Quantitative comparison of C-X-C chemokines produced by endotoxin-stimulated human alveolar macrophages

RICHARD B. GOODMAN,1 ROBERT M. STRIETER,2 CHARLES W. FREVERT,1 C. JAMES CUMMINGS,1 PATRICIA TEKAMP-OLSON,3 STEVEN L. KUNKEL,4 ALFRED WALZ,2 AND THOMAS R. MARTIN1

1Medical Research Service, Seattle Veterans Affairs Medical Center, and Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98108; 2Division of Pulmonary and Critical Care Medicine, Department of Medicine, and 4Department of Pathology, University of Michigan School of Medicine, Ann Arbor, Michigan 48109; 3The Chiron Corporation, Emeryville, California 94608; and 5The Theodor Kocher Institute, University of Bern, CH-3000 9, Switzerland

Goodman, Richard B., Robert M. Strieter, Charles W. Frevert, C. James Cummings, Patricia Tekamp-Olson, Steven L. Kunkel, Alfred Walz, and Thomas R. Martin. Quantitative comparison of C-X-C chemokines produced by endotoxin-stimulated human alveolar macrophages. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L87–L95, 1998.—The C-X-C chemokines are a structurally related and functionally redundant family of proteins with neutrophil chemotactic activity. Many of the C-X-C chemokines are produced by endotoxin-stimulated alveolar macrophages (AMs), but knowledge of their relative quantities and their relative contributions to the total chemotactic activity released from these cells is incomplete. Human AMs were stimulated with or without Escherichia coli endotoxin for 2, 4, 8, and 24 h. The mRNA sequences of interleukin (IL)-8, the 78-amino acid epithelial cell-derived neutrophil activator (ENA-78), growth-related protein (GRO) α, GROβ, and GROγ were cloned by PCR and identified by sequence analysis. The relative mRNA quantities were compared by Northern analysis, and IL-8 was found to predominate. Similarly, IL-8 protein concentrations in the cell supernatants were consistently higher than either the ENA-78 or GRO concentration, and by 24 h, IL-8 concentrations were 10-fold higher than those of the other C-X-C chemokines. Blocking polyclonal antibodies to IL-8 substantially reduced the chemotactic activity in the AM supernatants, whereas antibodies to ENA-78 and GRO had little or no effect. We conclude that IL-8 is the predominant C-X-C chemokine and the dominant neutrophil chemoattractant accumulating in 24-h supernatants of lipopolysaccharide-stimulated human AMs. These studies provide insight into potentially effective strategies of interrupting AM-derived inflammatory signals in the lungs.

chemotaxis; monocytes; neutrophils; lung

THE C-X-C CHEMOKINES are a functionally redundant family of proteins in that many of the family members have potent chemotactic activity for neutrophils (PMNs). A role for these chemokines has been implicated in a variety of human diseases characterized histologically by the presence of PMNs (23). Multiple members of the C-X-C chemokine family are present in inflammatory diseases, yet few studies have identified which one(s) predominate. Development of effective strategies to limit PMN-mediated inflammation depends on knowledge of which PMN chemoattractant predominates in specific inflammatory conditions.

As sentinel cells in the air space, alveolar macrophages (AMs) play a central role in the early host response to bacterial products such as endotoxin [lipopolysaccharide (LPS)]. AMs produce proteins with chemotactic activity specific for PMNs (30). The C-X-C chemokines interleukin-8 (IL-8), the 78-amino acid epithelial cell-derived neutrophil activator (ENA-78), and members of the growth-related protein (GRO) subfamily of C-X-C chemokines (GROα and GROγ) have all been identified as products of LPS-stimulated AMs (2, 36, 37); however, there are conflicting reports regarding GROβ (4, 24). Quantitative comparisons of the amounts of each of these C-X-C chemokines and their relative contributions to the total PMN chemotactic activity from LPS-stimulated AMs are lacking.

Therefore, our goals in these studies were to identify the spectrum of C-X-C chemokines produced by LPS-stimulated human AMs, to quantitate each of them, and to define the relative contribution of each C-X-C chemokine to the total chemotactic activity for PMNs. We found that in addition to IL-8, LPS-stimulated human AMs produce ENA-78 and all of the GRO subfamily members including GROβ. However, IL-8 is produced in logarithmically higher quantities than the other C-X-C chemokines, and, functionally, IL-8 constitutes the predominant PMN chemoattractant released from AMs under these conditions of stimulation. Thus, although AMs produce a spectrum of C-X-C chemokines, anti-inflammatory strategies aimed only at IL-8 may prove to be effective.

MATERIALS AND METHODS

Nomenclature. The GRO subfamily of C-X-C chemokines is composed of three proteins, GROα, GROβ, and GROγ, that are highly similar at both the amino acid level (>84% positional identity for secretory protein sequence) and the nucleic acid level (>90% positional identity for cDNA open reading frame sequence). As a result, antibodies directed at one may cross-react with the others. Similarly, Northern hybridization probes cannot be constructed that reliably distinguish them. When the methods used do not distinguish the three GRO subfamily members, the proteins are referred to generically as GRO.

Cell isolation. Human AMs were obtained from healthy volunteers by bronchoalveolar lavage. The subjects were premedicated with atropine, and the upper and lower airways were anesthetized with topical lidocaine. A Pentax FB-18X fiber-optic bronchoscope was inserted orally and advanced...
into the trachea. Suctioning of the upper airway was avoided. The bronchoscope was then advanced and gently wedged into a subsegmental airway of the right middle lobe or lingula. The distal lung segment was lavaged with five 30-ml aliquots of nonpyrogenic 0.9% saline. The first aliquot was discarded, and the subsequent four aliquots were combined and processed immediately. Cells in the lavage fluid were pelleted by centrifugation at 200 g for 30 min and washed two times with culture medium [serum-free RPMI 1640 medium (GIBCO BRL) containing 100 units/ml of penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, and 50 µg/ml of gentamicin]. Total cells were counted in a hemocytometer, and cytospin preparations were stained with Diff-Quik for differential cell counts. For these studies, all cell preparations contained >95% AMs, with >90% viability by trypsin blue exclusion. Less than 1% were bronchial epithelial cells.

Human blood was obtained by venipuncture of healthy volunteers. PMNs were isolated for use in the chemotaxis assay with MonoPoly resolving medium (ICN Pharmaceuticals, Costa Mesa, CA) according to the manufacturer's protocol and as previously described (11). PMNs were resuspended at a concentration of 3 × 10⁶ cells/ml in RPMI 1640 medium (GIBCO BRL) containing 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT). To isolate platelets and leukocytes for the preparation of blood RNA, whole blood was spun at 200 g for 10 min. The buffy coat and plasma were isolated and spun at 1,000 g for 30 min, and the platelet-rich cell pellet was collected.

Cell culture and stimulation. Human AMs were suspended at 1 × 10⁶ viable AMs/ml of culture medium (serum free) and distributed into culture flasks. Cells were incubated at 37°C in 5% CO₂ in air in the presence or absence of 1 µg/ml of Escherichia coli endotoxin (LPS 0111:B4, Sigma). This is a concentration of smooth-form endotoxin that Martin et al. (27) have shown in a prior study to be sufficient for cytokine release from AMs in the absence of LPS binding protein. Cell supernatants were aspirated, clarified by centrifugation, and the subsequent four aliquots were combined and processed immediately. Cells in the lavage fluid were pelleted by centrifugation at 200 g for 30 min and washed two times with culture medium [serum-free RPMI 1640 medium (GIBCO BRL) containing 100 units/ml of penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, and 50 µg/ml of gentamicin]. Total cells were counted in a hemocytometer, and cytospin preparations were stained with Diff-Quik for differential cell counts. For these studies, all cell preparations contained >95% AMs, with >90% viability by trypsin blue exclusion. Less than 1% were bronchial epithelial cells.

Table 1. PCR primer pairs

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>GenBank Accession No.</th>
<th>Primer Sequences</th>
<th>Expected Product Size</th>
</tr>
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<tbody>
<tr>
<td>IL-8 (29)</td>
<td>Y00787</td>
<td>5'-ATG ACT TCC AAG CGG GCC CTG-3'</td>
<td>300 bp</td>
</tr>
<tr>
<td>EOA-78 (40)</td>
<td>L37036</td>
<td>5'-TGA AAT TCC ACC CGG GAA TGT CAT CCC AAA-3'</td>
<td>162 bp</td>
</tr>
<tr>
<td>NAP-2 (41)</td>
<td>M54995</td>
<td>5'-TGG TTA GGT ATG TCC AAG GGA ACC-3'</td>
<td>144 bp</td>
</tr>
<tr>
<td>GROα (3)</td>
<td>J 03561</td>
<td>5'-AGC AGA TCC ATG ACC TGC CAA TTT-3'</td>
<td>341 bp</td>
</tr>
<tr>
<td>Common GRO</td>
<td></td>
<td>5'-CCC TCG TGG TGA GGT GAA TGT GCC AC-3'</td>
<td></td>
</tr>
<tr>
<td>GROα (3)</td>
<td>J 03561</td>
<td>5'-CAG CAA TCC CGG GCC CCT GC-3'</td>
<td>199 bp</td>
</tr>
<tr>
<td>GROβ (38)</td>
<td>X53799</td>
<td>5'-GCC TAC GAC TGG GTG GCC GC-3'</td>
<td></td>
</tr>
<tr>
<td>GROγ (38)</td>
<td>X53800</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nos. in parentheses, reference no. IL-8, interleukin-8; ENA-78, 78-amino acid epithelial cell-derived neutrophil activator; NAP-2, neutrophil-activating peptide-2; GRO, growth-related protein.
(NeuroProbe, Cabin John, MD) were used as described (11). In each assay, samples and controls (25 µl/well) were placed in the bottom chamber, and each was run in quadruplicate. Positive controls were zymosan-activated human serum (ZAS; 10% vol/vol) and AM supernatant (conditioned medium) with nonimmune goat IgG. Negative controls were fresh (unconditioned) culture medium with the appropriate added goat polyclonal IgG. PMNs (60 µl of 3 × 10^6 cells/ml in RPMI 1640 medium-10% fetal calf serum) were added to the top well, and the chambers were incubated for 90 min in a humidified Ziplock bag at 37°C. At the end of the incubation, the filters were removed and stained as described (11). Chemotaxis was measured by counting the number of PMNs in 10 high-powered fields (×450) at the leading front of migration. Data are expressed as a percentage of simultaneously assayed ZAS.

For dose-response comparisons of the C-X-C chemokines, PMN chemotaxis was performed with calcein-labeled (Molecular Probes, Eugene, OR) PMNs and polycarbonate filters in a 96-well chamber (NeuroProbe) as described (33). Briefly, the wells in the 96-well plate were filled (27 µl) with serial dilutions of C-X-C chemokines, 10% ZAS (positive control), or PBS containing 0.1% human serum albumin (negative control). Dilutions of the chemoattractants were made in PBS containing 0.1% human serum albumin. The 96-well plate was covered with a polycarbonate filter (8-µm pores; NeuroProbe). PMNs were incubated with calcein-AM (5 µg/ml final concentration) for 30 min, washed two times in PBS, and resuspended at 3 × 10^6 cells/ml in RPMI 1640 medium without phenol red (BioWhittaker, Walkersville, MD) containing 10% fetal calf serum. A 25-µl aliquot of the fluorescently labeled PMN suspension was placed on top of the filter over each well, and the plate was incubated at 37°C in 5% CO_2 in air for 1 h. At the end of the incubation, the cells remaining on top of the filter were removed, and the PMNs that had migrated into the filter and into the lower wells were measured by fluorescence (excitation 485 nm and emission 530 nm) in a Cytofluor II plate reader (PerSeptive Biosystems, Bedford, MA). Chemotaxis data are expressed as mean fluorescence in each well divided by total fluorescence of the PMNs available for migration multiplied by 100 (chemotactic index). The total fluorescence of the PMNs available for migration was measured on each plate by placing 25 µl of the calcein-labeled cell suspension directly into the bottom chamber and reading the total fluorescence at the end of the incubation.

Chemokine antibodies. Goat anti-human polyclonal IgG preparations specific for IL-8, ENA-78, and GRO were purchased commercially (R&D Systems, Minneapolis, MN). Although the IL-8 antibody has some (<5%) cross-reactivity with GROα, GROβ, and GROy by ELISA and Western blot, the IL-8 antibody does not block the biological activity of GROα nor does it cross-react with ENA-78 or a variety of other cytokines. The ENA-78 antibody has no cross-reactivity with IL-8, GROα, GROβ, GROy, or a variety of other cytokines. The GRO antibody has high cross-reactivity with GROα, GROβ, and GROy by Western blot and ELISA, but it does not recognize the other chemokines or a variety of other cytokines.

ELISA measurements. IL-8 was measured in duplicate by ELISA with commercially available reagents (R&D Systems). The threshold sensitivity of the IL-8 assay was 10 pg/ml, and there was no cross-reactivity with the other C-X-C chemokines or other known cytokines. ENA-78 was measured with a sandwich ELISA previously described (35). The ENA-78 ELISA had a threshold sensitivity of 10 pg/ml, and there was no cross-reactivity with the other C-X-C chemokines. GRO was measured in an ELISA developed at Chiron (P. Tekamp-Olson) that used a mouse monoclonal antibody to GROβ [macrophage inflammatory protein (MIP)-2b] and a polyclonal second antibody. The GRO ELISA recognized all three GRO proteins but did not cross-react with the other C-X-C chemokines. The threshold sensitivity in the GRO ELISA was 500 pg/ml.

Recombinant C-X-C chemokine proteins. Recombinant human (rh) IL-8 (72-amino acid form) was purchased from PeproTech (Rocky Hills, NJ) and from R&D Systems. rhGROα, rhGROβ, and rhGROy were purchased from R&D Systems. GROβ (MIP-2b) and GROy (MIP-2b) were also produced at Chiron (P. Tekamp-Olson). rhENA-78 was purchased from R&D Systems and also produced at the Theodor Kocher Institute (A. Walz).

Statistical methods. ANOVA for multiple comparisons with the Bonferroni post hoc test was used to compare the chemotactic activity in AM supernatants with and without the addition of blocking or control antibodies.

RESULTS

Identification of C-X-C chemokine mRNA by PCR. Human AMs were incubated for 4 h with 1 µg/ml of LPS. Total RNA was isolated from the AM monolayers, and the RNA was reverse transcribed with random hexamers. The signals for IL-8, ENA-78, GRO, and neutrophil-activating peptide (NAP)-2 were amplified by PCR. We observed single bands of the expected size for IL-8, ENA-78, and GRO (Fig. 1). Successful amplification depended on reverse transcription. In addition, there was no detectable signal for NAP-2 from human AMs, in contrast to the strong signal from platelet-enriched peripheral blood cells (Fig. 1). Each of the AM-derived PCR products was run on an agarose gel and stained with ethidium bromide. The identity of the PCR products was confirmed by sequence analysis. Thus human AMs transcribe the genes for IL-8, ENA-78, and GRO but not for NAP-2.

To identify which of the GRO subfamily genes are transcribed by LPS-stimulated AMs and to estimate their relative prevalence, we used a PCR primer pair directed at two regions of a nucleotide sequence that are conserved among the GRO subfamily members (Fig. 2). PCR amplification with these common GRO primers would be expected to amplify each of the GRO subfamily members in proportion to its initial mRNA prevalence. The amplified cDNAs between these two conserved primers include 13 base positions (Fig. 2, ★) that are divergent among the GRO subfamily members, allowing their identification by sequence analysis. We ligated the resultant mixture of PCR products into the plasmid vector, transformed E. coli, isolated eight clones, and completely sequenced them. Of the eight clones, four were GROβ and four were GROy. The GROα sequence was identified in a clone generated by the GROα-specific primers (Fig. 2, underline). Thus LPS-stimulated human AMs transcribe the mRNAs for all three GRO subfamily members, but GROβ and GROy appear to predominate.

Northern analysis. To compare the relative quantities of C-X-C chemokine mRNA accumulation, human AMs were incubated in the presence and absence of LPS, and the RNA was isolated after 2, 4, and 8 h (Fig. 3). LPS increased the steady-state levels of mRNA...
for all three C-X-C chemokines, but the relative amount of IL-8 mRNA at each time was greater than that of either GRO or ENA-78. Signals for each of the C-X-C chemokines were detectable at 2 h and persisted for 8 h. Non-LPS-stimulated cells also produced signals for all three C-X-C chemokines. Endotoxin levels in the non-LPS-stimulated AM supernatants were undetectable (<0.1 ng/ml). Because the cells were incubated in the absence of serum, augmentation of the effect of low levels of LPS by LPS binding protein was not a factor (27). Stimulation of AMs by adherence is the likely mechanism of activation of the non-LPS-stimulated AM supernatants.

Fig. 1. Amplification of interleukin (IL)-8, 78-amino acid epithelial cell-derived neutrophil activator (ENA-78), growth-related protein (GRO), and neutrophil-activating peptide-2 (NAP-2) signals by PCR. PCR products were amplified with primers specific for IL-8, ENA-78, GRO (generic), and NAP-2. Template RNA consisted of either lipopolysaccharide (LPS)-stimulated human alveolar macrophage (AM) RNA (lanes 1–8) or platelet-enriched peripheral blood cell RNA (lanes 9 and 10) that had (+) or had not (−) been reverse transcribed (R.T.). Nos. on left and right, size markers (in bp). Bands of the expected size were produced from AM RNA with primers for IL-8, ENA-78, and GRO (lanes 1, 3, and 5) but not with primers for NAP-2 (lane 7). The same primers for NAP-2 amplified a signal of the expected size from peripheral blood RNA (lane 9). No signals were detected in absence of reverse transcriptase (lanes 2, 4, 6, 8, and 10).

Fig. 2. Strategy for identifying the GRO subfamily cDNAs by PCR cloning and sequencing. Open reading frame (uppercase letters), flanking noncoding cDNA (lowercase letters), and deduced amino acid sequences (italic letters) of GROa, GROβ, and GROγ are shown. Sequence of upstream sense primer (Common GRO + Primer) and binding site of downstream antisense primer (Common GRO − Primer) for PCR are enclosed in boxes. Positions of PCR primers designed to be specific for GROa are underlined. The 13 positions where GRO subfamily sequences diverge are indicated by w (between common GRO primers), and divergence in deduced amino acids is indicated by showing each residue for GROa, GROβ, and GROγ in order. Numbering of base positions begins with start codon and ends with stop codon. Base sequences derived from sequence analysis are shown in bold italic letters, and they were 100% identical with reported sequences.
cells as previously described (12, 21). Thus LPS stimulates AMs to produce mRNA for all three C-X-C chemokines in a time-dependent manner.

Characterization of PMN chemotactic activity and C-X-C chemokine protein concentrations in human AM supernatants. We measured the time-dependent accumulation of PMN chemotactic activity in supernatants of LPS-stimulated AMs (Fig. 4A). Although a low level of chemotactic activity was detectable in unstimulated samples, LPS stimulated the release of significant additional PMN chemotactic activity. PMN chemotactic activity was detectable at 2 h and continued to accumulate for up to 24 h of incubation with LPS. The amount of chemotactic activity beyond 4 h was greater than that seen with a simultaneously assayed sample of 10% ZAS.

We also measured the time- and LPS-dependent accumulation of the individual C-X-C chemokines in the same supernatants by ELISA. As predicted by the steady-state mRNA levels, LPS augmented the accumulation of IL-8, ENA-78, and GRO proteins in the cell supernatants in a time-dependent manner (Fig. 4, B–D). IL-8 was the predominant C-X-C chemokine released at all times (Fig. 5). After 4 h, the relative concentrations were IL-8 > ENA-78 > GRO. At 24 h, the concentration of IL-8 was 12-fold greater than that of ENA-78 and 55-fold greater than that of GRO. The

![Figure 3](http://ajplung.physiology.org/) Time course and LPS inducibility of C-X-C chemokine mRNA in AMs. Human AMs were incubated in presence and absence of 1 µg/ml of LPS for 2, 4, or 8 h in vitro. Total RNA was isolated, and aliquots were electrophoresed in agarose in triplicate for transfer to 3 separate nylon blots. Each blot was hybridized with a radiolabeled cDNA probe specific for either IL-8, GRO, or ENA-78. A: phosphorimager scans. Migration positions of 28S and 18S rRNA bands are indicated. To clearly demonstrate time- and LPS-dependent effects on mRNA levels, display intensities of the 3 blots are different. IL-8 blot is similar to a 4-h autoradiogram, GRO blot is similar to a 12-h autoradiogram, and ENA-78 blot is similar to a 24-h autoradiogram. Blots were then hybridized with radiolabeled cDNA probe for 28S rRNA and scanned again (C). To allow for comparisons of relative mRNA quantities among probes, B displays original phosphorimage counts normalized for both lane loading and probe specific activity (see MATERIALS AND METHODS). y-Axis is a linear scale, and range is the same for each, starting at 0. Specific activity of IL-8 probe was $8.6 \times 10^6$ counts·min$^{-1}$·pmol$^{-1}$, GRO probe was $5.3 \times 10^6$ counts·min$^{-1}$·pmol$^{-1}$, and ENA-78 probe was $5.5 \times 10^6$ counts·min$^{-1}$·pmol$^{-1}$. Data are representative of experiments from 2 cell donors.

![Figure 4](http://ajplung.physiology.org/) Time course of accumulation of total chemotactic activity and C-X-C chemokine protein concentrations. Human AMs were incubated in presence (●) and absence (■) of 1 µg/ml of LPS for 2, 4, 8, or 24 h in vitro. Cell supernatants were collected and assayed for neutrophil (PMN) chemotactic activity (A). Each chemotaxis data point is mean ± SE of 3 assays performed in quadruplicate. IL-8 (IL-8; B), ENA-78 ([ENA-78]; C), and GRO ([GRO]; D) concentrations were measured by ELISA assays performed in duplicate. Results from a single macrophage donor are shown. Data are representative of experiments on macrophages from 3 donors.
concentration of ENA-78 was fivefold greater than that of GRO.

Comparison of chemotaxis dose response of C-X-C chemokines. rhIL-8 (72-amino acid form), rhENA-78, rhGROα, rhGROβ, and rhGROγ each have chemotactic activity for PMNs (Fig. 6). However, the total number of PMNs migrating toward each of the C-X-C chemokines at any given concentration was greatest for IL-8. This comparative relationship was seen in both chemotaxis assay systems. This suggests that, in vitro, IL-8 is a considerably more effective chemoattractant for PMNs than is ENA-78 or any of the GRO subfamily members, particularly when the concentrations of the C-X-C chemokines that are seen in LPS-stimulated AM supernatants are compared. For example, in supernatants from AMs stimulated with LPS for 24 h, the average measured quantity of IL-8 was 180 ng/ml (23 nM), of ENA-78 was 15 ng/ml (1.9 nM), and of GRO was 3.3 ng/ml (0.4 nM) (mean of 3 experiments; Fig. 5). By interpolation of these values onto the dose-response curves in Fig. 6, IL-8 would be expected to contribute the vast majority of the chemotactic activity.

Inhibition of chemotactic activity by C-X-C chemokine-specific antibodies. Using polyclonal antibodies specific for IL-8, ENA-78, and GRO, we found that antibody concentrations of 100 µg/ml were sufficient to inhibit the PMN chemotactic activity in solutions containing 300 ng/ml concentrations of the respective recombinant chemokines (data not shown). We then measured PMN chemotactic activity in supernatants of human AMs stimulated for 24 h with LPS. Chemotaxis was measured in the presence and absence of all combinations of the chemokine-specific antibodies and nonimmune IgG (Fig. 7). We found that the PMN chemotactic activity in these AM supernatants was significantly blocked whenever the IL-8 antibody was present (P < 0.05). In contrast, antibodies to ENA-78 and GRO had no inhibitory effect.

DISCUSSION

Our goals were to identify the spectrum and quantitatively compare the C-X-C chemokines produced by LPS-stimulated human AMs and to define the relative
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contribution of each to the total chemotactic activity released from AMs. We found that LPS-stimulated human AMs transcribe the genes for IL-8, ENA-78, GROα, GROβ, and GROγ but not for NAP-2. The secretion of the corresponding proteins is time and stimulus dependent. Immunoreactive levels of IL-8, ENA-78, and GRO are detectable in the cell supernatants by 2-4 h, but they accumulate in markedly different quantities over 24 h, with IL-8 >> ENA-78 >> GRO. In addition, we compared each of these chemokines in vitro PMN chemotaxis. At the concentrations measured in AM supernatant, we found that IL-8 is a considerably more effective chemoattractant for PMNs than the other AM-derived C-X-C chemokines. Finally, the PMN chemotactic activity in these cell supernatants was blocked by antibodies to IL-8, whereas antibodies to ENA-78 and GRO had no effect. Thus in addition to being released in considerably higher quantities, IL-8 is a more effective PMN chemoattractant than either ENA-78 or GRO, and IL-8 is responsible for virtually all of the PMN chemotactic activity accumulating in LPS-stimulated AM supernatants by 24 h.

AMs are known to produce a spectrum of C-X-C chemokines. IL-8 has been well characterized as a product of human AMs (36, 37). A homologue of ENA-78 has been identified from bovine AMs (2). We now report its production and its inducibility by LPS in human AMs. However, not all of the chemotactically active C-X-C chemokines are produced by AMs. We detected no transcription of the NAP-2 gene in AMs. The precursor protein of NAP-2 appears to derive solely from platelets, although macrophage products can convert the precursor protein into active NAP-2 (39).

Various members of the GRO subfamily of proteins have also been identified as products of human AMs, but there has been controversy regarding which of the subfamily members are produced under conditions of LPS stimulation (4, 24). Because of their remarkable similarity, part of this controversy likely results from the difficulty in designing strategies that reliably distinguish the three cDNAs. PCR primers that are unique at their 3′-end can be made. However, the binding kinetics of such forced primer sequences are suboptimal, and, consequently, a negative result is difficult to interpret. Because we sequenced the PCR products, our data show unequivocally that LPS-stimulated human AMs transcribe all three GRO subfamily genes.

There may be differences in the level of expression of each of the GRO genes. We designed common GRO PCR primers that targeted base sequences of 100% identity among the three GRO cDNAs, but they amplified fragments containing 13 positions where the base sequences are unique. We cloned and completely sequenced eight such PCR clones and found four of them were GROβ and four were GROγ. Only when we used primers specific for GROα did we identify its sequence. Thus, although LPS-stimulated human AMs transcribe all three GRO gene products, it appears that GROβ and GROγ predominate.

Even though IL-8, GROα, GROβ, GROγ, and ENA-78 are all chemotactically active, we found that IL-8 is the most effective in terms of the total PMNs migrating in vitro at any given concentration. PMNs express two different C-X-C chemokine receptors, CXCR1 (also known as IL-8 receptor A) and CXCR2 (IL-8 receptor B), that recognize these ligands with discrepant binding affinities. IL-8 binds with high affinity to both of the PMN cell-surface receptors, whereas the GRO subfamily members and ENA-78 bind with high affinity only to CXCR2 (1, 5, 6, 16, 25). The two IL-8 receptors are expressed in roughly equal quantities (8) on unstimulated PMNs. However, in the presence of a stimulating ligand, PMN cell-surface expression of CXCR2 rapidly downregulates, whereas CXCR1 is rapidly recycled (within minutes), and the net cell-surface expression of CXCR1 is maintained (7). Blockade of CXCR1 substantially reduces IL-8-mediated chemotaxis (15, 33), whereas blockade of CXCR2 has minimal effect (15). Taken together, these data suggest that CXCR1 is the principal receptor for mediating IL-8-induced chemotaxis and predict IL-8 to be the most effective chemoattractant of the C-X-C chemokines.

There have been several comparisons of IL-8 with one or more other PMN chemoattractants. In each of these studies, IL-8 was a more effective chemoattractant for PMNs than for NAP-2 (26, 40), ENA-78 (40), or the GRO subfamily members (10, 24). We directly compared all five of these C-X-C chemokines in the same assay system to establish the magnitude of their differences in chemotactic efficacy. We found that of these five AM-derived C-X-C chemokines, IL-8 is the most effective chemoattractant for PMNs. Together with our observation that LPS-stimulated AMs produce quantities of IL-8 that are an order of magnitude higher than those of either ENA-78 or GRO, these data suggest that IL-8 is the dominant PMN chemoattractant produced by these cells.

We observed that the majority (75%) of the PMN chemotactic activity in supernatants from human AMs stimulated for 24 h with LPS was inhibitable by a polyclonal antibody specific for IL-8. Sylvester et al. (37) reported a significant but incomplete reduction in PMN chemotactic activity after immunosorption of IL-8 with a monoclonal antibody. They concluded that other PMN chemoattractants were contributing. There are minimal levels of leukotriene B4 in 24-h supernatants of LPS-stimulated AMs (34), excluding it as a significant contributor. Other PMN chemoattractants that we did not measure exist, including granulocyte chemotactic protein-2 (32) and a 45-kDa chemoattractant produced by pleural macrophages (18) and mesothelial cells (14) that remains uncharacterized. We have now shown that the PMN chemotactic activity remaining after IL-8 blockade is not further inhibited by antibodies to either ENA-78 or GRO. This suggests the possibility that human AMs produce other chemoattractants that contribute to the total chemotactic activity in 24-h supernatants. We have also shown that antibodies to neither ENA-78 nor GRO affect the total PMN chemotactic activity measured. Thus, despite the presence of detectable levels of ENA-78 and GRO, the
quantities present are insufficient to contribute significantly to the total PMN chemotactic activity.

For these in vitro studies, we selected a concentration of endotoxin (1 µg/ml) that is clinically relevant. Previously, Martin et al. (28) reported endotoxin concentrations in bronchoalveolar lavage fluid (BALF) from patients with acute respiratory distress syndrome (ARDS) and from patients with pneumonia that were as high as 1–100 ng/ml, with an average concentration of 0.1 ng/ml. Because bronchoalveolar lavage in humans has been reported to dilute lung fluid by ~100-fold (31), we estimate the lung fluid concentrations of endotoxin in these patients to be as high as 0.1–10 µg/ml, with an average concentration of 10 ng/ml. Similarly, animals with E. coli pneumonia may have similar concentrations of endotoxin in their lungs (9).

We studied six rabbits given an intratracheal dose of live E. coli (1 x 10^8 colony-forming units/ml) that was sufficient to produce a sustained pneumonia (bacteria were cleared in animals given lower inocula). BALF obtained 24 h later showed a median endotoxin concentration of 12 ng/ml. Although it is difficult to know how much the lavage process diluted the original lung fluid in these animals, it is likely on the same order of magnitude as the 100-fold dilution seen in humans (31). We would therefore estimate endotoxin concentrations in the lung fluids of these animals to be 1.2 µg/ml, similar to the stimulating conditions studied in this paper.

The AM is one of the first cells in the alveolar space to encounter and respond to injurious stimuli. However, other cells in the alveolar environment can also produce C-X-C chemokines, particularly in disease states. In situ hybridization in animal trachea and bronchi stimulated by live Pseudomonas or with Pseudomonas culture supernatants demonstrates IL-8 mRNA expression in surface epithelial and gland duct cells as well as in recruited PMNs (17). In situ hybridization in lung sections of rabbits with E. coli pneumonia demonstrates GRO mRNA expression in bronchial epithelial cells as well as in parenchymal inflammatory leukocytes (19). Immunohistochemistry for ENA-78 in animal lungs with bronchopneumonia demonstrates that type II epithelial cells and endothelial cells are the predominant source of ENA-78 (2). Because of the comparatively large number of epithelial and endothelial cells present in the lungs, our data studying isolated macrophage supernatants may not reflect the relative quantities of C-X-C chemokines that are present in the lungs of patients with disease. In vivo or under different culture conditions, the profile of LPS-stimulated C-X-C chemokines may differ significantly from that observed here. For example, in contrast to our observations in AM supernatants, patients with ARDS have levels of ENA-78 in BALF that are higher than those of IL-8 throughout the course of the disease (13). Nevertheless, the majority of the PMN chemotactic activity in BALF from patients on day 3 of ARDS was blocked with antibodies to IL-8. The differences in chemotactic efficacy of the C-X-C chemokines that we describe here help explain why IL-8 was found to be the principal PMN chemoattractant in the BALF from patients on day 3 of ARDS (13).

This example highlights the importance of measuring the relative contribution of an individual chemokine to the total chemotactic activity in a biological fluid, in addition to measuring its absolute quantity. The data presented here demonstrate that despite the spectrum of C-X-C chemokines that are produced by endotoxin-stimulated human AMs, the dominant neutrophil chemoattractant is IL-8. IL-8 may be a rational single cytokine to target when the goal is to limit PMN emigration in the lungs.

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Address for reprint requests: R. B. Goodman, Pulmonary and Critical Care Section, Seattle VA Medical Center, 111B, 1660 S. Columbian Way, Seattle, WA 98108.

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