Cystic Fibrosis Ciliary Dyskinesia Substances and Pulmonary Disease

EFFECTS OF CILIARY DYSKINESIA SUBSTANCES ON NEUTROPHIL MOVEMENT IN VITRO

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ABSTRACT Cultured mononuclear cells (MNC) from individuals homozygous or heterozygous for the defective gene causing the inherited disease cystic fibrosis (CF) synthesize three unusual "mediators" termed ciliary dyskinesia substances (CDS), which markedly affect tracheal mucociliary systems in vitro. MNC cultures from normal healthy controls do not accumulate any CDS, whereas MNC cultures from non-CF patient controls with pulmonary disease synthesize at least one CDS. The possible involvement of the CDS in pulmonary disease is being investigated. In this study, we sought to determine whether the CDS could be chemoattractants for polymorphonuclear neutrophils (PMN), since they have characteristics in common with known chemoattractants generated by alveolar macrophages. Our analyses of crude MNC culture supernates indicated that cultures from both CF genotypes accumulate significantly higher levels of PMN chemoattractants than do analogous cultures from normal healthy controls. CF homozygote MNC also generated more activity than MNC from patient controls with chronic pulmonary disease. Fractionation of MNC culture supernates by gel permeation chromatography and characterization of active fractions demonstrated six distinct PMN chemoattractants in cultures from CF genotypes; five were also present in patient control and four in normal healthy control cultures. The excessive chemoattractant activity in MNC cultures from CF genotypes and patient controls was due to several different substances produced by monocytes: (*a*) two components of 1,000–3,500 mol wt, (*b*) two fragments of C5, and (*c*) a fragment of C3. One C5 fragment had ciliary dyskinesia activity, the other did not. The C3 fragment chemoattractant also had ciliary dyskinesia activity and was not found in MNC cultures from patient controls. A third CDS, which is CF-specific (5,000 mol wt), was neither chemotactic nor chemokinetic and did not inhibit random PMN migration; however, fractions containing this CF-specific CDS completely inhibited PMN chemotaxis in response to three different chemoattractants. We conclude that all of the CDS can potentially play a role in the pathophysiology of lung disease, as judged by their effects on PMN movement in vitro.

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INTRODUCTION

Chronic pulmonary disease is the major cause of morbidity and mortality in patients with cystic fibrosis (CF),[1] the most common inborn error of metabolism

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; CDS, ciliary dyskinesia substance; CF, cystic fibrosis; CI, chemotactic index; CKI, chemokinetic index; MI_C, cellular chemotactic migration inhibition index; MI_S, sample chemotactic migration inhibition index; MNC, mononuclear cells; Mϕ, macrophage(s); PBS, phosphate-buffered saline; PMN, polymorphonuclear neutrophils.

affecting the Caucasian population (1, 2). The pathogenesis of the obstructive phase of pulmonary disease in CF is complicated by the production of abnormal mucous secretions and recurrent bacterial infections (particularly with *Staphylococcus aureus* and *Pseudomonas aeruginosa*), but there is both direct and indirect evidence that much of the damage may be caused by excessive migration of polymorphonuclear neutrophils (PMN) into the lung (1, 3). According to Wood et al. (1), the extent of PMN infiltration does not correlate with the clinical state of the CF patient, suggesting the presence of a chronic inflammatory process even in those patients with clinically minimal disease and thus the possibility of some fundamental aberration in the inflammatory process.

The inflammatory response is an important normal host defense mechanism in the lungs for the containment and clearance of infectious agents, and alveolar macrophages (Mϕ) are generally considered to be the phagocytes that make initial contact with foreign material (4). PMN infiltration seems to occur after the activation of alveolar Mϕ by contact with foreign materials and is prompted by the elaboration of PMN chemoattractants by Mϕ (5–7). Mammalian Mϕ generate at least three PMN chemoattractants; one is a fragment of C5 (15,000 mol wt) derived by enzymatic cleavage of complement component C5 (5), and the other two are peptides of 10,000 and ~1,000–3,000 mol wt, respectively (5–7).

It is known that mononuclear cells (MNC) from both CF homozygotes and heterozygote carriers of the CF gene (CF genotypes) synthesize three unusual "mediators", termed "ciliary dyskinesia substances" (CDS) (8, 9). The CDS are similar in molecular weight to the Mϕ-derived PMN chemoattractants noted above. In addition, both groups of substances are peptides and are generated by purified monocytes (Mϕ) in vitro (5–9). One CDS (5,000 mol wt) found only in cultures of MNC of CF genotypes (the "CF-specific CDS"), is also synthesized by T lymphocytes (9). At least one of the other two CDS may be made by MNC from patient controls with pulmonary disease; however, they are not made by MNC from normal healthy controls (8, 9). In vitro these CDS promote disruption of normal ciliary synchrony (i.e., dyskinesia or ciliostasis) in rabbit tracheal epithelial explants (9), an effect that may be secondary to their action on metabolic systems governing intracellular levels of Ca^{++} and K^{+} (10). An analogy may be drawn between the CDS and the Mϕ-derived chemoattractants here also, since it is known that upon binding to PMN, chemoattractants induce several biochemical changes that lead to alterations in intracellular levels of K^{+} and Ca^{++} (11). The above similarities between the CDS and the chemoattractants produced by Mϕ prompted us to investigate whether the CDS could be PMN chemoattractants.

METHODS

Blood samples. Heparinized venous blood (50 U/ml Panheparin, preservative-free, Abbott Laboratories, North Chicago, Ill.) was obtained from 17 CF homozygotes, 17 heterozygote carriers for CF (parents of the patients with CF), 15 age-matched normal healthy controls, and 9 individuals (patient controls) with pulmonary disease (7 with chronic obstructive pulmonary disease, 1 with bronchial asthma, and 1 infected with *Mycobacterium fortuitum*) who were neither homozygous nor heterozygous for the CF gene as determined by analysis of their serum for the genetic marker cystic fibrosis protein (12), and by a negative family history for CF.

Cell culture. Short-term culture (6 d) of phytohemagglutinin-stimulated MNC or purified T lymphocytes (>99% pure) was performed by methods outlined in detail in recent publications (8, 9). T lymphocyte and MNC cultures consisted of 1.0-ml aliquots containing 10^6 cells per ml of standard culture medium (RPMI-1640 medium containing antibiotics and 1.0% bovine serum albumin, BSA) (9). Monocytes (>97% plastic adherent, phagocytic, acid-phosphatase-positive MNC) were purified from MNC and cultured for 4 d at 8×10^5 monocytes/2.0 ml in standard culture medium (RPMI-BSA) (9). Cell-free medium was obtained by filtering the cultures through a 0.45-μm prewashed (with unused medium) needle filter (Acrodisc, Gelman Sciences, Inc., Ann Arbor, Mich.). The medium was then split into multiple aliquots and frozen at −70°C until used for the experiments outlined below.

Detection of ciliary dyskinesia activity. Substances with ciliary dyskinesia activity were detected with a rabbit tracheal bioassay as described previously (8, 9). Unconcentrated samples were scored as active if they promoted disruption of normal ciliary synchrony (dyskinesia) within 35 min after application of the sample (8, 9).

Chromatography. Bio-gel P-10 gel (Bio-Rad Laboratories, Richmond, Calif.) permeation chromatography was performed using a calibrated 1.0 × 100-cm column and phosphate-buffered saline pH 7.2 (PBS) as eluent, as described previously (8, 9). Sephadex G-100 gel permeation chromatography was performed using a calibrated 1.2 × 100-cm column and 0.02 M ammonium bicarbonate acetic acid buffer, pH 7.2. Where noted in Results, acidification (pH 3.7) of culture supernates before chromatography was accomplished by adding an aliquot of 1.0 M glycine-HCl buffer (8). All of the CDS are normally complexed to macromolecular carriers (such as immunoglobulin (Ig)G when found in MNC cultures, but can be dissociated from their carriers by acidification and isolated by chromatography of the acidified mixture on a column of Bio-gel P-10; they are fully active and behave identically to the products made by CF monocytes and T lymphocytes (8, 9).

Individual tubes of the effluents were pooled as indicated in Results. These fractions were lyophilized and then reconstituted in water and dialyzed against PBS to equilibrium (24–36 h, except as noted below) before testing for chemotactic activity or ciliary dyskinesia activity (8). Dialysis tubing that retains substances of ≥3,500 mol wt was used exclusively, to prevent the loss of any CDS or low-molecular weight chemoattractants (Spectrapor 3 dialysis membrane tubing, Spectrum Medical Industries, Los Angeles, Calif.) (8). To ensure against the loss of potential chemoattractants in Bio-gel P-10 fractions VI and VII (see Results), these fractions were dialyzed for only 4 h against PBS. Under these conditions the phenol red marker (354 mol wt) present in fraction VII was not lost from the inside of the dialysis bag.

Unused cell-free culture medium (either fresh or incubated for 6 d at 37°C) and PBS were evaluated as controls in both

the rabbit tracheal bioassay and chemotaxis assays, as were column fractions from controls analogous to those generated from CF genotypes.

Neutrophil chemotaxis. Neutrophil chemotaxis under agarose was assayed using purified PMN (>99% pure) as described previously, differing only in that the agarose plates contained 0.5% BSA instead of human serum albumin and agarose-activated normal human serum served as the standard chemoattractant (agarose is as chemotaxigenic as zymosan) (13). The degree of chemotactic activity in each sample was calculated as a chemotactic index (CI), where

$$CI = \frac{\text{area of migration (square millimeters) toward attractant well (containing human serum or other test solutions)}}{\text{area of migration (square millimeters) toward control}} - 1.0.$$

A CI value of zero indicates no chemotactic activity. We routinely subtracted 1.0 from the raw CI values to eliminate the contribution of randomly migrating PMN to the area calculated for directed PMN migration. The control well was filled with either standard medium or PBS, depending on the mixture to be evaluated.

Effects of antisera and heating. To determine whether any of the CDS or chemoattractants were affected by antibodies specific for the fifth component of human complement (C5) or the third component of human complement (C3) (anti-C5 and anti-C3 respectively) or were inactivated by heating, the methods of Kazmierowski et al. (5) were followed with modification. Briefly, to study the effects of heating, aliquots of column-purified CDS or chemoattractants were heated in sealed tubes in a water bath at 56°C for 45 min. The samples were then allowed to cool to room temperature before testing. To study the effects of anti-C3 or anti-C5, 100-μl aliquots of column-purified CDS or chemoattractants were incubated for 60 min at 37°C and then for 240 min at 4°C with an excess (20 μl) of goat antiserum specific for either human C5 or C3 (Meloy Laboratories, Inc., Springfield, Va.). The anti-C3 and anti-C5 were heated at 56°C for 60 min before use. Control tubes contained (*a*) identical active aliquots incubated at 37°C and 4°C with 20 μl of PBS added rather than antiserum, and (*b*) 100 μl of PBS to which 20 μl of antiserum was added. The anti-C3 and anti-C5 were verified to be monospecific for human C3 or C5, respectively, by double immunodiffusion and immunoelectrophoresis.

Chemokinetic activity. To evaluate the possible chemokinetic activity of the CF-specific CDS [contained in Bio-gel P-10 fraction V from acidified culture medium of CF genotypes (see Results and ref. 9)], aliquots of fraction V from medium of MNC cultures of all donor types were dialyzed against water to remove electrolytes and then lyophilized and reconstituted in standard medium at a final concentration of either 2, 1, or 0.5 times compared to the original volume (i.e., the product from 10^6 cells in 1 ml). PMN were preincubated in the reconstituted aliquots for 15 to 30 min or mixed with the aliquots just prior to being placed in the central cell well (0-min preincubation) of each set on the agarose plates (13).

L-ascorbic acid (Ascorbate, Sigma Chemical Co., St. Louis, Mo.), which is chemokinetic (promotes enhanced random migration), but not chemotactic (14), was used (in medium) as a standard for evaluating the effects of fraction V components on random PMN migration. Chemokinetic activity was expressed as a chemokinetic index (CKI), where

$$CKI = \frac{\text{area of random migration (square millimeters) of PMN mixed with ascorbate or fraction V}}{\text{area of random migration (square millimeters) of PMN mixed with standard medium only}}.$$

Effects of CF-specific CDS on directed migration of PMN in response to chemotaxins purified from culture medium. To determine whether the CF-specific CDS present in Bio-gel P-10 fraction V of acidified culture medium could inhibit PMN chemotaxis, two types of experiments were performed. In one series of experiments, Bio-gel P-10 fraction V from untreated or acidified culture medium was mixed with other Bio-gel P-10 column fractions (I, II, or VI) containing PMN chemoattractants, and the resultant mixture was pipetted into the attractant well. To quantitate inhibitory effects, an MI_S value (sample chemotactic migration inhibition index) was calculated, where

$$MI_S = \frac{\text{CI obtained for PMN movement in response to the chemoattractant mixed with Bio-gel P-10 fraction V}}{\text{CI obtained for PMN movement in response to the chemoattractant alone}}.$$

In another series of experiments, PMN were incubated with aliquots of Bio-gel P-10 fraction V obtained by chromatography of either untreated or acidified culture medium, for 0, 15, or 30 min as described for evaluating potential chemokinetic activity, except that the ability of the PMN to respond to chemotaxins placed in the attractant well was evaluated concurrently with the effects of substances in fraction V on random migration (movement toward the medium or PBS well). To quantitate these inhibitory effects, an MI_C value (cellular chemotactic migration inhibition index) was calculated, where

$$MI_C = \frac{\text{CI obtained for PMN preincubated with Bio-gel P-10 fraction V}}{\text{CI obtained for PMN preincubated in standard medium}}.$$

All types of Bio-gel P-10 fraction V and various types of Bio-gel P-10 fractions I, II, and VI (i.e., from different MNC donors) were evaluated in both studies.

Statistical analysis. All data are expressed as mean±SD. Student's *t* test (two-tailed) was used to determine the significance of the results.

RESULTS

Chemotactic activity and ciliary dyskinesia activity in mononuclear cell culture medium. Significant amounts of PMN chemoattractant activity were found in MNC culture supernatants from all subjects studied (Fig. 1). Cultures from CF genotypes or patient controls, however, had significantly more activity than normal healthy control cultures ($P < 0.001$). In addition, the level of activity in CF homozygote cultures was significantly higher than in heterozygote carrier ($P < 0.001$) or patient control cultures ($0.01 < P < 0.025$). The level of activity in heterozygote carrier cultures was about the same as that found in patient control cultures (Fig. 1). When aliquots of the same MNC culture supernates were evaluated for the presence of CDS by recording the time required for each sample to promote ciliary dyskinesia [a measure of relative potency with respect to dyskinesia activity (8)], a trend similar to that noted above for the magnitude of the chemotactic activity was found for the same groups of subjects. MNC cultures from CF homozygotes contained more ciliary dyskinesia activity than cultures

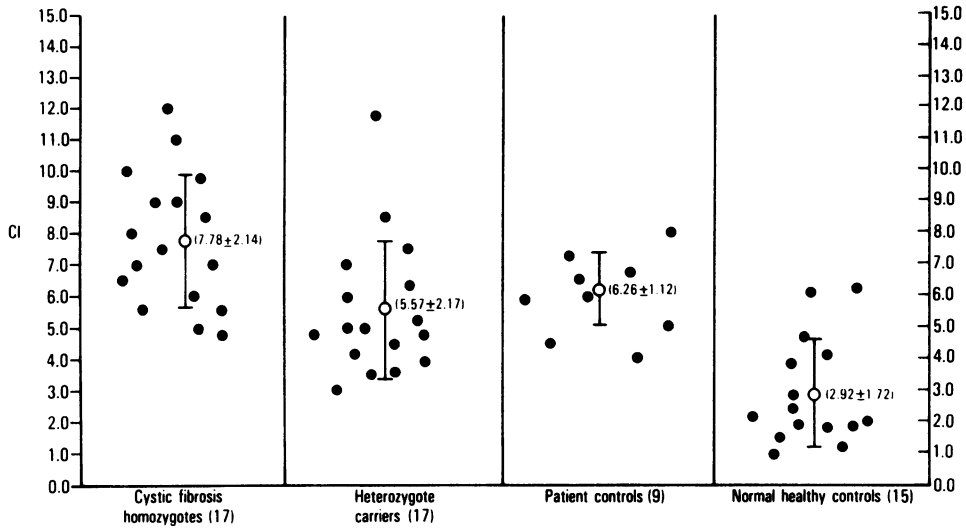
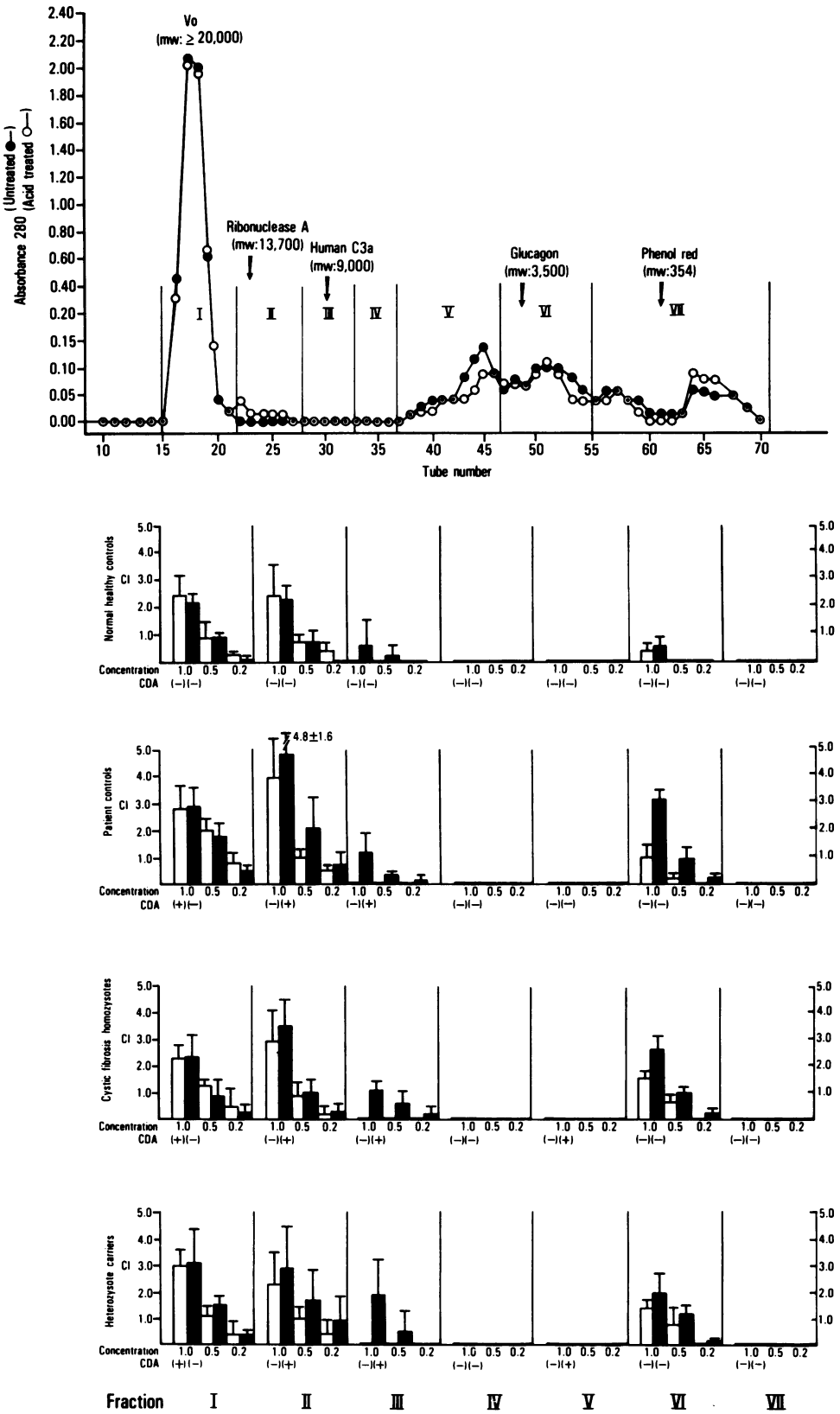


FIGURE 1 Neutrophil chemotactic activity in cell-free medium obtained from 6-d cultures of mononuclear leukocytes (10^6/ml) stimulated with phytohemagglutinin. The chemotatic activity is indicated as a CI. ●, represents the mean value for an individual culture analyzed on three separate occasions. ○, is the group mean (±SD). The CI value for the medium-only control was 0.02±0.01 (mean±SD for 50 determinations). Human serum which served as a control in each experiment was 8.93±1.60 (100 determinations).

from heterozygote carriers (mean±SD for time of onset of dyskinesia was 8.0±8.0 min vs. 20.0±10.0 min), whereas patient control cultures contained as much activity as heterozygote carrier cultures (23±7 min). As expected (8), normal healthy control cultures were routinely devoid of ciliary dyskinesia activity (no reaction by 35 min, the end of the observation period). This general concordance between the magnitude of chemoattractant activity and ciliary dyskinesia activity in each group of subjects tested seemed to support a notion that the CDS could be PMN chemoattractants. Further support for this supposition was sought by partially purifying the chemoattractants present in MNC culture supernates by gel permeation chromatography and evaluating each fraction obtained for chemoattractant and ciliary dyskinesia activities.

Gel permeation chromatography. A typical elution profile is shown in Fig. 2 (top panel) for the fractionation of untreated or acidified MNC culture medium on Bio-gel P-10. Significant levels of PMN chemoattractants were found in fraction I (≥20,000 mol wt), fraction II (15,000 mol wt), and fraction VI (1,000–3,500 mol wt) from untreated medium from all cultures. Fraction VI from normal control cultures contained only one-half to one-third the chemoattractant activity found in fraction VI from any of the other groups evaluated ($P < 0.001$); both the normal control and heterozygote carrier cultures contained less chemoattractant activity in fraction II than did cultures from CF homozygotes or patient controls (Fig. 2, lower panels). Acidification of aliquots of the same cultures prior to chromatography produced no significant changes in the levels of PMN chemoattractants found in fraction II or VI from normal controls but did result in the elution of increased levels of chemoattractants in these same fractions from CF genotypes or patient controls (Fig. 2). In addition, significant levels of chemoattractants were found in fraction III

FIGURE 2 Typical elution profiles for 2.0 ml untreated (●) or acidified (○) mononuclear leukocyte culture medium from a Bio-gel P-10 column (upper panel). Protein absorbance at 280 nm was recorded and the column was calibrated with substances of known molecular weight (mw) as indicated. Tube volumes of 1.8 ml were collected and the effluent was divided into seven fractions (I to VII) for analysis. Vo, void volume. The lower four panels show the results from assays of fractions I to VII from untreated (□) or acidified (■) culture medium for PMN chemoattractants or CDA. The ordinate for each panel indicates the magnitude of the PMN chemoattractant activity present, expressed as a CI. The abscissa indicates the concentration at which the reconstituted fraction was tested (relative to the original concentration, which is the activity from 1.0×10^6 cells/ml) and whether the fraction produced ciliary dyskinesia when tested at the highest concentration shown (+, activity present; −, no activity). Data shown are mean±SD for fractions from three different donors. Aliquots of culture medium from the same subjects were used to compare the results obtained from untreated and acidified culture medium.


Vo
(mw: ≥ 20,000)
Ribonuclease A
(mw:13,700)
Human C3a
(mw:9,000)
Glucagon
(mw:3,500)
Phenol red
(mw:354)
Absorbance 280 (Untreated ●—) (Acid treated ○—)
Tube number
Normal healthy controls
Patient controls
Cystic fibrosis homozysotes
Heterozysote carriers
CI
Concentration
CDA
4.8±1.6
Fraction

from acidified medium from both CF genotypes and patient controls. In contrast, almost no chemotactic activity was found in fraction III from normal control cultures (Fig. 2, lower panels). The levels of chemoattractants in fraction I were unaffected by acidification, and all cultures contained approximately the same amount of chemotactic activity in this fraction.

The results of our analyses of all Bio-gel P-10 column fractions for ciliary dyskinesia activity are also shown in Fig. 2 (lower panels). Only fraction I from untreated medium of cultures from CF genotypes and patient controls contained ciliary dyskinesia activity. Therefore, the fraction II and fraction VI chemoattractants from untreated culture medium are not CDS. In addition, it would appear that when the CDS are complexed to macromolecular carriers [as they are in untreated MNC culture medium (8)], they are not chemotactic for PMN, since the level of PMN chemoattractants in fraction I was similar for all cultures studied (Fig. 2).

Acidification of MNC culture medium is known to release the CDS from their macromolecular carriers and allows for their separation by Bio-gel P-10 chromatography (8; see Methods). CDS were found in Bio-gel P-10 fractions II, III, and V (5,000 mol wt) from acidified medium of CF genotypes and in fractions II and III from patient controls (Fig. 2, lower panels). The fact that fractions II and III both contained increased chemotactic activity when they contained CDS (compare CI values for fractions from untreated and acidified medium) indicated to us that at least two of the CDS may be PMN chemoattractants in their free state. In sharp contrast to this finding were the results for fraction V from CF genotypes containing the CF-specific-CDS (8, 9), which had no chemotactic activity when tested at a concentration of 0.20 to 1.0 (Fig. 2).

Effects of CF-specific CDS on random and directed PMN migration. To exclude the possibility that the CF-specific CDS (or other components in fraction V) might be present at such a high concentration that it could inhibit PMN movement by "deactivating" the PMN (15), we tested fraction V from acidified medium from CF genotypes over a range of concentrations from 2-fold to 1.0×10^{-4}-fold. No chemotactic activity could be detected at any concentration. We next explored the possibility that the CF-specific CDS might be chemokinetic for PMN. The CF-specific CDS was not chemokinetic and did not inhibit random PMN migration regardless of the concentration of fraction V (2-fold to 0.1-fold) or the incubation time (0, 15, or 30 min). (All CKI values were not significantly different from 1.0). In contrast, ascorbate promoted significant enhanced PMN migration when tested over a range of concentrations (1.0 to 10 mM). The magnitude of the CKI value was directly dependent on the concentration of ascorbate used and the time of preincubation. The most pronounced effect was observed when the PMN were preincubated in 5.0 M ascorbate for 30 min (mean CKI±SD for five experiments was 3.10±0.15). Ascorbate at 10 mM produced CKI values slightly <5 mM.

The effects of fraction V containing the CF-specific CDS on PMN migration were further evaluated in two series of experiments in which we studied the ability of the CF-specific CDS to inhibit directed PMN migration in response to the chemoattractants present in Bio-gel P-10 column fractions I, II, and VI. In the first series of experiments we tested the effects of mixing fraction V (unconcentrated or 2.0-fold concentrated) from untreated or acidified medium of all four types of donors with fraction I, II, or VI (unconcentrated). Aliquots of fraction V were dialyzed against water to remove electrolytes, lyophilized, and then reconstituted in an appropriate volume of the fraction containing the chemoattractant, and the mixture was placed in the attractant well. The results can be summarized as follow: (*a*) Fraction V from untreated cultures did not significantly affect PMN chemotaxis regardless of the concentration used, the identity of the cell donor, or the chemoattractant studied (Fig. 3; all MI_S values were >0.80. Data for normal healthy controls and patient controls is not shown). (*b*) Fraction V from acidified medium of normal control or patient control cultures did not contain significant inhibitory activity (all MI_S values ≥0.80), whereas fraction V from acidified medium of CF genotypes produced significant inhibition (see Fig. 3 for *P* values) of directed migration toward all the chemoattractants studied. In general, fraction V from CF homozygotes was more inhibitory than fraction V from heterozygote carriers. (*c*) Heating (56°C, 45 min) fraction V from CF genotypes eliminated the ability of all fractions to inhibit chemotaxis and also destroyed the CF-specific CDS. (*d*) The results presented in Fig. 3 were essentially identical for fractions I and II from acidified or untreated medium. However, in all instances fraction V from CF genotypes exerted a greater inhibitory effect on fraction VI from untreated medium than from acidified medium (even when the levels of chemoattractants in treated and untreated fraction VI were similar without fraction V added). We interpret these results as suggesting that the chemoattractants naturally occurring in fraction VI are more susceptible to inhibition than those additional chemoattractants eluting in fraction VI as a result of prior acidification of the culture medium.

In the second series of experiments, we evaluated the ability of components in fraction V to inhibit directed PMN migration when fraction V was mixed with the PMN instead of the chemoattractants. The experimental design was similar to that used to evaluate the chemokinetic activity of fraction V, except

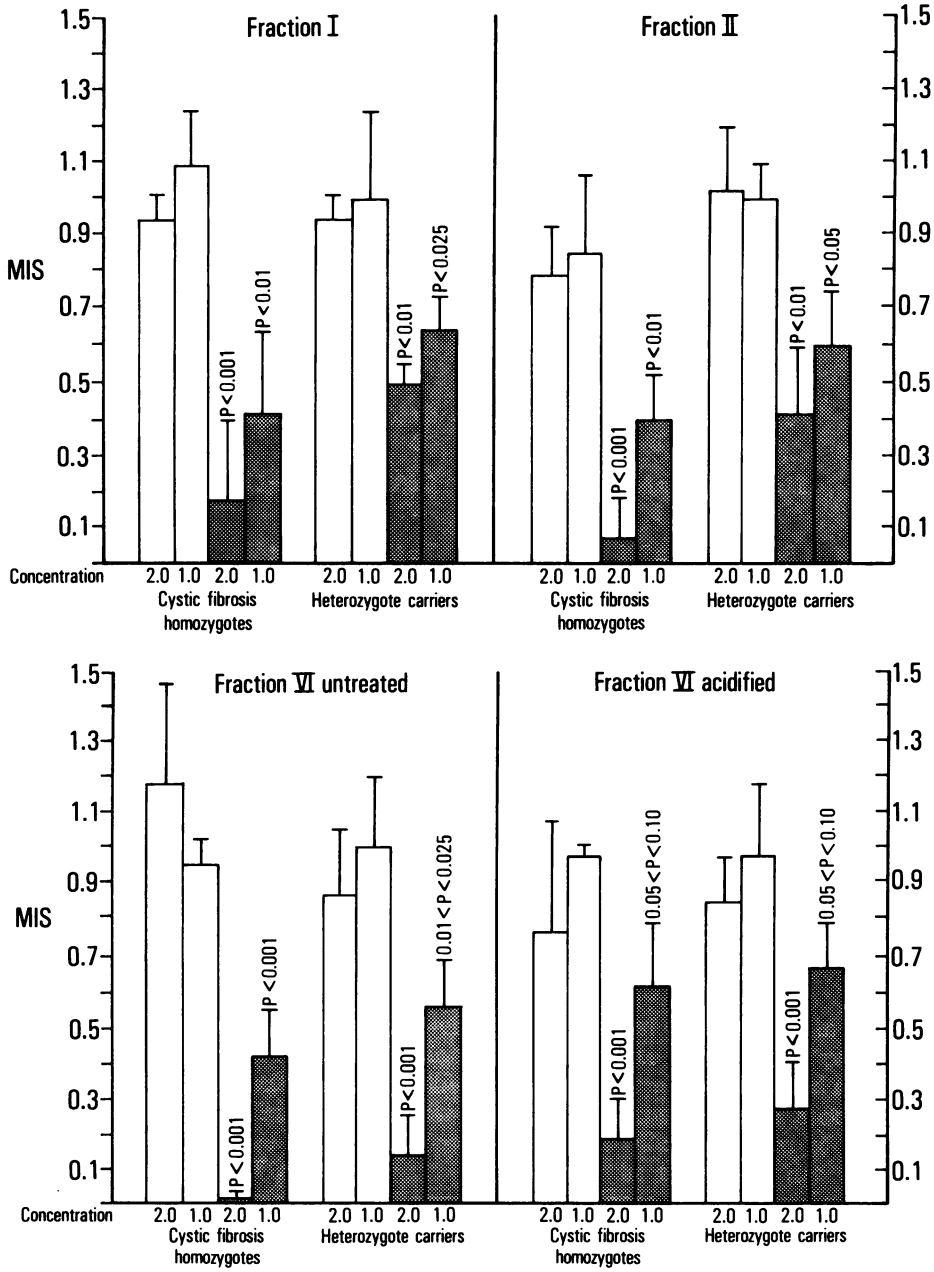


FIGURE 3 Inhibition of PMN migration in response to chemoattractants present in Bio-gel P-10 fraction I, fraction II, and fraction VI from untreated or acidified culture medium when Bio-gel P-10 fraction V was mixed with the above chemoattractant-containing fractions. The ordinate indicates the magnitude of inhibition of chemotaxis. The abscissa indicates the concentration of fraction V tested. Open bars are results for fraction V from untreated medium and shaded bars are results for fraction V from acidified medium. Data for normal healthy controls and patient controls are not shown (all mean MI_S values were ≥ 0.80). The significance of the inhibition observed relative to a control consisting of PMN incubated in medium only is noted above each column. Data shown in each column are mean±SD for four or more experiments.

that a chemoattractant was placed in the attractant well (see Methods). The results for this series of experiments directly paralleled those obtained for the first series with respect to (*a*) through (*d*) noted above. In addition, we found that the degree of inhibition was directly related to the concentration of fraction V used and the length of time the PMN were preincubated with fraction V.

Further characterization of the chemoattractants and ciliary dyskinesia substances. To obtain more information about the chemoattractants purified from untreated culture medium and to further evaluate whether or not the CDS present in fractions II and III from acidified culture medium were actually responsible for the increased chemotactic activity noted in these fractions (Fig. 2), we characterized the active components with respect to neutralization with anti-C3 or anti-C5, thermal stability, and cellular origin.

Effects of incubation with anti-C3 and anti-C5. Incubation with anti-C3 had no affect on the chemoattractant activities present in fraction I, II, or VI from either untreated or acidified culture medium (Table I) and did not neutralize the ciliary dyskinesia activity present in fraction II from acidified culture medium (data not shown). In contrast, both the chemoattractant activity (Table I) and the ciliary dyskinesia activity in fraction II were completely eliminated after incubation with anti-C5. The fraction I chemoattractant activity was partially reduced by incubation with anti-C5 (percent change −31.0%; $0.05 < P < 0.10$), whereas the fraction VI chemoattractants were unaffected by anti-C5 (Table I). When Bio-gel P-10 fraction I from normal control cultures was further fractionated on a column of Sephadex G-100, most of the chemotactic activity eluted at a position close to the BSA marker (66,000 mol wt) (Fig. 4). A second smaller peak of chemotactic activity eluted much later, with molecular weight close to the ribonuclease A marker (13,700 mol wt). The higher-molecular-weight activity was unaffected by treatment with anti-C5, whereas the 13,700 mol wt activity was completely eliminated by treatment with anti-C5 (data not shown). The results indicate that Bio-gel P-10 fraction I contains two chemoattractants, one of which is similar to the chemoattractant found in Bio-gel fraction II.

The chemotactic activity in fraction III was affected differently when incubated with anti-C3 or anti-C5, depending on the cell donor (Table II). Addition of anti-C3 to fraction III from normal healthy control or patient control cultures did not reduce chemotactic activity, whereas addition of anti-C5 completely neutralized the activity (percent change, −100% for both types; $P < 0.001$). In contrast, in culture medium from CF genotypes the chemoattractant activity in fraction III was significantly reduced by the addition of either anti-C3 or anti-C5 (Table II), suggesting the presence

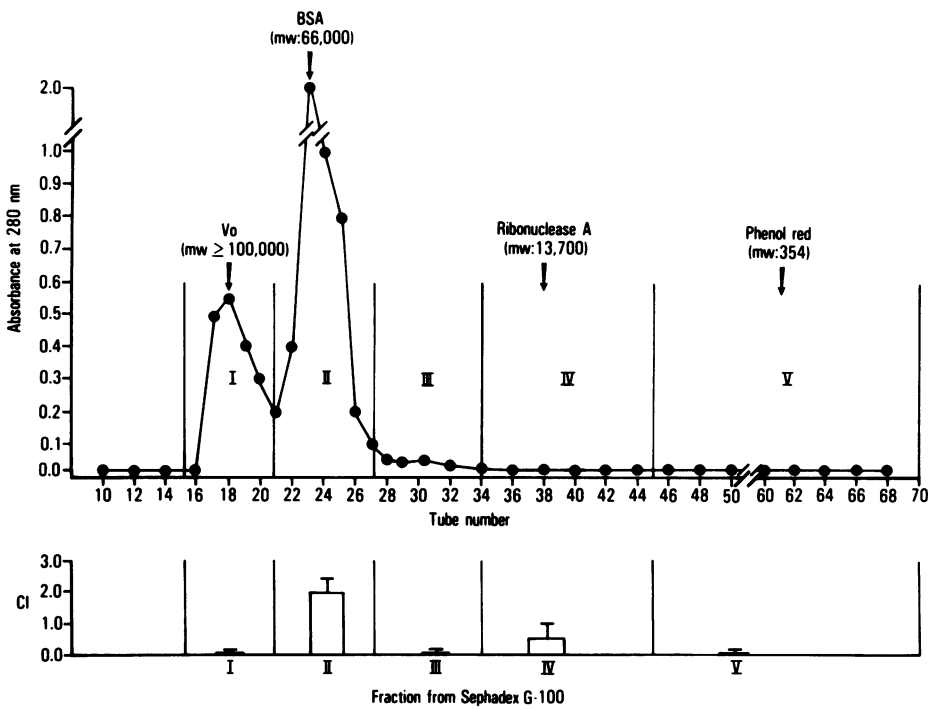


FIGURE 4 Elution profile for 2.0 ml of Bio-gel P-10 fraction I from a Sephadex G-100 column (upper panel). Protein absorbance at 280 nm was recorded and the column was calibrated with substances of known molecular weight (mw) as indicated. Tube volumes of 4.0 ml were collected and the effluent was divided into five fractions (I to V) for analysis of chemotactic activity. The lower panel shows the results from assays of each fraction reconstituted at twofold the original concentration. The ordinate is the same as in Fig. 2. Data shown are mean ± SD for three separate samples.

TABLE I

Effects of Incubation with Antibody-Specific for Human C3 or C5 on Substances Chemotactic for PMN Isolated from Culture Supernates of Mononuclear Leukocytes

Fraction*	Treatment	CI (units of activity‡	Percent change§
Fraction I	None	2.21±0.45	—
	Anti-C3	2.40±0.17	+9.0
	Anti-C5	1.53±0.35‖	−31.0
	37°C, 60 min	2.01±0.30	−9.0
Fraction II	None	3.33±0.37	—
	Anti-C3	2.75±0.10	−17.0
	Anti-C5	0.11±0.16¶	−97.0
	37°C, 60 min	3.21±0.47	−3.0
Fraction VI	None	1.89±0.10	—
	Anti-C3	1.95±0.10	+3.0
	Anti-C5	2.15±0.37	+14.0
	37°C, 60 min	2.00±0.20	+6.0

* From untreated or acidified culture medium after chromatography of crude culture supernate on Bio-gel P-10. Results were identical for both type of fractions.

‡ CI as defined in Methods. Data shown for each fraction are mean±SD for five separate experiments. Controls consisting of anti-C3 or anti-C5 in PBS only had CI values not significantly different from zero.

§ Percent change in activity relative to fraction not incubated with antibody.

‖ $0.05 < P < 0.10$.

¶ $P < 0.001$.

TABLE II

Effects of Incubation with Antibody Specific for Human C3 or C5 on Substances Chemotactic for PMN Present in Fraction III Obtained from Acidified Culture Supernates of Mononuclear Leukocytes

Culture type used to obtain fraction III	Treatment	CI (units of activity)*	Percent change‡
Normal healthy controls	None	0.81±0.31	—
	Anti-C3	0.69±0.11	−15.0
	Anti-C5	0.00±0.12§	−100.0
	37°C, 60 min	0.75±0.20	−7.0
Patient controls	None	1.38±0.18	—
	Anti-C3	1.17±0.55	−15.0
	Anti-C5	0.00±0.20§	−100.0
	37°C, 60 min	1.25±0.40	−9.0
CF homozygotes	None	1.01±0.33	—
	Anti-C3	0.37±0.10‖	−65.0
	Anti-C5	0.63±0.29¶	−38.0
	37°C, 60 min	1.05±0.20	+4.0
Heterozygote carriers for cystic fibrosis	None	0.94±0.12	—
	Anti-C3	0.49±0.18‖	−48.0
	Anti-C5	0.39±0.29‖	−59.0
	37°C, 60 min	0.96±0.10	+2.0

* CI as defined in Methods. Data are mean±SD for five separate experiments. Controls consisting of anti-C3 or anti-C5 in PBS only had CI values not significantly different from zero.

‡ Percent change in activity relative to fraction not incubated with antibody.

§ $P < 0.001$.

‖ $0.01 < P < 0.025$.

¶ $P < 0.10$.

of at least two types of PMN chemoattractants, one a fragment of C3 and the other a fragment of C5. The ciliary dyskinesia activity present in fraction III has been shown previously to be reduced by incubation with antibodies specific for human C3a (9). In the present study, we found that the ciliary dyskinesia activity present in fraction III from patient controls was neutralized by anti-C5, but not by anti-C3. In contrast, the dyskinesia activity in this fraction from cultures of CF genotypes was significantly affected by both anti-C3 and anti-C5. The CF-specific CDS present in fraction V was not affected by either anti-C3 or anti-C5.

Effects of heat treatment. The chemoattractants present in Bio-gel P-10 fractions I, II, and III from untreated or acidified culture medium were not significantly affected by heating (56°C for 45 min) regardless of the donor origin of the culture (Table III), although some reduction in activity was noted for fraction III (−24%, Table III). The ciliary dyskinesia activity present in fraction II was not significantly affected by heat treatment (active without concentration), whereas the activity in fraction III was significantly reduced (active only at threefold concentration) in samples from CF genotypes but not in samples from patient controls (active without concentration). In a single experiment using fraction III from a CF homozygote sample, we found that the activity remaining after heating the sample was markedly reduced by addition of anti-C5 but not anti-C3. The chemotactic activities in fraction VI from untreated culture medium were completely destroyed by heating, whereas the chemotactic activity in fraction VI from acidified culture medium was reduced by only 67% (Table III). The amount of activity remaining in fraction VI from acidified culture medium was significantly higher than the amount remaining in fraction VI from untreated culture medium ($P < 0.01$). We conclude that fraction VI from acidified medium contains two types of chemoattractants, which differ in thermal stability.

Cellular origin of chemoattractants and CDS. We have shown previously that purified T lymphocytes and monocytes from CF genotypes synthesize and secrete the CF-specific CDS found in Bio-gel P-10 fraction V from culture medium of MNC (9). Monocytes from these same subjects also synthesize and

TABLE III

Effects of Heat Treatment on Substances Chemotactic for PMN Isolated from Culture Supernates of Mononuclear Leukocytes

Fraction*	Treatment	CI (units of activity)‡	Percent change§
Fraction I	None	2.20±0.75	—
	56°C, 45 min	2.31±0.68	+5.0
Fraction II	None	2.86±0.80	—
	56°C, 45 min	2.36±0.67	−17.0
Fraction III (acidified culture medium)	None	1.19±0.95	—
	56°C, 45 min	0.90±0.44	−24.0
Fraction VI (untreated culture medium)	None	2.25±0.54	—
	56°C, 45 min	0.00±0.20‖	−100.0
Fraction VI (acidified culture medium)	None	2.80±0.64	—
	56°C, 45 min	0.91±0.24‖	−67.0

* Fractions as defined in Table I and Fig. 2. Data for Fractions I and II were similar for untreated and acidified culture medium.

‡ CI as defined in Methods. Data shown are mean±SD for five separate experiments. Medium only or PBS only controls had CI values not significantly different from zero.

§ Percent change in activity relative to fraction not treated by heating.

‖ $P < 0.001$.

secrete the CDS activities found in fraction II and fraction III from Bio-gel P-10 (Fig. 1; ref. 8). Thus, the cellular origin of each of the CDS found in culture medium from MNC is known.

Significant PMN chemoattractant activity ($P < 0.001$ as compared with medium-only controls) was found in all monocyte and T lymphocyte cultures established from either CF genotypes or normal control subjects (patient controls were not studied due to limitations in obtaining adequate amounts of cells) (Fig. 5). Although the number of each type of monocyte culture was small, a trend similar to that found for MNC cultures was evident with respect to the amount of activity in cultures for each group. In contrast, T lymphocyte cultures from all subjects contained similar amounts of chemoattractant activity.

Fractionation of untreated monocyte culture medium on a column of Bio-gel P-10 indicated that monocyte cultures from all donors studied contained significant chemotactic activity only in fractions II, III, and VI (Fig. 5). The chemoattractants in fractions II, III, and VI from monocyte cultures responded to incubation with anti-C5, anti-C3, or heating identically to analogous fractions from MNC, suggesting strongly that monocytes are the source of each of these chemoattractants. Fractionation of T lymphocyte culture medium on Bio-gel P-10 indicated that only fraction I contained significant activity (Fig. 5). This activity was resistant to heating, was not affected by treatment with anti-C3 or anti-C5, and was of 66,000 mol wt as determined by gel permeation chromatography on Sephadex G-100; thus, our data indicate that this activity is identical to the high molecular weight chemoattractant in Bio-gel P-10 fraction I from MNC cultures.

DISCUSSION

The possibility that three CDS, which are known to be abnormally produced by MNC from CF homozygotes and heterozygote carriers, are chemoattractants for PMN was examined. We first analyzed crude MNC culture supernatants and found general concordance between the amount of PMN chemoattractant activity and ciliary dyskinesia activity. The levels of both types of activity followed the order: CF homozygote cultures > patient control cultures ≥ heterozygote carrier cultures ≫ normal healthy control cultures. These results are consistent with the possibility that the CDS are PMN chemoattractants.

Our results obtained from the purification and characterization of the CDS and chemoattractants provided evidence that the excessive chemoattractant activity in MNC cultures from CF genotypes and patient controls could be attributed to elevated levels of three chemoattractants without ciliary dyskinesia activity (one of 15,000 and two of 1,000 to 3,500 mol wt, respectively), and of two CDS with chemoattractant activity (15,000 and 9,000 mol wt). In addition, our results also allow for a tentative identification of most of these substances or at least allow us to relate them to previously described PMN chemoattractants known to be synthesized by Mϕ (5–7). Our more important conclusions can be summarized as follows: (a) the 15,000-mol wt chemoattractant, which is elevated in both CF homozygote and patient control cultures (Fig. 2), appears to be identical to a fragment of C5 shown previously to be generated by rhesus monkey alveolar Mϕ (6), based on its cellular origin, thermal stability, and susceptibility to neutralization by anti-C5 but not anti-C3. (*b*) The thermal labile chemoattractant of 1000–3500 mol wt (found in Bio-gel P-10 fraction VI), which is produced in excess by monocytes from both CF genotypes and patient controls (Fig. 2), seems to be identical to a chemoattractant produced by human, guinea pig, and rhesus monkey alveolar Mϕ described previously by others (5–7). The identity of the thermal stabile chemoattractant(s) of 1,000–3,500 mol wt found in fraction VI from acidified culture medium is not known. (*c*) The 15,000-mol wt CDS and the 9,000-mol wt CDS both seem to be PMN chemoattractants. This conclusion is derived from two lines of evidence. First,

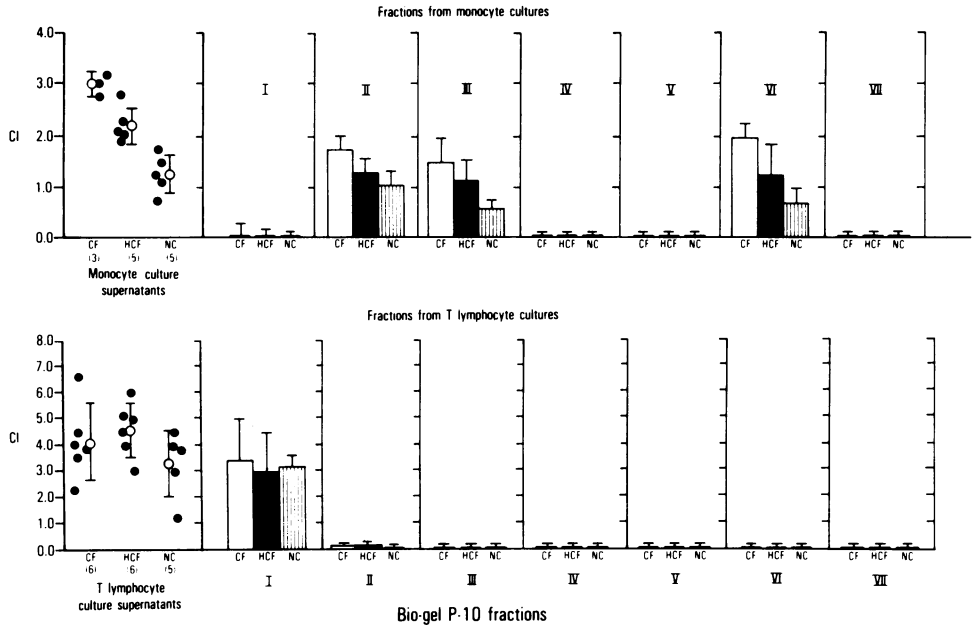


FIGURE 5 PMN chemotatic activity in cell-free medium obtained from 6-d cultures of purified T lymphocytes (10^6/ml) stimulated with phytohemagglutinin or 4-d cultures of purified monocytes (~8.0×10^5 monocytes per tube containing 2.0 ml medium), and in Bio-gel P-10 column fractions obtained from T lymphocyte or monocyte culture medium (fractions I to VII). Upper panel: Results for cultures of monocytes from CF homozygotes (CF), heterozygote carriers (HCF), and normal controls (NC). ●, mean values for individual cultures tested three or more times. ○, mean ± SD for the group. Data for column fractions are mean ± SD for three separate cultures. CI as defined in Fig. 1. Lower panel: Results for cultures of T lymphocytes from CF homozygotes, heterozygote carriers, and normal controls. Data for column fractions are mean ± SD for three separate cultures.

column fractions from acidified medium containing these CDS have increased chemotactic activity that can be accounted for by the CDS (especially fraction III from acidified culture medium). Secondly, treatments such as heating or incubation with anti-C3 or anti-C5 affect the chemotactic activities and dyskinesia activities in these fractions identically. The available evidence suggests that the 15,000-mol wt CDS is a fragment of C5, whereas the 9,000-mol wt CDS is a fragment of C3 uniquely found in cultures from CF genotypes (Table II).

All of the above substances that are fragments of C5 or C3 are most likely derived from cleavage of monocyte-produced C5 or C3 by proteolytic enzymes also secreted by monocytes (6, 16). They were not derived from serum, since all of our cultures were "serum-free" (containing only 1.0% BSA). Other investigators have also reported previously that proteolysis of C5 and C3 generates fragments with chemotactic activity (5, 17, 18). The excessive accumulation of chemotactic C3 and C5 fragments in CF genotype cultures might therefore reflect either hyperproduction of C5 or C3 or abnormal catabolism of C5 and C3 (involving either the generation or inactivation of chemotactic fragments). We feel that hyperproduction of C5 and C3 by MNC from CF genotypes is not a plausible explanation, for several reasons: (*a*) MNC from CF genotypes exhibit normal levels of [^{3}H]thymidine incorporation and normal or subnormal synthesis of protein, C5, and C3 when stimulated with PHA or pokeweed mitogen (9; manuscript in preparation); (*b*) CF genotypes have normal levels of serum complement activity (1, 2); and (*c*) normal cultures might be expected to produce significant amounts of both of the CDS chemoattractants since they contain detectable quantities of the C5 fragment without ciliary dyskinesia activity (Fig. 2). Thus, a more reasonable explanation seems to be that CF homozygote and heterozygote MNC have a metabolic defect involving proteolytic enzymes, which leads to the accumulation of unusual C3 (and C5) cleavage products (the CDS) that are chemotactic for PMN. A similar or related defect could also account for the excessive accumulation of the other chemoattractants (without dyskinesia activity) found in fraction II and fraction VI of cultures from CF genotypes.

In support of the above hypothesis are the recent findings of Walsh and co-workers (19 and references cited therein), which provide evidence that saliva,

serum, and cell culture medium from CF genotypes are deficient in serine protease activity (as compared to analogous fluids from normal controls), and the findings of Shapira and co-workers (20 and references cited therein) and Wilson and Fudenberg (21), which collectively indicate that alpha$_2$-macroglobulin, a major protease regulator synthesized by alveolar Mϕ and monocytes (22, 23), is abnormal in CF with respect to both its carbohydrate structure (20) and its ability to regulate serine proteases (20, 21). We speculate that the findings of Walsh and co-workers reflect a more fundamental defect in CF alpha$_2$-macroglobulin (24). For instance, reduced uptake of alpha$_2$-macroglobulin-protease complexes by CF Mϕ resulting from abnormal glycosylation of CF alpha$_2$-macroglobulin can promote decreased secretion of neutral proteases by CF Mϕ (25). In addition, abnormal metabolism of alpha$_2$-macroglobulin-enzyme complexes by CF Mϕ is at least partly responsible for CDS (26).[2]

The above evidence that at least two of the CDS may be chemoattractants prompted us to examine further the effects of the third, CF-specific CDS (8, 9) on PMN movement. We felt initially that the CF-specific CDS could be such a potent chemoattractant that when tested undiluted it "deactivated" the PMN (15). The CF-specific CDS, however, failed to promote chemotaxis when tested over a range of concentrations. In addition, it was not chemokinetic for PMN and did not inhibit random PMN migration. To our surprise, however, fraction V (containing the CF-specific CDS) completely "blocked" PMN responsiveness to all of the chemoattractants generated by T lymphocytes and monocytes when mixed with either the PMN or the chemoattractants. Evidence that the CF-specific CDS is the component in fraction V responsible for this inhibition is indirect, based on the facts that only fraction V from acidified medium from CF homozygote or heterozygote cultures had inhibitory activity, and that heating abolished the CF-specific CDS activity and the ability of the fraction to inhibit PMN responsiveness to chemoattractants. Thus, either the CF-specific CDS is responsible or another component made only by CF MNC is also present.

The possibility that the CF-specific CDS can inhibit PMN responsiveness is of special interest in regard to observations that cells from heterozygote carriers express the same biochemical abnormalities as those from CF homozygotes (although not to the same extent), yet these individuals are clinically asymptomatic. This is true not only in reference to the generation of chemoattractants and CDS activities (Figs. 1 and 2) but also for other biochemical abnormalities as well (reviewed in refs. 1 and 2). For years, many investigators have considered only two roles for the CF "factors" (the CDS and others) in the disease process: pathogenic or no role at all. We propose instead that the CF-specific CDS may have a "protective" role. In terms of the pathophysiology of lung disease, the CF-specific CDS may serve to counterbalance or block the action of some of the chemoattractants produced in carriers, thus preventing excessive infiltration of PMN (with subsequent tissue damage by lysosomal enzymes released from PMN and other related problems) as is observed in the lungs of CF patients (1). Thus, the CF-specific CDS in the heterozygote may produce a selective biological advantage (protection against obstructive pulmonary disease) analogous to that of carriers of the sickle cell trait (protection against malaria due to the presence of high levels of fetal hemoglobin [27]); this could explain why the CF gene has been maintained at such a high frequency in the Caucasian population. On the other hand, too much CF-specific CDS could be detrimental (i.e., in CF homozygotes). We will not attempt herein to speculate on mechanisms whereby the CF-specific CDS in carriers could modify or prevent the development of other clinical problems observed in CF homozygotes (1, 2). We do suggest a protective role for the CF-specific CDS, however, in the hope that this hypothesis will stimulate further investigations concerning the role of the CF-specific CDS in the disease process. Regardless of the interpretation of our results concerning the CF-specific CDS, we still feel that our data collectively indicate that all three CDS can potentially play a role in the pathogenesis of lung disease. In addition to their affects on PMN migration, when stimulating PMN and possibly Mϕ the CDS (and the other chemotoxins) could affect several aspects of phagocytic cell function or metabolism (e.g., phagocytic and bactericidal capacity, calcium and cyclic nucleotide metabolism, NADH-dehydrogenase activity, and oxygen consumption).

[2] Wilson, G. B., and E. Floyd. Submitted for publication.

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