

Differential Tumor Necrosis Factor α Expression by Astrocytes from Experimental Allergic Encephalomyelitis-susceptible and -resistant Rat Strains

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Summary

There is evidence that the cytokine tumor necrosis factor α (TNF- α) contributes to the pathogenesis of neurological autoimmune diseases such as multiple sclerosis (MS) and experimental allergic encephalomyelitis (EAE). TNF- α exerts damaging effects on oligodendrocytes, the myelin-producing cell of the central nervous system (CNS), and myelin itself. We have recently demonstrated TNF- α expression from astrocytes induced by lipopolysaccharide (LPS), interferon γ (IFN- γ), and interleukin 1 β (IL-1 β). Astrocytes secrete TNF- α in response to LPS alone, and can be primed by IFN- γ to enhance LPS-induced TNF- α production. IFN- γ and IL-1 β , cytokines known to be present in the CNS during neurological disease states, do not induce TNF- α production alone, but act synergistically to stimulate astrocyte TNF- α expression. Inbred Lewis and Brown-Norway (BN) rats differ in genetic susceptibility to EAE, which is controlled in part by major histocompatibility complex (MHC) genes. We examined TNF- α gene expression by astrocytes derived from BN rats (resistant to EAE) and Lewis rats (highly susceptible). Astrocytes from BN rats express TNF- α mRNA and protein in response to LPS alone, yet IFN- γ does not significantly enhance LPS-induced TNF- α expression, nor do they express appreciable TNF- α in response to the combined stimuli of IFN- γ /IL-1 β . In contrast, astrocytes from Lewis rats express low levels of TNF- α mRNA and protein in response to LPS, and are extremely responsive to the priming effect of IFN- γ for subsequent TNF- α gene expression. Also, Lewis astrocytes produce TNF- α in response to IFN- γ /IL-1 β . The differential TNF- α production by astrocytes from BN and Lewis strains is not due to the suppressive effect of prostaglandins, because the addition of indomethacin does not alter the differential pattern of TNF- α expression. Furthermore, Lewis and BN astrocytes produce another cytokine, IL-6, in response to LPS, IFN- γ , and IL-1 β in a comparable fashion. Peritoneal macrophages and neonatal microglia from Lewis and BN rats are responsive to both LPS and IFN- γ priming signals for subsequent TNF- α production, suggesting that differential TNF- α expression by the astrocyte is cell type specific. Taken together, these results suggest that differential TNF- α gene expression in response to LPS and IFN- γ is strain and cell specific, and reflects both transcriptional and post-transcriptional control mechanisms. The capacity for TNF- α production by Lewis astrocytes, especially in response to disease-related cytokines such as IFN- γ and IL-1 β , may contribute to disease susceptibility and to the inflammation and demyelination associated with EAE.

Astrocytes, the major glial cell in the central system (CNS),¹ were traditionally thought to act solely as a "support cell" for neurons, but recent evidence indicates that astrocytes have a wide range of functions, which include partici-

pation in immunological events occurring in the brain. The astrocyte can be stimulated to function in a manner analogous to monocytes/macrophages, and has been postulated to act as an immunocompetent cell in the CNS (1, 2). Among these immunologic functions, the astrocyte can be induced to express class II MHC antigens after exposure to IFN- γ or viruses (3, 4), and can present antigen to T cell clones in an MHC-restricted fashion upon expression of class II antigens (5). Class II MHC gene expression induced in the astrocyte by IFN- γ or virus is enhanced by TNF- α (6-8).

¹Abbreviations used in this paper: BN, Brown-Norway; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; GFAP, glial fibrillary acidic protein; GM-CSF, granulocyte/macrophage colony-stimulating factor; MBP, myelin basic protein; MS, multiple sclerosis; PKC, protein kinase C; RT, reverse transcription.

Cytokines, then, can regulate the ability of the astrocyte to function as an APC in the CNS.

Astrocytes can also be induced to express the following cytokines: IL-1 (9, 10); TNF- α (10–13); IL-6 (10, 14, 15); granulocyte colony-stimulating factor (G-CSF) (16); and granulocyte/macrophage colony-stimulating factor (GM-CSF) (16). The stimulatory agents that induce cytokine production by astrocytes include LPS, Ca²⁺ ionophore, viruses, and cytokines themselves (IL-1, TNF- α , IFN- γ).

The cytokine TNF- α has been postulated to have a central role in augmenting inflammatory demyelination and intracerebral immune responses. TNF- α has a diverse range of functions in the CNS, which include induction of class I MHC antigens on astrocytes (17, 18); induction of ICAM-1 on human fetal astrocytes (19); upregulation of class II MHC antigens induced by IFN- γ and/or virus on astrocytes (6–8); stimulation of IL-6 secretion by astrocytes (14, 15); proliferation of adult astrocytes (20); myelin damage (21); and lysis of oligodendrocytes (12). Astrocytes also express high affinity receptors for TNF- α (7), as well as produce TNF- α , which may represent an autocrine pathway of stimulation for these cells.

A variety of animal models exist for the study of CNS disease involving inflammatory demyelinating lesions. The best characterized experimental model for CNS autoimmune disease is experimental allergic encephalomyelitis (EAE). This disease is induced by injection of myelin basic protein (MBP) or transfer of encephalitogenic MBP-specific T cells to naive recipients. EAE is characterized by invasion of the CNS by T lymphocytes and macrophages, demyelination, and acute, chronic, or chronic relapsing paralysis. The mediators of this disease are MBP-reactive T helper cells that are class II MHC restricted (see for review, reference 22). It has been suggested that antigen-specific autoimmune T cells are responsible for initiation of disease, and that perpetuation of disease and subsequent demyelination may be the result of an influx of largely non-antigen-specific inflammatory cells of the recipient animal (23). There appears to be genetic control of susceptibility to EAE. In inbred rat strains, Lewis rats (RT-1^l haplotype) are susceptible to EAE, whereas Brown-Norway (BN) rats (RT-1ⁿ) are resistant. Lewis \times BN F₁ rats have disease severity one-tenth that of Lewis rats. Disease susceptibility appears to be linked to MHC alleles, although non-MHC genes may play a small role in contributing to EAE (24–26).

Several studies have examined what might contribute to the immunopathological reaction seen in the CNS of Lewis rats. Massa et al. (27, 28) demonstrated that astrocytes derived from Lewis rats express higher levels of class II MHC antigens upon treatment with either IFN- γ or virus compared with astrocytes obtained from BN rats. This hyperinduction of class II in EAE-susceptible Lewis rats was astrocyte specific, as both peritoneal macrophages and microglial cells of susceptible and resistant strains showed identical patterns for class II induction. Astrocyte class II MHC expression can contribute to antigen presentation in the brain, which is thought to enhance intracerebral immune responses.

Cytokine production has also been implicated in contributing to autoimmune diseases (see for review, reference

29). Since cytokines play a major role in regulating immune responses, aberrant expression may be a factor in the initiation and perpetuation of autoimmunity. Of particular interest is the fact that the genes for TNF- α and functionally related TNF- β (lymphotoxin) map within the MHC gene complex (30). Since many autoimmune diseases are strongly associated with class I and II MHC gene products, TNF- α /TNF- β are plausible candidates for cytokines involved with autoimmunity.

We have previously demonstrated that astrocytes from the outbred rat strain Sprague Dawley secrete TNF- α in response to LPS alone, IFN- γ plus LPS, and IFN- γ plus IL-1 β (11). IFN- γ alone does not induce TNF- α production by astrocytes but acts to enhance LPS-induced TNF- α synthesis and to synergize with IL-1 β for TNF- α production. The most potent TNF- α production is observed when astrocytes are pretreated with IFN- γ for 8–12 h before exposure to either LPS or IL-1 β , suggesting that IFN- γ provides a priming signal to the astrocyte. The aim of this study was to examine TNF- α expression by astrocytes from inbred BN and Lewis rats in response to the three different stimuli. We report that TNF- α expression at both the mRNA and protein level is differentially regulated in these strains depending upon the stimuli used for induction. Astrocytes from EAE-resistant BN rats are refractive to the priming effect of IFN- γ for enhanced LPS-induced TNF- α production, and produce very low levels of TNF- α in response to IFN- γ /IL-1 β . In contrast, astrocytes from EAE-susceptible Lewis rats are hyporesponsive to the LPS induction signal, and extremely sensitive to the priming effect of IFN- γ for subsequent TNF- α production. Also, Lewis astrocytes produce high levels of TNF- α when stimulated by IFN- γ and IL-1 β . Thus, Lewis and BN astrocytes are differentially regulated by LPS and IFN- γ with respect to TNF- α gene expression.

Materials and Methods

Primary Glial Cell Cultures. Primary glial cell cultures were established from neonatal rat cerebra as previously described (31). Meninges were removed before culture. Culture medium was Dulbecco's modified essential medium (DMEM), high glucose formula supplemented with glucose to a final concentration of 6 g/liter, 2 mM glutamine, 0.1 mM nonessential amino acid mixture, 0.1% gentamycin, and 10% FCS (Hyclone Laboratories, Logan, UT). Oligodendrocytes were separated from the astrocytes by mechanical dislodging after 14 d in primary culture, and then the astrocytes were obtained by trypsinization (0.25% trypsin, 0.02% EDTA). The astrocytes were monitored for purity by immunofluorescence. The cells were stained for glial fibrillary acidic protein (GFAP), an intracellular antigen unique to astrocytes, using a mAb to GFAP (1:4) for 30 min at room temperature, followed by a 30-min incubation with goat anti-mouse Ig-FITC (1:20). Astrocyte cultures were routinely >97% positive for GFAP, and <2% of the cells were microglia based on their positive staining for nonspecific esterase and MAC-1, a mAb that reacts with the C3b receptor. In subsequent experiments, astrocytes were purified by four repetitions of trypsinization and replating to remove contaminating microglia; after such manipulation, the astrocyte cultures were >99% positive for GFAP, and negative for nonspecific esterase and MAC-1 staining. Microglia were purified by a differential adhesion technique as described by Sasaki et al. (32). Confluent

mixed glial cultures were shaken at 270 rpm for 3 h, at which time floating cells were removed. The cells were plated in a 25-cm² tissue culture flask, and allowed to adhere for 1 h. Microglia adhere to plastic during this time period, while contaminating astrocytes and oligodendrocytes remain in the media. The adherent cells were positive for MAC-1 and nonspecific esterase (90%).

Rat Strains. The rat strains used for this study include Lewis (RT-1^l), BN (RT-1ⁿ), and the F₁ hybrid (Lewis × BN). All rats were purchased from Harlan Sprague-Dawley (Prattville, AL).

Peritoneal Macrophage Cultures. Rat peritoneal macrophages were isolated from adult Lewis and BN rats. Macrophages were removed by peritoneal lavage with ice-cold PBS, separated from contaminating red blood cells by a Ficoll-Hypaque gradient, and then plated onto 60-mm tissue culture dishes. Nonadherent cells were removed after 4 h, and the cells were cultured for an additional 48 h before stimulation by TNF- α production.

Reagents. Rat rIFN- γ (sp act, 4×10^6 U/mg) and human rIL-1 β (sp act, 5×10^8 U/mg) were obtained from AmGen Biologicals (Thousand Oaks, CA). Human rTNF- α (sp act, 5.6×10^7 U/mg) was the generous gift of Genentech, Inc. (South San Francisco, CA), and murine rIL-6 (sp act, 4×10^8 U/mg) was purchased from Biosource International, (Westlake Village, CA). Actinomycin D-mannitol, MTT [3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, LPS (*Escherichia coli*: 0127:B8), and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO).

Cytokine Production by Astrocytes. Primary rat astrocytes were resuspended in DMEM containing 10% FCS, and plated at 10^6 cells/well into six-well (35-mm) plates (Costar, Cambridge, MA). The plates were incubated overnight to allow recovery of the cells from trypsinization and to assure adherence of the astrocytes. When the astrocytes reached confluency (1–2 d after plating), the original media was aspirated off, and 2 ml of DMEM containing 2% FCS was added to the wells. Astrocytes were treated with LPS (0–10 μ g/ml), rat rIFN- γ (0–1,000 U/ml), human rIL-1 β (0–1,000 U/ml), or a combination of the above for various time periods (0–2 d). To induce cytokine production in astrocytes, a number of strategies were used that included the simultaneous addition of different agents or pretreatment with one agent before the addition of another. Supernatants were collected, centrifuged to remove contaminating cells, and stored at -70°C until use. Peritoneal macrophages and microglia were plated at 0.5×10^6 cells/well into 12-well plates, cultured for 48 h, then stimulated for TNF- α production as described above.

Measurement of TNF- α Activity. TNF- α activity in culture supernatants was determined in a biologic assay using WEHI 164 clone 13 mouse fibrosarcoma cells as previously described (11). TNF- α activity was expressed as TNF- α per culture supernatant (pg/ml). The absolute concentration of TNF- α (pg/ml) was determined by extrapolation from the standard curve, which was generated by using known amounts of human rTNF- α . The lower levels of TNF- α sensitivity in our assay system ranged from 4 to 20 pg TNF- α /ml. All samples were tested in triplicate and are presented as the mean \pm SD.

Measurement of IL-6 Protein Activity. IL-6 activity in culture supernatants was determined in a biologic assay using the IL-6-dependent B cell hybridoma 7TD1 as previously described (14, 33, 34). IL-6 activity was expressed as U/ml of IL-6 based on extrapolation from the standard curve, which was generated by using known amounts of murine rIL-6. All samples were assayed in triplicate.

Statistical Analysis. Levels of significance for comparisons between samples were determined using the student *t* test distribution.

RNA Isolation. Total cellular RNA was isolated from confluent

monolayers of astrocytes that had been incubated with culture media, LPS, IFN- γ plus LPS, and IFN- γ plus IL-1 β for various time intervals. RNA isolation followed the procedure of Chomczynski and Sacchi (35), as previously described (11). Briefly, cells were scraped and washed two times in PBS, and pelleted. RNA was extracted with guanidinium isothiocyanate and phenol, and precipitated with ethanol.

Polymerase Chain Reaction. PCR was performed as previously described (11, 36–38). Briefly, 2 μ g of total RNA isolated from astrocyte cultures was reverse transcribed by 200 U of Moloney mouse leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) for 10 min at room temperature, then 1 h at 42°C , using oligo(dT) as a primer, in a final volume of 20 μ l. The resulting cDNA was amplified with 2 U of AmpliTaq DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT) in a final volume of 100 μ l, containing 10 mM Tris-HCl, 50 mM NaCl, 1.5 mM MgCl₂, 0.01% gelatin, 1 mM of each deoxynucleotide triphosphate, and 100 pmol each of primers I and II. Primer I (ATGAG-CACAGAAAGCATGATC) is complementary to position 144–164 of the 5' end of mouse TNF- α cDNA (39), and primer II (TACAGGCTTGTCACCTCGAATT) is complementary to position 399–419 of the 3' end of the mouse TNF- α mRNA. Amplification was carried out in a twin block system (Ericomp Inc., San Diego, CA) for 30 cycles (one cycle = 94°C for 1 min, 55°C for 3 min, and 72°C for 3 min). Aliquots (1–16 μ l) of each resulting reaction mixture were applied to a 1% agarose gel, subjected to electrophoresis, and visualized by Southern blot hybridization with a 1,300-bp mouse TNF- α cDNA insert (40). The autoradiographs were quantitated by scanning densitometry with a video densitometer (620; Bio-Rad Laboratories, Richmond, CA).

cDNA Probes. Plasmid containing the 1,300-bp mouse TNF- α cDNA was the generous gift of Dr. Bruce Beutler (University of Texas at Dallas). The 1,300-bp insert was excised with PstI and EcoRI, purified and labeled with α -[³²P]deoxyCTP using a nick translation kit according to the manufacturer's instructions (Amersham Corp., Arlington Heights, IL).

Results

TNF- α Protein Production by Astrocytes from EAE-resistant and -susceptible Rat Strains. We examined TNF- α protein production by astrocytes from Lewis and BN rat strains in response to three different stimuli. Lewis and BN astrocyte cultures were treated with varying concentrations of LPS (1–10,000 ng/ml) with and without IFN- γ (100 U/ml), or IFN- γ (1–1,000 U/ml) plus IL-1 β (1,000 U/ml) for 18 h, at which point the supernatants were harvested and assayed for TNF- α production. As shown in Fig. 1, BN astrocytes produced TNF- α in response to LPS in a dose-dependent manner. Pretreatment of these cells with IFN- γ , then LPS, did not result in significant enhancement of TNF- α production. Even more striking was the observation that BN astrocytes secreted negligible amounts of TNF- α in response to the stimuli of IFN- γ /IL-1 β . This induction pathway was previously shown to be dependent on a priming signal generated by IFN- γ , then subsequent exposure to IL-1 β (11). Lewis astrocyte cultures exhibited a different induction pattern with respect to TNF- α production. Lewis astrocytes responded poorly to LPS alone at all concentrations tested, yet when pretreated with IFN- γ , then exposed to LPS, TNF- α protein production increased significantly (Fig. 2). Lewis astrocytes also produce TNF- α

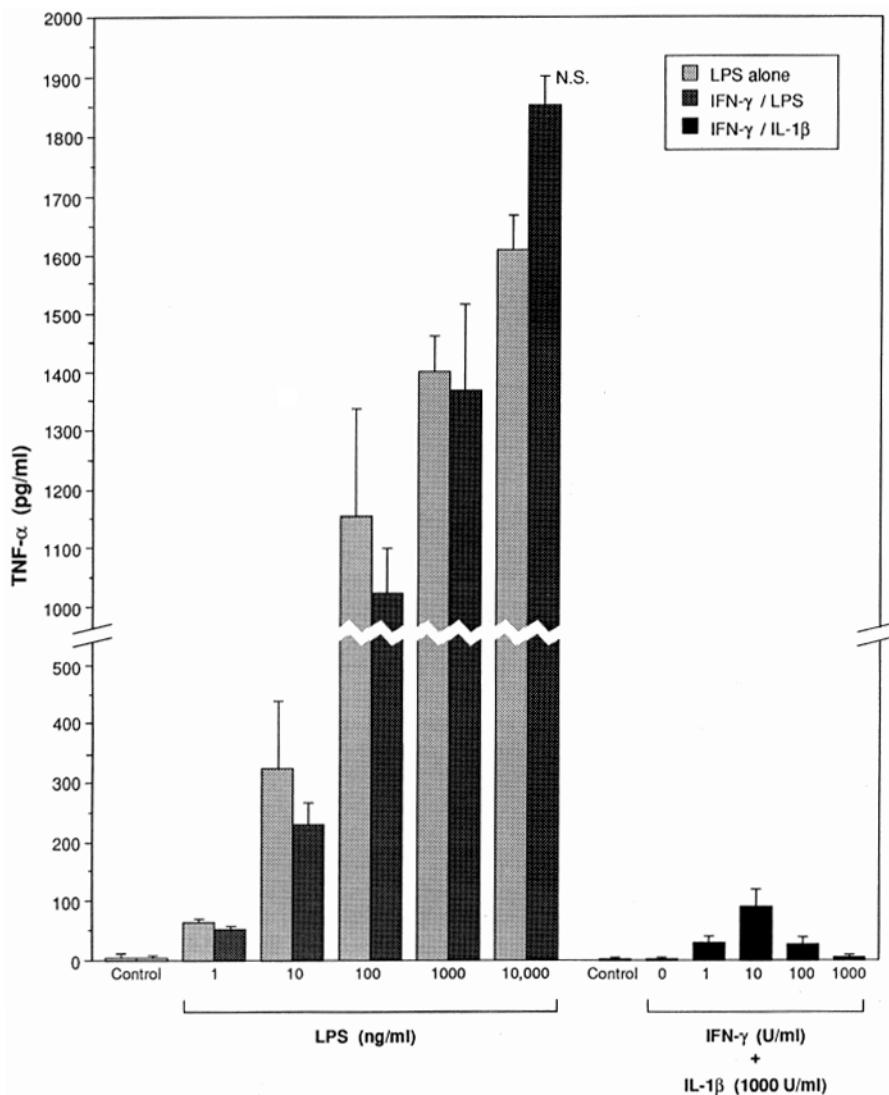


Figure 1. TNF- α production by BN astrocytes in response to LPS, IFN- γ , and IL-1 β . Rat astrocytes (10^6) were incubated with culture media for 18 h; LPS alone was incubated with culture media for 8 h, then LPS (1–10,000 ng/ml) for 10 h. IFN- γ /LPS was IFN- γ pretreatment (100 U/ml) for 8 h, then LPS (1–10,000 ng/ml) for an additional 10 h; and IFN- γ /IL-1 β was IFN- γ pretreatment (0–1,000 U/ml) for 8 h, then IL-1 β (1,000 U/ml) for an additional 10 h. Cell-free supernatants were assayed for TNF- α activity as described in Materials and Methods. These results are representative of four experiments. Statistical analysis was performed comparing LPS alone (10,000 ng/ml) to IFN- γ /LPS (10,000 ng/ml).

in response to IFN- γ /IL-1 β , in the range of what was previously observed for astrocytes from the outbred rat strain Sprague-Dawley (11). The Lewis \times BN F₁ rats show susceptibility to EAE compared with the fully resistant BN rat, however, disease severity in the F₁ strains is less than that observed for Lewis rats. The F₁ astrocytes produced low amounts of TNF- α in response to LPS, and IFN- γ pretreatment significantly enhanced LPS-induced TNF- α production (Fig. 3). The absolute levels of TNF- α in response to IFN- γ /LPS, though, were less than those observed for the Lewis astrocytes. The F₁ astrocyte TNF- α production in response to IFN- γ /IL-1 β was modest.

Prostaglandins have been demonstrated to inhibit LPS- and IFN- γ /LPS-induced TNF- α expression in murine macrophages (41–44). As astrocytes have the ability to secrete PGE₂ (45), we wished to determine if endogenous PGE₂ production contributed to strain differences in TNF- α production. Lewis and BN astrocytes were treated with 1 μ g/ml of indomethacin, which blocks PGE₂ synthesis, and then

TNF- α production was assessed. Treatment of cultures with indomethacin increased TNF- α production by both BN and Lewis astrocytes, but did not alter the overall pattern of TNF- α expression in response to LPS or IFN- γ /LPS (Table 1). Similar results were obtained for IFN- γ /IL-1 β -induced TNF- α production (data not shown). Most striking is the consistent low level of TNF- α production by Lewis rats in response to LPS plus indomethacin, and the enhancement with IFN- γ pretreatment. These results suggest that differential TNF- α production by astrocytes from BN and Lewis rats is not due to suppression by endogenous PGE₂.

TNF- α mRNA Expression by Astrocytes from EAE-resistant and -susceptible Strains. We next examined TNF- α mRNA levels from stimulated BN and Lewis astrocytes to assess if differences in TNF- α protein expression were reflected at the mRNA level. We had previously used the sensitive technique of reverse transcription (RT-PCR) to demonstrate levels of TNF- α mRNA in astrocytes because very low levels of this specific RNA are expressed (11). The astrocytes from BN and

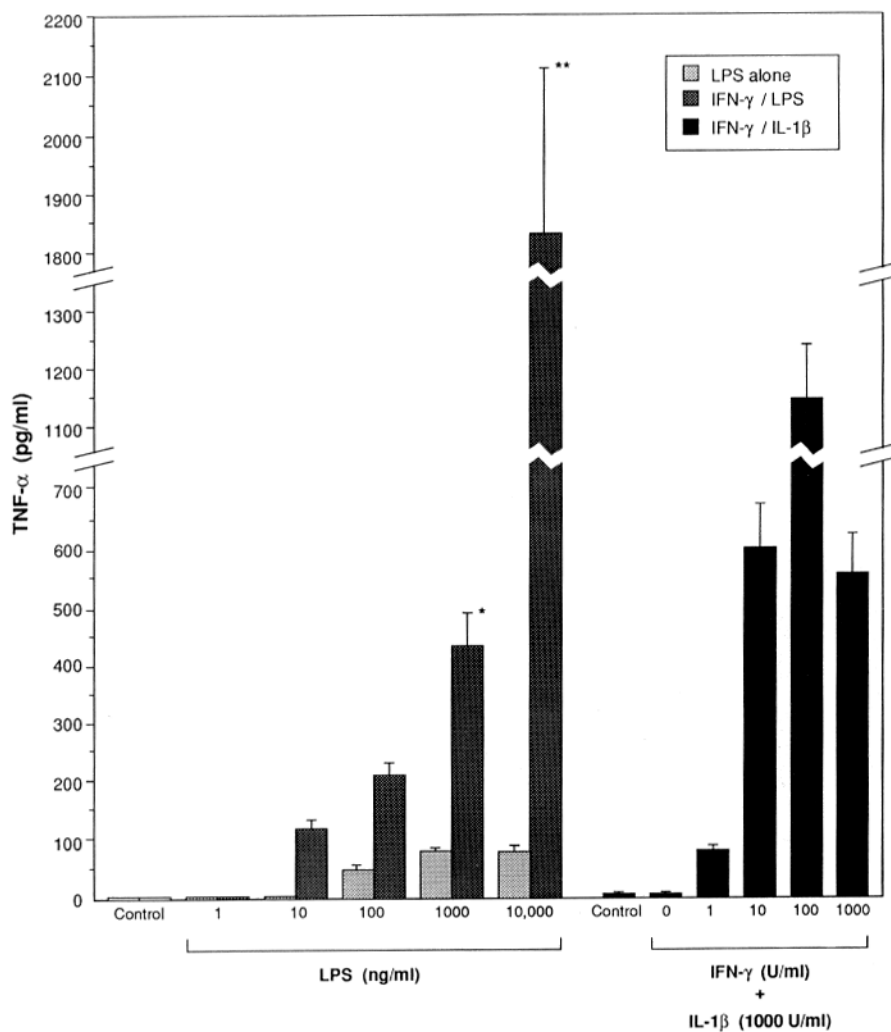


Figure 2. TNF- α production by Lewis astrocytes in response to LPS, IFN- γ , and IL-1 β . Rat astrocytes (10^6) were incubated with culture media for 18 h; LPS alone was incubated with culture media for 8 h, then LPS (1–10,000 ng/ml) for 10 h. IFN- γ /LPS was IFN- γ pretreatment (100 U/ml) for 8 h, then LPS (1–10,000 ng/ml) for an additional 10 h; and IFN- γ /IL-1 β was IFN- γ pretreatment (0–1,000 U/ml) for 8 h, then IL-1 β (1,000 U/ml) for an additional 10 hr. Cell-free supernatants were assayed for TNF- α activity as described in Materials and Methods. These results are representative of four experiments. Statistical analysis was performed comparing LPS alone (1,000 ng/ml; 10,000 ng/ml) to IFN- γ /LPS. (*) $p \leq 0.02$; (**) $p \leq 0.005$.

Table 1. BN and Lewis Astrocyte TNF- α Production in the Presence of Indomethacin

Rat strain	Cell treatment	Indomethacin (1 μ g/ml)	TNF- α activity*	Fold increase [†]
BN	LPS [§]	–	816.0 \pm 30	
	LPS	+	1,126.0 \pm 84	1.4
	IFN- γ /LPS	–	1,233.0 \pm 18	
	IFN- γ /LPS	+	3,546.0 \pm 55	2.8
Lewis	LPS	–	36.2 \pm 4	
	LPS	+	45.0 \pm 4	1.2
	IFN- γ /LPS	–	602.5 \pm 36	
	IFN- γ /LPS	+	2,137.5 \pm 77	3.5

* TNF- α activity was assessed as described in Materials and Methods.

[†] Fold increase compares the TNF- α concentration from cells in the presence of indomethacin to those in the absence of indomethacin.

[§] Control media for 8 h, then LPS (10 μ g/ml) for 10 h.

^{||} IFN- γ (100 U/ml) for 8 h, then LPS (10 μ g/ml) for an additional 10 h.

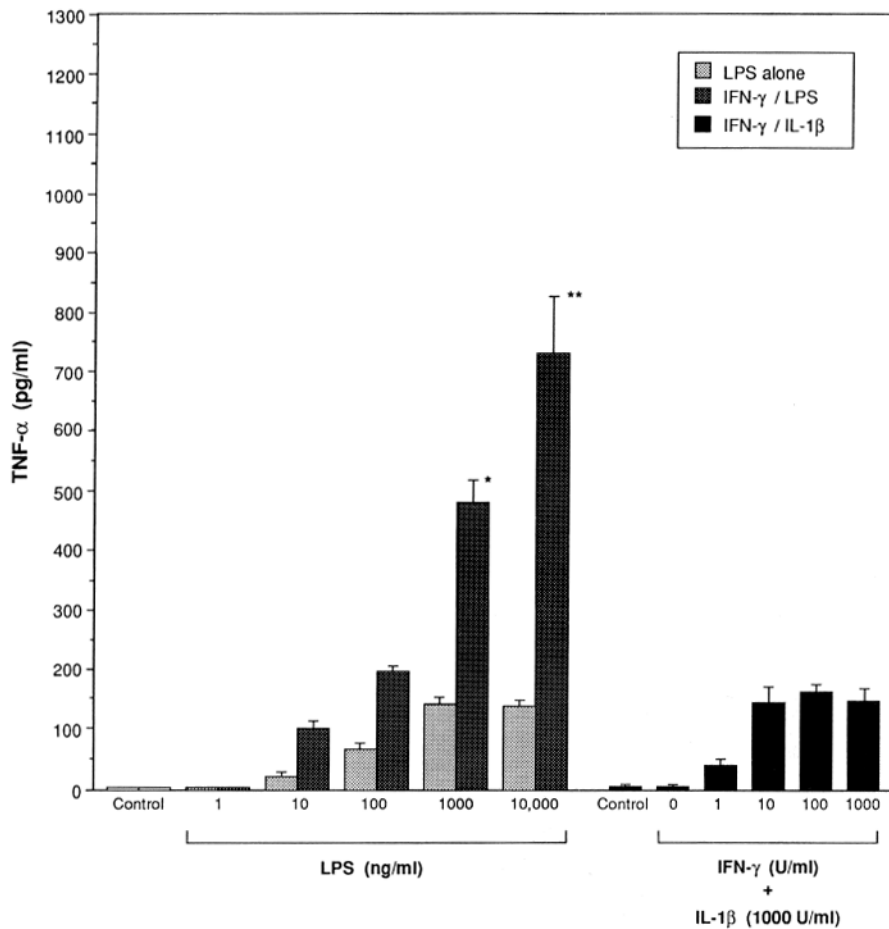


Figure 3. TNF- α production by Lewis \times BN F₁ astrocytes in response to LPS, IFN- γ , and IL-1 β . Rat astrocytes (10%) were incubated with culture media for 18 h; LPS alone was incubated with culture media for 8 h, then LPS (1–10,000 ng/ml) for 10 h. IFN- γ /LPS was IFN- γ pretreatment (100 U/ml) for 8 h, then LPS (1–10,000 ng/ml) for an additional 10 h; and IFN- γ /IL-1 β was IFN- γ pretreatment (0–1,000 U/ml) for 8 h, then IL-1 β (1,000 U/ml) for an additional 10 h. Cell free supernatants were assayed for TNF- α activity as described in Materials and Methods. These results are representative of four experiments. Statistical analysis was performed comparing LPS alone (1,000 ng/ml; 10,000 ng/ml) to IFN- γ /LPS. (*) $p \leq 0.02$; (**) $p \leq 0.01$.

Lewis were incubated with LPS (10 μ g/ml) for 4 h, IFN- γ (100 U/ml) for 8 h followed by LPS for an additional 4 h, or IFN- γ (100 U/ml) for 8 h followed by IL-1 β (100 U/ml) for an additional 4 h; then RNA was isolated. Using this RNA, we initially synthesized the corresponding cDNA by RT, and then used PCR to amplify a specific sequence of the TNF- α cDNA as described in Materials and Methods. As shown in Fig. 4, the amplified TNF- α cDNA sequence is detected in astrocytes stimulated with LPS alone, IFN- γ /LPS, or IFN- γ /IL-1 β , but not in unstimulated astrocyte cultures. The amplified sequence, hybridizing with a mouse TNF- α cDNA probe, had the expected size of 275 bp. To insure linearity of the assay, varying amounts of the PCR product (1–16 μ l) were run on a Southern blot. Scanning of the autoradiographs was performed on the exposure shown in Fig. 4, as well as ones developed for less time. Differences in TNF- α mRNA expression were observed within each rat strain, depending on the stimulus used, as well as between BN and Lewis astrocytes. Comparing TNF- α mRNA levels in BN astrocytes, our results indicate that these cells express TNF- α mRNA in response to LPS alone, and slightly more upon pretreatment with IFN- γ (1.5-fold increase). Low levels of TNF- α mRNA are expressed in response to stimulation with IFN- γ /IL-1 β compared with mRNA levels from LPS-

or IFN- γ /LPS-treated astrocytes. When examining TNF- α mRNA expression by Lewis astrocytes, we observed that low levels of TNF- α mRNA are detected in cells stimulated with LPS alone, enhanced expression with the combined stimuli of IFN- γ /LPS (2.5-fold increase), and even higher levels of TNF- α mRNA upon stimulation with IFN- γ /IL-1 β . These differences within each strain are consistent with the TNF- α protein results (Figs. 1 and 2), although the differences in the mRNA levels are not as pronounced. When TNF- α mRNA levels are compared between BN and Lewis astrocytes, the most striking difference is in response to IFN- γ /IL-1 β (Fig. 5).

TNF- α Protein Production by Peritoneal Macrophages from EAE-resistant and -susceptible Rat Strains. To determine if the differences in TNF- α production by Lewis and BN astrocytes were restricted to this cell type, we examined TNF- α production by peritoneal macrophages from these same strains. Peritoneal macrophages were obtained from adult Lewis and BN rats as described in Materials and Methods, and exposed to the stimuli of LPS, IFN- γ , IFN- γ /LPS, and IFN- γ /IL-1 β . As shown in Table 2, peritoneal macrophages from both strains produce TNF- α in response to LPS, and IFN- γ pretreatment enhances TNF- α production. In addition, these cells produce TNF- α in response to IFN- γ /IL-1 β in a comparable manner.

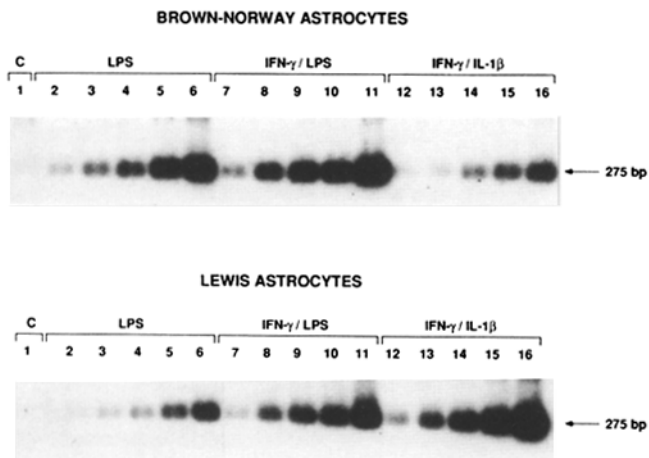


Figure 4. Demonstration of TNF- α mRNA in BN and Lewis astrocytes by reverse transcription and PCR. Astrocytes were incubated with culture media alone (control) for 12 h; LPS (10 μ g/ml) for 4 h; IFN- γ (100 U/ml) for 8 h followed by LPS (10 μ g/ml) for an additional 4 h; or IFN- γ (100 U/ml) for 8 h followed by IL-1 β (100 U/ml) for an additional 4 h. Total cellular RNA was isolated and processed for RT-PCR as described in Materials and Methods. Varying amounts of the PCR product (1, 2, 4, 8, and 16 μ l) were analyzed for LPS (lanes 2-6), IFN- γ /LPS (lanes 7-11), and IFN- γ /IL-1 β (lanes 12-16). 16 μ l of the PCR product from unstimulated control astrocytes was analyzed (lane 1). Analysis was by Southern blot hybridization with a labeled 1,300-bp mouse TNF- α cDNA. The blots were exposed to X-Omat film for 7.5 h at -70°C .

Similar results were also obtained when testing neonatal microglia (data not shown). This suggests that TNF- α production in these rat strains is regulated differently in astrocytes vs. peritoneal macrophages and microglia.

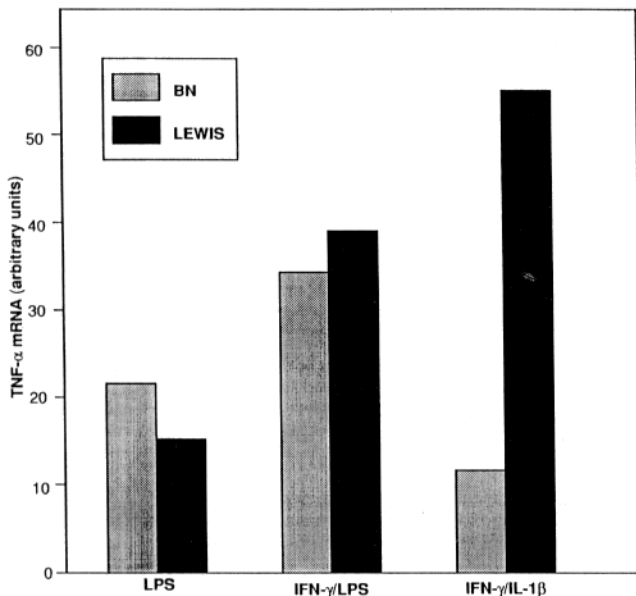


Figure 5. Comparison of TNF- α mRNA levels between BN and Lewis astrocytes. Astrocytes were incubated and processed as described for Fig. 4. mRNA values are expressed in arbitrary units as determined from densitometric scanning of autoradiographs. Each bar graph represents the mean of two experiments (data shown in Fig. 4 and one additional experiment).

Table 2. Analysis of TNF- α Production by Peritoneal Macrophages from EAE-susceptible and -resistant Rat Strains

Cell treatment	TNF- α *	
	BN	Lewis
	<i>pg/ml</i> 5×10^5 cells	
Control [†]	120 \pm 20	150 \pm 42
LPS [§]	1,200 \pm 121	1,220 \pm 430
IFN- γ	710 \pm 37	1,040 \pm 135
IFN- γ /LPS [¶]	4,009 \pm 339	4,210 \pm 1210
IFN- γ /IL-1 β **	1,000 \pm 300	1,250 \pm 125

* TNF- α activity was assessed as described in Materials and Methods.

[†] Culture media alone for 18 h.

[§] LPS (1 μ g/ml) for 18 h.

^{||} IFN- γ (100 U/ml) for 18 h.

[¶] IFN- γ (100 U/ml) for 8 h, then LPS (1 μ g/ml) for an additional 12 h.

** IFN- γ (100 U/ml) for 8 h, then IL-1 β (1,000 U/ml) for an additional 12 h.

IL-6 Protein Production by Astrocytes from EAE-resistant and -susceptible Rat Strains. We examined IL-6 production by Lewis and BN astrocytes in response to LPS, IFN- γ , and IL-1 β to determine if differential cytokine expression by these cells extended to IL-6. We have recently demonstrated that primary rat astrocytes secrete IL-6 upon stimulation with LPS or the cytokines TNF- α and IL-1 β . IFN- γ alone has no effect on IL-6 production, but synergizes with IL-1 β for enhanced IL-6 expression (14). Lewis and BN astrocytes were treated with LPS (1 μ g/ml) with or without IFN- γ (100 U/ml), IFN- γ (100 U/ml), and IL-1 β (1,000 U/ml), or with IFN- γ (100

Table 3. BN and Lewis Astrocyte IL-6 Protein Production

Cell treatment	IL-6*	
	BN	Lewis
	<i>U/ml</i>	
Control [†]	<5	<5
LPS [§]	82 \pm 20	67 \pm 15
IFN- γ	<5	<5
IFN- γ /LPS [¶]	1,354 \pm 47	1,240 \pm 62
IL-1 β **	75 \pm 30	123 \pm 38
IFN- γ /IL-1 β ^{##}	976 \pm 25	1,058 \pm 72

* IL-6 activity was assessed as described in Materials and Methods.

[†] Control media alone for 18 h.

[§] Control media for 8 h, then LPS (1 μ g/ml) for 10 h.

^{||} Control media for 8 h, then IFN- γ (100 U/ml) for 10 h.

[¶] IFN- γ (100 U/ml) for 8 h, then LPS (1 μ g/ml) for an additional 10 h.

** Control media for 8 h, then IL-1 β (1,000 U/ml) for 10 h.

^{##} IFN- γ (100 U/ml) for 8 h, then IL-1 β (1,000 U/ml) for an additional 10 h.

U/ml) plus IL-1 β (1,000 U/ml) for 18 h, at which point the supernatants were harvested and assayed for IL-6 production. Both Lewis and BN astrocytes secrete low levels of IL-6 protein in response to LPS alone, and IFN- γ synergizes with LPS to enhance IL-6 secretion. IFN- γ alone does not induce IL-6 production, but enhances IL-1 β -induced IL-6 expression (Table 3). These findings demonstrate that Lewis and BN astrocytes make comparable amounts of IL-6 protein in response to LPS, IFN- γ /LPS, IL-1 β , and IFN- γ /IL-1 β , and indicate that differences in TNF- α production appear to be selective.

Discussion

We have demonstrated that astrocytes from EAE-susceptible and -resistant rat strains differ in their ability to express TNF- α mRNA and protein. These differences are especially pronounced for IFN- γ priming of TNF- α gene expression. Endogenous levels of PGE₂, a known inhibitor of TNF- α , did not differ in astrocytes from the two strains. Differential TNF- α expression is selective for the astrocyte, as both peritoneal macrophages and microglia from Lewis and BN rats express TNF- α protein in response to all of the stimuli used. In addition, IL-6 production by Lewis and BN astrocytes is comparable in response to induction by LPS, IFN- γ , and IL-1 β , indicating that cytokine production in general is not altered. Taken together, these results indicate that the differential expression of TNF- α mRNA and protein in Lewis and BN astrocytes may involve transcriptional and/or post-transcriptional events.

The pattern of responsiveness of astrocytes from both strains to IFN- γ priming for TNF- α production is consistent with data from Massa et al. (28) on IFN- γ inducibility of class II MHC antigens in these same strains; i.e., Lewis astrocytes express higher levels of class II MHC antigens in response to IFN- γ than do BN astrocytes. The lack of IFN- γ responsiveness in the BN astrocyte could be the result of differences in (a) the number and/or affinity of IFN- γ receptors; (b) intracellular second messengers activated by IFN- γ ; (c) astrocyte-specific transcriptional factors activated or modified by IFN- γ ; or (d) TNF- α DNA regulatory regions responsive to factors induced by IFN- γ . Our results would suggest that BN astrocytes express functional IFN- γ receptors capable of binding ligand and generating a biological response as evidenced by IL-6 production in response to IFN- γ priming, thus ruling out inherently defective IFN- γ -induced signal transduction. Macrophages from the A/J strain of mice are deficient in their response to IFN- γ for acquisition of tumoricidal competence (46). These macrophages do not respond to IFN- γ by activation of protein kinase C (PKC) or by efflux of intracellular Ca²⁺, indicating a defect in the transduction signals initiated by IFN- γ . Studies on astrocytes from outbred Sprague Dawley rats indicate that IFN- γ induction of class II MHC and TNF- α production utilize different intracellular pathways. IFN- γ induction of class II MHC appears to involve the Na⁺/H⁺ antiporter system (Benveniste, E.N., et al., manuscript in preparation), while TNF- α production occurs via activation of PKC (Chung, I.Y., and E.N. Benveniste, unpublished obser-

vation). Future studies will be required to determine if these two intracellular signaling pathways are operational in the BN astrocyte in response to IFN- γ .

It is also possible that BN astrocytes exhibit a defect in some aspect of IFN- γ -mediated signal transduction that is distal to the activation of second messengers, such as IFN- γ -induced transcription factor(s) that interact with regulatory elements in the TNF- α promoter. Recently, LPS and IFN- γ were shown to activate transcription of the mouse TNF- α gene in murine peritoneal macrophages via the activation of NF- κ B (47). TNF- α expression in these cells is different from that of rat astrocytes, as LPS and IFN- γ alone induce macrophage TNF- α production, whereas IFN- γ alone has no effect in astrocytes, but enhances LPS-induced expression. The priming signal mediated by IFN- γ may not be expressed or expressed in an aberrant manner in BN astrocytes, resulting in minimal enhancement of LPS-induced TNF- α expression, and minimal expression of IFN- γ /IL-1 β -induced TNF- α mRNA and protein. Since the rat TNF- α gene has not been cloned, we do not know whether the rat TNF- α promoter region contains similar regulatory elements.

The implication of hyporesponsiveness to LPS in Lewis astrocytes is not clear. LPS is capable of inducing IL-6 protein production by Lewis astrocytes, and we have also found that LPS inhibits the expression of IFN- γ -induced class II antigens on these same cells (Chung, I.Y., and E.N. Benveniste, unpublished observation). Thus, Lewis astrocyte hyporesponsiveness to LPS is not global, but seems to be restricted to TNF- α gene expression. In C3H/HeJ mice (endotoxin resistant), peritoneal macrophages produce no detectable TNF- α protein, even when expressing TNF- α mRNA (40). It has been suggested that a dual defect prevents TNF- α expression in these mice; high concentrations of LPS are required to induce TNF- α mRNA levels within the cell, and a post-transcriptional defect prevents the translation of the mRNA to TNF- α protein. Our findings are somewhat similar, although low levels of TNF- α mRNA are detected in LPS-treated Lewis astrocytes even when high concentrations of LPS (10 μ g/ml) are used for stimulation. This would suggest a partial transcriptional block, which can not be overcome by using high concentrations of LPS, as well as a post-transcriptional defect.

When comparing TNF- α mRNA and protein expression in the two strains, the differences in TNF- α mRNA levels are not as striking as those for TNF- α protein, especially in response to either LPS alone or IFN- γ /LPS. There are several explanations for these findings, one being the sensitivity of RT-PCR. Using conventional Northern blot analysis, we were unable to detect mRNA from LPS-stimulated astrocytes from outbred rat strains (11), as well as Lewis and BN rats. The analysis by PCR has increased our level of sensitivity, and we are now able to detect TNF- α mRNA from LPS-stimulated astrocytes. It is also possible that the TNF- α gene is effectively transcribed in Lewis and BN astrocytes in response to all the stimuli tested, but there are differences in mRNA stability. We have observed that although TNF- α protein levels are comparable for astrocytes stimulated with IFN- γ /LPS and IFN- γ /IL-1 β from Lewis and Sprague Dawley

strains (11), RNA levels are consistently higher from IFN- γ /IL-1 β -stimulated astrocytes. This may reflect TNF- α mRNA instability in LPS-stimulated astrocytes, as observed by Lieberman et al. (10). Alternatively, transcriptional activity in response to LPS for Lewis astrocytes, and IFN- γ /IL-1 β for BN astrocytes, may occur, with a subsequent block in either translation or secretion of mature TNF protein.

Our studies were performed on astrocytes derived from neonatal rats to define differences that might contribute to susceptibility for subsequent development of EAE. The different profile of responsiveness in Lewis and BN astrocytes (especially in response to IFN- γ) is not a generalized feature of this developmental stage, as neonatal microglia from these two strains respond equally well to all stimuli tested for TNF- α production. We propose that the ability of the Lewis astrocyte to respond to IFN- γ , a cytokine not normally present in the CNS except during inflammatory disease states, by expression of class II MHC antigens and TNF- α production, may contribute selectively to intracerebral immune responses and inflammation in this rat strain.

As mentioned previously, EAE is characterized by the infiltration of mononuclear cells into the CNS, with the predominant cell types being activated T cells and macrophages. Activated T cells in the Lewis CNS could serve as an endogenous source of the cytokine IFN- γ , and prime astrocytes for TNF- α expression. Infiltrating macrophages and resident astrocytes/microglia represent sources of IL-1 that could interact with IFN- γ -primed astrocytes, resulting in TNF- α production. TNF- α production by Lewis astrocytes in response to IFN- γ and IL-1 may perpetuate the influx of non-antigen-specific inflammatory cells into the CNS by increasing the

permeability of endothelial cells (48), and by enhancing expression of adhesion molecules on both brain endothelium and astrocytes (19, 49). Additionally, astrocyte TNF- α production may kill or damage neighboring oligodendrocytes (12) and directly damage the myelin sheath (21), contributing to demyelination.

There are reports suggesting a causal relationship between cytokine production and disease development. LPS-induced TNF- α production by macrophages from the autoimmune-prone NZB \times NZW F₁ strain is significantly less than that from MHC-matched normal mice, and the administration of TNF- α protects this strain from developing lupus nephritis (50). These data suggest that depressed production of TNF- α may contribute to development of autoimmunity. In contrast, Boswell et al. (51) found that local production of TNF- α by kidney macrophages may accelerate renal disease in MRL-lpr mice. These differences may reflect the different strains of mice under study. Overproduction of TNF- α plays a central role in the pathogenesis of cerebral malaria, as in vivo treatment with anti-TNF antibody prevents disease (52). An association of TNF- α with MS is suggested by the observation of TNF- α -positive astrocytes in the MS brain (53). A recent report by Ruddle et al. (54) demonstrated that an antibody to TNF- α /lymphotoxin could prevent the transfer of EAE by encephalitogenic T cells. These findings indicate that inhibition of the biological activities of these two cytokines could prevent neurological disease, although the mechanism(s) of action is as yet unknown. Thus, a number of in vivo and in vitro studies implicate TNF- α and the cells that produce it as contributors to both the initiation and perpetuation of EAE.

We thank Drs. John Whitaker and Casey Morrow for helpful discussions, and Ms. Melissa Mabowitz for excellent secretarial assistance.

This work was funded in part by grants AI-27290 from the National Institutes of Health (E. N. Benveniste), grant RG 2269-A-4 from the National Multiple Sclerosis Society (E. N. Benveniste), and grant RG 2205-A-3 from the National Multiple Sclerosis Society (E. N. Benveniste).

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Received for publication 10 December 1990.

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