and localized hybridization signal was found for the mRNA encoding TLR2 and MCP-1 in the brain of mice challenged with SNP into the basal ganglia and killed 24 h afterward (Figs. 1 and 2). The message for TLR2, MCP-1, and \(\alpha\) transcripts increased 2 days later and remained detectable up to 21 days posttreatment with SNP. The hybridization signal was moderate to low and remained mostly in the caudate putamen of ipsilateral side, although few positive cells were also found in the hippocampus and cortex. Administration of SNP along with IFN-\(\gamma\) or TNF-\(\alpha\) increased gene expression as soon as 24 h postadministration, but the signal intensity and distribution patterns were similar to those already described for mice challenged only with both cytokines. The marked differences in gene expression clearly took place at 3 and 7 days; whereas infusion of both cytokines caused a transient innate immune response, the mixture of SNP, IFN-\(\gamma\), and/or TNF-\(\alpha\) led to a prolonged increase in TLR2, MCP-1, \(\alpha\), \(\alpha\), iNOS, and IL-12p40 gene expression in regions ipsilateral to the injection site (Fig. 3). The gene encoding TLR2 remained quite strong 21 days following injection of SNP plus both cytokines, and \(\alpha\) transcripts were still detectable at this time after all SNP treatments (Fig. 3). Expression of all the genes reported in this study was restricted to damaged areas near the injection site. This localized inflammatory response seems therefore associated with neuronal cell death and cellular infiltration (see below). It is of interest to note that both NO inhibitor and donor exacerbated the immune reaction to IFN-\(\gamma\) and TNF-\(\alpha\), but only SNP provoked neurodegeneration. The hybridization signal was not due to unspecific labeling because damaged tissues never depicted positive messages when hybridized with sense probes (data not shown). Both cytokines also increased expression of proinflammatory genes in response to a lower dose of SNP (500 ng, data not shown).

**Consequences of inflammation and NO on the neuronal integrity**

LFB staining was used for the histologic analysis of myelinated fibers (blue) and neurons (purple) in the CNS. This staining preparation also allowed identification of infiltrating cells, such as neutrophils and monocytes/macrophages. Except for the lesion caused by implantation of the infusion cannula, the brain of saline-injected mice never exhibited signs of neurodegeneration at any of the times evaluated in this study. Fibers of the corpus callosum and myelinated fibers in the caudate putamen in proximity of injection site were unchanged compared with contralateral side, and the injection did not affect the shape and number of neurons (Fig. 4). Clusters of blood cells were detected along the cannula track in the meninges, cortex, and caudate putamen, but not within the brain parenchyma. Similarly, a single intrastratal bolus of IFN-\(\gamma\), TNF-\(\alpha\), or both cytokines together failed to cause neurodegeneration and demyelination at all the times evaluated. This contrasts with the brain of animals that received the NO donor SNP, which

**FIGURE 4.** Effect of cytokines and NO donor SNP on the neuronal integrity in the mouse brain. LFB staining was used to label the myelinated fibers, neurons, and immigrating cells in the mouse brain. Examples of LFB-stained coronal sections of mice (top row) that were killed 3 days after a single intrastriatal infusion with solutions containing saline, IFN-\(\gamma\), TNF-\(\alpha\), and SNP. The degenerated areas are depicted by the dashed lines. Confirmation of neuronal death was performed via the FJB method, whereas cell death by apoptosis was detected by means of a TdT-FragEL DNA Fragmentation Detection kit (bottom row). Photomicrographs (second row) result from the merge of six different pictures taken on the same brain section to cover the entire ipsilateral side at the level of the injection site. The images were merged using Canon PhotoStitch 3.1. Magnification: LFB, \(\times 3.25\); FJB, \(\times 10\) (second row) and \(\times 100\) (third row); and FragEL, \(\times 250\) (bottom row). Scale bar: FJB, 400 \(\mu\)m (second row) and 25 \(\mu\)m (third row); and FragEL, 10 \(\mu\)m (bottom row). Depictions of FragEL cells are laser scanning confocal microscopic images (Fluoview SV500; Olympus America, Melville, NY).
causes neuronal death, demyelination, and infiltration of circulating leukocytes in the infused areas (Fig. 4 and Fig. 5B). The cerebral tissue in the cortex and caudate putamen was partially destructed and filled with cellular debris and damaged neurons, which have a different shape than healthy neurons (Fig. 5, I and J).

The degeneration process spread out to more caudal regions, including the hippocampus and striatum. IFN-γ did not modify notably the effects of SNP, especially at time 3 days. (Figs. 4 and 5). In contrast, the ability of NO donor to cause neurotoxicity was clearly exacerbated by the

### Table III. Number of FJB neurons in the brain of cytokine-infused and NO donor-infused mice

<table>
<thead>
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<td>SNP</td>
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</tr>
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<td>IFN-γ + TNF-α</td>
<td>3</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>SNP</td>
<td>3</td>
<td>32.80 ± 6.84</td>
</tr>
<tr>
<td>SNP + IFN-γ</td>
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<td>42.50 ± 11.85</td>
</tr>
<tr>
<td>SNP + TNF-α</td>
<td>3</td>
<td>50.33 ± 6.12</td>
</tr>
<tr>
<td>SNP + IFN-γ + TNF-α</td>
<td>3</td>
<td>84.00 ± 9.98</td>
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</tbody>
</table>

*All the FJB-positive cells were counted manually and the results were reported as the mean number of positive cells per area ± SEM values. The digitized areas were taken at the same rostrocaudal level ipsilateral to the injection site.

†Three different areas were delimited and quantified; surrounding the infusion site in the caudate putamen (area a), dorsal to the infusion site in the cortex (area b), and lateral to the infusion site in the cortical region (area c). †, Significantly different (p < 0.05) from the group of SNP mice. ††,Significantly different (p < 0.05) from the IFN-γ + TNF-α group of mice. See Materials and Methods for details.

IFN-γ + TNF-α, intrastriatal (IS) infusion of IFN-γ (100 ng) and TNF-α (100 ng); SNP, IS infusion of SNP (1 μg); SNP + IFN-γ, IS infusion of SNP (1 μg) and IFN-γ (100 ng); SNP + TNF-α, IS infusion of SNP (1 μg) and TNF-α (100 ng); SNP + IFN-γ + TNF-α, IS infusion of SNP (1 μg), IFN-γ (100 ng) and TNF-α (100 ng).
proinflammatory cytokine TNF-α. In these brains, myelinated fibers of the corpus callosum and neurons of the cerebral cortex, hippocampus, and dorsal striatum were injured by the solution containing SNP and TNF-α. However, neurodegeneration took place essentially within the ipsilateral side and the contralateral side remained intact, at least as revealed by this staining procedure (Figs. 4 and 5D). Several clusters of infiltrating neutrophils were detected in the injured areas. These cells were associated with blood vessels at time 3 days postinjection and progressively immigrated across the brain parenchyma (Fig. 5, K–M). The neurotoxic effects of the solution containing SNP and TNF-α were not exacerbated, but slightly attenuated by IFN-γ. Indeed, the extent of the degenerated structures was more restricted in the brains of SNP/IFN-γ/TNF-α-administered mice than in those of mice challenged only with SNP and TNF (Figs. 4 and 5). Although l-NAME enhanced expression of several inflammatory genes in response to intrastriatal IFN-γ and TNF-α infusion, these brains did not show any anatomical signs of neuronal cell death (data not shown).

As for the LFB histologic preparations, the brain of mice that received intraparenchymal administration of saline solution, IFN-γ, TNF-α, or both cytokines exhibited only background FJB staining, except along the cannula track (Fig. 4 and Table III). This localized staining decreased at 3 days and essentially vanished 7 days after the infusion. One day after the single bolus of SNP in the basal ganglia, few positive FJB neurons were found along the cannula track and surrounding areas. The signal greatly increased and spread across the cortex and dorsal striatum 3 days after the infusion with the NO donor (Fig. 4). Dying neurons were also found more rostrally in the lateral septum and more caudally in the hippocampus, dentate gyrus, and striatum. Once again, TNF-α either alone or combined with IFN-γ greatly exacerbated the neurodegenerative processes caused by SNP (Fig. 4 and Table III). Positive FJB neurons were detected almost across the entire ipsilateral side of the brain as well as in the lateral septum and hippocampus of the contralateral side. This was particularly the case in the CNS of mice that were killed 3 days after being injected with SNP and TNF-α. FJB-positive cells were quantified in different areas ipsilateral to the infusion site (Table III). The number of degenerating neurons was highest in all the areas of mice that were killed 1 day after the intracerebral challenge with both SNP and TNF-α. At time 3 days however, it is the mixture of SNP combined with both cytokines that led to a more severe neuronal death in all the areas quantified (Table III). The FJB signal was no longer detectable in the brain of all mice at times 7–21 days postinjection. Administration of l-NAME either alone or together with cytokines did not provoke neuronal cell death as revealed by both LFB and FJB methods (data not shown).

To determine the potential contribution of apoptosis in mediating the effects of different treatments on cell death, we used a TdT-FragEL DNA fragmentation detection kit. As for the other histologic procedures, this method failed to detect apoptotic cells in the brain of mice injected with l-NAME alone or together with either IFN-γ or TNF-α or both cytokines at all postinjection times. SNP alone did not yet induce apoptosis at time 24 h, but apoptotic cells were already numerous in the cortex just above the corpus callosum of mice challenged with the solution containing SNP and cytokines. As seen in Fig. 4 (bottom row), the number of positive nuclei greatly increased 3 and 7 days after the infusion of SNP alone, but the NO-releasing substance caused more cell death via apoptosis when combined with TNF-α. It is interesting to note that although LFB and FJB revealed a massive neurodegeneration at 3 days, the number and spread of FragEL-positive cells peaked 7 days after the different treatments using SNP. The brains of these animals exhibited a similar pattern of cleaved caspase-3 immunoreactive cells across the ipsilateral side (Fig. 6A). These cells were also immunoreactive to the neuronal marker NeuN (Fig. 6B). Although these data show that neuronal death by apoptosis clearly took place in these brains, cellular necrosis may also contribute to the neuronal and glial cell loss in mice treated with SNP alone or together with TNF-α and/or IFN-γ. Astrocytes and microglia did...
not exhibit positive cleaved caspase-3 immunoreactive signal (data not shown).

Discussion
The present study provides solid evidence that parenchymal administration of TNF-α alone or combined with IFN-γ provoked a robust inflammatory reaction in the mouse brain. These proinflammatory signaling events were transient and did not lead to demyelination or neurodegeneration. This suggests that these immune molecules are not by themselves capable of causing cell death in the CNS, at least when administered acutely as performed in the present study. However, these cytokines contributed greatly to exacerbate the cerebral damage caused by the NO donor SNP. The treatment combining TNF-α and SNP was particularly harmful to cerebral elements. In other words, TNF-α clearly accelerated and accentuated the devastating effects of NO donor in the mouse CNS. The brains of these animals exhibited demyelination (LFB), neurodegeneration (positive FJB neurons), and cellular apoptosis (positive TUNEL and cleaved caspase-3 neurons) across the ipsilateral side and sometime in regions of the contralateral side. This cerebral damage was also associated with a robust increase in expression of genes encoding proinflammatory molecules in a pattern similar to that of degenerating groups of cells. Some transcripts were still expressed 21 days after the treatment combining SNP and cytokines, which suggest that SNP-induced neurotoxicity leads to a subsequent inflammatory response and microglial reactivity. The latter is not directly responsible for the neurodegeneration, but it contributed to expand the damage in presence of exogenous TNF-α. In this regard, inhibition of NO pathway by 1-NAME increased transcriptional activation of TNF-α, iNOS, and IL-12p40 in IFN-γ plus TNF-α-injected mice, but these brains did not exhibit any signs of neurodegeneration. Therefore, inhibiting and stimulating NO enhanced the ability of both cytokines to trigger the inflammatory response in the brain, although neurotoxicity took place only in the groups of SNP-treated mice. In this case, inflammation is believed to be a consequence and not a direct cause of cell death, but once engaged this process becomes more susceptible to TNF-α.

Inflammatory molecules have been studied in the context of several neurodegenerative diseases. Lee et al. (26) have demonstrated that neutralizing TNF-α Ab was able to reduce the number of dying neurons and oligodendrocytes in response to spinal cord injury. They have also observed a better cell survival in animals treated with a NO synthesis inhibitor. Besides, constitutive production of soluble TNF-α type I receptor in transgenic mice significantly inhibited the neurodegenerative events following motoneuron axotomy (27). Moreover, this cytokine plays a critical role in the profound neurodegeneration taking place in animals treated with the glucocorticoid receptor inhibitor RU486 before the intrastratal bolus of LPS (28). Inhibition of TNF-α is indeed able to totally abolish the neurotoxic effects of the endotoxin and TNF-α-induced neuronal damage is dependent on both NO and caspase pathways (28).

Taken together these data weigh in favor of TNF-α in playing a key role in neurodegenerative events. Despite this finding, conflicting results exist in regard to the role of TNF-α in either exacerbating brain damages or ameliorating brain recovery during different pathologic situations. In a very elegant study, Arnett et al. (16) have used mice lacking TNF-α and its associated receptors to study a model of demyelination and remyelination. Surprisingly, the lack of TNF-α led to a significant delay in remyelination, which was associated with a reduction in the pool of proliferating oligodendrocyte progenitors followed by a reduction in the number of mature oligodendrocytes. This reparative role for TNF-α in the CNS is mediated via the TNFR2, not TNFR1 (16), indicating that the dual role of TNF-α in demyelination and remyelination may depend on the receptor type and/or the cellular source of the cytokine. It is also important to mention that a single bolus of TNF-α either alone or combined with IFN-γ did not provoke neurodegeneration despite the profound inflammatory response.

The binding of TNF-α to its cognate receptors leads to the formation of the TNFR1-associated death domain/TNFR1-associated factor (TRAF)2 complex, which activates the NF-κB signaling events. TNF-α is actually one of the most potent effectors of NF-κB activity through the 55 kDa TNF type I receptor in most types of cells in the systemic immune system as well as in the CNS (29). FADD/MORT1, TRAF2, and the death domain kinase receptor-interacting protein are recruited and may also interact directly with TNFR1-associated death domain (30). Whereas FADD/MORT1 is essential for TNF-induced apoptosis, receptor-interacting proteins and TRAF2 seem to be the key molecules for activating both NF-κB and mitogen-activated protein kinases (31).

The latter is a major survival pathway (32); it leads to the synthesis of several antiapoptotic proteins, and inhibiting NF-κB provokes cell death in presence of TNF-α (33–35). However, the increase in the activity of this pathway by TNF-α clearly failed to prevent the neurotoxic effects of SNP, it actually further increased demyelination and neuronal cell death. These data, together with the robust immunoreactive signal for the cleaved caspase-3 and positive TUNEL nuclei, support the concept that apoptotic signaling events circumvented the protecting activity of NF-κB in animals challenged with SNP in the CNS.

IFN-γ was also found to play a critical role in mediating cell death, because transgenic mice expressing IFN-γ in the hippocampus showed increased susceptibility to neuronal damage (4) and IFN regulatory factor-1 (a transcription factor activated by IFN-γ) knockout mice are more resistant to experimental autoimmune encephalomyelitis (36). On the contrary, Furlan et al. (37) have suggested a protective role of IFN-γ in this autoimmune demyelinating disease commonly used to model the pathogenetic mechanisms involved in multiple sclerosis. They showed that administration of a vector expressing IFN-γ in the fluid of the spinal chord attenuated the symptoms. In this particular case, IFN-γ was responsible of the induction of TNFR inhibitor (p55) on infiltrating T cells, helping TNF-α to induce apoptosis of these cells. In the same line, the single injection of IFN-γ either alone or combined with TNF-α failed to cause demyelination and neuronal loss, but the mixture of both cytokines causes a strong and transient transcriptional activation of proinflammatory genes. This response is therefore not detrimental for the cerebral elements, although it may depend on the dose and duration of the treatment. Indeed, we have recently found that chronic TNF-α infusion in the basal ganglia led to a profound neurodegeneration via both NO and caspase pathways (28). We have yet to determine whether chronic IFN-γ infusion may also be associated with such damage in the brain or the cytokine has to be combined with TNF-α to induce cell death in the CNS.

The exact mechanisms involved the ability of TNF-α and IFN-γ to enhance the neurotoxic effects of SNP remain unclear at this point. NO is able to reduce ATP generation and induce apoptosis of PC12 cells (38). Reduction of mitochondrial respiration and neurotoxicity also take place in response to antifungal drug amphotericin B, especially when combined with IFN-γ (39). These authors have shown that amphotericin B causes a dose-dependent increase of NO generation in IFN-γ-stimulated rat and mouse astrocytes, as well as in IFN-γ plus TNF-α-activated rat astrocytoma cell line C6. These data indicate that both cytokines may further increase NO production and seriously compromise the oxidative
phosphorylation and mitochondrial chain in neurons and other cells of the brain. It remains however important to keep in mind that the NO concentration reached with the dose of SNP used in these experiments may not represent the endogenous levels normally achieved during pathologic conditions. It is therefore possible that IFN-γ and TNF-α have the ability to amplify cerebral damages in other models of neurotoxicity.

In conclusion, intrastriatal infusion of TNF-α provoked a robust and transient innate immune response in the brain, especially in the presence of IFN-γ. Surprisingly, such an inflammatory response and microglial reactivity was not associated with neurodegeneration and demyelination. These molecules (in particular TNF-α) were however able to enhance the neurotoxic effects of SNP that were associated with a sustained transcriptional activation of TLR2 gene up to 21 days postinjection. Cooperation between TNF-α and NO may therefore play a critical role in neurological disorders that have an immune etiology.

Acknowledgments

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References