Inflamm Res 45: 386–392 (1996)

1023-3830/96/080386-07 $ 1.50+0.20/0

Interleukin-8 (IL-8) is a major neutrophil chemotaxin from human alveolar macrophages stimulated with staphylococcal enterotoxin A (SEA)

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Received 16 October 1995; returned for revision 12 December 1995; accepted by I. Ahnfelt-Rønne 30 April 1996

Abstract. Since Staphylococcus aureus is an important human pathogen, and infection of the lungs is characterized by neutrophil infiltration we studied the role of a staphylococcal toxin, enterotoxin A (SEA) on the synthesis and secretion of IL-8 by human alveolar macrophages. As SEA concentration was increased, the IL-8 accumulation in the macrophage conditioned medium increased. The concentration of mRNA encoding IL-8 was also elevated in the macrophage in response to increases in SEA concentration. Although the monocytic cell line U937 was able to respond to SEA and secrete IL-8, treatment with PMA prior to SEA stimulation increased the IL-8 accumulation around fifty fold indicating that maturation of the undifferentiated cell to a more macrophage-like cell facilitated IL-8 accumulation. Stimulating human alveolar macrophages with high concentrations of SEA caused an increase in IL-1 accumulation. However, when the cells were incubated with SEA in the presence of IL-1 receptor antagonist, there was no decrease in IL-8 accumulation. Addition of a neutralizing anti-IL-8 monoclonal antibody to the culture medium of SEA-stimulated macrophages significantly reduced the neutrophil chemotactic activity of the medium. These studies showed that IL-8 is a major neutrophil chemotaxin from human alveolar macrophages stimulated with SEA.

Key words: Interleukin-8 – Staphylococcal enterotoxin A – Macrophages – Neutrophils – Inflammation

Introduction

There are approximately 500,000 cases of sepsis annually in the United States, with an associated mortality of between 20 and 50%. Tissue damage can occur directly due to bacterial toxins [1, 2], but may also result from the inflammatory response triggered by components of the bacterial cell [3, 4]. The systemic inflammatory response to infection frequently precipitates acute lung injury. Neutrophils have been implicated in the cause of the initial lung damage, and neutrophils accumulate within the lungs in the early phase of acute lung injury [5, 6]. The mechanisms of recruitment of neutrophils to the lung have not been fully elucidated. However, recently IL-8 was shown to be an important chemotaxin within the lung [7, 8], and this cytokine can be synthesized by several cell types present within the lung [9, 12]. In addition to its chemotactic activity, IL-8 can cause neutrophil degranulation [13, 14] and the release of oxidants [15, 16], functions which could cause or worsen lung injury [6, 17, 18]. Gene expression of IL-8 from human alveolar macrophages can be induced by cytokines such as tumor necrosis factor-α (TNF-α) and Interleukin-1 (IL-1) as well as by endotoxin from Gram negative bacteria, lipopolysaccharide (LPS) [10].

Staphylococcus aureus (S. aureus) is a common pathogen both in the community and in hospitalized patients [19]. Infection can take many forms ranging from minor skin infections to septicemia [20]. *S. aureus* is also associated with pneumonia [20] and the adult respiratory distress syndrome [21]. Since *S. aureus* is usually associated with a polymorphonuclear leukocytosis we wished to determine whether the bacterial protein SEA is able to stimulate alveolar macrophages (the predominant phagocytic cell within the alveoli) to synthesize and release IL-8. SEA is a single chain polypeptide superantigen [22] secreted by strains of *S. aureus*, and is known to induce interleukin-1 (IL-1) and tumor necrosis factor-α and -β (TNF-α and -β) in some cells [10, 23]. SEA is a stable superantigen [24], consisting of 233 amino acids with a molecular weight of 27,078 and known primary sequence [25]. It binds directly to

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monocytes and B cells [26], and is a potent polyclonal T cell activator [27].

In this study we examined the role of SEA in the stimulation of IL-8 from human alveolar macrophages.

Methods

Human subjects. All work was approved by the Institutional Human Subjects Committee at the University of Texas Health Center.

Isolation of human alveolar macrophages. Human alveolar macrophages were washed from human lungs that were resected as part of the treatment of cancer. Briefly the lungs were lavaged with saline containing sodium heparin (1 USP unit/ml). The cells were then pelleted by centrifugation, and the red cells disrupted by hypotonic lysis. The cells were again pelleted, and the red cell stroma was gently washed from the surface of the pellet. A white cell differential count was then performed, and only those preparations containing less than 5% neutrophils were used. The cells ($5\text{-}20 \times 10^5$ cells/ml) were then diluted in RPMI 1640 culture medium (GIBCO, Grand Island, NY, USA) containing gentamicin sulphate, 2.5 μg/ml (MA Bioproducts, Walkersville, MD, USA). After 4 h the non-adherent cells were washed from the surface of the plastic. Typically between 1×10^8 and 1×10^9 cells were obtained from each resected lung.

Stimulation of cells. Two types of cells were used. 1) Human alveolar macrophages, 2) U937 (ATCC CRL 1593) a human histiocytic lymphoma cell line [28].

Stimulation of macrophages. Adherent macrophages plated at a concentration of 5×10^5 cells/ml in 96 well tissue culture plates were incubated for 16 h at 37°C in 5% CO_2 with either SEA (Serva Inc., Westbury, NY, USA) 0.1 ng-10 μg/ml or an equal concentration of endotoxin from Escherichia coli serotype 026:B26 (LPS; Sigma Chemical Co., St. Louis, MO, USA). Control cells were treated with media alone. After 16 h incubation, the cells were removed from the fluid by centrifugation, and the supernatant was stored at −70°C until it was analyzed. At the time of sampling the media, a standard dye-exclusion cell viability test was performed using trypan blue. Some cultures (as shown) were grown in the presence of recombinant human interleukin-1 receptor antagonist protein (IL-1ra, 20 ng/ml; R & D Systems, Minneapolis, MN, USA).

Stimulation of U937 cells. Cells were plated at a density of 5×10^5 cells/ml in 96 well tissue culture plates. They were then allowed to stabilize for 3 h before being treated with phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co., St. Louis, MO, USA) for 3 h. The cells were then incubated in the presence of bacterial toxin as described for stimulation of macrophages.

Isolation of RNA. RNA was extracted from human alveolar macrophages using a slight modification of the procedure of Evans and Kamdar [29]. Briefly macrophage monolayers were cooled to 4°C for 20 min before being disrupted with guanidine hydrochloride (8M) containing sodium acetate (0.3M) and sarcosyl (10%). The resulting viscous liquid was passed through a 25 gauge needle four times to shear DNA. The material was then centrifuged at 12,000 rpm at 4°C for 20 min and the gelatinous pellet discarded. The RNA was precipitated with absolute alcohol and rapidly redissolved in guanidine hydrochloride (8M) containing 1/10th volume sodium acetate (3M) to protect the RNA from residual RNAase activity. The sample was then extracted x2 with chloroform and phenol, and the RNA precipitated and stored dry at −70°C until further analysis.

Northern blot analysis. The method was adapted from that of Manniatis [30]. Samples of total cellular RNA were treated with ethidium bromide and electrophoresed through a 1.2% agarose gel containing 1.1% formaldehyde as a denaturant. After soaking the gel in water to remove the formaldehyde, the RNA was partially hydrolyzed using an excess of sodium hydroxide (50 mM) containing sodium chloride (10 mM). The gel was neutralized with Tris-HCl (0.1M pH 7.5) and the separated RNA photographed under UV light. The RNA was then transferred to nitrocellulose by suction. The nitrocellulose blot was then baked and prehybridized. The RNA was hybridized overnight with a nick-translated, full length cDNA probe encoding IL-8 [31]. After washing with a solution of 0.15M sodium chloride containing 0.015M sodium citrate and 0.1% sodium dodecyl sulphate (SDS); pH 7.0, the nitrocellulose was washed with 0.25 × SSC containing 0.1% SDS. The blot was dried and subjected to autoradiography. The relative density of each band was measured at 450 nm using a Zeineh scanning densitometer (Biomed Instruments, Fullerton, CA, USA).

Quantitation of IL-8. IL-8 was quantitated using an ELISA as previously described [32]. The assay employed a monoclonal murine IgG_1 antibody purified from ascites developed with hybridoma HB9647 [32] (ATCC, with permission from Dr. E. J. Leonard), and a rabbit anti-human IL-8 polyclonal antiserum (Upstate Biotechnology Incorporated, Lake Placid, NY, USA). The assay has a lower limit of detection of 150 pg/ml, and shows no cross reactivity at 2000 pg/ml with β-thromboglobulin, human MIP 2α, GRO, MCAF or RANTES.

Quantitation of IL-1. IL-1 was quantitated using a commercially available ELISA (R&D Systems, Minneapolis, MN, USA).

Isolation of human neutrophils. Human blood was anticoagulated with heparin. For chemotactic experiments neutrophils were separated by dextran sedimentation and erythrocyte lysis by the method of Boyum [33] as modified in our earlier studies [34–36].

Analysis of chemotactic activity. An aliquot of media from macrophages stimulated with SEA was assayed for IL-8 concentration. Additional aliquots of the media were then treated overnight at 4°C with a 1000 fold molar excess of either an IL-8 specific monoclonal antibody [32, 49] or an irrelevant monoclonal antibody that was produced and purified in an identical manner. Chemotaxis was then performed using the leading front method as described by Zigmond and Hirsch [37]. The test material was placed in the lower well of a Boyden chamber [38]. A five micron pore size, 100 μm thick cellulose nitrate filter (Sartorius Filter, Inc., San Francisco, CA, USA) was placed on the surface and the chamber was then assembled. A 200 μl aliquot of the neutrophil preparation (1×10^6 cells/ml) in RPMI-1640 containing 1% albumin was added to the top of the filter and incubated at 37°C for 40 min. The filter was then fixed and stained and mounted on a glass microscope slide. The leading front was determined by the position of the leading two cells. The distance that the leading two cells had moved through the filter was measured for six fields on each filter. The measurements were made with two filters for each set of conditions. Controls of media alone (not conditioned by the growth of cells), and recombinant IL-8 were assessed in the same experiments.

Statistical analysis. Each experiment was performed at least three times. The data were analyzed for statistical significance using a one way analysis of variance followed by Dunnett's multiple comparison procedure.

Results

IL-8 accumulates in the culture medium of human alveolar macrophages even in the absence of any added soluble stimulus. This basal level of accumulation is increased in a concentration dependent manner when the cells are incubated in the presence of SEA (Fig. 1). In similar experiments using U937 cells, there was also a concentration dependent relationship between the

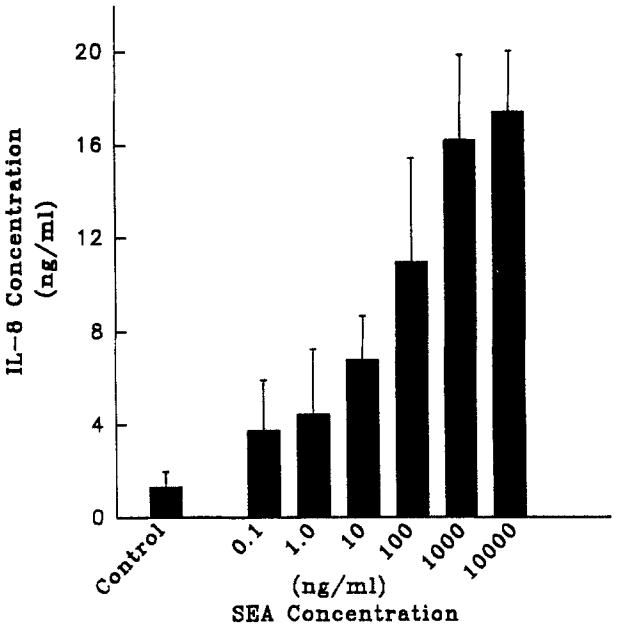


Fig. 1. IL-8 accumulation in media of human alveolar macrophages. Culture medium of human alveolar macrophages incubated for 16 h in the presence or absence of SEA was assayed for its IL-8 concentration using an ELISA. Each bar represents the mean ± SEM and SD of 8 replicate cultures. The results are from a typical experiment.

amount of SEA or LPS added to the macrophages and the IL-8 accumulation (Fig. 2). Maximal IL-8 accumulation in media of U937 cells was lower than in the media of macrophages. The U937 cells were much less responsive to SEA than LPS, and only a concentration of 10,000 ng/ml SEA was able to stimulate any significant increase in IL-8 accumulation ($p < 0.001$). When U937 cells were cultured in the presence of PMA for 3 h prior to SEA stimulation, many of the cells became adherent to the plastic culture vessels. Furthermore, when the cells were incubated in the presence of SEA (10,000 ng/ml), the accumulation of IL-8 in the culture medium increased approximately fifty fold from 1 ng/ml (in the absence of PMA) to nearly 50 ng/ml in the presence of a PMA concentration of 100 ng/ml (Fig. 3). This increase approximated the IL-8 accumulation achieved with human alveolar macrophages. PMA primed U937 cells responded to much lower concentrations of SEA. A dose of 1 ng/ml SEA was able to induce a significant increase in the IL-8 accumulation as compared to control cultures ($p < 0.001$).

Since IL-1 is a potent stimulator of IL-8 secretion in macrophages [10], we determined whether the IL-8 accumulation in the media was a direct response to SEA and LPS, or was secondary to IL-1 release by the cells. When the macrophages were stimulated with LPS, IL-1 production increased significantly even at low concentrations of the toxin (Fig. 4). However, when the cells were stimulated with SEA, IL-1 accumulation at SEA concentrations below 1,000 ng/ml did not increase significantly (Fig. 4). At SEA concentrations of 10,000 ng/ml the IL-1 accumulation was approximately five times less than with an equivalent dose of LPS. Furthermore, recombinant human IL-1 receptor antagonist a protein which inhibits the binding of IL-1 to its cellular receptor, had no effect on the IL-8 accumulation in the media of macrophages stimulated with either SEA or LPS (Fig. 5).

To determine if mature macrophages retained the

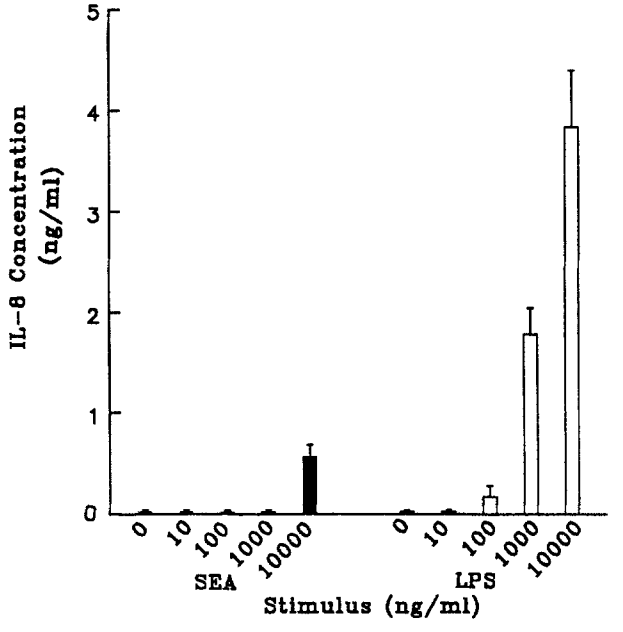


Fig. 2. IL-8 accumulation in media of U937 cells. U937 cells incubated for 16 h in the presence of SEA ■ or LPS □ were assayed for their IL-8 concentration using an ELISA. Each bar represents the mean ± SEM and SD of 8 replicate cultures. The results are from a typical experiment.

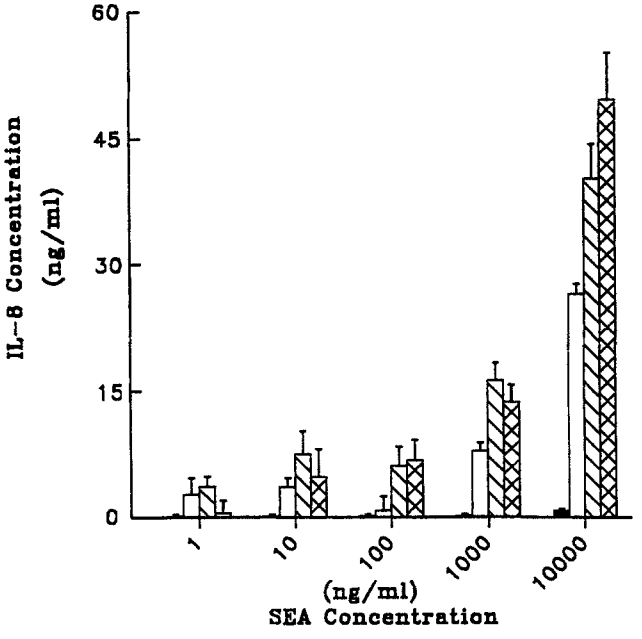


Fig. 3. Effect of PMA on U937 response. U937 cells were incubated for three hours with PMA 0 ng/ml ■; 1 ng/ml □; 10 ng/ml ▨; 100 ng/ml ▩; and for a further 16 h in the presence of SEA (0–10000 ng/ml). The IL-8 accumulation in the culture medium was assayed using an ELISA. Each bar represents the mean ± SEM and SD of 8 replicate cultures. IL-8 concentration in the media of cells grown in the absence of both SEA and PMA were below the detection limit of the assay. The results are from a typical experiment.

ability to synthesize IL-8, RNA was isolated from human alveolar macrophages stimulated for 12 h with SEA. The RNA samples were separated on electrophoresis and Northern blots of the gels were probed for RNA encoding IL-8. Figure 6 shows that although equivalent amounts of

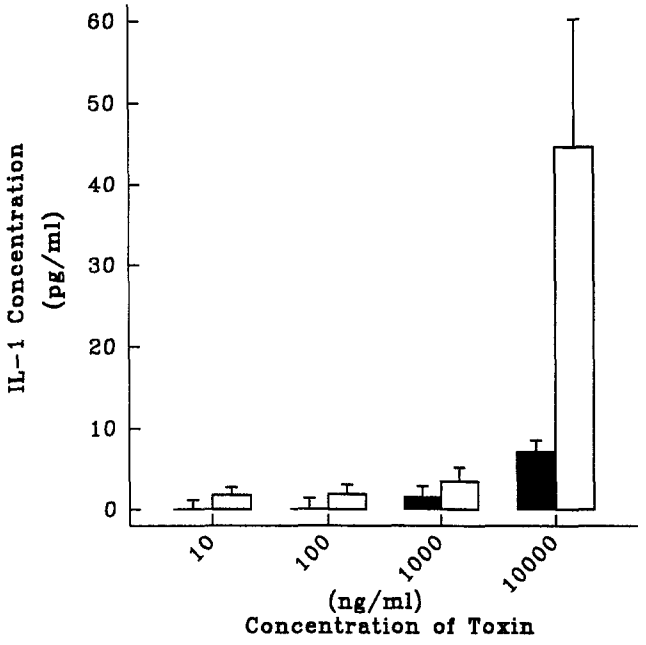


Fig. 4. Evaluation of IL-1 production. Human alveolar macrophages were incubated for 16 h in the presence of SEA (closed bars) or LPS (open bars) at concentrations of 10–10000 ng/ml. The conditioned medium was then assayed for IL-1 using an ELISA. Each bar represents the mean ± SEM and SD of 8 replicate cultures. The results are from a typical experiment.

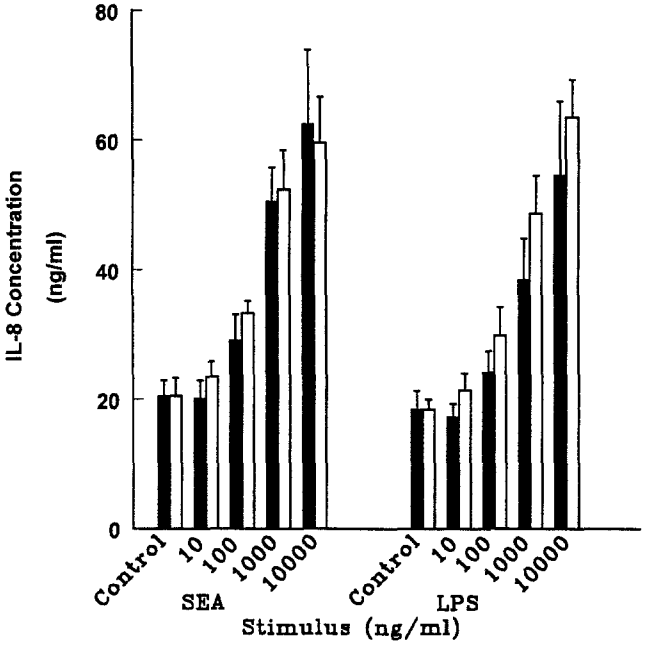
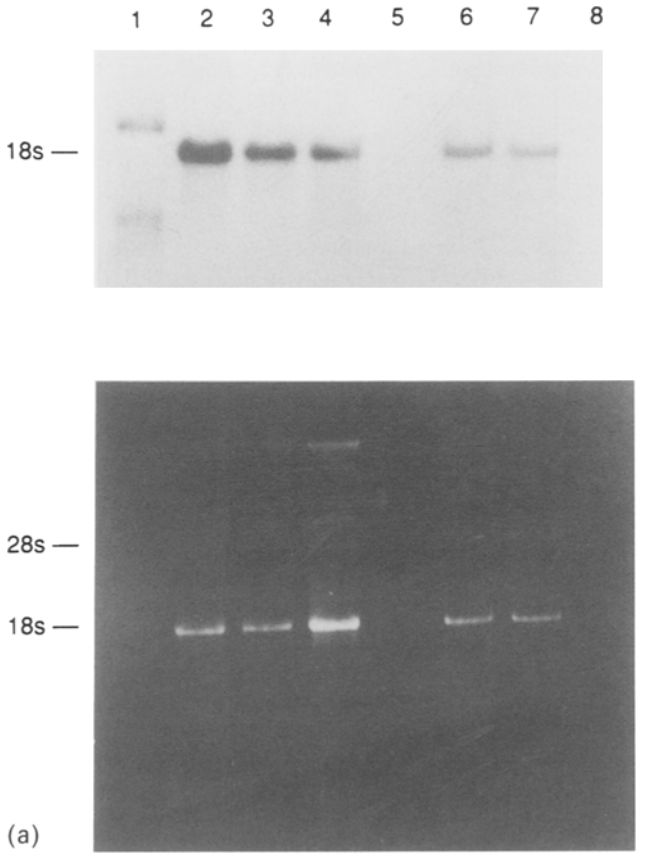


Fig. 5. IL-8 accumulation in media of human alveolar macrophages in the presence of IL-1ra. Macrophages were incubated with for 16 h in the presence (open bars) or absence (closed bars) of IL-1ra 20 ng/ml and stimulated with SEA or LPS. The culture media were then assayed for their IL-8 concentration using an ELISA. Each bar represents the mean ± SEM and SD of 8 replicate cultures. The results are from a typical experiment.



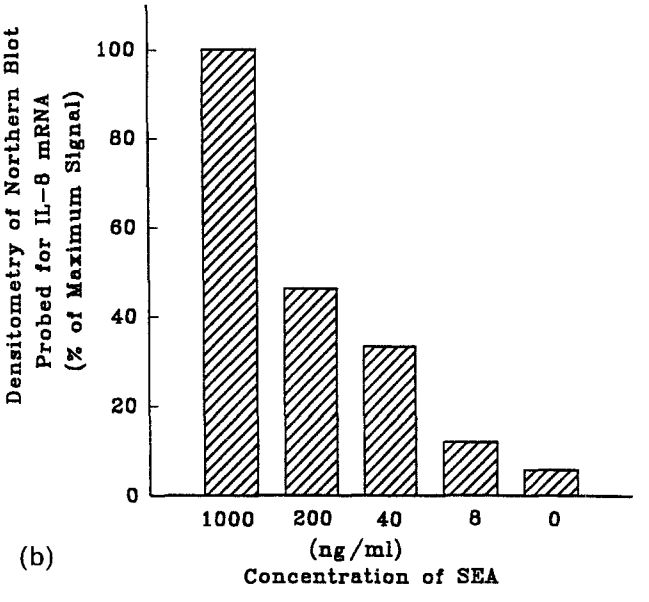


Fig. 6. Induction of mRNA encoding IL-8. Human alveolar macrophages were incubated in the presence or absence of SEA for 12 h. RNA was extracted from the cells and subjected to Northern blot analysis in which the RNA was probed for mRNA encoding IL-8. Lane 1) Kb standards, 2-4) RNA from cells stimulated with 1000, 200, & 40 ng/ml SEA respectively, 5) buffer alone, 6, 7) RNA from cells stimulated with 8 and 0 ng/ml SEA respectively, 8) buffer alone. The resultant autoradiograph was quantitated using a scanning laser densitometer. (a) Shows the probed Northern blot (upper panel) and the unprobed gel showing that each well contained approximately the same amount of mRNA (lower panel). (b) Shows the densitometric analysis of the Northern blot.

RNA were loaded onto the gel, there was an increase in mRNA, of approximately 1.8 kb in size, encoding IL-8.

Since IL-8 is a neutrophil chemotaxin, we examined the relative contribution of IL-8 to the total neutrophil chemotactic activity in the media of SEA stimulated macrophages. There was detectable neutrophil chemotactic activity in the macrophage conditioned medium from cells grown in the absence of bacterial toxin. However, there was a significant increase in the chemotactic activity when the cells were treated with SEA ($p < 0.01$). Treatment of the culture medium with an antibody which neutralizes IL-8 activity significantly reduced ($p < 0.01$) the neutrophil chemotactic activity (Fig. 7).

Discussion

The cause of the neutrophil influx into the lungs in various disease states is not well understood. In some conditions such as bacterial pneumonia the stimulus most probably originates from bacteria within the lung. However in other bacteria related disease states this may not be the case, as in the *S. aureus* induced toxic shock syndrome, where the lung injury which frequently occurs is presumably due to release of a toxin from an infection site distant from the lung. The adult respiratory distress syndrome (ARDS), is often associated with septicemia or with a constellation of findings characteristically associated with septicemia [39, 40]. In a prospective study which examined 40 patients it was found that many individuals developed ARDS less than six hours after sepsis [41]. Furthermore, early and late mortality in ARDS is related primarily to sepsis [42]. *E. coli* was identified as the causal infectious agent in approximately 14% of the cases of ARDS staphylococci caused a further 11 [43]. In addition, we have shown that IL-8 is a major neutrophil chemotaxin in the lungs of patients with the ARDS [7], and is particularly important in ARDS associated with sepsis [48].

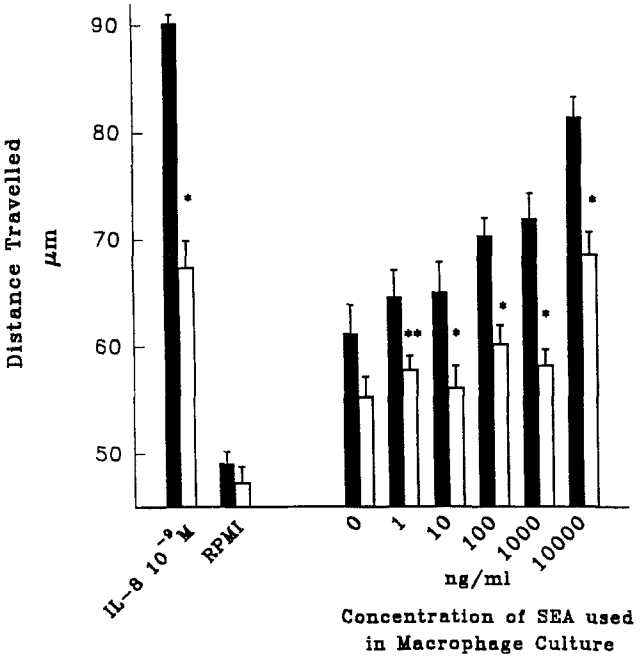


Fig. 7. Neutrophil chemotactic activity of macrophage conditioned medium. Medium from human alveolar macrophages cultured in the presence of SEA 0-10,000 ng/ml was assayed for IL-8 concentration and then aliquots were treated with a thousand fold molar excess of either an anti-IL-8 (open bars) or an irrelevant monoclonal antibody (closed bars). The resulting fluids were assessed for chemotactic activity. The first 4 bars show the chemotaxis controls of recombinant IL-8 or media. The following 12 bars show the chemotactic activity in media conditioned by macrophages grown in the presence or absence of SEA. Each point represents the mean ± SEM of 12 readings. There are significant differences between the samples treated with anti-IL-8 compared to treatment with the irrelevant antibody (*$p < 0.01$, **$p = 0.02$). There was no significant difference between the two antibody additions in the media conditioned by macrophages in the absence of SEA.

In this study we showed that staphylococcal enterotoxin A acted directly on human alveolar macrophages to induce the release of IL-8. While both SEA and LPS increased the IL-1 accumulation in the culture media, LPS was much more potent. These findings are in broad agreement with those of Bjork et al. [44], who used immunocytochemical techniques to show that after a 12 h stimulation of peripheral blood monocytes there were 2-3 times as many cells producing IL-1 after LPS treatment than after treatment with SEA. While the gene expression of IL-8 can be induced in alveolar macrophages by IL-1 [10] the inability of IL-1ra to diminish the production of IL-8 by alveolar macrophages suggests that SEA does not increase IL-8 production by increasing IL-1.

IL-8 accumulates in the culture medium of human alveolar macrophages in the absence of any added stimulus [45] and is most probably induced by the adhesion of the cell to the plastic of the culture vessel [46]. The secretion of chemotactic activity by macrophages increased significantly in parallel with the concentration of SEA added. When the medium was treated with an antibody which neutralized IL-8 [49], the chemotactic activity was greatly reduced showing that IL-8 was the most potent neutrophil chemotaxin in the medium.

The U937 cell line was used as a model of an immature monocytic cell. It expresses many of the monocyte-like characteristics, but can be differentiated to a macrophage-like cell by incubation with PMA [47]. PMA treatment of U-937 cells prior to SEA stimulation caused the cells to become adherent and induced a profound increase in the IL-8 accumulation. These data suggest either that PMA and SEA have a direct synergistic affect on IL-8 stimulation, or that undifferentiated U937 cells do not produce as much IL-8 as more mature macrophage-like cells. These data raise the possibility that SEA could be carried, in the blood, to the lungs where significant secretion of IL-8 could occur due to the presence of the high responding alveolar macrophages.

In summary, we have shown that SEA can stimulate both the transcription of the gene encoding IL-8, and the accumulation of IL-8 in the medium of human alveolar macrophages. Furthermore, IL-8 is the predominant neutrophil chemotaxin secreted by SEA stimulated macrophages. Therefore, if methods of inhibiting SEA activity can be developed, the lung damage associated with staphylococcal infections may be reduced.

Acknowledgments. We thank Drs. Jerry McLarty and David Holiday (Dept. of Epidemiology/Biomathematics, University of Texas Health Center at Tyler) for their help with the statistical analyses. The work was supported by grants from the American Heart Association, Texas Affiliate Inc., and the Lung Division of the National Heart, Lung, and Blood Institute (No. R01-HL43650) and the Gugenheim Fund. E. J. Miller is a Parker B. Francis Fellow.

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