Alveolar Macrophage-derived Chemotactic Factor

KINETICS OF IN VITRO PRODUCTION AND PARTIAL CHARACTERIZATION

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ABSTRACT Alveolar macrophages are the initial phagocytic cells that encounter foreign material and particulates deposited in the terminal airways. We have examined a mechanism by which these cells, after phagocytic challenge, may control or amplify the inflammatory response in lung parenchyma. Normal human alveolar macrophages (AM) were studied from eight subjects. With in vitro culture, AM produced and released two substances into culture media which have potent chemoattractant activity for blood polymorphonuclear granulocytes(PMN) and negligible activity for mononuclear cells. Release of these factors is maximally stimulated by aggregated human immunoglobulin (Ig)G or zymosan particles; however, simple adhesion of the macrophages to plastic surfaces is also sufficient to stimulate release of these chemotactic substances.

The larger substance (10,000 daltons) is immunologically distinct from C5a and interacts with a different PMN membrane receptor than that known to exist for formyl-methionyl-leucyl-phenylalanine. Its chemotactic activity is sensitive to the enzymatic effect of trypsin. Although producing a single elution peak on gel-filtration chromatography, electrofocusing in polyacrylamide gels yielded five peaks of radioactivity. Chemotactic activity was localized to a fraction with a pI = 5.0. The smaller molecular weight substance has been less well characterized. Thus, the human AM can produce at least two factors which attract PMN and this capability may augment the local inflammatory response in the lung.

INTRODUCTION

In the lungs, the inflammatory response is an important host defense mechanism for the containment and clearance of infectious agents and toxic particles which, by aspiration or inhalation, reach the air-exchange surface (1, 2). The obvious part of this response consists histologically of an accumulation of fluid and cellular exudate in alveoli and adjacent airways. In reality, however, this is a later phase of the inflammatory response and develops after initiating factors have triggered a biologic sequence. Increasingly, research interest is focusing on the control of the early phases of the response. In certain experimental models of lung injury, specific antibody challenge, airway injection of preformed immune complexes (containing immunoglobulin [Ig]G), and certain complement fragments quickly produce parenchymal hemorrhage and inflammation and appear to be relevant ways to elicit the response. A pertinent question must now be raised: do infectious agents such as bacteria, airway-formed immune complexes in hypersensitive hosts, or inhaled particulates directly stimulate fluid and cellular components in alveoli and airway secretions to elicit the inflammatory response, or do other intermediate and modulating steps occur? Does the above sequence by-pass the pulmonary alveolar macrophage (AM)[1] or does this phagocyte cell play a role in the mediation of the response? Generally, AM are considered to be the phagocytes that make initial contact with foreign materials that reach the terminal airways and function to remove or contain them. This is an important part of efficient lung host defense. Although much is known about the phagocytic ability of AM (3–6), and the armamentarium of enzymes and secretory products they possess (7), less is known about the interrelation of stimulated or "challenged" macrophages with other arms of the inflammatory response (8). Recent evidence suggests that through the elaboration of chemo-

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[1] *Abbreviations used in this paper*: AM, alveolar macrophage; CBZ-phe-meth, carbobenzyloxy-phenylalanyl-methionine; FMLP, formyl-methionyl-leucyl-phenylalanine; GBSS, Gey's balanced salt solution; HAIgG, heat-aggregated IgG; MNC, mononuclear cells; PMN, polymorphonuclear granulocytes; STI, soybean trypsin inactivation.

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tactic factors, AM have the capability of attracting secondary phagocytic cells such as polymorphonuclear granulocytes (PMN). This poses an alternate way for initiating or controlling the influx of circulating inflammatory cells into alveoli from the airway side of the lungs.

In a subhuman primate model (*Macaca mulatta*), the accumulation of PMN in respiratory secretions was induced in part by two factors which were isolated from lung lavage fluid: an activated fragment of the fifth component of complement (C5a) and a smaller molecular weight protein (~5,000 daltons) which was free in lavage fluid. This smaller noncomplement factor was secreted by in vitro-cultured AM and has selective activity for PMN (9), which suggested that the alveolar macrophage might initiate or amplify the local lung inflammatory response by selective release of a chemotactic factor. Hunninghake and colleagues (10) reported confirmatory findings that guinea pig AM produced a similar factor and noted again its preferential activity for PMN. They made two important additional observations. First, after intratracheal inoculation of heat-killed staphylococci, the chemotactic factor was generated in lung lavage fluid, demonstrating in vivo production. Second, when the partially purified factor was injected intratracheally, a PMN-cellular infiltrate developed, thus showing in vivo activity and specificity for the factor.

In the present study, chemotactic substances produced by human AM have been identified and characterized. The results with in vitro-cultured human cells are not as simple as those found in the animal experiments. Instead of isolating a single factor with chemoattractant activity, the human AM appear to produce several chemotactic molecules.

METHODS

Bronchoalveolar lavage. After informed consent, fiberoptic bronchoscopy and bronchoalveolar lavage were performed on healthy human volunteers. The nose and upper airway were anesthetized with 4% lidocaine before the bronchoscope (model 5 BF2, Olympus Corporation of America, New Hyde Park, N. Y.) was passed transnasally; 1% lidocaine was used as necessary in the lower airways. The bronchoscope was wedged in a subsegmental bronchus of the right middle lobe or lingula and 150–200 ml of sterile normal saline was instilled in 50-ml aliquots and aspirated by syringe (11).

Processing of lavage material and establishment of in vitro cell cultures. Lavage fluid was filtered immediately through several layers of coarse gauze to remove mucus and then centrifuged at 500 *g* for 10 min at 4°C to sediment the respiratory cells. The supernate was decanted and the cell pellet was resuspended with modified Hanks' balanced salt solution (Ca^{++} and Mg^{++} free, Grand Island Biological Co., Grand Island, N. Y.). A cell count by hemocytometer, viability by trypan blue dye exclusion (12), neutral red uptake (13), and differential count of Wright's-stained cytocentrifuged-prepared cells were performed. Respiratory cells were then adjusted to 10^6 viable cells per milliliter in serum-free McCoy's 5A medium (Grand Island Biological Co.), supplemented with 100 U penicillin, 100 μg streptomycin, and 5 μg gentamicin/ml, and added to culture containers (6). The cells were incubated in air/5% CO_2 at 37°C in 25-cm^2 plastic flasks (Corning Glass Works, Science Products Div., Corning, N. Y.) or in 4-cm^2 glass-bottom chambers (Lab-Tek Products, Div. of Miles Laboratories Inc., Naperville, Ill.). The culture medium was changed daily. In some experiments the cell supernate was sampled at intervals, and when such an aliquot of fluid was removed, fresh McCoy's 5A was replaced in an equal amount so that flask volume remained unchanged. In some experiments indomethacin and 5, 8, 11, 14-eicosatetraynoic acid (14) were added to media at the initiation of cell culture.

Column chromatography. Supernatant fluid from AM cultures was concentrated by lyophilization or by positive-pressure ultrafiltration (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) using UM05 membranes at 4°C. The concentrated material was gel filtered through columns containing Sephadex G-50 SF or Sephadex G-25 SF (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and eluted with sterile Dulbecco's phosphate-buffered saline, pH 7.4 (Grand Island Biological Co.) at 4°C. Gel columns were calibrated with the following marker substances: blue dextran (2×10^6 daltons), chymotrypsin (25×10^3-daltons), Ribonuclease A (13.7×10^3 daltons) (which were supplied by Pharmacia Fine Chemicals), bovine insulin (5.1×10^3 daltons) (Sigma Chemical Co., St. Louis, Mo.), and bacitracin (1.5×10^3 daltons).

Isoelectric focusing. Cell supernate was purified by gel chromatography and effluent fractions with chemotactic activity were pooled and concentrated twofold by positive-pressure ultrafiltration (Amicon Corp., UM 05 membranes). An aliquot of this concentrated column pool was iodinated with ^{125}I-Na (supplied carrier-free, New England Nuclear, Boston, Mass.) by the method of Hunter and Greenwood (15) and desalted by filtration through a column of Sephadex G-25 coarse (Pharmacia Fine Chemicals) with phosphate-buffered saline.

Chemotactically active material comixed with a ^{125}I trace-labeled specimen was electrofocused in 100-mm, 5% polyacrylamide gel cylinders in ampholyte gradients having a 3–10 pH range (Ampholyne, Pharmacia Fine Chemicals [16, 17]). The anode solution was 0.2% H_2SO_4 and the cathode was 0.4% ethylenediamine. Electrophoresis was performed at 1.5 mA/gel until voltage reached 400 V, then continued at 400 V for a total of 5 h. Gels were immediately frozen and cut into 2-mm sections with a gel slicer (Bio-Rad Laboratories, Richmond, Calif.); gel sections were collected in plastic tubes and counted in a gamma counter. Samples with radioactivity were suspended in Gey's balanced salt solution (GBSS) (Flow Laboratories, Inc., Rockville, Md.) and assayed for chemotactic potency. The pH gradients were measured from gels run simultaneously without protein, similarly sliced into 2-mm sections, and eluted with 10 mM NaCl.

Preparation of blood leukocytes. Heparinized venous blood was drawn from human volunteers. Mononuclear cells (MNC) were prepared from whole blood on Ficoll-Hypaque (Ficoll-paque, Pharmacia Fine Chemicals) density cushions and PMN were obtained by dextran (275,000-daltons, Sigma Chemical Co.) sedimentation of the erythrocyte-PMN pellet (18). Erythrocytes were lysed with 0.2% saline and tonicity was reconstituted with 1.6% saline. Resultant cell preparations contained ~95% viable cells by trypan blue dye exclusion; the MNC fraction consisted of 25–45% monocytes whereas PMN fractions contained >99% PMN by differential count of Wright's-stained, cytocentrifuged cell smears. Cells were finally suspended in

GBSS which was supplemented with 2% bovine serum albumin.

Chemotaxis assays. PMN chemotaxis was performed by the method of Zigmond and Hirsch (19) using Boyden chambers; alternatively, 100-μl "blind well" chambers (Neuro Probe Inc., Bethesda, Md.) were used. In a typical assay PMN (1.5×10^6/ml in GBSS) were separated from buffer or from a chemotactic stimulus by 3-μm nitrocellulose filters (Millipore Corp., Bedford, Mass.) and incubated for 30 min at 37°C in air/5% CO_2. The experiment was terminated by aspirating the cell suspension and fixing the filter in 75% methanol. Subsequently, filters were stained with Gill I hematoxylin, (Lerner Laboratories, Stamford, Conn.), dehydrated in alcohol and cleared in xylene. Chemotaxis was calculated by measuring with the micrometer on the microscope's fine focus (microscope series 10, American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.) the distance the leading front of cells had migrated into the filter.

For MNC chemotaxis a modification of the population study, reported by Swanson and Becker (20), was used. Briefly, a suspension of 5×10^6 MNC, separated from buffer or stimulant by an 8-μm nitrocellulose filter (Millipore Corp.), was incubated for 90 min at 37°C in air/5% CO_2. Thereafter an experiment was terminated and filters prepared as described for PMN. Chemotaxis was calculated by counting the nuclei of cells that had migrated 15 μm into the filter in each of 10 hpf. All MNC and PMN chemotaxis experiments were performed in duplicate.

Trypsinization of chemotactic factor material. Trypsin and soybean trypsin inactivator (Calbiochem, San Diego, Calif.) were prepared in stock solutions as 20 mg/ml in Hanks' balanced salt solution and stored at −30°C until used. Trypsin was added to chemotactic substances in a final concentration of 0.4% and then incubated for 1 h at 37°C. Soybean trypsin inactivation (STI) was then added in an equivalent amount to terminate the enzymatic activity and the samples were placed in blind well chambers for analysis of chemotactic potency. Controls included GBSS with 2% bovine serum albumin incubated with trypsin for 1 h, then inactivated with STI; and activated serum incubated with trypsin for 1 h. Trypsin and STI were also added simultaneously to activated serum to determine if these compounds added nonspecifically inhibited serum chemotaxins.

Chemical and immunological reagents. Rabbit antisera to human C5 and C3a were obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. The IgG fraction was prepared from these antisera by 33% ammonium sulfate precipitation of the antiserum and subsequent DEAE-cellulose anion-exchange chromatography of the dissolved precipitate. Pooled IgG-containing fractions were further purified by Sephadex G-200 gel filtration in borate saline buffer, pH 8.0; and the IgG-enriched fraction was finally equilibrated in Hanks' balanced salt solution at 1 mg/ml.

Heat-aggregated human IgG (HAIgG), which had been gel filtered through Sephadex G-200, was a gift from Dr. John Hardin, Rheumatology Section, Yale University. Formyl-methionyl-leucyl-phenylalanine (FMLP) (21) and its competitive antagonist, carbobenzyloxy-phenylalanyl-methionine (CBZ-phe-meth) (22) were obtained in powdered form from Bachem Fine Chemicals, Torrance, Calif. These reagents were dissolved in dimethyl sulfoxide and diluted to a working concentration with GBSS. The final concentration of dimethyl sulfoxide was <1%. Zymosan particles were from Difco Laboratories (Detroit, Mich.), washed three times in saline, autoclaved, and stored in Hanks' balanced salt solution (1 mg/ml) at −30°C until used. Activated human serum was prepared by incubating fresh serum diluted 1:10 with Veronal gel buffer at 37°C for 1 h in the presence of *Pseudomonas aeruginosa* lipopolysaccharide (Pseudogen, Parke, Davis & Company, Detroit, Mich.) (23). The diluted serum was heated to 56°C for 30 min to inactivate inhibitors of chemotaxis and then further diluted 1:1 with GBSS so that the final concentration of serum was 5%. Indomethacin was obtained from Sigma Chemical Co., and 5,8,11,14-eicosatetraynoic acid was the kind gift of Dr. B. G. Hamilton, Roche Laboratories, Div. of Hoffmann-La Roche Inc., Nutley, N. J. These reagents were dissolved in 95% ethanol sterilized by filtration (Millex, Millipore Corp.) and stored at −30°C until used. When such agents were added to cell cultures final ethanol concentration was <0.5%.

Statistical evaluation. The data from each experiment were subjected to analysis by Student's t test, and P values reported are for two-tailed test.

RESULTS

Experimental subjects. Eight subjects, mean age 26 yr, (range 18–33 yr) participated in the bronchoalveolar lavage procedure. These included six nonsmokers (two female, four male) and two smokers (male). The mean recovery of lavage fluid was 65% of instillate for the group. An average of 72×10^6 cells was recovered from the smokers and a mean of 15.8×10^6 was recovered from nonsmokers. Mean values for differential counts of recovered cells were: 92% AM, 8% lymphocytes, and <1% PMN.

Kinetics of chemotactic factor release—effect of various biologic substances. Respiratory cells (>90% AM) were placed in culture in flat-bottom plastic dishes. Cells were cultured with media alone or with media containing HAIgG, chosen as a model of immune complex stimulation (24, 25), or with zymosan. Both stimulants were added at 6×10^5 particles/viable cell (26). Some respiratory cells were cultured in plastic cylinders and continuously rotated to inhibit the macrophages from adhering to the plastic surface. Aliquots of cell culture supernate were sampled at intervals and tested for chemotactic activity. Results (Fig. 1) indicated that a chemotactically active material was detected earliest and, in greatest amount in culture supernates from cell monolayers stimulated with HAIgG. Maximal activity was evident 3 h after the cells were challenged. The supernate from cells stimulated with zymosan also contained chemotactically active material, but the peak of activity was noted at 6 h. Supernatant samples obtained from AM which were only allowed to adhere to plastic surfaces also contained chemotactic activity. However, the activity found in stimulated cultures was considerably greater than that found in unstimulated cell cultures ($P < 0.001$ at 3 and 6 h). Moreover, if cells were mechanically rotated to inhibit cell adhesion to plastic (▼), chemotactic factor production was significantly inhibited ($P < 0.05$ at 3 h and $P < 0.001$ at 6 h) compared with the unstimulated adherent cells (■), as shown in Fig. 1. In separate

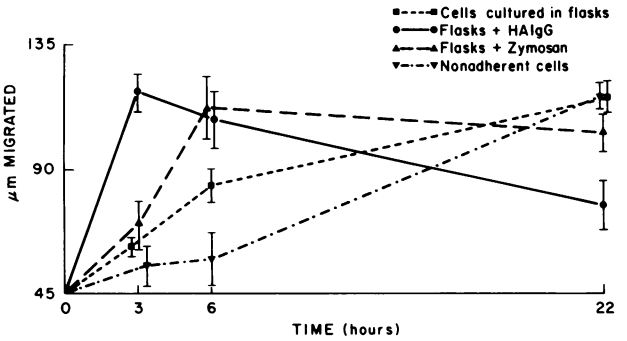

FIGURE 1 Effect of zymosan, HAIgG, and adhesion on chemotaxin released by AM in vitro. Relative chemotactic activity (micrometers of migration) for PMN is shown at intervals during 22 h of in vitro culture in supernatant samples from AM obtained from a cigarette smoker. Duplicate respiratory cell cultures were stimulated with either HAIgG or left unstimulated; in some control cultures, cells were not allowed to adhere. Each point recorded is the mean of four determinations ±SD (vertical bar). PMN migration at time 0 h, when cultures were initiated, was <45 μm for all samples.

experiments, freshly lavaged AM suspended in 1 ml McCoy's 5A were rapidly frozen in dry ice/acetone then thawed at 37°C. Such cell lysates from two nonsmokers failed to promote chemotaxis (migration toward cell lysate from subject I was 35±5 μm and subject II 32±4 μm; buffer control was 30±5 μm).

Results from six experimental subjects are shown in Fig. 2. Here the chemotactic potency of culture supernates from cells stimulated with zymosan is represented as a percent of the chemotactic potency of a simultaneously sampled unstimulated cell culture supernate. The findings for the group are similar to those noted in the initial experiment (Fig. 1). Activity was present at 3 h, and at 3 and 6 h, the stimulated cultures were significantly greater than simultaneous control cultures. Again noted is the decline in chemotactic activity in stimulated culture supernate at 24 h. As shown in Fig. 1, this relative decline was the result of an absolute decrease in chemotactic potency of supernate obtained from 24-h stimulated cells as well as an increase in the chemotactic potency noted in unstimulated cultures at 24 h. Serial dilutions of 24-h poststimulation supernate failed to reveal an increase in the chemotactic potency.

To further investigate this decreased chemotactic potency noted in stimulated supernates, supernatant fluid from 24-h poststimulation was mixed in equal parts with supernate from 24-h unstimulated cell monolayers. This admixture resulted in a significant decrease in the observed potency of the unstimulated supernate (migration toward 24-h unstimulated supernate 117.5±4.3 μm, migration toward 24-h stimulated supernate 78.1±5.6 μm, migration toward mixture 67.8±8.2 μm $P < 0.001$). This decrease was much greater than that caused by a comparable (50%) dilution of active material with buffer (Table II).

Release of chemotactic substance from AM in long-term culture. The preceding experiments demonstrated that AM, if allowed to adhere to plastic surfaces, would release a chemotactically active material into culture supernate. If AM-rich monolayers were unmanipulated, except for a daily change of protein-free culture media, detectable chemotactic activity in the supernate declined linearly with time, and was not significantly different from migration toward GBSS control at ~10 d after initial culture (Fig. 3). The fall in chemotactic activity in the supernates can be attributed in part to cell death and drop off from culture. However, in 14-d-old cultures, the adherent AM were all viable (tryphan blue dye exclusion) and when stimulated with HAIgG (Table I) they responded by releasing chemotactic activity into the supernate with a time-course similar to that shown in Fig. 1. Activity was present at 3 h and chemotactically active material persisted in the supernate of stimulated cells for several days after stimulation.

Molecular characterization of the chemotactically active material from AM cultures. To further characterize this chemotactically active material, 150 ml of supernate from 30 culture flasks of unstimulated AM

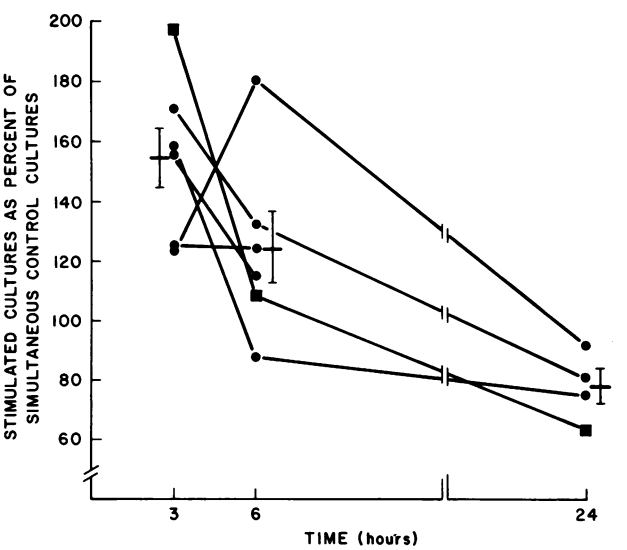

FIGURE 2 Temporal profile of chemotaxin release by zymosan-stimulated AM from six subjects. Here results are expressed as: (Chemotactic potency of stimulated AM supernate)/(Chemotactic potency of unstimulated AM supernate) × 100. Supernate was obtained from stimulated and unstimulated cultures at times shown. ■ is the smoker and ● denotes the nonsmokers. Dash and vertical bars represent mean ±SEM of data points. At time 0 h activity of stimulated and unstimulated supernates is the same and all points fall at 100%.

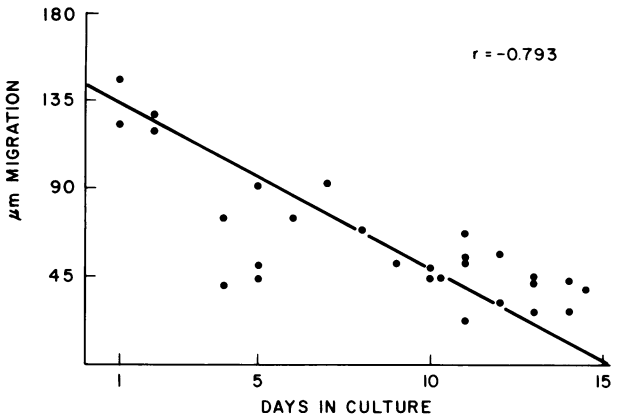

FIGURE 3 Production of chemotactic activity for PMN by AM in long-term culture. The spontaneous release of chemotactic activity into culture supernate of adherent but otherwise unstimulated respiratory cells was monitored during 14 d of in vitro culture. PMN migration toward a Gey's medium control was <40 μm each day. Migration >50 μm was significantly different than resting migration ($P < 0.05$) and indicated the presence of some chemotactic activity in the supernate.

monolayers (24 h in culture) was concentrated 20-fold by positive-pressure ultrafiltration and filtered through a calibrated Sephadex column. Fig. 4 illustrates a representative chromatogram. Chemotactic activity was detected in two parts of the elution pattern, a larger peak corresponding to an elution position for a 9,500-dalton material and a smaller peak centered at <1,000 daltons location. The larger molecular weight peak was found to contain <1 ng/ml of endotoxin in the pooled and concentrated fractions from this peak, and the smaller had no endotoxin by the limulus lysate test. The position of both chemotactic peaks has been reproducible on four replicate gel filtrations. Eluant fractions from the larger molecular weight peak were pooled and concentrated twofold. An aliquot of this material was trypsinized and the chemotactic potency was compared with that of the native material. Trypsin significantly diminished chemotactic potency (migration toward native material

TABLE I
*Effect of HAIgG on Chemotactic Factor Production from AM in Long-Term Culture**

	Intervals sampled poststimulation		
	3 H	24 H	48 H
Stimulated cells	63.6±5‡	75.5±7‡	73.6±4.8‡
Unstimulated cells	45±7	50.7±8.9	48.4±5.7
GBSS	47.2±5	—	—

* AM in culture 14 d before stimulation.
‡ $P < 0.01$ stimulated vs. unstimulated cells.

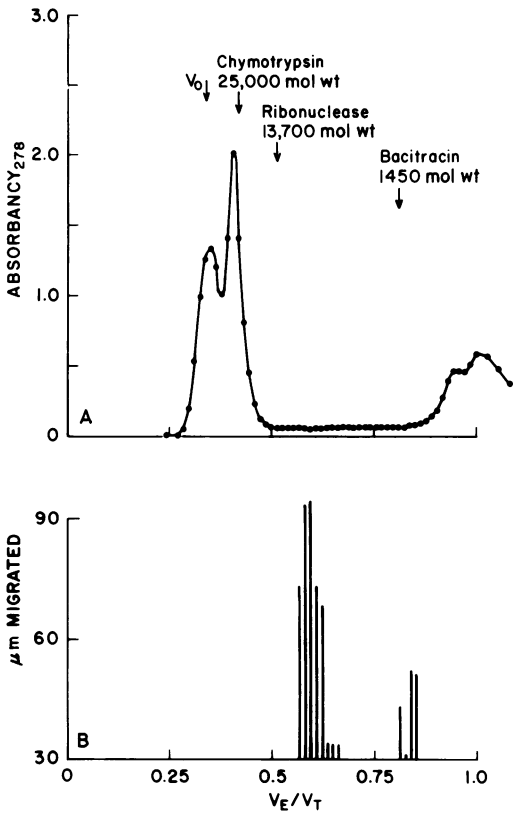

FIGURE 4 Gel filtration of pooled AM culture supernates. 150 ml of unstimulated AM culture supernate from nonsmokers were concentrated to 7 ml and filtered through Sephadex G-50 SF (column dimensions 2.6 (i.d.) × 61.4 cm with gel bed volume of 324.6 ml) in phosphate-buffered saline, pH 7.2. The position of eluant fractions (4.5-ml fractions were collected) is expressed as V_E/V_T. In A, the relative protein content (A_{278}) of the fractions and the elution position of calibration markers are given. In B, migration of PMN in micrometers toward these fractions is shown. Control cell migration in the buffer was 30 μm. The column apparatus was sterile and eluant fractions were free of detectable endotoxin material.

111.2±11.9 μm, whereas, migration toward trypsinized material was 83.0±9.2 μm, $P < 0.001$). Control experiments demonstrated that 1 h of trypsinization of 5% activated serum significantly decreased its chemotactic potency as well (migration toward native activated serum was 105±6.2 μm, and migration toward trypsinized activated serum was 75±7.2 μm, $P < 0.001$). The simultaneous addition of trypsin and STI to GBSS buffer significantly increased cell migration (migration toward GBSS was 47±5.2 μm vs. migration toward GBSS plus trypsin plus STI which was 69.6±12 μm, $P < 0.01$). Simultaneous addition of trypsin and STI to activated serum resulted in a slight increase in PMN migration (127±8.6 μm). The above experiments demonstrate that the chemotactic potency of the column fractionated material

was sensitive to trypsin and therefore presumably contained some protein material.

An aliquot of this column-prepared material (Fig. 1) was iodinated to provide a trace marker. This material (50 μl/gel) was subsequently electrofocused simultaneously on eight polyacrylamide gels. After freezing and slicing the gels radioactivity was determined in 2-mm sections. The distribution of radioactivity in a focused gel and simultaneous pH gradient is displayed in Fig. 5. Five peaks of activity were noted. Identical but replicate gel fractions were solubilized with GBSS and eluate of the gel fractions from each of the five peaks of radioactivity were pooled. PMN migration toward pool B (pI = 5.0) was significantly greater than resting migration (pool B 57.2 ±12 μm vs. GBSS 37.2±3.3 μm $P < 0.01$); whereas, migration toward pools from the other four peaks did not differ from control migration.

To determine if the concentrated material from pooled macrophage supernates was representative of the chemotactic substance assayed in individual stimulated and unstimulated cell cultures, we fractionated chemotactically active supernates (5-ml samples) from individual AM cultures on a small calibrated Sephadex G-25 SF column (Fig. 6). Fig 6A represents chemotactic activity of effluent fractions from a 3-h post-stimulation culture supernate and the elution positions of column standards. The chromatograph shows activity in two positions, one in the void volume of the column and one with an elution position beyond the bacitracin marker. Fig. 6B represents chemotactic activity in fractions from a similar column run using supernate from a 24-h unstimulated culture. These filtration patterns indicated that both peaks of activity are present in supernates from stimulated and unstimulated cell cultures and compare favorably with results obtained with pooled material (Fig. 4).

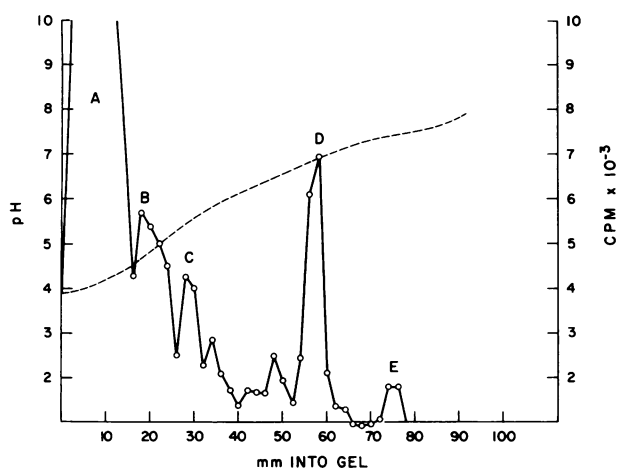

FIGURE 5 Isoelectric focusing of ^{125}I-labeled Sephadex column-purified chemotactic material. Active fractions eluting at V_E/V_T of 0.6 (Fig. 4) were trace labeled with ^{125}I and subjected to electrophoresis in 5% polyacrylamide gel columns with 2% ampholyte concentration, pH gradient 3–10. Gels were snap frozen, sliced, and radioactivity of 2-mm sections determined in a gamma counter. Results are shown from a representative gel. The dashed line gives the pH of 2-mm gel sections and the solid line depicts radioactivity in counts per minute.

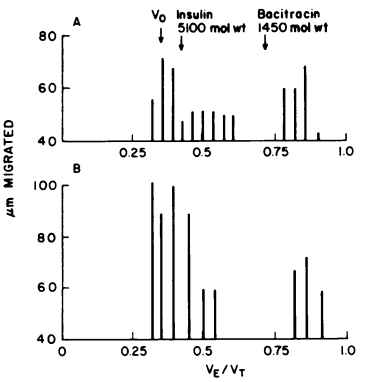

FIGURE 6 Gel filtration of supernates from individual AM cell cultures. 5 ml of supernate, concentrated to 1.8 ml, was chromatographed on a Sephadex G-25 SF column [dimensions 1.1 (i.d.) × 25 cm] in phosphate-buffered saline. The same column was used for both samples and the elution positions of the molecular weight markers were identical for each run. (A) The elution profile of a stimulated monolayer (1.5×10^6 AM) supernate obtained 3 h after zymosan challenge. PMN migration (micrometers) denotes relative chemotactic activity in the eluant fractions. The elution positions of known molecular weight markers are shown by arrows. Protein (A_{278}) was not detected spectrophometrically in the column fractions. (B) The elution of chemotactic activity in an unstimulated culture supernate, sampled after 24 h of culture, is contrasted.

Activity of purified chemotactic factor against PMN and MNC. The pooled eluate of the larger molecular weight material (Fig. 4) was tested for activity against PMN in a standard "checkerboard" format. In this assay PMN are suspended in varying concentrations of pooled eluate material and varying concentrations of this material are placed below the filter. In this way, the response of the cells in a known gradient can be studied and true chemotaxis separated from increased random migration (chemokinesis). Results (Table II) indicated that the PMN were sensitive to changes in the gradient and responded to the macrophage-released factor in a manner described for other known chemotaxins (19).

When the column-purified larger molecular weight factor was similarly tested against MNC targets, little cellular response was noted. In contrast to PMN which demonstrated a brisk, gradient-depended migration toward this factor, MNC failed to respond. Results showed MNC migration toward GBSS to be 16±8 (mean±SD) nuclei/hpf, migration toward macrophage factor to be 21±7 nuclei/hpf ($P > 0.1$), and

TABLE II

Effect of a Concentration Gradient of Macrophage-derived Factor on PMN Migration

	Relative concentration of chemoattractant in upper well of chamber			
	0%*	25%	50%	100%
Relative concentration of chemoattractant in lower well of chamber				
0%	49‡	—	—	53
25%	64.2	58‡	57	—
50%	83	57	49‡	—
100%	84.2	—	—	42‡

* Vertical column demonstrates the effect of an increasing concentration of chemotactic factor in the lower well on PMN migration in micrometers.

‡ Diagonal column demonstrates the micrometers migrated by PMN when concentrations of chemoattractant above and below the filter are equal (i.e., no gradient).

migration toward a positive control of 5% activated serum to be 110±27 nuclei/hpf ($P < 0.001$).

Effects of CBZ-phe-meth and antisera to C5 and C3a. Table III demonstrates the effects of known inhibitors of chemotaxis on two known chemotactic substances used as controls and on the larger molecular weight macrophage-derived factor. As demonstrated, anti-C5 antiserum (IgG fraction) had a significant ($P < 0.01$) effect on activated serum but no effect on PMN migration induced by the macrophage-released factor. In addition, CBZ-phe-meth significantly ($P < 0.01$) inhibited the chemotactic effect of FMLP, but had no significant effect ($P > 0.1$) on the macrophage-released factor.

DISCUSSION

When in vitro-cultured human respiratory cells (predominantly alveolar macrophages) are stimulated with HAIgG or with zymosan, they release potent substances which have chemoattractant activity for PMN. These presumptive factors are not preformed but are rapidly synthesized after cell stimulation. Consistently, we have isolated in the supernatant medium from stimulated and adherent, but otherwise unstimulated cell cultures, a material with a molecular weight of ~10,000 daltons, determined with calibrated column chromatography. A smaller molecular weight factor has also been detected which elutes from dextran gels at a position suggesting a molecular weight smaller than bacitracin (1,400 daltons).

The larger molecular weight material has been evaluated in some detail. The presumptive factor is not C5 (or C5a) because its chemotactic activity for PMN was not inhibited by specific antibody to C5, which was found to inhibit the chemotactic effect of endotoxin-activated human serum. Moreover, because the activity of this factor was undiminished by CBZ-phe-meth, it must bind to a different PMN membrane receptor than the one which interacts with FMLP. Isoelectric focusing of this material, which had appeared quite homogenous in dextran gels (Fig. 4), produced five peaks of radioactivity. However, chemotactic activity was limited to a single peak (B, Fig. 5) with a corresponding pI = 5.0. PMN were found to respond to this factor in a manner similar to their response to other known chemotaxins in the checkerboard gradient analysis (Table II). The chemoattractant activity of this large molecular weight factor was more potent for PMN than mononuclear inflammatory cells. This finding was predicted by the results obtained in the monkey (9) and the guinea pig (10) and was clearly demonstrated in the present study. Monocytes, although responsive to a positive control of endotoxin-activated serum, were unresponsive to the column-purified material.

Although at least two chemotactically active factors were isolated from cell monolayer supernates, the precise relationship between these two factors has not been defined. At least two possible explanations exist for the occurrence of these two factors. First, the two peaks of chemotactic activity (Fig. 4) may in fact represent only one active substance. The smaller molecular weight factor could be an active fragment of the larger molecular weight substance. Conversely, the larger molecular weight substance could represent an aggregate of the smaller. However, we feel that the consistent isolation of the identified peaks from multiple supernatant specimens on replicate column runs makes random aggregation or degradation unlikely. We favor the possibility that the alveolar macrophage does produce two factors of markedly different molecular weight after cell activation. The

TABLE III

Effect of CBZ-Phe-Meth and Anti-Complement Antisera on the Activity of AM-Derived Chemotactic Factors

Stimulant	Inhibitors			
	None	CBZ-Phe-Meth*	Anti-C_5‡	Anti-C_3‡
Activated serum	100§	80	46	95
FMLP‖	85	44	—	—
AM-derived factor	73	64	74	69
GBSS (control)	38	—	—	—

* CBZ-phe-meth is present in 0.2 mM final concentration.

‡ Antisera (IgG fraction) were preincubated with chemoattractants for 1 h at 23°C in all cases.

§ Distance migrated by target PMN in micrometers.

‖ FMLP is present in 0.1 μM final concentration.

larger molecular weight material has been well characterized, but the nature of the smaller molecular weight material remains unclear.

Others have demonstrated the presence of 12-hydroxy 5,8,10,14-eicostetraenoic acid in macrophage culture supernates (27). This compound is known to be a potent attractant for PMN (28). Because the solvents and reagents necessary to isolate this compound produce severe chemical conditions which denature other biological substances, this has precluded our direct determination of 5,8,10,14-eicostetraenoic acid activity levels in supernatant fluids. In preliminary studies using the cells from one smoking subject, we found that early release of chemotactic factor is inhibited by 5,8,11,14-eicosatetraynoic acid (an inhibitor of both cyclooxygenase and lipoxygenase (14) pathways of prostaglandin metabolism) but unaltered by indomethacin (an inhibitor only of the cyclooxygenase enzyme). This suggests that 12-hydroxy 5,8,10,14-eicostetraenoic acid, a product of the lipoxygenase pathway may be the smaller molecular weight factor isolated. Moreover, prostaglandins are closely associated with the plasma membranes of cells and may be metabolized quite early in the process of phagocytosis, perhaps yielding 12-hydroxy 5,8,10,14-eicostetraenoic acid, a compound with molecular weight similar to that of the smaller compound identified in the present study (Figs. 4 and 6).

With this in vitro culture system which uses a mixed cell population, it is possible that any factor released into the culture supernate could originate from contaminating lymphocytes or perhaps require a preliminary lymphocyte-monocyte interaction for release. For several reasons, we feel that these possibilities are unlikely, in our experiments. First, the cells harvested from normal lungs were overwhelmingly (>92%) alveolar macrophages. Second, one nonsmoker did have 20% lymphocytes and 80% macrophages in the recovered cell population. Despite the potential increase in lymphocytes present in the cultured cell monolayers, results obtained from this cell population were no different from those of the other subjects in whom a greater percentage of cells were AM. Finally, most lymphocytes are not strongly adherent cells and may be removed from plastic surfaces with repeated washings. AM in long-term culture (14 d) released chemotactically active material after stimulation with a time-course which paralleled that of freshly cultured cells (Table I). In these cultures, lymphocytes had been essentially eliminated by frequent media changes and none were visible on stained fixed smears of 14-d AM cultures. All of the above support our thesis that AM are solely responsible for the release of these chemotactic substances.

Our data show that supernates from zymosan-stimulated cell cultures were significantly more potent than control cell supernates obtained 3 and 6 h after initiation of cultures. By 24 h of culture, the chemotactic potency of control supernates had increased and that of stimulated supernates had diminished (Figs. 1 and 2) such that the potency of control culture supernates now significantly exceeded that obtained from stimulated cells. There are several possible explanations for this. First, the factors may be degraded while in solution. Secondly, the factors may continue to increase with time but PMN migration toward the active supernate may be decreased because of the peculiar sigmoidal shape of the dose-response curve of some known chemotactic factors (21). However, serial dilution of supernatant material from 24-h poststimulation cultures failed to demonstrate an increase in chemotactic activity. Finally, it is possible that activated macrophages may produce an inhibitor of PMN migration at this time (24 h) after stimulation. Our preliminary experiments involving mixing of supernate obtained 24 h after stimulation with that obtained from unstimulated monolayers at 24 h seem to support the last of these possibilities. The presence of an inhibitor which modulates or suppresses the inflammatory response has been previously demonstrated by Ward and co-workers (29, 30). We have not yet purified this inhibitor material from the 24-h supernate.

In summary, AM, after exposure to HAIgG and zymosan will release substances which have potent chemoattractant activity for PMN. This suggests that the AM can amplify the phagocytic capacity of the lung after airway bacteriologic or immune complex challenge. Preliminary data indicate that the stimulated macrophage may later release an inhibitor of chemotaxis into its culture medium by 24 h after challenge, which supports the concept that the AM is important in the control of cellular inflammation in the lung.

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