Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung.

T J Standiford, … , J Westwick, R M Strieter

J Clin Invest. 1990;86(6):1945-1953. https://doi.org/10.1172/JCI114928.

Research Article

Cellular constituents of the alveolar-capillary wall may be key participants in the recruitment of polymorphonuclear leukocytes to the lung through the generation of the novel neutrophil chemotactic peptide interleukin-8 (IL-8). This interaction appears to occur via the ability of human alveolar macrophage (AM)-derived monokines, tumor necrosis factor (TNF), and interleukin-1 (IL-1) to induce gene expression of IL-8 from pulmonary type II-like epithelial cells (A549). Northern blot analysis demonstrated that steady-state IL-8 mRNA expression, by either TNF- or IL-1 beta-treated A549 cells, occurred in both a dose- and time-dependent fashion. Similarly, extracellular antigenic IL-8, as assessed by specific ELISA, was expressed from TNF- or IL-1 beta-stimulated epithelial cells in a time-dependent fashion with maximal IL-8 antigen detected at 24 h poststimulation. Immunohistochemical staining utilizing rabbit anti-human IL-8 antibody identified immunoreactive, cell-associated IL-8 antigen as early as 8 h post-TNF or IL-1 beta stimulation. A549-generated neutrophil chemotactic bioactivity paralleled IL-8 steady-state mRNA levels. Signal specificity was demonstrated in this system as IL-8 mRNA or protein expression by lipopolysaccharide (LPS)-treated A549 cells was not different from unstimulated cells. Although LPS did not serve as a direct stimulus for the production of IL-8 by type II-like epithelial cells, the condition media from LPS-challenged AM induced a significant expression of IL-8 mRNA by the A549 cells. 24-h conditioned media from LPS-treated cells was as potent […]

Interleukin-8 Gene Expression by a Pulmonary Epithelial Cell Line

A Model for Cytokine Networks in the Lung

Theodore J. Standiford,* Steven L. Kunkel, Michael A. Basha,‡ Stephen W. Chensue, Joseph P. Lynch III,* Galen B. Toews,* John Westwick,§ and Robert M. Strieter*

*University of Michigan Medical School Departments of Pathology and *Internal Medicine, Division of Pulmonary and Critical Care Ann Arbor, Michigan 48109-0602; ‡Henry Ford Hospital, Division of Pulmonary and Critical Care, Detroit, Michigan 48202; §Department of Pharmacology, Hunterian Institute, Royal College of Surgeons, London, United Kingdom*

Abstract

Cellular constituents of the alveolar-capillary wall may be key participants in the recruitment of polymorphonuclear leukocytes to the lung through the generation of the novel neutrophil chemotactic peptide interleukin-8 (IL-8). This interaction appears to occur via the ability of human alveolar macrophage (AM)–derived monokines, tumor necrosis factor (TNF), and interleukin-1 (IL-1) to induce gene expression of IL-8 from pulmonary type II–like epithelial cells (A549). Northern blot analysis demonstrated that steady-state IL-8 mRNA expression, by either TNF- or IL-1β–treated A549 cells, occurred in both a dose- and time-dependent fashion. Similarly, extracellular antigenic IL-8, as assessed by specific ELISA, was expressed from TNF- or IL-1β–stimulated epithelial cells in a time-dependent fashion with maximal IL-8 antigen detected at 24 h poststimulation. Immunohistochemical staining utilizing rabbit anti–human IL-8 antibody identified immunoreactive, cell-associated IL-8 antigen as early as 8 h post-TNF or IL-1β stimulation. A549-generated neutrophil chemotactic bioactivity paralleled IL-8 steady-state mRNA levels. Signal specificity was demonstrated in this system as IL-8 mRNA or protein expression by lipopolysaccharide (LPS)–treated A549 cells was not different from unstimulated cells. Although LPS did not serve as a direct stimulus for the production of IL-8 by type II–like epithelial cells, the condition media from LPS-challenged AM induced a significant expression of IL-8 mRNA by the A549 cells. 24-h conditioned media from LPS-treated cells was as potent as either IL-1β or TNF in generating steady-state IL-8 mRNA by A549 cells. Preincubation of LPS-treated AM-conditioned media with anti–human TNF or IL-1β neutralizing antibodies resulted in significant abrogation of IL-8 gene expression by A549 pulmonary epithelial cells. These findings demonstrate potential cell-to-cell communication circuits that may be important between AMs and pulmonary epithelial cells during the recruitment phase of acute lung inflammation. (*J. Clin. Invest.* 1990. 86:1945–1953.) Key words: interleukin-8 • monokine • tumor necrosis factor

Address correspondence to Steven L. Kunkel, Ph.D., Department of Pathology, M-5214 Medical Science I, Box 0602, University of Michigan, Ann Arbor, MI 48109-0602.

Received for publication 23 January 1990 and in revised form 8 August 1990.

J. Clin. Invest.

0021-9738/90/12/1945/09 $2.00
Volume 86, December 1990, 1945–1953

Introduction

Sequestration of polymorphonuclear leukocytes (PMN)[1] within the lung is characteristic of a number of acute and chronic pulmonary diseases (1–3). The presence of PMNs is determined by the local generation of chemotactic agents, which direct PMN migration from the vascular compartment to the alveolar space along established chemotactic gradients. Although an array of chemotactic factors have been isolated from the lung, none have shown leukocyte specificity (2, 4, 5). Furthermore, the cellular origins of these chemoattractants have not been fully elucidated. Substantial investigation has focused on the human alveolar macrophage (AM) as a primary source of these chemotactic factors (6–8). Recently, however, neutrophil chemotactic activity has been found to be produced by endothelial cells (9), fibroblasts (10), and pulmonary epithelial cells (11–13). While the AM may be a principal cell in orchestrating the elicitation of PMN to the air space, other cellular constituents of the alveolar-capillary wall may participate in mediating this recruitment event.

Recently, a polypeptide with specific neutrophil chemotactic activity, interleukin-8 (IL-8), has been cloned (14) and isolated from a number of cells including: peripheral blood monocytes (15, 16), endothelial cells (9), fibroblasts (10), and synovial cells (17). Stimulus specificity for the elaboration of this cytokine has been demonstrated using both exogenous and endogenous stimuli. Mononuclear phagocytes and endothelial cells have the ability to generate IL-8 in response to an exogenous stimulus such as lipopolysaccharide (LPS). In contrast, a host-generated stimulus, such as tumor necrosis factor (TNF) or interleukin-1 (IL-1), is required for the production of fibroblast and synovial cell-derived IL-8. This latter interaction is demonstrative of cytokine-networking, whereby one cell population is dependent upon mediators synthesized by a neighboring cell. This interaction may be exemplified in the lung where the alveolar space is in contact with the ambient environment, which may provide the exogenous stimulus (e.g., bacterial products) for AM-derived cytokines. These polypeptide mediators may then act in a paracrine fashion to stimulate nonimmune cellular components of the alveolar-capillary wall to produce additional effector molecules. In this study we demonstrate that stimulated pulmonary epithelial-like cells possess effector activity in the inflammatory process by generating IL-8. Furthermore, cellular communication between

1. *Abbreviations used in this paper:* AM, alveolar macrophage; FMLP, formylmethionyleucylphenylalanine; IL, interleukin; LPS, lipopolysaccharide; PMN, polymorphonuclear; TNF, tumor necrosis factor.

A549 pulmonary epithelial cells and AMs appear to operate via the generation of AM-derived TNF and IL-1, as these cytokines are essential for the production of epithelial cell-derived IL-8.

Methods

Reagent preparation. Human recombinant IL-1α and IL-1β with a specific activity of 20 U/ng and 30 U/ng, respectively, and murine antihuman monoclonal IL-1β antibodies were the generous gift of the Upjohn Co. (Kalamazoo, MI). Human recombinant TNF with a specific activity of 22 U/ng was kindly provided by the Cetus Corp. (Berkeley, CA). Human recombinant IL-8 was the generous gift of Sandoz Pharmaceutical Co. (Hanover, NJ). Polyclonal antihuman TNF and IL-8 antiserum were produced by immunization of rabbits with recombinant TNF or IL-8 in multiple intradermal sites with complete Freund's adjuvant. IL-8 antisera, in concentrations used in the ELISA or immunohistochemical localization of antigenic IL-8, reacted with recombinant IL-8 in Western blot analysis, and failed to cross-react with CTAP-III or platelet factor 4. Polyclonal antihuman TNF utilized in this study was capable of neutralizing 10 U of TNF at a dilution of 1:25,625, whereas 1 μg of the murine antihuman monoclonal IL-1β antibody was capable of inhibiting 95% of the IL-1β-induced production of IL-2 by 1A5 murine T cells. Efficacy of neutralizing antihuman IL-8 antibodies has been previously reported (10). Serial dilutions of pyrogen free cytokines were prepared in RPMI 1640 (Whittaker Bio products, Walkersville, MD) containing 1 mM glutamine, 25 mM Hepes, 100 U/ml penicillin 100 ng/ml streptomycin (Hazleton Biologics, Inc., St. Lenexa, KS) (complete media). Stock LPS (*E. coli* 0111:B4, Sigma Chemical Co., St. Louis, MO) was prepared at a concentration of 200 μg/ml in complete media.

Preparation of A549 pulmonary epithelial cells. A549 pulmonary epithelial cells (American Type Culture Collection, Rockville, MD) are a cell line derived from a patient with alveolar cell carcinoma of the lung. These cells retain features of type II alveolar epithelial cells, including cytoplasmic multilamellar inclusion bodies and the synthesis of surfactant (18). The A549 cell line was used in these studies since human type II cells are both difficult to obtain in pure culture and are often contaminated with resident mononuclear phagocytic cells. Human A549 pulmonary epithelial cells were grown to confluency on 100 mm petri dishes (Corning Glass Works, Corning, NY) in complete RPMI 1640, plus 10% FCS. On the day of use, A549 monolayers were washed free of FCS with complete media and either cytokines or LPS was added for the specified times and doses. Cell-free supernatants were collected, and total cellular RNA was extracted as described below.

Recovery and isolation of human alveolar macrophages (AM) and generation of conditioned media. Five normal, nonsmoking volunteers agreed to undergo flexible fiberoptic bronchoscopy with bronchoalveolar lavage (BAL) by standard techniques (19). The subjects had no evidence of recent respiratory tract infection in the preceding 6 wk and were not taking any medications. The recovered BAL fluid/cell suspension was filtered through gauze and centrifuged at 600 *g*. The cells were resuspended in complete media and washed three times. Total cell count per BAL was $33\pm4 \times 10^6$ and cell differential revealed 92±3% AM, 7±3% lymphocytes, and 1±0.5% PMN. Lavaged cells were > 95% viable as assessed by trypan blue exclusion. Freshly isolated human AM were plated on 100-mm culture plates at a concentration of 10×10^6 AM/plate. LPS at a concentration of 10 μg/ml was added to some plates, while the remainder were unstimulated. Conditioned media was collected after a 24-h incubation period at 37°C. 24-h AM-conditioned media (LPS-stimulated and -unstimulated) was placed over A549 monolayers in 60-mm culture plates. Total A549 cellular RNA was isolated at 4 h and Northern blot analysis for IL-8 mRNA performed. In neutralizing studies, LPS-stimulated AM-conditioned media was preincubated for 30 min with either (*a*) rabbit polyclonal antihuman TNF antiserum or control preimmune rabbit polyclonal serum (1:100 dilution), (*b*) murine monoclonal antihuman IL-1β antibody or control murine IgG (Sigma Chemical Co.) (75 μg/ml), or (*c*) both anti-TNF and anti-IL-1β antibodies or the combination of controls. Antibody-treated conditioned media was placed over A549 monolayers and RNA isolated at 4 h as described below.

Northern blot analysis. Total cellular RNA from A549 cells was isolated using a modification of Chirgwin et al. (20) and Jonas et al. (21). Briefly, A549 cell monolayers were scraped into a solution of 25 mM Tris, pH 8.0, containing 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, the above suspension was added to an equal volume of 100 mM Tris, pH 8.0, containing 10 mM EDTA and 1.0% SDS. The mixture was then extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was alcohol-precipitated and the pellet dissolved in 10 mM Tris, 0.1 mM EDTA (TE) buffer with 0.1% Sarkosyl. RNA was separated by Northern blot analysis using formaldehyde, 1% agarose gels, transblotted to nitrocellulose, baked, prehybridized, and hybridized with a ^{32}P 5′ end-labeled oligonucleotide probe. A 30-mer oligonucleotide probe was synthesized using the published cDNA sequence for human-derived NCF/IL-8 (22). The probe was complementary to nucleotides 262–291 and had the sequence 5′-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3′. Blots were washed and autoradiographs were quantitated using laser densitometry (Ultrascan XS, LXB Instruments, Inc., Houston, TX). Equivalent amounts of total RNA/gel were assessed by monitoring 28S and 18S rRNA.

Immunohistochemistry. Immunolocalization of IL-8 was performed by fixing A549 cell monolayers in 4% paraformaldehyde in PBS for 10 min, then rinsing twice with PBS. Before staining, the slides were treated for 15 min in 1:1 absolute methanol and 3% H_2O_2, rinsed in PBS then nonspecific binding sites were blocked with a 1:50 dilution of normal goat serum. Normal serum was removed, followed by the addition of 1:2,000 concentration of either control (rabbit) or rabbit antihuman IL-8 serum. After 15 min of incubation at 37°C, the slides were rinsed with PBS, overlaid with biotinylated goat antirabbit IgG (1:200; Vector Laboratories Inc., Burlingame, CA), incubated 15 min, and rinsed two times with PBS. The slides were treated with Streptavidin conjugated to peroxidase for 15 min at 37°C, rinsed three times, overlaid with substrate chromogen (3-amino 9-ethylcarbazole) for 7 min at 37°C to allow color development, and rinsed with distilled water. Mayer's hematoxylin was used as counterstain. To demonstrate antibody specificity, in competitive inhibition studies, immunostaining for human IL-8 showed 100% inhibition by the addition of exogenous recombinant IL-8.

Interleukin 8 ELISA. Antigenic IL-8 was quantitated using a double ligand method as previously described by Bacon et al. (23). Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F) were coated with immunosorbent purified goat anti-IL-8 antibody (0.5 μg in 100 μl 0.1 M $NaHCO_3/NaCO_3$ buffer, pH 9.6) for 16 h at 4°C and then washed four times with PBS, pH 7.5 with 0.5% Tween-20 (washing buffer). Samples and standards, both in duplicate, were added as 100-μl aliquots in washing buffer containing 2% FCS and incubated for 90 min at 37°C. Plates were then washed four times and incubated with biotinylated goat anti-IL-8 antibody (25 ng per well) for 30 min at 37°C. Plates were washed four times and incubated with avidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) for 30 min at 37°C. Plates were then washed four times and incubated with the peroxidase substrate (Bio-Rad Laboratories) at room temperature to the desired extinction, and the reaction terminated with 3% oxalic acid. Plates were read at 405 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant IL-8, from 100 ng to 30 pg per well. This ELISA method consistently detected IL-8 concentrations in a linear fashion > 300 pg/ml.

Chemotactic assay. Human neutrophils were prepared from peripheral blood by Ficoll-Hypaque density gradient centrifugation followed by sedimentation in 5% dextran in 0.9% saline (Sigma Chemical Co.). Neutrophils were separated from erythrocytes by hypotonic lysis and then suspended in HBSS with calcium/magnesium (Gibco, Grand Island, NY) at 2×10^6 cells/ml with > 95% viability by trypan blue

exclusion. Chemotaxis was performed as previously described (10). Briefly, 225 μl of diluted (1:1) supernatant specimen, 10^{-7} M formylmethionyleucylphenylalanine (fMLP, Sigma Chemical Co.) or HBSS alone was placed in duplicate bottom wells of a blind-well chemotaxis chamber. A 3-μm pore size polycarbonate filter (polyvinylpyrrolidone-free, Nucleopore Corp., Pleasanton, CA) was placed in the assembly and 333 μl of neutrophil suspension placed in each of the top wells. Chemotaxis chamber assemblies were incubated at 37°C in humidified 95% air and 5% CO_2 for 1 h. The filters were removed, fixed in methanol, and stained with 2% toluidine blue (Sigma Chemical Co.). Neutrophils that had migrated through the bottom of the filter were counted in 10 high-power fields (1,000×). Chemotactic bioactivity was standardized to and expressed as the percent of the positive control (10^{-7} fMLP). In neutralization studies, A549 24-h conditioned media was incubated with a 1:100 or 1:1,000 dilution of either control (rabbit preimmune serum) or neutralizing rabbit antihuman IL-8 antiserum for 30 min at 37°C and then assayed for chemotactic activity.

Statistical analysis. Data are expressed as means±SEM. Groups of data were evaluated by analysis of variance (ANOVA). Data that appeared statistically significant were compared by Student's *t* test for comparing the means of multiple groups, and considered significant if *P* values were < 0.05.

Results

A549 cell-derived chemotactic activity. Confluent A549 cell monolayers were stimulated at time 0 with either 20 ng/ml TNF, 20 ng/ml IL-1β, or 10 μg/ml LPS and assessed for the time-dependent generation of chemotactic activity. At specific time intervals, supernatants were harvested and analyzed for chemotactic bioactivity. As depicted in Fig. 1, TNF-treated A549 cells yielded significant neutrophil chemotactic activity as early as 1 h posttreatment, plateauing by 8 h, with maximal neutrophil chemotactic activity seen at 24 h (64±4.2% fMLP control, $n = 3$). Likewise, IL-1β-treated cells generated significant neutrophil chemotactic activity as early as 2 h, the production plateaued by 4 h, with maximal chemotactic activity observed at 24 h posttreatment (41.5±3.2% fMLP control). In contrast, LPS-stimulated A549 cells generated only modest chemotactic activity that was 26±2.3% fMLP control at 24 h. Neither TNF, IL-1β, nor LPS were directly chemotactic for neutrophils in the chemotactic assays.

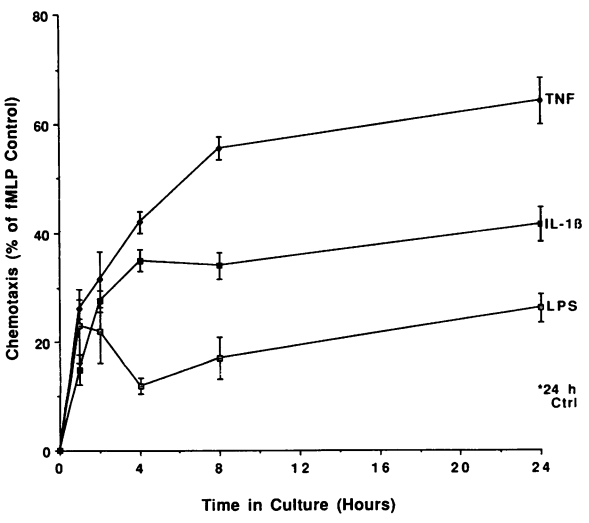

Figure 1. Neutrophil chemotactic activity of monokine-stimulated A549 pulmonary epithelial cells. Time-dependent increase in neutrophil chemotactic bioactivity from LPS, TNF- and IL-1β-stimulated A549 epithelial cells. Neutrophil chemotaxis on the ordinate is expressed as a percent of fMLP (10^{-7} M) positive control. The 24-h negative control is represented by the asterisk.

Time-dependent expression of steady-state IL-8 mRNA by A549 cells. To determine whether A549 epithelial cells expressed mRNA for IL-8, A549 cell monolayers were stimulated at time 0 with TNF (20 ng/ml), IL-1β (20 ng/ml), or LPS (10 μg/ml), and at specific time intervals total RNA was extracted. As shown in Fig. 2, IL-1β caused a rapid rise in steady-state IL-8 mRNA levels by 1 h (43±2.9% maximum, $n = 4$) with peak mRNA accummulation by 4 h (100% maximum). Continued IL-8 mRNA expression was observed at 8 h (93±2.9% maximum) and 24 h (74±7.4% maximum). TNF-treated A549 cells similarly expressed IL-8 mRNA in a time-dependent fashion, with peak steady-state mRNA levels observed between 2 h (85±8% maximum) and 4 h (73±14.4% maximum) poststimulation. IL-8 mRNA levels continued to be detected at 8 h (60±5.2% maximum) and 24 h (46±15.3% maximum) posttreatment with TNF. In contrast, LPS failed to induce expression of A549-derived IL-8 mRNA (data not shown).

Dose-dependent expression of IL-8 mRNA by A549 cells. Confluent A549 cells were stimulated for 4 h with either 20 pg/ml to 20 ng/ml of TNF, IL-1α, or IL-1β or 1 pg/ml to 10 μg/ml LPS, then assessed for the expression of IL-8 mRNA. As

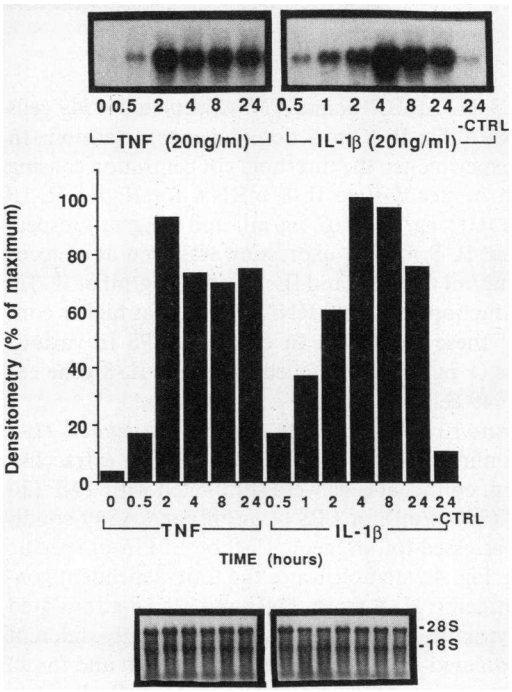

Figure 2. Kinetic expression of IL-8 mRNA from TNF- and IL-1β-treated A549 epithelial cells. Northern blot analysis of IL-8 mRNA expression (*top*), laser densitometry of respective Northern blots (*middle*), and 28 S and 18 S ribosomal RNA (*bottom*). Steady-state IL-8 mRNA levels increased in a kinetic fashion with maximal gene expression post TNF (20 ng/ml) and IL-1β (20 ng/ml) stimulation occurring at 2 and 4 h, respectively, and continued for the next 20 h. Data are represented as the percent of maximum response (4-h time point, IL-1β). The data depicted is representative of three individual experiments.

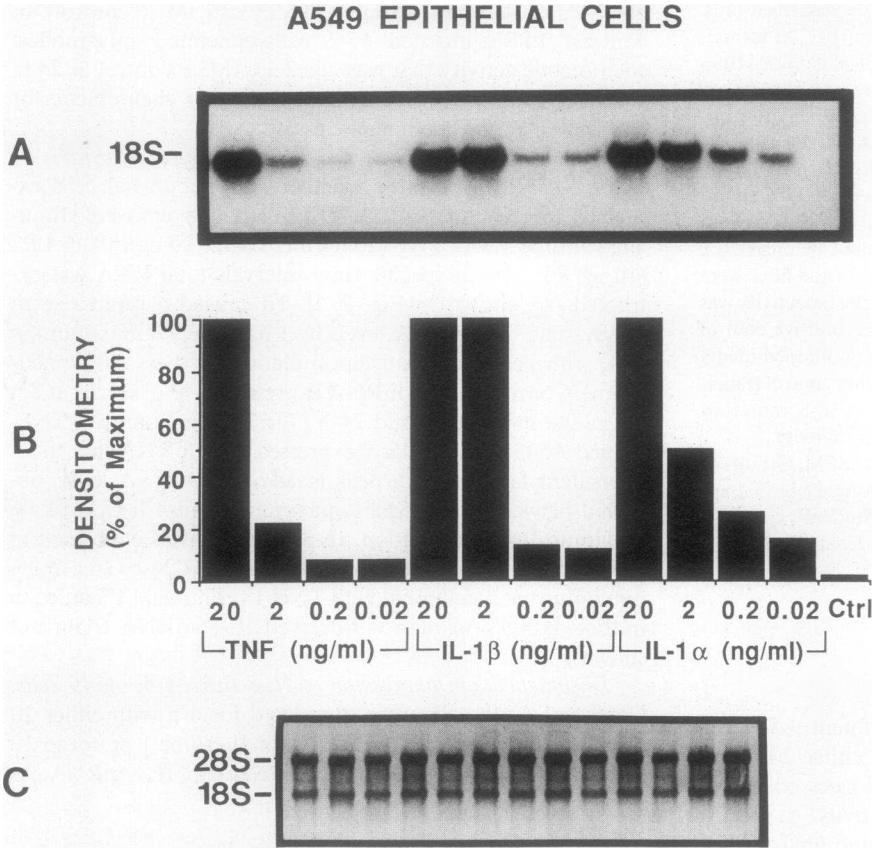

Figure 3. Expression of A549-derived IL-8 mRNA in response to graded doses of TNF, IL-1β, or IL-1α. A549 epithelial cells treated with graded doses of TNF, IL-1β, or IL-1α (20 pg-20 ng/ml) demonstrated a dose-dependent expression of IL-8 mRNA at 4 h. A plateau in IL-8 mRNA expression was observed above a dose of 20 ng/ml for all three monokines. The threshold concentration for the induction of IL-8 gene expression by TNF, IL-1β, and IL-1α was 2 ng/ml, 0.02 ng/ml, and 0.02 ng/ml, respectively. The data shown are representative of three individual studies. *Ctrl,* control.

shown in Fig. 3, IL-1α, IL-1β, and TNF-stimulated A549 cells expressed mRNA for IL-8 in a dose-dependent fashion. In each of three experiments, the threshold concentration causing the expression of steady-state IL-8 mRNA for IL-1α, IL-1β and TNF was 0.02 ng/ml, 0.02 ng/ml, and 2 ng/ml, respectively. Maximal IL-8 mRNA expression was seen at concentrations of 20 ng/ml for TNF and IL-1α, and 2 ng/ml of IL-1β. No further induction of IL-8 mRNA was seen at higher concentrations of these cytokines. In contrast, LPS in various concentrations (1 pg to 10 μg) failed to induce IL-8 gene expression by A549 cells.

Demonstration of extracellular and cell-associated IL-8 antigen. To confirm the presence of A549-derived extracellular IL-8 protein, epithelial cells were stimulated with TNF (20 ng/ml), IL-1β (20 ng/ml), or LPS (1 μg/ml) and A549-conditioned media assessed for antigenic IL-8 by ELISA at specific time intervals. Fig. 4 *I* demonstrates the time-dependent generation of antigenic IL-8 from TNF and IL-1β-stimulated A549 cells. Cytokine induced IL-8 was significantly different from LPS-stimulated cells ($P < 0.05$, $n = 3$) by 4 h and for all subsequent time points to 24 h poststimulation. Peak extracellular levels of IL-8 were observed at 24 h. At this time point conditioned media from either TNF, IL-1β, or LPS-stimulated and unstimulated A549 cells were assessed for antigenic IL-8 ($n = 5$). As shown in Fig. 4 *II,* untreated and LPS-treated cells produced 4.9±0.5 ng/ml and 5.1±0.2 ng/ml IL-8 ($P = 0.65$), respectively. In contrast, TNF-stimulated epithelial cells generated 29.1±4.7 ng/ml of IL-8, a fivefold rise over untreated or LPS-treated cells ($P < 0.001$). IL-1β-stimulated A549 cells produced 33.9 ng/ml of IL-8, which was not significantly different from TNF stimulation ($P = 0.087$), but dramatically different from LPS-stimulated or unstimulated cells ($P < 0.01$). Immunohistochemical analysis was performed to confirm the presence of cell-associated antigenic IL-8 protein. A549 cells were cultured in Labtek chambers (25,000 cells/well) and treated with 20 ng/ml of IL-1β or TNF at time 0. At specific time intervals, the cells were fixed and immunostained for antigenic IL-8. Maximal IL-8 reactivity by either IL-1β or TNF stimulated A549 cells occurred at 24 h, with 38 and 36% of cells demonstrating cell-associated IL-8 antigen, respectively. No immunoreactive IL-8 was present in unstimulated A549 cells. Fig. 4 *III* is a representative photomicrograph of the immunohistochemical localization of A549 cell-derived antigenic IL-8 at 24 h. Fig. 4 *IIIA* is control (untreated) demonstrating the absence of specific antigenic IL-8, whereas Fig. 4 *IIIB* represents IL-1β-treated (20 ng/ml) A549 cells showing cell-associated antigenic IL-8. Both Fig. 4 III *A* and *B* were incubated with immune sera (1:2,000 dilution). TNF stimulation (20 ng/ml) resulted in a similar staining pattern as that seen in Fig. 4 *B* (picture not shown). The immunolocalization was specific for A549-derived IL-8, as 100% inhibition of immunostaining occurred in the presence of exogenous recombinant IL-8.

Inhibition of A549-derived chemotactic activity by rabbit anti-human IL-8 antibody. To determine the relative contribution of IL-8 to A549-derived neutrophil chemotactic activity, 24-h conditioned media was harvested from TNF, IL-1β, LPS-stimulated and unstimulated A549 cells and preincubated with control (rabbit preimmune serum) and polyclonal rabbit anti-human IL-8 antibody (Table I). Preincubation

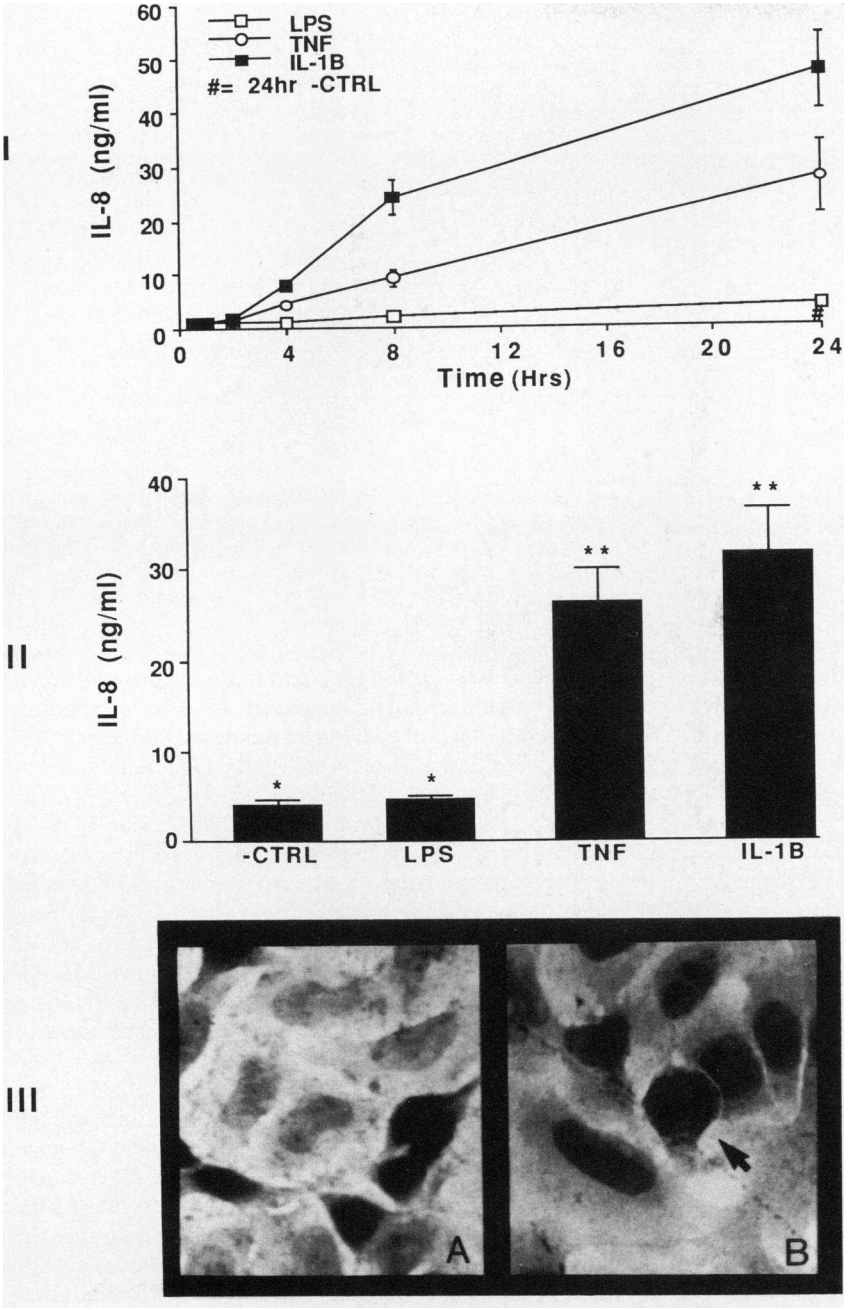

Figure 4. Expression of immunoreactive extracellular and cell-associated IL-8 by A549 pulmonary epithelial cells. *I* demonstrates the time-dependent generation of extracellular IL-8 from TNF and IL-1β-stimulated A549 cells as assessed by ELISA. *II* displays antigenic IL-8 levels from 24-h TNF- or IL-1β stimulated A549 conditioned media as compared to extracellular IL-8 derived from unstimulated or LPS-stimulated epithelial cells. *III* is a representative photomicrograph of untreated and IL-1β-treated A549 epithelial cells. (*A*) unstimulated A549 cells without detectable IL-8, and (*B*) IL-1β-stimulated A549 epithelial cells revealing the presence of immunoreactive IL-8 (as demonstrated by arrow).

with either preimmune or anti-human IL-8 antibody (1:1,000) did not significantly alter the chemotactic activity of unstimulated or LPS-stimulated A549 conditioned media as compared with control serum. In addition, these antibodies did not alter fMLP-induced chemotaxis. In contrast, the chemotactic activity of IL-1β-treated A549 conditioned media was reduced by 53% in the presence of anti-human IL-8 antibody ($P < 0.001$). TNF-stimulated A549-derived chemotactic activity was also significantly reduced by anti-human IL-8 antibody, but to a lesser extent (19% suppression, ($P < 0.01$). Also shown in Table I, anti-human IL-8 antisera treatment resulted in 49, 66, and 33% suppression of the neutrophil chemotactic activity induced by 1, 10, and 100 ng/ml of recombinant IL-8, respectively. When background chemotactic activity was subtracted, IL-8 neutralization was even more complete.

Induction of A549 cell IL-8 gene expression by LPS-stimulated human AM-condition media. Previous work in our laboratory and others have demonstrated IL-1α, IL-1β, and TNF production by LPS-stimulated human AM (2, 19, 20). To ascertain whether human alveolar macrophages could secrete a soluble factor(s) that would induce the gene expression of IL-8 from A549 epithelial cells, cell-free human AM-conditioned media (24 h LPS-stimulated or unstimulated) was incubated with A549 cell monolayers. As depicted in Fig. 5, control media alone caused no IL-8 gene expression by A549 epithelial cells, while LPS-stimulated human AM-conditioned media caused substantial IL-8 gene expression by the treated epithelial cells. Steady-state IL-8 mRNA accumulation was also expressed by A549 epithelial cells in the presence of unstimulated AM-conditioned media, but was only 19±5% ($P < 0.01$, $n = 5$) of that seen with LPS-stimulated conditioned

Table I. Neutralization of IL-8 Activity with Polyclonal Rabbit Anti-Human IL-8 Antibody

	Mean cells/HPF (1000×)			
	Control serum (1:1000)	Immune serum (1:1000)	Suppression	*P* value
			%	
24° CTRL	28.8 (17.0)	29.8 (18)	NC‡	0.8
24° IL-1B (20 ng/ml)	65.9 (54.2)	30.7 (19.0)	53 (61)	0.0001*
24° TNF (20 ng/ml)	70.6 (58.9)	57.4 (45.7)	19 (22)	0.006*
24° LPS (1 μg/ml)	39.7 (28)	42.0 (30.3)	NC‡	0.61
fMLP (10^{-7} M)	128.3 (116.6)	126.6 (114.9)	NC‡	0.858
rIL-8 (1 ng/ml)	27.9 (16.2)	14.2 (2.5)	49 (85)	0.0002*
rIL-8 (10 ng/ml)	49.6 (37.9)	16.7 (5)	66 (87)	0.0001*
rIL-8 (100 ng/ml)	76.3 (64.6)	49.2 (37.5)	36 (42)	0.0001*
HBSS	11.2	12.3	NC‡	0.465

Human A549 lung epithelial cells were treated as described, supernatant collected, and incubated with control or immune serum for 30 min at 37°C before chemotaxis analysis. Data in parenthesis represents subtraction of HBSS control. * Data are statistically significant. ‡ NC, no significant change.

media. To better define the relative contributions of the specific AM-derived monokines TNF and IL-1β, neutralization studies were performed (n = 3). LPS-stimulated AM-conditioned media was preincubated for 30 min with appropriate control antisera and/or IgG. In addition, AM-conditioned media was treated with either murine antihuman IL-1β monoclonal antibody (75 μg/ml), rabbit antihuman TNF polyclonal antibody (1:100), or both antibodies in combination and then overlaid on A549 cell monolayers. Total RNA was extracted at 4 h. As shown in Fig. 6, antihuman IL-1β or antihuman TNF antibodies alone, or in combination resulted in a 44±8%, 28±7%, and 83±4% reduction in steady-state IL-8 mRNA levels, respectively. In contrast, LPS-stimulated AM-conditioned media induced maximal A549 epithelial cell-derived IL-8 mRNA in the presence of control rabbit sera, control murine IgG, or both controls in combination (data not shown).

Discussion

The influx of neutrophils is a predominant finding in many forms of acute and chronic lung injury. Enhanced neutrophil chemotactic activity has been seen in the bronchoalveolar lavage fluid from patients with several diverse pulmonary disease states including ARDS (4), idiopathic pulmonary fibrosis (2), and asbestosis (5). The mechanism(s) by which neutrophils and other inflammatory cells are recruited to the lung remain unknown, although the local generation of chemotactic factors is essential in the initiation and evolution of this process. A number of locally generated chemotactic substances include: bacterial peptide fMLP (24), extracellular matrix components such as laminin (25), elastin, and collagen fragments (26, 27), and fibronectin (28). In addition, the AM can secrete several inflammatory cell chemoattractants including LTB_4 (29), PDGF (30), and PAF (6). Furthermore, AMs can generate TNF and IL-1 (19, 20), monokines which have been shown to stimulate IL-8 production by several nonimmune cells, including human endothelial cells (9) and fibroblasts (10).

The pulmonary epithelium, an integral component of the alveolar-capillary wall, serves multiple functions in the lungs, including maintenance of barrier-to-solvent and solute fluxes (31), synthesis of surfactant (32), and the metabolism of xenobiotics (33). In the setting of lung injury, however, the pulmonary epithelium has generally been considered a target of inflammatory cells rather than an active participant in the generation of an inflammatory response (34, 35). Recently, it has been shown that bovine bronchial epithelial cells (11) and bleomycin-treated rat type II cells (12) can generate inflammatory cell chemoattractants. Additional studies by Suzuki et al. (13) have described a neutrophil chemotactic factor generated from lung giant cell carcinoma (LU65C) that has a similar molecular weight (10,000 D) and significant amino acid homology to IL-8. We now demonstrate that A549 pulmonary epithelial cells generate chemotactic activity for neutrophils in the presence of specific monokines.

We have demonstrated the pulmonary epithelial cell–derived gene expression of IL-8 by Northern blot analysis, the production of chemotactic activity by bioassay, and the presence of antigenic extracellular and cell-associated IL-8 protein by ELISA and immunohistochemical techniques. IL-8 gene expression by A549 cells displays signal specificity in that the macrophage-derived cytokines TNF and IL-1, but not LPS, stimulate A549 cells to express mRNA for IL-8. Human fibroblasts display similar signal specificity (10), whereas monocytes, AM, and endothelial cells produce IL-8 in response to TNF, IL-1, or LPS (9). These observations suggest that an initial host response is needed to elicit the appropriate signals before A549 epithelial cells or fibroblasts can generate IL-8. In contrast, cells which are more likely to come in direct contact with a primary stimulus such as LPS (AM and endothelial cells) can express IL-8 in the absence of additional signals.

Because of the close proximity of alveolar macrophage to the pulmonary epithelium within the airspace, and the observation that A549 cells produce IL-8 only in response to specific monokines, it would appear that the AM may be critical in the induction of pulmonary epithelial cell IL-8 gene expression. To further define the role of the AM in this regard, we found that LPS-stimulated AM-conditioned media induced steady-state IL-8 mRNA by A549 pulmonary epithelial cells. This response was significantly inhibited by neutralizing the AM-

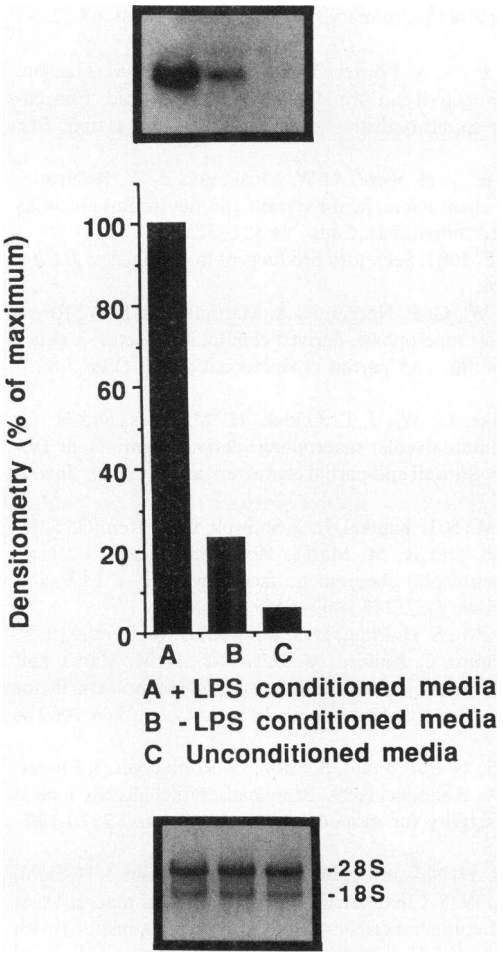

Figure 5. IL-8 gene expression by A549 epithelial cells in response to AM-conditioned media. A549 epithelial cell IL-8 gene expression posttreatment with LPS-stimulated AM-conditioned media (*A*), unstimulated AM-conditioned media (*B*), and unconditioned media (*C*). Substantial IL-8 mRNA accumulation was generated from LPS-stimulated AM-conditioned media. Treatment with unstimulated AM-conditioned media resulted in only 20% of the A549 cell-derived IL-8 mRNA levels as compared to LPS-stimulated AM-conditioned media. No A549 cell IL-8 gene expression was seen posttreatment with unconditioned media. Data are representative of five individual experiments.

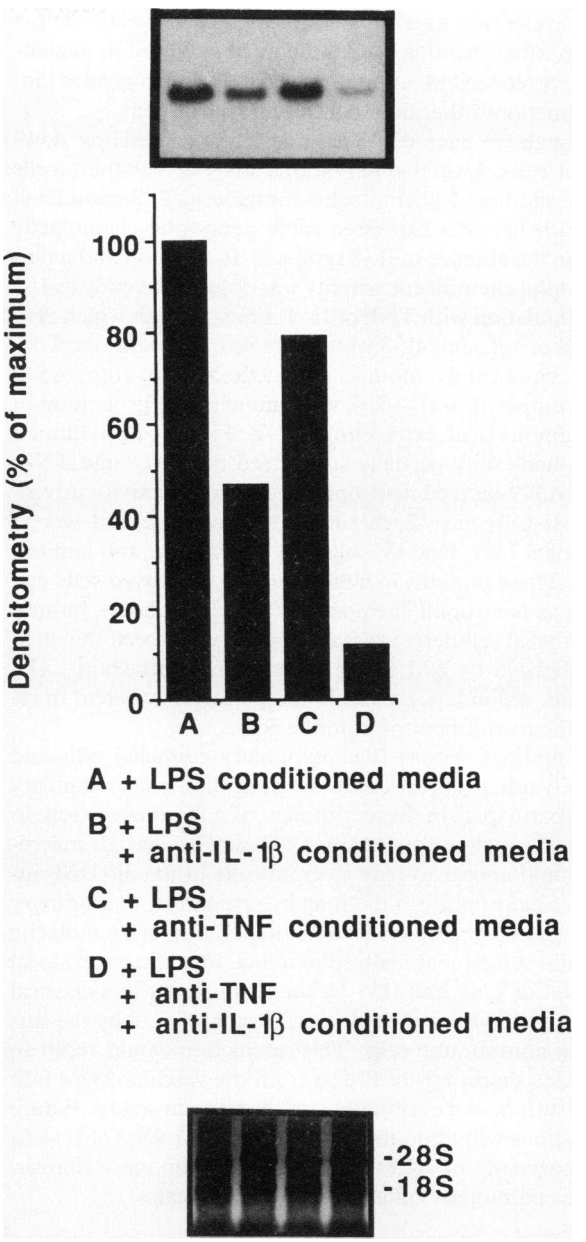

Figure 6. AM-conditioned media induced IL-8 gene expression by A549 epithelial cells in the presence and absence of TNF and IL-1β neutralizing antibodies. Northern blot analysis representing steady-state IL-8 mRNA by A549 epithelial cells 4 h postaddition of LPS-stimulated AM-conditioned media in the absence (*A*), or presence of anti-IL-1β neutralizing antibody (75 μg/ml) (*B*), in the presence of anti-TNF neutralizing antibody (1:100) (*C*), or in the presence of both anti-IL-1β and anti-TNF neutralizing antibodies (*D*). Preincubation of LPS-stimulated AM-conditioned media with anti-IL-1β or anti-TNF antibodies alone or in combination resulted in significant abrogation of steady-state IL-8 mRNA levels. Preimmune sera and/or control IgG did not alter IL-8 gene expression by LPS-stimulated AM-conditioned media nor did they alter the TNF or IL-1β-induced IL-8 gene expression from these cells (data not shown).

derived TNF and IL-1β. These findings establish direct cell-to-cell communication between alveolar macrophages and pulmonary epithelial cells via the generation of these soluble mediators.

IL-8 is a recently described peptide that has been referred to as monocyte-derived neutrophil chemotactic factor (MDNCF) (15), neutrophil-activating peptide-1 (NAP-1) (14), neutrophil chemotactic factor (NCF) (9), and most recently IL-8 (14). In addition to being a potent neutrophil chemoattractant, IL-8 also stimulates neutrophil oxygen radical generation, degranulation (36), and leukotriene synthesis (37). The active form of IL-8 is a 72–amino acid peptide with an estimated molecular weight of 8,000 D, and belongs to a unique supergene family that includes murine macrophage inflammatory peptide-2, platelet factor 4, human platelet basic protein, human inducible protein IP-10, 9E3/pCEF-4 from Rous sarcoma transformed fibroblasts, and Gro (38). Monocytes and macrophages appear to be the predominant sources of IL-8, although endothelial cells, fibroblasts, and synovial cells have also been shown to generate IL-8 (9, 10, 17). IL-8 has a relatively long half-life and is somewhat resistant to proteolytic

enzymatic cleavage as compared to other chemotactic factors (14), suggesting that this cytokine may be involved in mediating more prolonged inflammatory cell influx or may have biological functions other than neutrophil recruitment.

Although we have demonstrated IL-8 synthesis by A549 epithelial cells, several observations suggest that these cells generate additional chemotactic substances. LPS-stimulated A549 epithelial cells expressed some neutrophil chemotactic activity in the absence of IL-8 synthesis. In addition, induction of neutrophil chemotactic activity was observed as early as 1–2 h poststimulation with TNF or IL-1, time points at which very low levels of antigenic IL-8 were expressed. Furthermore, TNF induced significantly more chemotactic activity from A549 cells as compared to IL-1β, despite inducing the generation of similar amounts of extracellular IL-8. Finally, anti-human IL-8 antibody only partially suppressed the IL-1β and TNF-induced A549-derived neutrophil chemotactic activity. Alveolar type II cells have been shown to synthesize and secrete complement (39), type IV collagen, fibronectin, and laminin (40, 41). These proteins in either a native or cleaved state can function as neutrophil chemoattractants. In addition, pulmonary epithelial cell-derived arachidonic acid has been shown to be metabolized by AM to the potent chemoattractant LTB_4 (42). Thus, redundancy exists in the pulmonary system in regard to the recruitment of immune cells.

Our findings suggest that pulmonary epithelial cells and potentially other cellular components of the alveolar-capillary wall can participate in the recruitment of inflammatory cells to the lungs through the generation of IL-8. The alveolar macrophage would appear to play a central role in the directed migration of neutrophils to the lung by responding to a primary signal (e.g., LPS) which leads to the elaboration of chemotactic factors and subsequent neutrophil influx. In addition, the local generation of TNF and IL-1 by the AM can act in a classical paracrine fashion to induce the expression of IL-8 by the surrounding nonimmune cells. This interaction would result in the directed diapedesis of PMNs from the vascular space into the interstitium and eventually into the alveolar spaces. Future investigations will undoubtedly establish the role(s) of IL-8 in the pathogenesis of ARDS, sarcoidosis, pulmonary fibrosis, and other pulmonary inflammatory disease states.

Acknowledgements

We acknowledge Holly Evanoff for her cell culture expertise. We thank Peggy Weber and Suzanne Miller for their secretarial assistance.

This work was supported in part by National Institutes of Health grants HL02401, DK-38149, HL31963, HL35276, and the American Lung Association. Dr. Kunkel is an Established Investigator of the American Heart Association. Dr. Strieter is a RJR Nabisco Research Scholar.

References

1. Weiland, J. E., W. B. Davis, J. F. Holter, J. R. Mohammed, P. M. Dorinsky, and J. E. Gadek. 1986. Lung neutrophils in the adult respiratory distress syndrome. Clinical and pathophysiologic significance. *Am. Rev. Respir. Dis.* 133:218–225.

2. Hunninghake, G. W., K. C. Garrett, H. B. Richerson, J. C. Fantone, P. A. Ward, S. I. Rennard, P. B. Bitterman, and R. G. Crystal. 1984. Pathogenesis of the granulomatous lung diseases. *Am. Rev. Respir. Dis.* 130:476–496.

3. Hunninghake, G. W., J. E. Gadek, T. J. Lawley, and R. G. Crystal. 1981. Mechanisms of neutrophil accumulation in the lungs of patients with idiopathic pulmonary fibrosis. *J. Clin. Invest.* 68:S259–S269.

4. Parsons, P. E., A. A. Fowler, T. M. Hyers, and P. M. Henson. 1985. Chemotactic activity in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 132:490–493.

5. Hayes, A. A., A. H. Rose, A. W. Musk, and B. W. Robinson. 1988. Neutrophil chemotactic factor release and neutrophil alveolitis in asbestos-exposed individuals. *Chest.* 94:521–525.

6. Nathan, C. F. 1987. Secretory products of macrophages. *J. Clin. Invest.* 79:319–326.

7. Merrill, W. W., G. P. Naegel, R. A. Matthay, and H. Y. Reynolds. 1980. Alveolar macrophage-derived chemotactic factor: kinetics of in vitro production and partial characterization. *J. Clin. Invest.* 65:268–276.

8. Hunninghake, G. W., J. E. Gadek, H. M. Fales, and R. G. Crystal. 1980. Human alveolar macrophage-derived chemotactic factor for neutrophils. Stimuli and partial characterization. *J. Clin. Invest.* 66:473–483.

9. Strieter, R. M., S. L. Kunkel, H. J. Showell, D. G. Remick, S. H. Phan, P. A. Ward, and R. M. Marks. 1989. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-α, LPS, and IL-1β. *Science (Wash. DC).* 243:1467–1469.

10. Strieter, R. M., S. H. Phan, H. J. Showell, D. R. Remick, J. P. Lynch III, M. Genord, C. Raiford, M. Eskandari, R. M. Marks, and S. L. Kunkel. 1989. Monokine-induced neutrophil chemotactic factor gene expression in human fibroblasts. *J. Biol. Chem.* 264:10621–10626.

11. Koyama, S., S. I. Rennard, S. Shoji, D. Romberger, J. Linder, R. Ertl, and R. A. Robbins. 1989. Bronchial epithelial cells release chemoattractant activity for monocytes. *Am. J. Physiol.* 257:L130–L136.

12. Suwabe, A., H. Nakamura, N. Yakuwa, K. Igarashi, J. Higuchi, and K. Takahashi. 1988. Chemotactic activity of alveolar macrophages to alveolar type II epithelial cells isolated from rat lung instilled with bleomycin. *J. Jpn. Med. Soc. Biol. Interface.* 19:78–86.

13. Suzuki, K., H. Miyasaka, H. Ota, Y. Yamakawa, M. Tagawa, A. Kuramoto, and S. Mizuna. 1989. Purification and partial primary sequence of a chemotactic protein for polymorphonuclear leukocytes derived from human lung giant cell carcinoma LU65C cells. *J. Exp. Med.* 169:1895–1901.

14. Baggiolini, M., A. Walz, and S. L. Kunkel. 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest.* 84:1045–1049.

15. Matsushima, K., K. Morishita, T. Yoshimura, S. Lavu, Y. Obayashi, W. Lew, E. Appella, H. F. Kung, E. J. Leonard, and J. J. Oppenheim. 1988. Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin-1 and tumor necrosis factor. *J. Exp. Med.* 167:1883–1893.

16. Yoshimura, T., K. Matsushima, J. J. Oppenheim, and E. J. Leonard. 1987. Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin-1 (IL-1). *J. Immunol.* 139:788–793.

17. Watson, M. L., G. P. Lewis, and J. Westwick. 1988. Neutrophil stimulation by recombinant cytokines and a factor produced by IL-1-treated synovial cell culture. *Immunology.* 65:567–572.

18. Lieber, M., B. Smith, A. Szakal, W. Nelson-Rees, and G. Todaro. 1976. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer.* 17:62–70.

19. Strieter, R. M., D. G. Remick, J. P. Lynch III, R. N. Spengler, and S. L. Kunkel. 1989. Interleukin-2-induced tumor necrosis factor-alpha (TNF-α) gene expression in human alveolar macrophages and peripheral blood monocytes. *Am. Rev. Respir. Dis.* 139:335–342.

20. Strieter, R. M., D. G. Remick, J. P. Lynch III, M. Genord, C.

Raiford, R. Spengler, and S. L. Kunkel. 1989. Differential regulation of tumor necrosis factor-alpha in human alveolar macrophages and peripheral blood monocytes: a cellular and molecular analysis. *Am. J. Respir. Cell Mol. Biol.* 1:57–63.

21. Chirgwin, J. M., A. E. Przybyca, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

22. Jonas, E., T. D. Sargent, and I. B. Davis. 1985. Epidermal keratin-gene expressed in embryos of xenopus laevis. *Proc. Natl. Acad. Sci. USA.* 82:5413–5416.

23. Bacon, K. B., J. Westwick, and R. D. R. Camp. 1989. Potent and specific inhibition of IL-8, IL-1α, and IL-1β-induced in vitro human lymphocyte migration by calcium channel antagonists. *Biochem. Biophys. Res. Commun.* 165:349–354.

24. Showell, H. J., R. J. Freer, S. H. Zigmond, E. Schiffman, S. Aswanikumar, B. Corcoran, and E. L. Becker. 1976. Structure-activity relationships of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. *J. Exp. Med.* 143:1154–1165.

25. Terranova, V. P., R. DiFlorio, and E. S. Hujanen. 1986. Laminin promotes rabbit neutrophil motility and attachment. *J. Clin. Invest.* 77:1180–1186.

26. Laskin, D. L., T. Kimura, S. Sakakibara, D. J. Riley, and R. A. Berg. 1986. Chemotactic activity of collagen-like polypeptides for human peripheral blood neutrophils. *J. Leukocyte Biol.* 39:255–266.

27. Senior, R. M., A. Hinek, G. L. Griffin, D. J. Pipoly, E. C. Crouch, and R. P. Mecham. 1989. Neutrophils show chemotaxis to type IV collagen and its 7S domain and contain a 67 kD type IV collagen binding protein with lectin properties. *Am. J. Respir. Cell Mol. Biol.* 1:479–487.

28. Clark, R. A. F., N. E. Wikner, D. E. Doherty, and D. A. Norris. 1988. Cryptic chemotactic activity of fibronectin for human monocytes resides in the 120-kDa fibroblastic cell-binding fragment. *J. Biol. Chem.* 263:12115–12123.

29. Martin, T. R., L. C. Altman, R. K. Albert, and W. R. Henderson. 1984. Leukotriene B_4 production by the human alveolar macrophage: a potential mechanism for amplifying inflammation in the lung. *Am. Rev. Respir. Dis.* 129:106–111.

30. Mornex, J. F., Y. Martinet, K. Yamauchi, P. B. Bitterman, G. R. Grotendorst, A. C. Weir, G. R. Martin, and R. G. Crystal. 1986. Spontaneous expression of the *c*-sis gene and release of a platelet-derived growth factor-like molecule by human alveolar macrophages. *J. Clin. Invest.* 78:61–66.

31. Gorin, A. B., and P. A. Stewart. 1979. Differential permeability of endothelial and epithelial barriers to albumin flux. *J. Appl. Physiol.* 47:1315–1324.

32. Rooney, S. A. 1985. The surfactant system and lung phospholipid biochemistry. *Am. Rev. Respir. Dis.* 131:439–460.

33. Jones, K. G., J. F. Holland, G. L. Foureman, J. R. Bend, and J. R. Fouts. 1983. Xenobiotic metabolism in Clara cells and alveolar type II cells isolated from the lungs of rats treated with β-naphthoflavone. *J. Pharmacol. Exp. Ther.* 225:316–321.

34. Ayars, G. H., L. C. Altman, H. Rosen, and T. Doyle. 1984. The injurious effect of neutrophils on pneumocytes in vitro. *Am. Rev. Respir. Dis.* 130:964–973.

35. Simon, R. H., P. D. DeHart, R. F. Todd III. 1986. Neutrophil-induced injury of rat pulmonary alveolar epithelial cells. *J. Clin. Invest.* 78:1375–1386.

36. Peveri, P., A. Walz, B. Dewald, and M. Baggiolini. 1988. A novel neutrophil-activating factor produced by human mononuclear phagocytes. *J. Exp. Med.* 167:1547–1559.

37. Schroder, J. 1989. The monocyte-derived neutrophil activating peptide (NAP/interleukin 8) stimulates human neutrophil arachidonate-5-lipoxygenase, but not the release of cellular arachidonate. *J. Exp. Med.* 170:847–863.

38. Wolpe, S. D., and A. Cerami. 1989. Macrophage inflammatory proteins 1 and 2: members of a novel superfamily of cytokines. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:2565–2573.

39. Strunk, R. C., D. M. Eidlen, and R. J. Mason. 1988. Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. *J. Clin. Invest.* 81:1419–1426.

40. Sage, H., F. M. Farin, G. E. Striker, and A. B. Fisher. 1983. Granular pneumocytes in primary culture secrete several major components of the extracellular matrix. *Biochemistry.* 22:2148–2155.

41. Crouch, E. C., M. A. Moxley, and W. Longmore. 1987. Synthesis of collagenous proteins by pulmonary type II epithelial cells. *Am. Rev. Respir. Dis.* 135:1118–1123.

42. Chauncey, J. B., R. S. Simon, and M. Peters-Golden. 1988. Rat alveolar macrophage synthesize leukotriene B_4 and 12-hydroxyeicosatetraenoic acid from alveolar epithelial cell-derived arachidonic acid. *Am. Rev. Respir. Dis.* 138:928–935.