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Cytokine Regulation of Human Microglial Cell IL-8 Production

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IL-8 involvement in neutrophil activation and chemotaxis may be important in inflammatory responses within the central nervous system, secondary to meningitis, encephalitis, and traumatic injury. The source of IL-8 within the brain during these inflammatory processes, however, is unknown. To explore the role of microglia in the production of IL-8, human fetal microglia, which are the resident macrophages of the brain, were treated with LPS and pro- and anti-inflammatory cytokines to determine their effects on IL-8 production. We found that IL-8 protein levels increased in response to LPS or IL-1β, or to TNF-α, which also corresponded to elevated IL-8 mRNA levels by RT-PCR. Pretreatment with IL-4, IL-10, or TGF-β1 potently inhibited the stimulatory effects of these proinflammatory agents. These findings indicate that human microglia synthesize IL-8 in response to proinflammatory stimuli, and that anti-inflammatory cytokines down-regulate the production of this chemokine. These results may have important therapeutic implications for certain central nervous system insults involving inflammation. *The Journal of Immunology, 1998, 160: 1944–1948.

A cute bacterial (purulent) meningitis occurs when bacteria gain access to the subarachnoid space, usually via the nasopharynx or systemic circulation, prompting the entry of neutrophils. Although effective antibiotics are available, substantial mortality due to bacterial meningitis persists and high rates of neurologic sequelae occur in patients who survive the initial infection. Recent advances have shown that the inflammatory response in the brain, triggered by bacterial products such as LPS, a major cell wall component of *Neisseria meningitidis* (meningococcus) and *Haemophilus influenzae* type b (Hib), may be partially responsible for the morbidity. (2).

Bacteria induce the production of cytokines by many cell types including leukocytes and endothelial cells (3) that form the vascular component of the blood-brain barrier (BBB). Additionally, astrocytes, which are the predominant cell type on the brain side of the BBB, and microglial cells (contributing up to 20% of the brain cells of the BBB (4)) produce a wide variety of cytokines and β-chemokines (5–7) when activated. Cytokines appear to either play a protective role or initiate an irreversible over-reaction of the immune system ultimately leading to cell death (8). Both IL-1β and TNF-α are produced during meningitis or the release of this chemokine. These results may have important therapeutic implications for certain central nervous system insults involving inflammation.

Materials and Methods

Reagents

TNF-α, IL-1β, IL-4, IL-8, IL-10, TGF-β1, and anti-IL-8 Abs were kindly provided by R&D Systems, Inc. (Minneapolis, MN). Anti-goat horseradish peroxidase conjugate was obtained from Jackson ImmunoResearch (West Grove, PA). LPS and Tween-20 were acquired from Sigma Chemical Co. (St. Louis, MO).

Brain cell cultures

Human fetal brain tissues were obtained from 16- to 22-wk-old aborted fetuses under a protocol approved by the Human Subjects Research Committee at our institution. These cultures were prepared using a previously described technique (34). Briefly, brain tissues were dissociated after a...
45-min trypsinization (0.125%) and were seeded into 75-cm² Falcon culture flasks (Fisher Scientific, Pittsburgh, PA) in DMEM (Sigma) containing 10% heat-inactivated serum (HyClone Laboratories, Logan, UT) and penicillin/streptomycin (Sigma). On day 10 to 12 of culture, harvested cells (microglia) were seeded into 96-well plates.

Adult brain specimens (≥0.01 g) were obtained from patients (with informed consent) undergoing elective or emergency neurosurgical procedures at Hennepin County Medical Center (Minneapolis, MN) and prepared following a slightly modified protocol from that used for processing fetal microglia. Following trypsinization, cells were resuspended in 35.6 ml PBS, layered over a gradient containing 9.6 ml of 100% Percoll (Sigma) and 4.8 ml of 1.25 M sucrose (Sigma), and centrifuged at 14,000 rpm for 45 min. Collected cells were washed and plated into 75-cm² flasks. On day 14 of culture, microglia (5 × 10⁴ cells/well) were seeded into 96-well plates. Purified fetal or adult microglia were composed of cell populations, >99% of which stained with CD68 Abs (Dako, Carpenteria, CA), a microglial cell marker, and <1% of which stained with Abs to glial fibrillary acidic protein (Dako), an astrocyte marker.

Fetal microglia were prepared as previously described (35). More than 99% of the cells were positive for glial fibrillary acidic protein. Astrocytes were seeded into 96-well plates at a density of 5 × 10⁶ cells/well.

Monocyte-derived macrophage (MDM) cultures

Whole blood was drawn from healthy laboratory donors and diluted with sterile PBS. This mixture was added to a Ficoll-Hypaque gradient (Lymphocyte Separation Medium, Organan Teknika Corp., Durham, NC) and centrifuged at 1400 rpm for 30 min. PBMC were collected from the gradient and washed three times in PBS. The cells were then resuspended in RPMI 1640 (Sigma) with antibiotics, and monocytes were allowed to adhere to the bottom of a 24-well plate for 2 to 3 h. Nonadherent cells were washed off vigorously several times, and RPMI 1640 containing 10% FBS and antibiotics was re-added. Monocytes were allowed to differentiate into MDMs for 5 days in a 5% CO₂ environment at 37°C.

Experimental protocol

In all experiments testing IL-8 protein production, cells were washed once and treated the day after plating. Controls, which were cells incubated in medium alone, were included in all experiments. Supernatants were collected at 16 to 18 h and frozen at −20°C to be tested later by ELISA. To evaluate the effects of LPS, IL-1β, and TNF-α on IL-8 production by microglia, cells (5 × 10⁴ per well) were treated with LPS, IL-1β, or TNF-α at the indicated doses. Cytokine specificity was determined by pretreating microglial cells (5 × 10⁴ per well) with 10 µg/ml rabbit anti-human IL-1β Ab (Genzyme, Cambridge, MA) or anti-human TNF-α Ab (R&D Systems) for 45 min followed by 16 h of stimulation with either IL-1β (10 ng/ml) or TNF-α (20 ng/ml), respectively, and then measuring a decrease in IL-8 production in the Ab-treated wells compared with the stimulated wells alone. The effects of anti-inflammatory cytokines were tested by pretreating cells with IL-4, IL-10, or TGF-β1 for 6 h. Then LPS, IL-1β, or TNF-α were added alone or in combination with IL-4, IL-10, or TGF-β1, and the cells were allowed to incubate overnight. Adult microglia, MDM, and astrocytes (all at 5 × 10⁶ cells/well) were treated with LPS, IL-1β, or TNF-α and compared with fetal microglial cells treated in an identical manner.

RT-PCR

Total RNA was extracted from microglia using guanidinium isothiocyanate as previously described (36). Reverse transcription of 1.5 µg of RNA was performed with 50 µM oligo(dT)₁₂₋₁₈ followed by the addition of a reaction mixture containing 5× first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂, 0.1 MDTT), SuperScript II RNase H⁻ Reverse Transcriptase (Life Technologies, Gaithersburg, MD), and 10 mM deoxyinososide triphosphates mix (10 mM each of dATP, dGTP, dCTP, and dTTP). (HP Pharmacia Biotech, Piscataway, NJ) in a final volume of 20 µl. The mixture was incubated at 42°C for 1 h followed by termination at 95°C for 5 min. Amplification of IL-8 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (as a control) was performed with an automatic thermocycler (Stratagene, La Jolla, CA) in a reaction mixture containing 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C), and 1% Triton X-100), 25 mM MgCl₂, 10 mM deoxynucleoside triphosphates mixture, 5 U of Taq DNA polymerase (Promega, Madison, WI) per µl with 0.22 µg/µl TaqStart Ab (Clontech, Palo Alto, CA), 25 µM primer (sense and antisense), CDNA, and H₂O. Amplification was set at 94°C for 45 s, 65°C for 45 s, and 72°C for 90 s followed by a 10-min extension at 72°C. The IL-8 PCR product (289 bp) was amplified for 17 to 19 cycles and the GAPDH (600 bp) for 22 cycles. Both PCR products were viewed under UV light after 2% agarose gel electrophoresis and staining in ethidium bromide. The IL-8 primer sets (Clontech) were 5’-ATG ACT TCT ACC AAG

**FIGURE 1.** Dose-response effects of LPS, IL-1β, and TNF-α on human fetal microglial cell IL-8 production. Microglial cells were treated with the indicated concentrations of proinflammatory mediators for 16 h, and supernatants were collected and assayed for IL-8. Data (mean ± SEM) are representative of three separate experiments with microglia from different brain tissue specimens.

CTG GCC GTG GCT-3’ (sense) and 5’-TCT CAG CCC TCT TCA AAA ACT TCT C-3’ (antisense). The GADPH primer sets (Stratagene) were 5’-ACC ACA GTC CAT GCC ATC AC-3’ (sense) and 5’-TCC ACC ACC CTG TTA CGT TA-3’ (antisense).

**ELISA**

IL-8 protein was detected by an ELISA developed in our laboratory. Briefly, 96-well culture plates were coated with 0.1 µg/ml mouse anti-human IL-8 mAb and stored overnight at 4°C. The following day, the plates were washed with 0.05% PBS/Tween-20 and nonspecific binding was blocked by treatment with 1% BSA for 1 h at 37°C. After washing, a standard series of diluted human rIL-8 was added to each plate along with supernatant samples and incubated for 2 h at 37°C. Next, goat anti-human IL-8 Ab (2 µg/ml) was added for 1.5 h at 37°C followed by anti-goat IgG horseradish peroxidase conjugate (1:2,000 in PBS) for 1 h. After extensive washing with PBS/Tween-20, 3,3,5,5-tetramethylbenzidine (TMB, Neogen Corporation, Lexington, KY) was added for 10 to 20 min at room temperature for color development, and then the development was stopped by the addition of 100 µl of 1 M H₂SO₄. OD was read at 450 nm and compared with standard values for quantification. The sensitivity of this assay is 10 pg/ml, and is specific for IL-8.

**Statistical analysis**

When appropriate, data were analyzed for comparison of multiple means using ANOVA followed by Scheffe’s F test. Statistical significance was determined at the level of 95% confidence.

**Results**

Initially, IL-8 production by human fetal microglial cells was tested at 8, 24, and 48 h following LPS (1 µg/ml) stimulation. In the culture supernatants, IL-8 increased over time with maximal production observed at 48 h in both the control (67 ± 21 ng/ml) and LPS-stimulated (360 ± 10 ng/ml) cultures. IL-8 production was minimal at 8 h in the control cultures (4 ± 1 ng/ml) but was markedly increased in the LPS-treated cultures (150 ± 2 ng/ml). After 24 h, LPS-stimulated microglia released more IL-8 (288 ± 10 ng/ml) with the control values remaining low (13 ± 1 ng/ml). Based on these findings and on subsequent experiments at 16 h, a time point of 16 h poststimulation was selected for the remaining experiments.

After treatment with varying doses of LPS, IL-1β, and TNF-α, fetal microglial cell culture supernatants were tested by ELISA for IL-8 protein (Fig. 1). LPS and IL-1β stimulated IL-8 in a
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FIGURE 2. Effects of LPS, IL-1β, and TNF-α on human fetal microglial cell IL-8 mRNA expression. Microglia were treated with 1 µg/ml LPS, 10 ng/ml IL-1β, and 20 ng/ml TNF-α for 6 h before RNA extraction. The RT-PCR product shown was amplified for 17 cycles. Lane M shows a 100 bp marker, lane C shows medium-only treated cells, and lane H₂O contains no cDNA for an internal control. Results are representative of three separate experiments.

FIGURE 3. Cytokine regulation of IL-8 production. Human fetal microglia cells were pretreated with the indicated concentrations of IL-4 (A), IL-10 (B), or TGF-β1 (C) for 6 h and then stimulated for 16 h with LPS (1 µg/ml), IL-1β (10 ng/ml), or TNF-α (20 ng/ml). Data, expressed as percent inhibition of cytokine-stimulated IL-8 production, are representative of five separate experiments.

FIGURE 4. Effects of IL-4, IL-10, and TGF-β1 on IL-8 mRNA expression. Cells were pretreated with IL-4 (30 ng/ml), IL-10 (10 ng/ml), or TGF-β1 (10 ng/ml) for 16 h and then stimulated with LPS (10 ng/ml), IL-1β (10 ng/ml), or TNF-α (20 ng/ml) for 4 h. Controls were treated either with media (–) or with LPS, IL-1β, or TNF-α alone (+). GADPH primer was used for an internal control. The IL-8 PCR product was amplified for 17 to 19 cycles, while the GADPH product was amplified for 22 cycles.

dose-dependent manner (ED₅₀ = 20 pg and 2 ng, respectively) with a maximal production of 180 ± 20 ng/ml and 161 ± 15 ng/ml, respectively. TNF-α also increased IL-8 production over the control level (40 ± 1 ng/ml compared with 7 ± 1 ng/ml; ED₅₀ = 200 pg/ml) but the maximal stimulation of IL-8 by TNF-α was less than that observed with LPS and IL-1β (Fig. 1). All data points in Figure 1 were greater than control values (p < 0.05) with the exception of the 0.01 and 0.1 ng/ml IL-1β-stimulated and 0.01 ng/ml LPS-stimulated treatments. IL-8 release induced by IL-1β and TNF-α was cytokine specific, as mAbs against each cytokine inhibited IL-8 production by 75 ± 6% and 92 ± 9%, respectively (data from two separate experiments). These proinflammatory stimuli also increased IL-8 mRNA levels in human fetal microglia (Fig. 2). A low level of constitutive expression of IL-8 mRNA was seen in the control cells, but only at higher amplification.

Next, we studied the effect of anti-inflammatory cytokines on fetal microglial IL-8 production. The production of IL-8 in response to all three proinflammatory stimuli (i.e., LPS, IL-1β, and TNF-α) was inhibited by IL-4, IL-10, and TGF-β1 at levels of 59% to 86% inhibition at maximal doses (Fig. 3, A–C). These anti-inflammatory cytokines were most effective in decreasing IL-1β- and TNF-α-stimulated IL-8 production. IL-4, IL-10, or TGF-β1 alone had a minimal effect on constitutive expression relative to the amount of control production of IL-8. All the data in Figure 3, A–C, were different (p < 0.05) than the values obtained with the stimulus alone (i.e., LPS, IL-1β, or TNF-α) with the exception of the IL-4 (0.3 ng/ml)/TNF-α-stimulated and the IL-10 (0.1 ng/ml)/IL-1β-stimulated points. When using RT-PCR at the transcriptional level, microglial cells treated with LPS, IL-1β, and TNF-α exhibited smaller bands when pretreated with IL-4 or IL-10 overnight (Fig. 4). TGF-β1 appeared to have no effect on LPS- and IL-1β-induced IL-8 mRNA expression, but potentially decreased IL-8 mRNA expression in the TNF-α-treated cells. These observed decreases may have been due to differing transcription rates and/or altered mRNA message stability, neither of which were analyzed in the present study.

Finally, the ability of fetal microglia to produce IL-8 was compared both with that of microglia isolated from adult brain tissue and with MDM, as well as with fetal astrocytes. IL-8 production by microglia from fetal and adult brain tissue was comparable with IL-8 produced by MDM (Table I). Compared with control values at 16 h, LPS and IL-1β increased IL-8 production (p ≤ 0.05), but the TNF-α-stimulated increase was not significant. In sharp contrast to the mononuclear phagocytes, astrocytes produced no IL-8 in response to LPS. However, IL-1β was a potent stimulator of IL-8 production by astrocytes (80 ± 15 ng/ml), and TNF-α elicited relatively low amounts of IL-8 (5 ± 1 ng/ml) (Table I).

Discussion

The results of this study indicate that both human fetal and adult microglial cells (as well as MDM) produce IL-8 in response to LPS, IL-1β, and TNF-α, two cytokines that play a key role in the initiation of an inflammatory response. This production appears to be dose dependent in fetal microglia and is inhibited by the anti-inflammatory cytokines IL-4, IL-10, and TGF-β.

The role of chemokines such as IL-8 in CNS diseases is not understood completely. Chemokines attract specific cell types...
search performed on endothelial cells (22, 48), neutrophils (49), and in vivo models (50); however, our results could be due to altered message stability or differing rates of transcription. Additionally, IL-10 and TGF-β1 have been found in the CSF of patients with bacterial meningitis (51–53), suggesting these cytokines could modulate IL-8 production in vivo.

In conclusion, the interactions between microglia, IL-8, and neutrophils appear important in understanding the pathogenesis of acute bacterial meningitis and other CNS insults involving inflammation including brain trauma (54, 55). Further studies to delineate the mechanisms through which LPS, IL-1β, and TNF-α induce IL-8 and the production of other chemokines and to determine whether microglia and astrocytes produce IL-8 in vivo (leading to a migration of neutrophils across the BBB or to further activation of neutrophils once they cross the BBB) would contribute to greater knowledge and potential therapies for brain insults involving inflammation.

References


