

Mechanism for the Inflammatory Response in Primate Lungs

DEMONSTRATION AND PARTIAL CHARACTERIZATION OF AN ALVEOLAR MACROPHAGE-DERIVED CHEMOTACTIC FACTOR WITH PREFERENTIAL ACTIVITY FOR POLYMORPHONUCLEAR LEUKOCYTES

JOHN A. KAZMIEROWSKI, JOHN I. GALLIN, and HERBERT Y. REYNOLDS

*From the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases,
National Institutes of Health, Bethesda, Maryland 20014*

ABSTRACT Approximately 4 h after an initial bronchoalveolar lavage (BAL) of a primate's lung, an appreciable number of polymorphonuclear leukocytes (PMNs) were noted to accumulate in respiratory fluids when lavage was repeated. Whereas, alveolar macrophages (90%) and lymphocytes (7%) were the principal respiratory cells recovered initially from lavage fluid, later samples contained 45–90% PMNs. To explain the observed ingress of PMNs into lung fluids, concentrated BAL fluid was tested for chemoattractant activity. Such fluid obtained 4 and 24 h after an initial lavage contained material that produced directed migration (chemotaxis) for PMNs and mononuclear cells isolated from peripheral blood of normal donors. Gel filtration chromatography of BAL disclosed two peaks of chemotactic activity in the effluent fractions. Material from the column with an estimated molecular weight of 15,000 daltons was chemotactic for both PMNs and mononuclear cells. Because it was susceptible to inactivation with antiserum against the fifth component of complement, resistant to heating, and unaffected by antiserum against C3, this factor was considered analogous to the cleavage product of the fifth component of complement, C5a. In addition chemotactic activity for PMNs only was contained in an effluent peak having a molecular weight of about 5,000 daltons. This material was heat labile but unaffected by antisera to complement components. To locate the possible source of these factors in respiratory fluid, *in vitro* cultures of alveolar macrophages were established. These cells, whether stimulated by phago-

cytosis of opsonized bacteria or merely by attachment to a glass surface, produced chemotactic material which had physical characteristics similar to the small molecular weight material in BAL. Moreover, it induced preferential chemotaxis for PMNs.

Thus, in primate lungs, at least two chemotactic substances may generate an inflammatory response; one which is a fragment of the complement component C5 and another small molecular weight factor which is released from alveolar macrophages.

INTRODUCTION

The bronchopulmonary tract has numerous ways to protect itself from various noxious materials contained in respired ambient air (1). When infectious particles of critical size, usually bacteria, are inhaled and deposited in the distal airways and alveoli, it is generally accepted that resident alveolar macrophages engage these particles, and by phagocytosis and intracellular killing preserve sterility of the lower respiratory tract. However, failure of this cellular mechanism may lead to establishment of infection and the development of an inflammatory response, characterized by an accumulation of polymorphonuclear leukocytes (PMNs).¹ Although this PMN response to acute bacterial infection was elegantly described by Wood (2, 3) years ago, little is known about the mechanisms of accumulation or ingress of these cells into lung tissue and broncho-

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¹ *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; ECFA, eosinophil chemotactic factor of anaphylaxis; HBSS, Hanks balanced salt solution; PMNs, polymorphonuclear leukocytes.

alveolar secretions or about chemotactic factors which might be involved in this reaction (4).

The present study was prompted by an observation noted in earlier experiments (5) that a second bronchoalveolar lavage (BAL) performed in normal monkeys within 24 h of a previous lavage yielded respiratory cells which consisted primarily of PMNs and not of alveolar macrophages and lymphocytes as was customary. Monkeys at the time of second BAL had no evidence of infection and no auscultatory or chest X-ray abnormalities. To evaluate the possibility that in vivo production of chemotactic factors was responsible for the accumulation of PMNs, we have examined BAL fluid from serial bronchoalveolar lavages for chemotactic activity and have compared these results with supernatant fluids from alveolar macrophage cultures stimulated by phagocytosis of bacteria. These studies were designed to evaluate the physical characteristics and cellular specificity of chemotactic factors generated in primate lungs.

METHODS

Animals. Normal rhesus monkeys (*Macaca mulatta*), aged 1–3 yr, weighing 10–20 pounds, and of both sexes, were used. Adults were born and raised in the breeding facilities of the National Institutes of Health and were maintained in individual cages on a diet of monkey chow supplemented with liquid multivitamins and fresh fruit.

Bronchoalveolar lavage. BAL was performed as described previously (5). After light general anesthesia with 0.5 ml Ketamine hydrochloride (Bristol Laboratories Div., Syracuse, N. Y.) and local anesthesia of the lower pharynx and vocal cords with 2% lidocaine (Med-Tech, Inc., Elwood, Kans.), a fiberoptic bronchoscope (model FO-8800, American Cystoscope Makers Inc., Stamford, Conn.) was positioned in one of the main-stem bronchi; 400 ml of 0.9% saline was infused in 50-ml aliquots and aspirated. Bacterial cultures in representative animals revealed 100 colonies or less of mixed flora per lavage concentrate. Animals tolerated the procedure well, remained afebrile (rectal temperature), and chest X ray showed no pneumonic infiltrates. When repeated lavages were performed in an animal, the same portion of lung was lavaged each time.

Preparation of BAL fluid and respiratory cells. Lavage fluid was immediately centrifuged at 500 g and 15 min at 25°C, and the supernate was decanted from the cell pellet. The percentage of erythrocytes in the pellet was consistently less than 3% so the fluid was considered to contain material recovered from the air side of the lower respiratory tract. The supernatant lavage fluid was concentrated to a final volume of 5 ml at 4°C with positive pressure ultrafiltration (Diaflo, UM-05 membrane, Amicon Corp., Scientific Sys. Div. Lexington, Mass.) and then frozen at –70°C until used. The cell pellet was resuspended in modified Hanks' balanced salt solution² (HBSS) and washed twice before a final cell suspension was made.

Cell viability was assessed by eosin Y dye exclusion (6). Leukocyte counts were performed with a particle counter (Coulter Electronics Inc., Hialeah, Fla.), and differential

counts were made from cytocentrifuge smears stained with Wright-Giemsa stain. Phagocytic cells were identified in wet preparations by staining with neutral red dye (7).

Establishment of short-term alveolar macrophage cell cultures and phagocytosis experiments. Respiratory cells were cultured on glass surfaces in 250-ml Erlenmeyer flasks containing McCoy's 5A medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 0.3% vol/vol L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. About 35×10^6 viable macrophages were added to each flask and allowed to adhere in an atmosphere of moist air and 5% CO₂ at 37°C. Monolayers were incubated for 24 h; then washed three times with HBSS with added calcium and magnesium, and reconstituted with 50 ml of HBSS/flask. Adherent cells consisted of approximately 95% alveolar macrophages. To insure comparability of cell cultures, individual flasks were checked for uniformity of the cell monolayers by examination with an inverted microscope at 200×. Viability of an aliquot of cells was determined with eosin Y dye exclusion before and after each experiment.

Alveolar macrophages in culture were stimulated by phagocytosis of opsonized heat-killed *Staphylococcus albus*. Bacteria were opsonized by incubation for 1 h at 37°C with an equal volume of fresh monkey serum, followed by four washes in 0.9% normal saline. Opsonized, washed bacteria were added to monolayers in a ratio of bacteria to macrophages of 50:1. After 1 h of incubation at 37°C in a shaking water bath, monolayers were washed four times and reconstituted with 50 ml HBSS (protein-free culture media). In all cases macrophages obtained from these flasks showed extensive ingestion of bacteria. 4-ml samples of bathing media were obtained from the flasks at 0, 1, 3, 5, and 8 h after phagocytic challenge and chemotactic activity was measured in these undiluted samples. With each sampling, an equivalent amount of media was replaced to keep the volume of media in the flask constant.

Preparation of blood leukocytes. PMNs were prepared from heparinized peripheral blood by Ficoll-Hypaque (8) and dextran sedimentation (Dextran T-250, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). Residual erythrocytes were removed by hypotonic lysis (9). Preparations with greater than 95% PMNs were obtained with these methods.

The mononuclear cell suspensions obtained from the Ficoll-Hypaque gradient centrifugation contained over 95% mononuclear cells of which about 80% were lymphocytes and 20% were monocytes.

Assay of chemotactic activity. Chemotaxis was measured with a modification of the morphologic assay described by Zigmond and Hirsch (10). For PMNs the leukocytes were placed in the upper compartment of a chemotactic chamber which was separated from a lower compartment by a 5-µm micropore filter (Millipore, Millipore Corp., Bedford, Mass.). For mononuclear cell chemotaxis an 8-µm micropore filter (Sartorius, Beckman Instruments, Inc., Science Essentials Co., Mountainside, N. J.) was used. Cells were suspended at a density of 2.3×10^6 PMNs/ml or 3.0×10^6 mononuclear cells/ml in Gey's medium which contained 2% of bovine albumin, penicillin, and streptomycin (Microbiological Associates, Bethesda, Md.). The chemotactic stimulus was added to the lower compartment. Incubation times were 30 min for PMNs and 60 min for mononuclear cells. Micropore filters were removed, fixed in methanol, stained with hematoxylin and eosin, dehydrated in increasing concentrations of alcohol, and cleared in xylene (9). The distance (in micrometers) that the leading front of cells had migrated into the filter was determined with a microscope micrometer as previously described (10). Five individual measurements

² Prepared without calcium and magnesium ions or phenol red.

were made on each of duplicate filters and the data were pooled. Pooling of such data for each point is acceptable since we have shown previously (11) that the variability of measurement within a given filter is greater than the variability among several filters. Chemoattractants used as positive controls included sodium caseinate, 5 mg/ml in 0.9% saline (Difco Laboratories, Detroit, Mich.) (12), and normal monkey serum activated with endotoxin (*Escherichia coli*: 0127:B8 lipopolysaccharide B, Difco Laboratories) as previously described (13). Other controls included culture media from alveolar macrophage monolayers incubated without bacteria or from incubation of bacteria alone. In certain experiments cycloheximide (The Upjohn Co., Kalamazoo, Mich.) was added to macrophage culture media at a final concentration of 15 μ g/ml.

Gel filtration of concentrated bronchial lavage and alveolar macrophage culture media. Pooled specimens were concentrated by positive pressure ultra filtration (Amicon, UM-05 membrane) and then fractionated by gel filtration through Sephadex G-75 columns, dimensions 2.5 \times 100 cm (Pharmacia Fine Chemicals, Inc.). 5-ml fractions were eluted with 0.9% saline buffered with 0.04 M phosphate, pH 7.4, at 4°C. The elution of protein was estimated spectrophotometrically (280 nm) and the chemoattractant activities of the eluant fractions were measured with undiluted samples in the chemotactic chambers. For molecular weight estimates, columns were calibrated with blue dextran (2×10^6 daltons), bovine albumin (67,000 daltons), ovalbumin (45,000 daltons), cytochrome C (12,384 daltons) and bacitracin (1,450 daltons).

Antisera and immunological methods. Goat antisera prepared against the fifth component of human complement (C5) (Melroy Laboratories Inc., Springfield, Va.) and the third component of human complement (C3) (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) were heated at 56°C for 30 min before use. Partially purified C5a was obtained by Sephadex G-75 chromatography of human serum activated with *E. coli* endotoxin as previously described (13).

Statistical analysis. All data are expressed as the mean \pm SEM. Student's *t* test (two-tailed) was used to compare means of different experiments.

RESULTS

Cellular content and chemotactic activity in repeated BAL. Typically, from an initial BAL, the respiratory cell differential count consisted of 88–92% alveolar macrophages, 5–8% lymphocytes, and 2–4% PMNs, whereas cell preparations obtained at the second lavage 24 h later contained 90–93% PMNs, 4–9% alveolar macrophages, and 1–2% lymphocytes. Eosinophils were seen very infrequently.

Since BAL with sterile saline consistently resulted in a marked accumulation of PMNs in bronchial secretions within 24 h of the initial lavage, studies were done to monitor changes in the cellular composition of BAL and to measure chemotactic activity in this fluid from serial lavages. In a representative experiment, a monkey underwent four lavage procedures spaced during a 24-h interval and a final lavage at 72 h (Fig. 1). The total number of respiratory cells recovered and the absolute number of PMNs and alveolar macrophages are given (upper panel, Fig. 1) and compared with the chemoattractant activity for PMNs and mononuclear

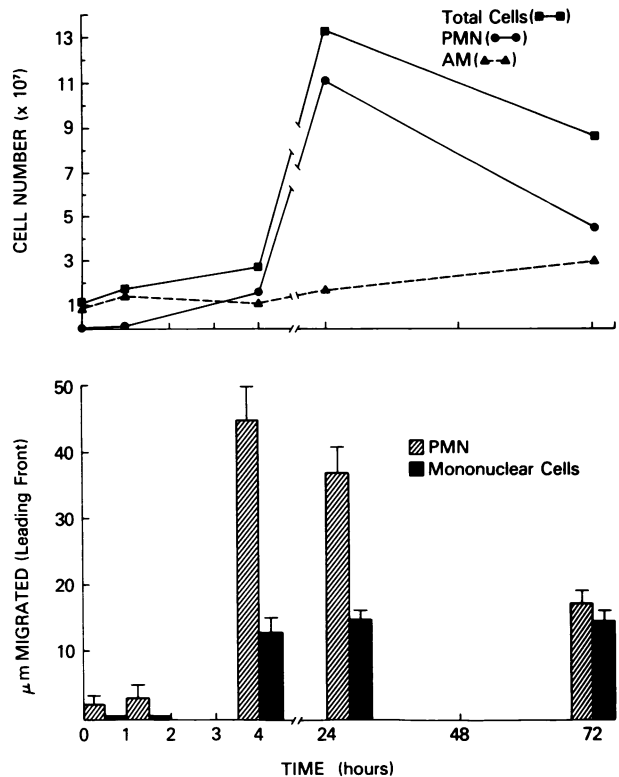


FIGURE 1 A monkey underwent BAL at intervals during a 72-h period to illustrate changes in the total number of recoverable respiratory cells and in the number of PMNs and alveolar macrophages (AM) (upper panel). Simultaneous chemotactic activity, expressed as migration of the leading front of cells into a micropore filter, in concentrated lavage fluid was measured for two cell preparations (vertical bars).

cells in the concentrated BAL (lower panel). The recovery of BAL fluid in each instance was comparable ($75 \pm 4.0\%$) and the animal had no evidence of infectious complications. As shown in Fig. 1, after base-line values were obtained, repeat lavage 1 h later did not yield significantly altered cell counts. However, at 4 h the total cell recovery had more than doubled and PMNs now accounted for about 45% of the cell population, a marked increase from the 2–4% in initial lavages. At this time, potent chemotactic activity, especially for PMNs, was present in the concentrated BAL. With the lavage at 24 h, approximately a 10-fold increase in total recoverable cells had occurred and PMNs accounted for 90% of the cells. Chemotactic activity in BAL remained high although lower than at 4 h, possibly related to chemotactic factor inactivation by local secretion of neutrophil products. With the final lavage 2 days later, the acute cellular response was abating, fewer PMNs were present, and chemotactic activity for this cell type had diminished as well. It is noteworthy that serial chest X rays obtained on the monkey showed no recognizable abnormalities.

TABLE I
Chemotactic Activity in Concentrated BAL Fluid

Specimen	Migration into filter of leading cell front	
	PMN chemotaxis*	Mononuclear cell chemotaxis*
	μm	
Buffer (phosphate-buffered saline)	40.8±0.6	36.6±0.7
Activated serum‡	81.2±1.6	62.0±2.8
Casein (5 mg/ml)	91.6±3.1	68.8±5.8
BAL fluid§		
Control	45.8±1.5	38.1±1.4
4 h	84.0±1.1¶	55.2±1.6**
24 h	76.2±2.0**	59.1±4.0**

* Data (mean±SEM) combined from four individual experiments with PMN and mononuclear cells from different donors in each case.

‡ Fresh whole monkey serum activated with endotoxin.

§ All specimens in each experiment were adjusted to the same protein concentration.

¶ $P < 0.001$ compared with control lavage.

** $P < 0.01$ compared with control lavage.

The relative chemotactic activity for donor monkey PMNs and mononuclear cells was measured in concentrated BAL fluid obtained from four animals relavaged at 4 and 24 h after an initial lavage (Table I). Concentrated BAL fluid obtained at 4 and 24 h showed marked chemotactic activity for both PMN and mononuclear cells compared with that measured in base line or control fluid ($P < 0.001$). These activities were comparable to the activity obtained with activated monkey serum and casein, which are both known to be potent chemotactic agents.

To determine if the lavage fluid obtained by repeated bronchial washings was causing directed migration (chemotaxis) or merely increasing random migration of cells, experiments were performed with the active material placed in various compartments of the chemotactic chambers (Table II). For both PMN and mononuclear cells there was significantly less migration by cells exposed to a uniform concentration of BAL (activated random migration) as opposed to cells exposed to a gradient of stimulus (directed migration). Both of these conditions, however, produced significantly more cell migration than when cells were exposed only to buffer (random migration). Thus, the bronchial lavage fluid fulfilled accepted criteria for being a chemotactic factor in that it stimulated random migration and when used under conditions that created a gradient, it converted random migration to directed migration (chemotaxis) (10).

Partial characterization of the chemotactic activity

in bronchial washings. To evaluate further the chemotactic activity of bronchial secretions, BAL fluids were pooled from five monkeys in whom lavages were done 24 h after an initial lavage. The concentrated pool of bronchial lavage was fractionated by gel filtration (Sephadex G-75) and the chemotactic activity measured in the effluent fractions (Fig. 2). Two peaks of chemotactic activity for PMNs were noted, one eluting at 320 ml (peak A) and another eluting at 410 ml (peak B). Mononuclear cell chemotactic activity was also present in the fractions eluting at 320 ml (designated peak C), but was not detected in other fractions recovered at 410 ml. Calibration of the Sephadex G-75 column with proteins of known molecular weight provided an estimate of the molecular size of the chemotactic factors: 15,000 daltons for peak A and peak C, and less than 5,000 daltons for peak B. Fractions with chemotactic activity were pooled and concentrated 10-fold (Amicon, UM-05 membrane) for further characterization of the PMN-chemotactic activity (Table III). Peak A material was heat stable after incubation at 56°C for 45 min. However, the chemotactic activity was diminished by 45% ($P < 0.001$) with treatment with goat antiserum to human C5 but not by similar exposure to antiserum against C3. Peak B, in contrast, was heat labile ($P < 0.02$) and was not affected by treatment with goat antisera to the third and fifth components of human complement.

Chemotactic activity obtained from alveolar macrophages stimulated in vitro. In experiments designed

TABLE II
Evidence That Concentrated BAL* Produces Directed Cell Migration (Chemotaxis)

Parameter	Contents of chamber compartment		Migration into filter of leading cell front	
	Upper	Lower	PMN migration	Mononuclear cell migration
	μm			
Random migration‡	PBS and cells	PBS	38.7±0.5	36.0±0.6
Activated random migration‡§	BAL and cells	BAL	56.0±1.0	47.4±1.7
Directed migration§	PBS and cells	BAL	83.0±1.3	62.0±1.5

PBS, phosphate-buffered saline.

* BAL was obtained 24 h after an initial lavage.

‡ $P < 0.001$ for activated random migration of cells compared with PBS for both PMNs and mononuclear cells.

§ $P < 0.001$ for directed migration of cells compared with activated random migration for both PMNs and mononuclear cells.

to investigate the possible source of the chemotactic activity measured in concentrated BAL fluid, monkey alveolar macrophages were established in *in vitro* cultures. Monolayers were stimulated by phagocytosis of heat-killed opsonized *S. albus* and chemotactic activity in the culture fluid from the monolayers was measured in chemotactic chambers. Results from a representative experiment are shown in Fig. 3A; data similar to that in Fig. 3 were obtained in three separate experiments. Both the alveolar macrophage monolayer incubated with bacteria (○) and a monolayer of alveolar macrophages from the same cell pool but not incubated with bacteria (●) developed considerable chemotactic activity for PMNs in culture supernates by 1 h of incubation, and this activity increased linearly over the 8-h interval of the experiment. The supernatant fluid of a cell-free suspension (□) of alveolar macrophages (not glass adherent), obtained from the same macrophage

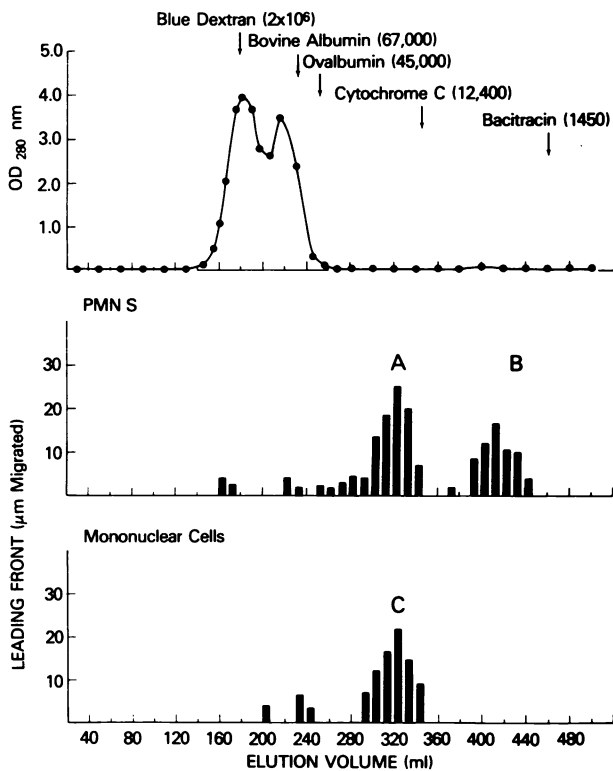


FIGURE 2 The elution profile of concentrated BAL fluid from a Sephadex G-75 column is shown. Protein is located in the upper panel (OD 280 nm) and the elution positions of proteins with known molecular weights are given. Chemotactic activity in the column fractions, depicted on the ordinate as micrometers of migration of the leading cell front into a micropore filter, is shown for PMNs (middle panel) and for mononuclear cells (lower panel). Concentrated BAL fluid has two peaks of chemotactic activity for PMNs which eluted at 320 ml (peak A) and at 410 ml (peak B), and one peak of chemotactic activity for mononuclear cells which also eluted at 320 ml (peak C).

TABLE III
Characterization of Substances Chemotactic for PMNs Isolated by Column Fractionation from Pooled BAL

Specimen	PMN chemotaxis*	Percent change†
	μm	%
Buffer (PBS)	37.0 \pm 1.0	—
Peak A		
No treatment	83.8 \pm 0.7§	—
Heated at 56°C for 45 min	82.8 \pm 1.0	-1.2%
Anti-C3	79.7 \pm 2.1	-5.0%
Anti-C5	46.4 \pm 1.9§	-44.6%
Peak B		
No treatment	57.9 \pm 3.9¶	—
Heated at 56°C for 45 min	37.7 \pm 2.9¶	-35.0%
Anti-C3	57.4 \pm 4.1	-1.0%
Anti-C5	56.1 \pm 4.9	-3.0%

PBS, phosphate-buffered saline.

* Data (mean \pm SEM) measuring PMN migration (migration into filter of leading cell front) are presented from four experiments each performed in duplicate with PMNs from a different monkey donor in each case. Five high power fields were counted per filter.

† Percent decrease in activity compared with untreated specimen.

§ $P < 0.001$.

¶ $P < 0.02$.

pool and given the phagocytic stimulus with bacteria, showed significantly less ($P < 0.01$) chemotactic activity than either of the adherent monolayer preparations. As a control, heat-killed opsonized bacteria incubated in media without cells (▲) had little chemotactic activity.

Further studies were done to determine if the chemotactic material released from alveolar macrophages into the culture supernatant was preformed in the cell or synthesized after stimulation. Cell lysates prepared from alveolar macrophages regularly had extensive chemotactic activity for both PMNs and monocytes, suggesting there is some storage of the factor within the cell. Bacteria-stimulated alveolar macrophage monolayers (Fig. 3B) were incubated with 15 $\mu\text{g}/\text{ml}$ of cycloheximide (■) to study the effects of this protein synthesis inhibitor on the generation of chemotactic activity. Cycloheximide significantly inhibited release of the chemotactic activity into the supernatant fluid ($P < 0.001$) for all points compared with normally stimulated (□) cells. Control experiments, not depicted, showed no reduction in PMN migration when the same concentration of cycloheximide was added to a casein chemotactic stimulus. Thus, the effect of cycloheximide was on the production and (or) release of chemotactic factor(s) from the cell monolayer and not due to a toxic or inhibiting influence of cycloheximide per se on the chemotactic assay.

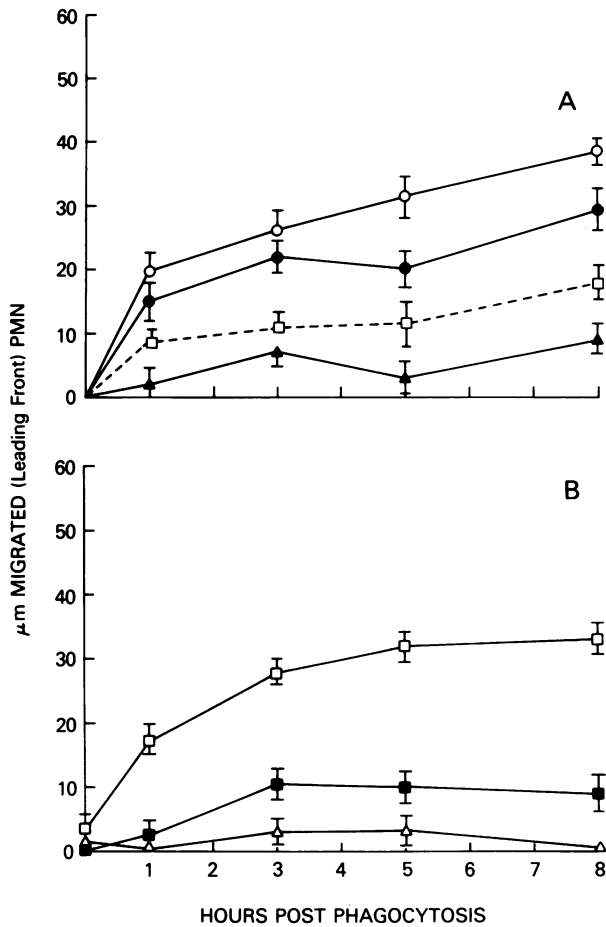


FIGURE 3 The generation of chemotactic activity for PMNs, depicted as micrometers of migration into a micropore filter, from alveolar macrophage monolayers is shown during 8 h of incubation. Panel A shows that both the alveolar macrophage monolayer incubated with bacteria (○) and a cell monolayer from the same pool but not incubated with bacteria (●) generate considerable chemotactic activity in the culture media when compared with that from a nonadherent alveolar macrophage cell suspension (□) or that from opsonized bacteria alone incubated without macrophages (▲). Chemotactic activity is plotted as the response with the stimulus minus the response with buffer controls (random migration). Panel B shows that incubation of the cell monolayer with 15 $\mu\text{g}/\text{ml}$ of cycloheximide in the culture medium inhibits release of the chemotactic material from bacteria-stimulated macrophages (■) compared with normal activity generated from stimulated monolayers (□). Background activity from bacteria and culture media only is minimal (Δ).

Alveolar macrophage incubation experiments measuring the chemotactic activity for mononuclear cells in monolayer's supernatant fluid showed similar findings. Chemotactic activity was measured after 1 h of incubation and this increased over the duration of the experiment. Considerable activity was again measured in fluid from monolayers not stimulated with bacteria.

Partial characterization of the chemotactic activity released from alveolar macrophages in vitro. Supernatant fluids from five alveolar macrophage monolayers, initially stimulated with heat-killed *S. albus* and incubated for 8 h, were pooled, concentrated 50-fold (Amicon, UM-05 membrane) and gel filtered through Sephadex G-75 (Fig. 4). No change in optical density readings (280 nm) denoted minimal protein elution from the column (contrast with BAL in Fig. 2). However, a prominent peak of chemotactic activity for PMNs eluted at about 400 ml, corresponding to a molecule of less than 5,000 daltons in weight. No significant chemotactic activity for mononuclear cells were detected in the elution fractions. Column fractions with chemotactic activity (Fig. 4) were pooled and concentrated 10-fold (Amicon, UM-05 membrane); this material was further characterized in Table IV. The activity was heat labile and was not affected by prior incubation with goat antiserum to human complement components C3 and C5. This concentrated material induced directed migration of PMNs as

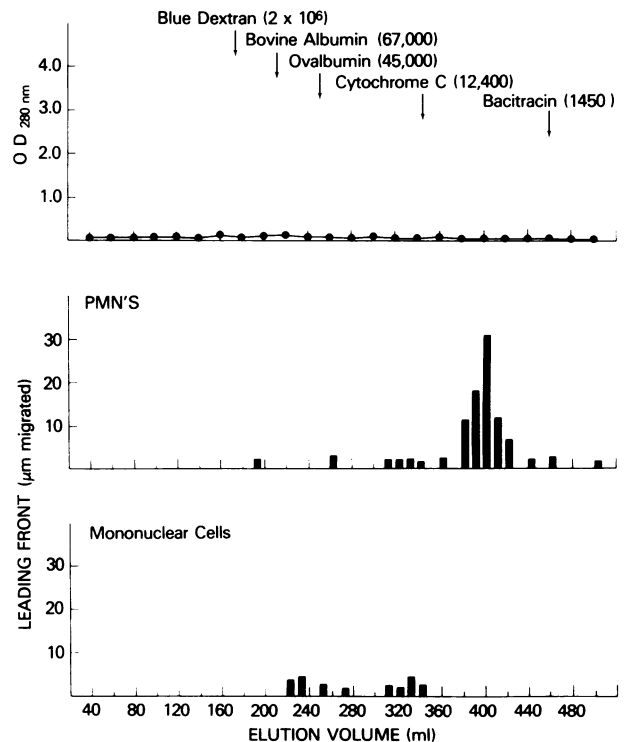


FIGURE 4 The elution profile of concentrated alveolar macrophage culture media, obtained after 8 h of incubation of stimulated macrophages, is depicted after fractionation through a column of Sephadex G-75. Optical density readings for protein and the elution positions of various calibration proteins are shown in the upper panel. Effluent fractions with chemotactic activity, given in terms of migration of the leading front of cells into a micropore filter, are noted by vertical bars for PMNs (middle panel) and for mononuclear cells (bottom panel).

outlined previously (Table II) but had no detectable chemotactic activity for mononuclear cells. In essence this material had characteristics similar to those found for Peak B (Fig. 2 and Table III) material in BAL.

DISCUSSION

BAL of the primate lung was a sufficient stimulus to induce an accumulation of PMNs in the distal airways and alveoli within 24 h after a single lavage. With repeated lavages, such an accumulation of PMNs became evident in approximately 4 h. Concentrated BAL fluid promoted directed movement or chemotaxis of PMNs and of mononuclear cells as well. Further analysis has shown this chemotactic activity to consist of at least two distinct factors which have different physical characteristics and different chemoattractant specificities. As demonstrated by gel filtration separation (Fig. 2), the chemotactically active material in concentrated BAL fluid eluted as two major peaks. One peak, with an estimated molecular weight of 15,000 daltons, had chemotactic activity for both PMN and mononuclear cells, was heat stable, and was diminished in activity by treatment with antiserum to the fifth component of human complement. The other active peak, corresponding to material of about 5,000 daltons, had detectable chemotactic activity only for PMNs, was heat labile, and was not affected by treatment with goat antiserum to the third of fifth complement components (Table III).

To investigate the possible origin of these chemotactic factors in the respiratory tract, *in vitro* cultures of normal monkey alveolar macrophages were studied. These cells, when adherent to glass surfaces or stimulated by phagocytosis of opsonized killed bacteria, secreted material into culture media (protein-free medium) which was chemotactically active for PMNs, was heat labile, unaffected by treatment with antiserum to human C3 or C5, and had a molecular weight less than 5,000 daltons (Table IV and Fig. 4). Moreover, macrophage synthesis and (or) release of this material was prevented by cycloheximide (Fig. 3B).

It was of interest that the chemotactic activity localized in Peak A after gel filtration of concentrated BAL fluid (Fig. 2) was heat stable and diminished by incubation with antiserum to human C5. It would appear that this material represents the complement-derived chemoattractant, C5a, which also has a molecular weight of approximately 15,000 daltons, is heat stable and is diminished in activity by anti-C5 antiserum. C5a has been found to be chemotactic for neutrophils (14) mononuclear cells (11, 14–16), and eosinophils (17). Chemotactically active complement components present in pulmonary secretions may originate from local activation of the complement pathway in the lower respiratory tract. Although low levels

TABLE IV
Characterization of Material Chemotactic for PMNs
in Supernatant Fluid for Alveolar
Macrophage Monolayers

Specimen	PMN chemotaxis*	Percent change†
	μm	%
Buffer (PBS)	41.7 \pm 1.4	—
Casein (5 mg/ml)	101.1 \pm 6.9	—
Column peak§		
No treatment	69.2 \pm 1.9	—
56°C, 45 min	46.7 \pm 2.4¶	-32.5
Anti-C5	67.9 \pm 2.1	-2.0
Anti-C3	68.9 \pm 1.6	-0.5

PBS, phosphate-buffered saline.

* Data (mean \pm SEM) measuring PMN migration (migration into filter of leading cell front) are presented from four experiments each performed in duplicate with PMN cells from different donors in each case. Five highpower fields were counted per filter.

† Percent decrease in activity compared with untreated specimen.

§ Gel filtration on Sephadex G-75 of pooled monolayer supernatant fluids (Fig. 4).

¶ Heating significantly reduced chemotactic activity ($P < 0.001$) compared to untreated material.

of complement activity can be detected in rabbit (18) and human (19) BAL fluid, appreciable amounts of serum factors could enter the lung with extensive inflammation and be activated by locally secreted material. Several studies (16, 17, 20, 21) have shown granulocytes to contain a material that generated chemotactic activity from whole serum and from isolated C5. Ward and Zvaifler (22) have demonstrated that the enzyme responsible for direct cleavage of C5 to C5a is released *in vitro* from rabbit PMNs after phagocytosis of immune complexes. In addition it has been shown that human PMNs, during phagocytosis of latex particles, release an enzyme capable of generating chemotactic activity from whole serum primarily by activating the classical and the alternate complement pathways, and by the direct cleavage of C5a from C5 (23). This enzyme is located in specific granules and released rapidly after phagocytosis (24). A recent report (25) has shown that activated complement components in turn can induce lysosomal enzyme release from macrophages. Thus, an amplification system mediated through locally released activated complement components in the lung could permit persistence of an inflammatory state.

Complement components have been reported to be synthesized by macrophages and their monocyte precursors by several investigators. Primate peritoneal macrophages synthesize the third and fourth components of complement (26), and human monocytes

synthesize and secrete the second component of complement (C2) (27). It is conceivable that yet another complement protein, C5, might be produced by alveolar macrophages and secreted locally into lung secretions. The localization of C5 in alveolar macrophages would help to prove this point.

In addition to complement-related mediators, our experiments establish that another heat labile, small molecular weight chemotactic factor is produced locally in vivo and is apparently derived from alveolar macrophages as shown by in vitro experiments. The requirement of cells for production of this factor was clearly established. Little chemotactic activity was measured in supernates from stimulated macrophage cultures incubated with 15 $\mu\text{g/ml}$ of cycloheximide (Fig. 3B), demonstrating that protein synthesis is involved in the generation or the release of this factor (28, 29). Alveolar macrophages, whether cultured on glass surfaces or stimulated by phagocytosis of bacteria, produced considerable chemotactic activity compared to the same number of macrophages incubated as a free cell suspension (Fig. 3B). This suggests that just the attachment to a glass surface is enough stimulation to induce release of a sizeable amount of active factor.

The relationship between the active material, described in these experiments, produced by macrophage monolayers and chemotactic materials described by others is unclear at present. This chemotactic factor which preferentially attracts PMNs has properties similar to those described for dialyzable transfer factor (30). Although this chemotactic factor may also have properties in common with the small molecular weight eosinophil-tactic tetrapeptides (ECFA) (31), the material appears to differ from ECFA. Eosinophils were not prominent among the respiratory cells recovered with repeated lavage. Moreover, in studies using eosinophil-enriched cell preparations and measuring leukocyte locomotion under agarose (32), which enables precise identification of cell types, our chemotactic factor did not cause preferential attraction of eosinophils in the mass of migrating cells.³ Macrophages have been shown to secrete a number of biologically active substances, including lysozyme (33), collagenase (34), plasminogen activator (35), elastase (36), endogenous pyrogen (37), and the complement components C3 and C4 (26). The small molecular weight of the macrophage chemotactic factor we have described distinguishes it from some of the other macrophage products listed above. Thus, this factor is another biologically active molecule, secreted by alveolar macrophages, which may be important in lung defenses.

³ Klempner, M. A., and J. I. Gallin. Unpublished observation.

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