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Tumor Necrosis Factor Alpha Upregulates Human Microglial Cell Production of Interleukin-10 In Vitro

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Interleukin (IL)-10 appears to play an important regulatory role in the systemic inflammatory response; however, production of IL-10 within the human central nervous system has not been described. Using cultures of human fetal microglial cells, the resident macrophages of the brain, we investigated the production and regulation of bioactive IL-10. Lipopolysaccharide stimulated acute release of tumor necrosis factor (TNF)-α (peak by 8 h) and delayed production of IL-10 (over a 48-h period) in microglial cell cultures. Treatment of microglial cell cultures with TNF-α and IL-6 resulted in a dose-dependent release of IL-10. These cytokines also induced expression of IL-10 mRNA. Treatment of microglial cell cultures with IL-10 markedly inhibited TNF-α and IL-6 production. These findings suggest that during inflammation within the brain, acute release of TNF-α and IL-6 by activated microglia could promote subsequent release of IL-10, which functions to minimize the potential neurotoxic effects of proinflammatory cytokines.

Interleukin (IL)-10, initially identified as a T-cell product which inhibits synthesis of proinflammatory cytokines (17), is produced following activation by many different cell types, including mononuclear phagocytes (15, 18, 31). IL-10 downregulates the production of several proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1, and IL-6 (15). In vivo studies have demonstrated a protective role of IL-10 in endotoxemia in rodents (22, 25, 28) by inhibiting the release of TNF-α, a major mediator involved in the pathogenesis of septic shock (23).

In brain cell cultures, activated microglial cells have been shown to be neurotoxic (4, 10, 11). Production of TNF-α by activated microglia plays a key role in the generation of nitric oxide, a free radical with neurotoxic activity (10, 12). Activated microglial cells and cytokines have been implicated in the pathogenesis of bacterial meningitis (34) and in neurodegenerative diseases, including Alzheimer's disease (9) and acquired immunodeficiency syndrome dementia (16). Since IL-10 is found to potently inhibit TNF-α production by somatic macrophages (3, 18), endogenous production of IL-10 within the brain could minimize inflammatory responses. Recent studies have demonstrated that IL-10 inhibits major histocompatibility class II expression and production of IL-6 and granulocyte-macrophage colony-stimulating factor by murine glial cells (19). Other investigators have demonstrated IL-10 mRNA expression by using reverse transcription (RT)-PCR (5, 27) and protein by immunocytochemistry (26) in mouse brain tissue in the presence of certain inflammatory or infectious diseases, although the phenotype of the cells producing IL-10 was not determined. IL-10 has been detected in cerebrospinal fluid of some patients with viral meningitis and other neurologic diseases (21), but production of IL-10 within the human central nervous system has not been described. In the present study, we investigated the release of IL-10 from human fetal microglial cell cultures and the potential regulation of IL-10 production by certain proinflammatory cytokines.

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MATERIALS AND METHODS

Reagents. Recombinant human TNF-α, IL-6, and IL-10 were obtained from R&D Systems (Minneapolis, Minn.). All of the cytokines contained <2 pg of lipopolysaccharide (LPS) per 20 ng of cytokine protein. LPS (O111:B4) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Avian myeloblastosis virus reverse transcriptase-5× RT buffer and *Taq* DNA polymerase-10× PCR buffer-$MgCl_2$ were purchased from Promega Co. (Madison, Wis.). Oligo$(dT)_{12–18}$, deoxynucleoside triphosphate mix, RNase inhibitor, and sodium PP_i were purchased from ClonTech (Palo Alto, Calif.), Boehringer Mannheim (Boston, Mass.), Pharmacia Biotech (Piscataway, N.J.), and Sigma, respectively.

Microglial cell cultures. Human fetal brain tissue was obtained from aborted fetuses (16 to 20-weeks old) under a protocol approved by the Human Subjects Research Committee at our institution as previously described (6). In brief, brain tissues were dissociated, trypsinized, and plated in culture flasks containing Dulbecco modified Eagle medium with 10% fetal calf serum and antibiotics. Culture medium was replenished on days 1 and 4, and cells were harvested and filtered through a 70-μm cell strainer on day 12 of culture. These cells (>99% stained with anti-CD68 [Dako Corp., Carpinteria, Calif.], a marker of human macrophages) were plated in wells of 24-well plates at a density of 5×10^4 cells per well.

Experimental protocol. To evaluate the effects of dose and time of LPS exposure on IL-10 production, microglial cells (5×10^4 cells per 500 μl per well) were stimulated either with LPS (1 μg/ml) for 6, 24, or 48 h or with various concentrations of LPS ranging from 1 pg/ml to 10 μg/ml for 48 h. IL-10 levels in culture supernatants were measured by a bioassay. To determine the effects of TNF-α and IL-6 on IL-10 release, microglial cells were stimulated with various concentrations of TNF-α and IL-6 ranging from 0.16 to 100 ng/ml for 48 h, and culture supernatants were assayed for IL-10. To investigate the effects of LPS, TNF-α, and IL-6 on IL-10 gene expression, microglial cells were incubated with 1 μg of LPS per ml, 20 ng of TNF-α per ml, or 100 μg of IL-6 per ml for 8 h and then total RNA was extracted. To study the inhibitory effect of IL-10 on TNF-α or IL-6 release, microglial cell cultures were treated with LPS (1 μg/ml) in the absence or presence of IL-10 (10 ng/ml) for 8 and 24 h prior to collection of supernatants for TNF-α and IL-6 bioassays, respectively.

Cytokine bioassays. IL-10 was measured by a proliferation assay using the MC/9-2 murine mast cell clone kindly provided by M. Tsang (R&D Systems). The sensitivity of the IL-10 assay was 40 pg/ml with a 50% effective dose of 670 ± 42 pg/ml ($n = 10$). Antibodies specific to IL-10 (10 μg/ml) blocked about 90% of the sample-induced growth activity of MC/9-2. TNF-α was measured by a cytotoxicity assay using the L929 mouse fibrosarcoma cell line, as previously described (8). The 50% effective dose of the TNF-α assay was 19 ± 2 pg/ml ($n = 20$) with a sensitivity of 1 pg/ml. Antibodies to TNF-α (10 μg/ml) completely blocked TNF-α bioactivity in the samples. IL-6 was determined by using IL-6-dependent T1165 plasmacytoma cells as previously described (8). The sensitivity

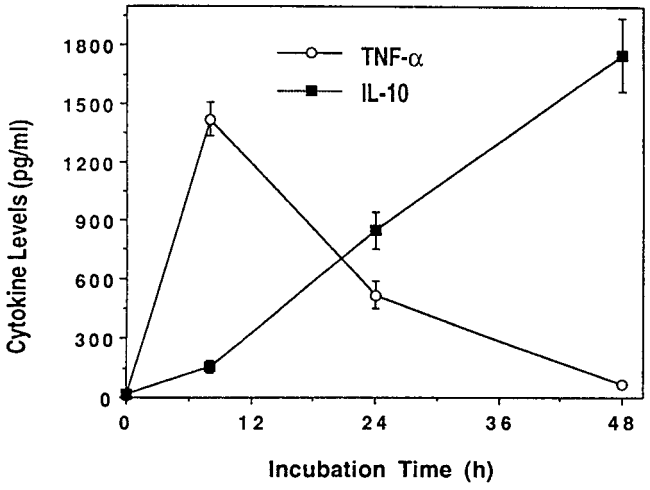

FIG. 1. Time course of TNF-α and IL-10 release. Human fetal microglial cell cultures were treated with LPS (1 μg/ml), and supernatants were harvested at the times indicated for TNF-α and IL-10 determination. The data shown (mean ± standard error) are representative of three separate experiments.

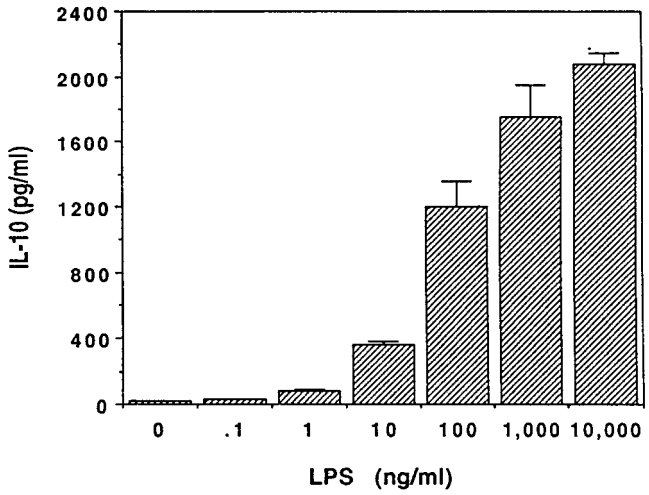

FIG. 2. Dose-response relationship of LPS with IL-10 release. Microglial cell cultures were treated with medium alone or medium containing the concentrations of LPS indicated for 48 h. Supernatants were harvested for IL-10 assay. The data shown (mean ± standard error) are representative of three separate experiments.

of the IL-6 assay was 1 pg/ml with a 50% effective dose of 49.6 ± 2.1 pg/ml (n = 14). Antibodies to IL-6 (10 μg/ml) completely suppressed T1165 cell proliferation induced by culture samples.

RT-PCR. Total RNA was extracted by guanidinium isothiocyanate from microglial cells as previously described (13). RT of 1 μg of RNA was performed with 50 μM oligo$(dT)_{12-18}$. A reaction mixture containing 15 U of avian myeloblastosis virus reverse transcriptase 25 U of RNase inhibitor, 5× RT buffer (250 mM Tris-HCl [pH 8.3] at 42°C, 250 mM KCl, 50 mM $MgCl_2$, 50 mM dithiothreitol, 2.5 mN spermidine), 10 mM deoxynucleoside triphosphate mix (dATP, dCTP, dGTP, and dTTP), and 40 mM sodium PP_i was added to the RNA, and then the mixture was subjected to incubation at 42°C for 1 h, followed by termination at 95°C for 5 min. Amplification of IL-10 or glyceraldehyde-3-phosphate dehydrogenase (as a control) cDNA was performed with an automatic thermocycler (Coy Lab, Ann Arbor, Mich.), and the reaction mixture contained 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0 at 25°C], 1% Triton X-100), 25 mM $MgCl_2$, 10 mM deoxynucleoside triphosphate mixture, 5 U of *Taq* DNA polymerase per ml, 25 μM primer (sense and antisense), cDNA, and H_2O. Amplification was set at 94°C for 45 s, 60 to 65°C for 45 s, and 72°C for 90 s followed by a 10-min extension at 72°C.

Starting from cycle 10, an aliquot of the IL-10 PCR product was collected at intervals of three cycles, blotted onto a nylon membrane (MSI, Westborough, Mass.), hybridized with an α-^{32}P end-labeled IL-10 internal probe (5′-GGC CTT GCT CTT GTT TTC AC-3′), and analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The IL-10 primer sets (ClonTech, Palo Alto, Calif.) were 5′-AAG CTG AGA ACC AAG ACC CAG ACA TCA AGG CG-3′ (sense) and 5′-AGC TAT CCC AGA GCC CCA GAT CCG ATT TTG G-3′ (antisense). The glyceraldehyde-3-phosphate dehydrogenase primer sets (Stratagene Cloning Systems, La Jolla, Calif.) were 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ (sense) and 5′-TCT ACA CGG CAG GTC AGG TCC ACC-3′ (antisense). The amplified PCR products for IL-10 and glyceraldehyde-3-phosphate dehydrogenase were 328 and 600 bp, respectively.

Statistical analysis. Student's t test (two tailed) was used to compare the differences between the means of two groups. To compare means of multiple groups, analysis of variance, followed by Scheffe's F test, was used.

RESULTS

Human fetal microglial cells produced IL-10 following stimulation by LPS. Release of IL-10 from microglial cells was detectable at 6 h and continued to rise over the ensuing incubation period (48 h), while the release of TNF-α was detectable at 2 h and peaked at 8 h (Fig. 1). LPS dose dependently stimulated microglial cell release of IL-10 with a 50% effective dose of approximately 100 ng per ml of LPS (Fig. 2). A marked increase ($P < 0.05$) in IL-10 release was observed when 10 ng of LPS per ml was used, whereas maximal IL-10 production was found at an LPS concentration of 10 μg/ml. No toxicity to the microglial cells was observed at these LPS concentrations, as determined by a trypan blue dye exclusion assay.

Since TNF-α induces IL-10 production by human monocytes (42), we evaluated the stimulatory effects of TNF-α and IL-6 on microglial cell release of IL-10. Microglial cells released substantial amounts of IL-10 after stimulation with TNF-α or IL-6. The stimulatory effects of these cytokines were dose dependent (Fig. 3). After 48 h of treatment, TNF-α (100 ng/ml) induced a 20-fold increase ($P < 0.01$) in microglial cell release of IL-10 (Fig. 3A), whereas IL-6 (100 ng/ml) stimulated a threefold increase ($P < 0.05$) in IL-10 release (Fig. 3B).

Expression of IL-10 mRNA was markedly upregulated by LPS, TNF-α, and IL-6 compared with the control cultures (Fig. 4). None of these stimuli affected glyceraldehyde-3-phosphate dehydrogenase mRNA expression (data not shown).

We next tested the inhibitory effect of IL-10 on microglial cell release of TNF-α and IL-6. IL-10 (10 ng/ml) dramatically inhibited ($P < 0.01$) LPS-stimulated release of TNF-α (Fig. 5A) by 8 h and IL-6 (Fig. 5B) by 24 h in microglial cell cultures.

DISCUSSION

Although other investigators have demonstrated IL-10 expression in mouse brains in certain inflammatory conditions (5, 21, 26, 27), the phenotype of the cells producing IL-10 was not characterized. This study provides evidence that human microglial cells produce IL-10 and that the regulation of expression of this anti-inflammatory cytokine involves proinflammatory cytokines TNF-α and IL-6. We found in the present study that LPS-stimulated microglial cells released substantial amounts of TNF-α, immediately followed by a significant release of IL-10. We also found that IL-10 markedly inhibited TNF-α release. These findings suggest a potential reciprocal relationship between these two cytokines. Indeed, TNF-α, and also IL-6, potently triggered microglial cell production of IL-10 at both the mRNA and protein levels. On the basis of these findings, we propose that the acute release of proinflammatory cytokines such as TNF-α and IL-6 may be critical for host defense of the central nervous system against infectious agents and that subsequent production of IL-10 downregulates these

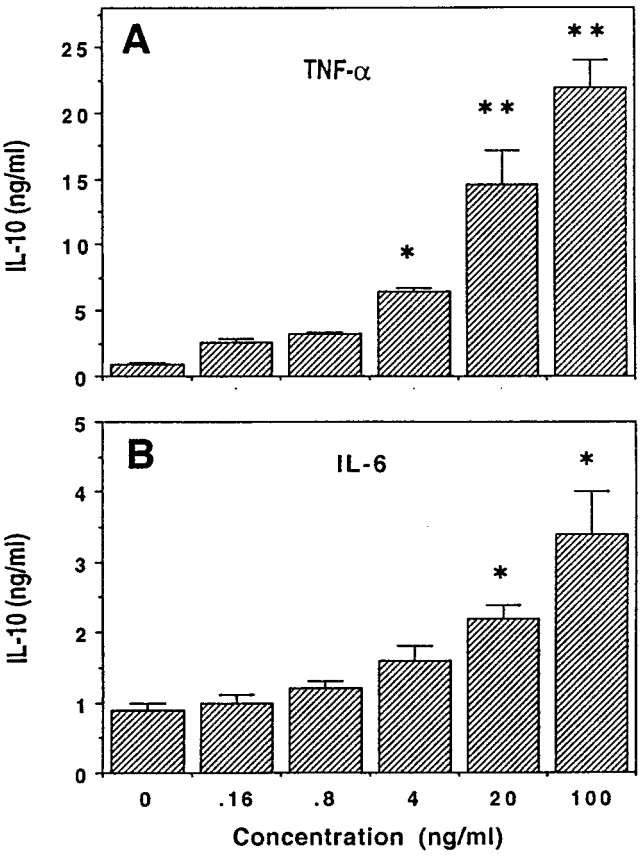

FIG. 3. Dose-response relationship of TNF-α and IL-6 with IL-10 release. Microglial cell cultures were treated with medium alone or medium containing the concentrations of indicated TNF-α (A) or IL-6 (B) for 48 h. Supernatants were then harvested for IL-10 assay. The data shown (mean ± standard error) are representative of three separate experiments. *, $P < 0.05$ versus the control value; **, $P < 0.01$ versus the control value.

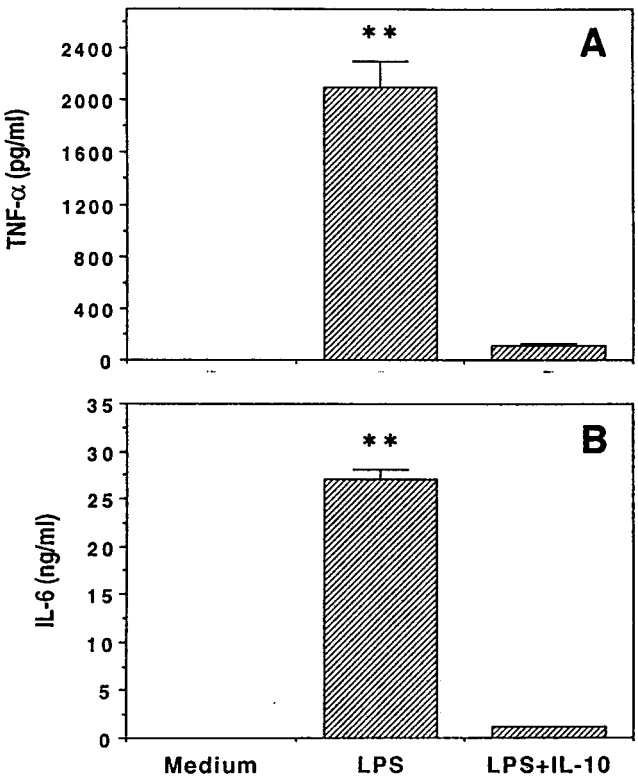

FIG. 5. Effects of IL-10 on TNF-α and IL-6. Microglial cell cultures were treated with LPS (1 μg/ml) in the absence or presence of IL-10 (10 ng/ml) for 8 and 24 h before supernatants were harvested for TNF-α (A) and IL-6 (B) determination, respectively. The data shown (mean ± standard error) are representative of three separate experiments. **, $P < 0.01$ versus the control value.

cytokines, which minimizes neuronal damage due to overproduction of proinflammatory cytokines (11).

TNF-α was a more potent stimulus than IL-6 for the induction of IL-10 release by microglial cell cultures. Since TNF-α did not appear to induce higher IL-10 mRNA expression in microglial cell cultures than did LPS or IL-6, this suggests a differential posttranscriptional regulation of IL-10 release by these stimuli. In human monocyte cultures, LPS treatment resulted in a maximal release of immunologically detectable IL-10 while stimulation with TNF-α induced greater synthesis of IL-10 mRNA (42). The differences between our findings with microglial cells and those reported with monocytes could be due to different tissue sources of mononuclear phagocytes (i.e., brain versus blood), assays (i.e., biologic versus immunologic), or experimental conditions.

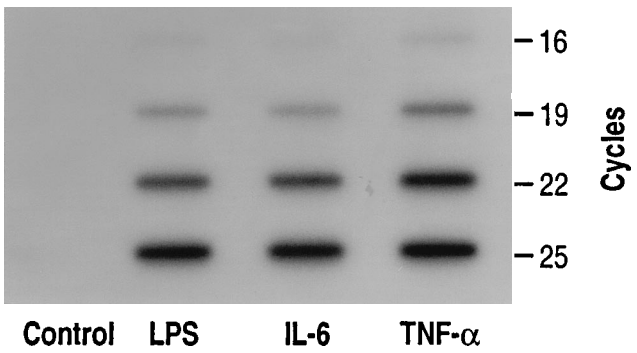

FIG. 4. Effects of LPS, TNF-α, and IL-6 on IL-10 mRNA induction assessed by slot blot analysis of the RT-PCR product hybridized with an IL-10 internal probe at the amplification cycles indicated. Microglial cell cultures were treated with medium or medium containing LPS (1 μg/ml), TNF-α (20 ng/ml), and IL-6 (100 μg/ml) for 8 h. The results shown are representative of two separate experiments.

After treatment of microglial cell cultures with LPS, TNF-α was produced relatively early (peak by 8 h) and the TNF-α concentrations achieved (>2 ng/ml) could potentially induce IL-10 production by three- to fourfold over control cultures. IL-10 release during sepsis or endotoxic shock has been reported (14, 24, 29). In an in vivo study of humans and chimpanzees administered recombinant human TNF-α and endotoxin, a transient rise in circulating IL-10 in plasma was detected (40). These findings also support the role of TNF-α in the stimulation of IL-10 production.

The acute release of TNF-α under pathologic conditions may have either deleterious or beneficial effects (2). Feedback regulation of this cytokine by other cytokines, such as IL-10 (41), could operate to minimize TNF-α-induced damage, thereby maintaining homeostasis. For example, TNF-α has been shown to upregulate HIV-1 expression in the chronically infected promonocytic cell line U1 (33), and IL-10, induced during HIV-1 infection, is capable of decreasing viral replication in human macrophages (1, 37) and in peripheral blood mononuclear cells (30). Results of the present study revealed

that TNF-α treatment of microglial cell cultures induced release of substantial amounts of IL-10 and that IL-10 potently suppressed TNF-α production in LPS-stimulated microglial cell cultures.

In contrast to a protective role of IL-10 against tissue injury or human immunodeficiency virus replication, suppression of inflammatory cytokines by IL-10 under some circumstances may be detrimental to the host. It has been suggested that inflammatory cytokines, such as TNF-α and IL-6, released by activated human microglial cells play a critical role in controlling the growth of the intracellular parasite *Toxoplasma gondii* (6). In addition, IL-10 has been reported to inhibit macrophage microbicidal activity against *Schistosoma mansoni* (32) and to mediate in vivo susceptibility to *Trypanosoma cruzi* infection (35). Thus, the balance between host defense and tissue injury appears to be critical for host survival during infectious disease insults.

Elevation of TNF-α is a hallmark of bacterial meningitis (36, 38, 39). Treatment of human brain cell cultures with TNF-α has been found to potentiate glutamate receptor-mediated neurotoxicity, suggesting a potential detrimental effect of TNF-α within the central nervous system (7). Thus, treatment with IL-10 to reduce TNF-α production could theoretically be of therapeutic value in bacterial meningitis. Given our findings that TNF-α stimulated IL-10 release by microglial cell cultures, it is possible that elevated IL-10 levels could be detected in the cerebrospinal fluid of patients with bacterial meningitis. Indeed, IL-10 has been found in the cerebrospinal fluid of 95% of patients with bacterial meningitis (20). This could be of diagnostic value in the evaluation of patients with central nervous system infections.

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