Interleukin-10 Inhibits Interleukin-8 Production in Human Neutrophils

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In highly purified human polymorphonuclear leukocyte (PMN) preparations containing less than 0.1% contaminating monocytes, significant amounts of interleukin-8 (IL-8) and small amounts of IL-1α, IL-1β, and tumor necrosis factor-alpha (TNF-α) were produced by lipopolysaccharide (LPS) stimulation. Contrary to published reports, IL-8 production could not be detected. IL-10 inhibited the production of IL-1α, IL-1β, IL-8, and TNF-α in LPS-stimulated PMNs, as it did in human blood mononuclear cell (MNC) preparations enriched in monocytes. Subsequent investigation of cytokine synthesis inhibitory effect of IL-10 on PMNs was focused on IL-8. IL-10 inhibited IL-8 synthesis in a dose-dependent manner and, in this regard, it was more potent than IL-4 and transforming growth factor-β1 (TGF-β1). In both MNCs and PMNs, degradation of LPS-induced IL-8 mRNA was enhanced by IL-10. Furthermore, as determined by nuclear run-on assays, IL-10 inhibited LPS-induced transcription of IL-8 gene in MNCs. However, in PMNs, run-on assays could not reliably detect IL-8 gene transcription. These results provide the first evidence that the human peripheral neutrophil is a target for inhibition of cytokine synthesis by IL-10, and that IL-10 acts by affecting both gene transcription and mRNA stability.

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INTERLEUKIN-10 (IL-10) is a newly identified cytokine with a molecular weight of 18 kD, which was initially described as cytokine synthesis inhibitory factor. IL-10 inhibits production of a wide range of cytokines in various cell types, such as IL-1, IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α) in macrophages/montocytes11,12; IL-3, lymphotoxin, interferon-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in Th1 cells13,14; IL-4 and IL-5 in Th2 cells; and interferon-γ and TNF-α in natural killer cells.15,16 Because many of these cytokines (IL-1, IL-6, IL-8, and TNF-α) are important inflammatory mediators, IL-10 may offer clinical utility as an antiinflammatory agent.17

It has been reported that human polymorphonuclear leukocytes (PMNs) produce various cytokines, including IL-6, IL-1, IL-13,15 TNF-α,11,16,18 and IL-8.11,19,20 On a cell basis, the production of these cytokines from PMNs is much less than that from monocytes, which are the principal producers of all these cytokines. Nevertheless, there may be a significant contribution of PMNs to the overall cytokine production during inflammation, because of the quantitative predominance of PMNs over monocytes in the peripheral blood and at sites of acute inflammation.

In the present report, we show that human PMNs respond to lipopolysaccharide (LPS) by generating significant amounts of IL-8 and small amounts of IL-1 and TNF-α, and that IL-10 inhibits this cytokine production by affecting both mRNA synthesis and degradation.

MATERIALS AND METHODS

Preparation of MNCs and PMNs. Fifty milliliters of venous blood from a healthy male donor was mixed with 5 mL of a buffer consisting of 3.8% sodium citrate and 2% dextrose. Mononuclear cells (MNCs) and PMNs were prepared as described,21 with some modifications. Briefly, after dextran sedimentation of red blood cells, the plasma was layered carefully onto Ficoll-Paque (Pharmacia, Piscataway, NJ). After centrifugation, MNCs at the interface were carefully transferred to a clean tube with a Pasteur pipette, and washed with phosphate-buffered saline (PBS) and then with cell culture medium (see below). The concentrations of monocytes in MNC preparations were 15% to 30% depending on the donors, as determined by nonspecific esterase cytochemical method using an anaphylactic acetate esterase staining kit (Sigma, St Louis, MO).

To further purify PMNs, the remainder of the Ficoll-Paque phase was completely removed with a fresh Pasteur pipette. The tube wall was carefully washed with PBS, and the cell pellet on the bottom of the tube was suspended in 5 mL of PBS. The suspension was transferred to a new tube and erythrocytes were lysed by adding 40 mL of ice-cold water. PMNs were recovered by centrifugation, washed, and resuspended in the cell culture medium (see below).

Cell culture. Cells were cultured in RPMI 1640 medium supplemented with 1% each of penicillin and streptomycin, nonessential amino acids, sodium pyruvate, glutamine (all from Gibco, Grand Island, NY) and 5% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT) in a humidified atmosphere of 5% CO2 at a cell density of 1 × 10^6/mL, in the presence of appropriate agents as specified. The incubation was performed for 14 and 12 hours for cytokine protein assays and Northern blot analysis, respectively.

Assays for cytokine protein production. Cytokine protein amounts in cell culture supernatants were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (IL-6 and TNF-α kits from Biosource, Camarillo, CA; IL-1α, IL-1β, and IL-8 kits from R & D Systems, Minneapolis, MN).

Northern blot analysis. Total cellular RNA was isolated using RNeasy solution (Biotest Laboratories, Houston, TX) according to the manufacturer’s instructions. RNA electrophoresis was performed on a 1% agarose-formaldehyde gel, and fractionated RNA was then transferred to Duralon UV Membranes (Stratagene, La Jolla, CA) with a PosiBlot pressure blower (Stratagene). Prehybridization was performed at 42°C for 2 hours in a solution of 5X SSC, 5X Denhardt’s reagent, 50 mmol/L sodium phosphate, 0.1% sodium dodecyl sulfate (SDS), 5 mmol/L EDTA, 50% formamide, 50 μg/mL denatured salmon sperm DNA, and 50 μg/mL denatured torula yeast RNA. IL-8 and IL-6 mRNAs were detected by hybridization with respective cDNA probes (R & D Systems) labeled with 32P by the random primer labeling method. The hybridization was performed at 42°C for 18 hours in a solution of 5X SSC, 1X Denhardt’s reagent, 20 mmol/L sodium phosphate, 0.1% SDS, 5 mmol/L EDTA, 50% formamide, 50 μg/mL denatured salmon sperm DNA, and 50 μg/mL denatured torula yeast RNA. After the hybridization, membranes were washed with 2× SSC/0.1% SDS twice at room temperature, each for 10 minutes, then once at 50°C for 30 minutes, and finally with 0.1X SSC/0.1% SDS at room temperature for 5 minutes.

β-Actin cDNA probe (Clontech, Palo Alto, CA) was labeled with biotin, and detected by the streptavidin-alkaline phosphatase method.
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Nuclear run-on assay. MNCs (0.3 to 0.5 × 10⁶ cells per sample) or PMNs (1 × 10⁶ cells per sample) were cultured in the absence or the presence of various agents. After selected times, cells were collected, nuclei prepared, and nuclear run-on assays performed with some minor modifications. Briefly, nuclei from each sample were suspended in 0.2 mL of reaction buffer, which contained 10 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA (40% glycerol). Elongation of nascent RNA chains was initiated by mixing this nuclear suspension with 0.2 mL of reaction buffer, which contained 10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.3 mM KCl, 12.5 mM dithiothreitol, 2.5 mM EDTA, and 25 mM each of adenosine triphosphate (ATP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP). 80 U RNasin (Promega, Madison, WI), and 0.1 mL [³²P]uridine triphosphate (UTP) (specific activity, 3,000 Ci/mmol; New England Nuclear, Boston, MA). After incubation at 37°C for 1 hour, [³²P]-labeled RNA was isolated and dissolved in 0.4 mL of TES buffer (10 mM Tris-HCl, pH 7.9, 10 mM EDTA, 0.2% SDS, 0.3 mM NaCl, and 5% dextran sulfate), then analyzed as follows. Cytokine cDNA inserts and β-actin cDNA probe, 250 ng each, were immobilized on Nytran membrane (Schleicher & Schuell, Keene, NH) and the membrane was prehybridized overnight at 60°C in a solution of 5X SSC, 5X Denhardt’s reagent, 50 mM NaCl, sodium phosphate, 1X Blocking Solution (Bio-Max), 5% SDS, 5 mM EDTA, and 5% dextran sulfate. Labeled RNA was hybridized with immobilized cDNA probes at 65°C for 3 days and the membrane was washed three times, each for 30 minutes, with 2X SSC at 60°C, then twice, each for 20 minutes, with 2X SSC containing 10 µg/mL RNase A (Sigma) at 37°C, and finally once for 1 hour with 2X SSC at 37°C.

Other reagents. Recombinant human IL-10 and IL-4 were from Schering-Plough Research Institute (Union, NJ). Human natural transforming growth factor-β (TGF-β1) was obtained from Genzyme (Cambridge, MA). LPS was purchased from Sigma.

Data presentation. Assays for cytokines were performed in duplicate. Each set of experiments were performed at least twice and the data presented are from representative experiments. Data points are the mean of two determinations, which were within 10%, mostly 5%, of the mean.

RESULTS

LPS-stimulated PMNs produce IL-1, IL-8, and TNF-α, but not IL-6. It has been reported that PMNs produce IL-1, IL-6, IL-8, and TNF-α. To investigate the effect of IL-10 on cytokine production by PMNs, it is critical to use PMN preparations free of contaminating monocytes, because monocytes are the principal producers of all these cytokines, and also because monocytes are the primary target for IL-10 action. To Table 1 shows the effect of monocyte contamination on LPS-induced cytokine production by PMN preparations. In a highly purified PMN preparation containing only approximately 0.1% contaminating monocytes, significant amounts of IL-8 and small amounts of IL-1α, IL-1β, and TNF-α were produced, but IL-6 was undetectable. In contrast, PMN preparations obtained using standard procedures, which deferred from ours in that we exercised extreme caution in removing the Ficoll-Paque phase and washed the tube wall to minimize monocyte contamination, contained approximately 0.8% monocytes and produced IL-8 in significant amounts. Because IL-6 is a marker for monocyte contamination, it would appear that the IL-1α, IL-1β, TNF-α, and IL-6 were derived from PMNs, rather than from contaminating monocytes in our highly purified PMN preparation.

When MNCs were added in known increments to the highly purified PMN preparation then stimulated with LPS, there were proportional increases in both IL-6 (Fig 1A) and IL-8 (Fig 1B). Extrapolation of IL-6 and IL-8 curves to the MNC-free condition indicated release of IL-8, but not IL-6, supporting the contention that, under our experimental conditions, LPS-stimulated human neutrophils do not produce IL-6.

MNCs responded to low concentrations of LPS by producing significant amounts of IL-8 (ED₅₀ < 0.02 ng/mL) (Fig 2A), while IL-8 production in PMNs required relatively higher LPS concentrations (ED₅₀ ≈ 6 ng/mL) (Fig 2B). IL-8 production in MNCs plateaued at 0.64 ng/mL of LPS, a concentration at which there was no IL-8 or IL-6 production in PMNs, indicating, again, that there was no significant monocyte contamination in our PMN preparation. This difference in sensitivity of MNCs and PMNs to LPS may well be due to the fact that monocytes produce IL-1 and TNF-α in large quantities and that these cytokines act synergistically with LPS to stimulate IL-8 production from monocytes. IL-6 was produced in large amounts by MNCs at all LPS concentrations, while PMNs produced IL-6 at barely detectable levels, even at the highest LPS concentration (400 ng/mL). These results further indicate that our PMN preparations were essentially devoid of contaminating monocytes, and that, under our experimental conditions, PMNs generate IL-8, but not IL-6.

IL-10 inhibits LPS-induced cytokine production in human PMNs. The effects of IL-10 on IL-8 and IL-6 synthesis were studied in both PMNs and MNCs (Fig 3). In all cases, IL-10 inhibited cytokine synthesis in a dose-dependent manner, although PMNs were less sensitive to IL-10 than were MNCs. In the same set of experiments, we also investigated the effects of IL-4 and TGF-β1 on cytokine production, because IL-4 and TGF-β1 inhibited cytokine synthesis in monocytes/macrophages. Since optimal concentrations of IL-4 and TGF-β1 for cytokine synthesis inhibition were...
Fig 1. LPS-stimulated production of cytokines by PMNs mixed with MNCs. MNCs and highly purified PMNs were both suspended at a cell density of 1 x 10^6/mL in culture medium, then MNC suspension was mixed quantitatively with PMN suspension. The mixtures containing MNCs at the indicated concentrations were cultured in the presence of 80 ng/mL of LPS for 14 hours. Supernatants were used to determine released IL-6 (A) and IL-8 (B). For other details, see Table 1. Note that the horizontal axis shows concentrations of mononuclear cells, and that monocytes account for approximately 20% of the total cell population.

FIG 2. LPS dose responses for the production of IL-8 and IL-6 in PMNs and MNCs. Cells were cultured in the presence of various concentrations of LPS for 14 hours, and then IL-6 (A) and IL-8 (B) in the supernatants were measured. For further details, see Table 1. (A) MNCs, (B) PMNs. In MNCs, the amounts of IL-6 and IL-8 in the absence of LPS were 229 and 4,444 pg/mL, respectively, and they were subtracted from values obtained in the presence of various concentrations of LPS. In PMNs, there was no detectable IL-8 or IL-6 production in the absence of LPS.

DISCUSSION

Using highly purified neutrophil preparations containing less than 0.1% monocytes, we conclude in the present study that human peripheral neutrophils respond to LPS by generating several inflammatory cytokines, including IL-1, IL-8, and TNF-α. IL-8 is produced in large quantities, while the production of IL-1 and TNF-α is rather limited (Table 1).
A previous study has used an immunocytochemical approach to demonstrate that LPS-stimulated PMNs do generate both IL-1α and IL-1β. Several previous studies have also claimed IL-6 production by LPS- and GM-CSF-stimulated neutrophils. However, in the present study, we have found no indication of IL-6 production as evaluated by protein ELISA at a detection level of 5 pg/mL or by Northern blotting upon stimulation with LPS (Table 1) or with GM-CSF (data not shown). Recent in situ hybridization experiments have concluded that neutrophils do produce IL-6, but only when they are in circulation, and that they lose this ability following their withdrawal from the blood stream. These observations indicate that PMN preparations used in the previous studies may have contained monocytes in sufficient numbers to account for the IL-6 production.
Indeed, IL-6 is readily detected in neutrophil preparations containing 0.6% to 0.8% monocytes (Table 1).

That IL-10 inhibits cytokine synthesis in monocytes and T cells is well established. Our present results show for the first time that IL-10 also acts on PMNs to inhibit LPS-induced cytokine synthesis. This effect of IL-10 on IL-8 production is dose-dependent (Fig 3) and involves a reduction in the cytokine mRNA level (Fig 4). In these regards, PMNs bear striking similarities with monocytes (Figs 3 and 4). The exquisite sensitivity of neutrophils to IL-10 (1 ng/mL = 56 pmol/L) clearly indicates that IL-10 exerts its inhibitory action on PMNs by interacting with specific receptors.

Mechanisms by which IL-10 reduces cytokine mRNA accumulation in stimulated cells are largely unknown. A recent study in murine peritoneal macrophages has indicated that IL-10 may act by enhancing the degradation of cytokine mRNA. In support of this observation, we find that IL-10 enhances IL-8 mRNA degradation in both MNCs and PMNs (Fig 6). We further find that IL-10 is capable of inhibiting IL-8 gene transcription (Fig 5). Thus, the inhibitory action of IL-10 on IL-8 mRNA appears to be the consequence of both inhibition of gene transcription and enhancement of mRNA degradation.

Compared with monocytes, PMNs are poor producers of inflammatory cytokines, including IL-1, IL-8, and TNF-α. Nevertheless, the overall contribution of PMNs to the in vivo production of inflammatory cytokines may be significant, because of quantitative predominance of PMNs over monocytes in the peripheral blood and at the sites of acute inflammation. Thus, the current finding that IL-10 inhibits cytokine production by PMNs underscores the potential importance of IL-10 in the regulation of PMN-mediated inflammatory processes.

REFERENCES

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