CHEMOTAXIS AND COTTON EXTRACTS

W. S. Lynn, Sylvia Muñoz, J. A. Campbell, and P. W. Jeffs

Departments of Biochemistry and Chemistry
Duke University Medical Center
Durham, North Carolina 27710

Introduction

Inhalation of dust found in cotton mills is associated with the clinical syndrome, byssinosis. Neither the chemical cause nor the pathophysiology of this pulmonary symptomatology is understood. Recent studies from this laboratory have indicated that inhalation of cotton dust extracts or of many polyphenols by animals produces, in a few hours, a marked outpouring of polymorphonuclear cells (PMN) into the lumen of airways.[1] To facilitate identification of the chemotactic material(s) in cotton dust and to ascertain the possible role of complement, *in vitro* chemotactic assays have been done, using the method of Keller and Sorkin.[2–5] A fluorescent substance soluble in organic solvents has been isolated from cotton dust and purified by gas chromatography which in low concentrations (2 μg/ml) accelerates rate of penetration of 8-micron filters by PMN in the absence of added serum or complement. In fact, serum inhibits the migration of the cells, especially when added to the same side of the chamber as the cells.[6] This material appears to be the major chemotactic agent in cotton bracts as assayed under the conditions of this report.

Materials and Methods

Isolation of the Chemotactic Factor

The material could be isolated either by steam distillation under atmospheric pressure or by continuous extraction with organic solvents (ethyl acetate) of dried cotton bracts of two commercial species or of respirable ducts obtained from cotton mills in North Carolina. The organic phase is washed twice with aqueous 1% KOH and after removal of the solvent anerobically, the residue is dissolved in hexane, and applied to a column of silicic acid 100 mesh (20 × 1 cm). The column is washed first with 1% ethyl acetate in hexane (300 ml). The fluorescent material is eluted with hexane containing 3% ether acetate. Since the green fluorescence of the material is easily observed on the column, using an ultraviolet light, it can be readily fractionated. This fluorescent peak, after concentration is then dissolved in benzene chloroform, 4 to 1, applied to an alumina column and washed thoroughly with same solvent. The fluorescent material is then eluted with benzene/chloroform 3 to 2, is applied to a Hewlett-Packard® Gas Chromatogram 5700 equipped with a stream splitter and with a column (6 ft × ⅛ in.) packed with UCW982 (10%) on chromosorb and eluted at 175°. The fluorescent material emerges as one symmetrical peak at 16 min and is collected in hexane (0°). This eluant is fed directly into a gas chromatograph-mass spectrometer combination employing a (10 ft × ⅛ in.) 1% OV-17

column. This resolved it into 2 isomeric components having a molecular weight of 260. High resolution mass spectral data is not yet definitive. Infra red spectra in chloroform indicates presence of two carbonyls, at 1740 and 1715 nm and a smaller band at 1675 nm. Further structural studies are in progress.

The unknown material can also be separated by thin layer chromatography using Supelco Silica Gel plates (05–8032) and solvents (benzene 72.5%, acetone 2.5% and hexane 25% by volume or benzene 90 and methanol 10%). Rf is 0.23 and 0.71 respectively.

Chemotactic Assays

Polymorphonuclear cells were obtained from mature guinea pig peritoneum 13 hours after injection of 30 ml of 0.1% oxyster glycogen. We placed 2×10^5 cells in the top chamber of Borden chambers fitted with 8 μ Sartorius filters (obtained from Science Essentials Co., Anaheim, Calif.) for 2½ hours at 37°. Both chambers contained 1 ml of Gey's buffer containing 2% bovine serum albumin. It is not necessary to wash these filters before use. We were unable to obtain consistent results using other brand filters probably due to the presence of detergent and to variations in thickness.[7, 8] All assays were done in triplicate. The filters were fixed and stained by the method of Boyden,[9] and both sides of the filters counted. At least 10 high-power fields were counted in each filter and all experiments were done in triplicate. All triplicates using this assay vary no more than 12%. With short incubations, essentially no cells penetrate the filter in the absence of added chemotaxins. At these cell concentrations, a solid sheet of cells (over 500 per field) were fixed to the starting side, except when serum was present on the starting side. In the presence of serum, macrophages only adhered to the starting side. Incubations were all done in sterile Gey's buffer containing 2% bovine serum albumin, pH 7.4. Reagents were all added either in isotonic NaCl or in 2 μ/ml of ethanol. The ethanol was evaporated before addition of the buffer. Differential counts indicated that over 95% of these cells are PMN's.

Isolation of Chemotactic Agents from Crude Casein and from Eschericia coli *Broth*

Crude Casein

Crude bovine casein (obtained from Fisher) was partially dissolved in water (20 mg/ml) by raising the pH to 10.0 with NaOH for 1 hour at 30°. The insoluble material was removed by centrifugation at 40,000 $\times$ g av for 30 min. The soluble material was dialyzed exhaustively, dried by lyophilization and the dry powder extracted with ethanol first and then with chloroform/methanol 2 to 1 by volume. The organic solvents were removed and the dry lipid then extracted with hexane. The hexane removed most of the brown color. The white residue was dissolved in ethanol and used directly for assay.

The lipid-free extracted protein was dissolved in 2 mM Tris pH 8.2 and applied to an anion exchange column (DE52, Sigma) and eluted with a NaCl linear gradient, 0 to 0.5 M. Four discrete protein peaks were obtained. All four peaks were somewhat chemotactic, but most of the activity was in peak I,

FIGURE 1. No further protein purification was attempted. All peaks after reduction with 0.1% mercaptoethanol in 1% dodecyl sulfate at 100° for 4 min were then applied to acrylamide gels containing 1% sodium dodecylsulfate and electrophoresed (Weber and Osborn).[10] Only one peptide (23,000 MW) could be observed by staining with Coomassie dye on the gels in all fractions.

Alpha casein (Worthington CASA 21A) was also purified as above. Very little of this protein was in true solution since all solutions were somewhat cloudy even after centrifugation at 100,000 × g av. No brown material could be extracted from this preparation by chloroform/methanol. By gel electrophoresis, this protein also contained essentially one peptide, 23,000 MW.

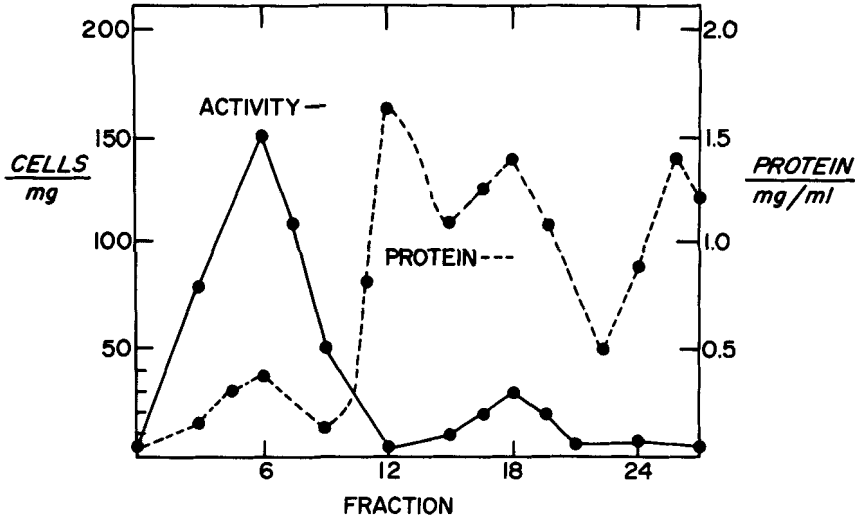

FIGURE 1. Anion exchange chromatography of soluble protein from crude bovine casein. Extracts of crude casein, pH 8.2 were adsorbed on DE52 columns and eluted with a NaCl gradient, 0 to 0.5 M equilibrated with 5 mm Tris, pH 8.2. Protein was measured where indicated (Lowry Method) and chemotactic assays run (see METHODS). Each assay contained 0.2 mg of the protein.

E. Coli

E. coli (obtained from human colon) were inoculated into 199 media, containing phenol red and glucose but no protein, and grown for 70 hours at 37°. The cells were then removed by centrifugation at 40,000 × g av and the supernatant exhaustively dialyzed against 1 mM Tris, pH 8.0. The protein was applied to a DE52 column 2 cm x 20 cm, equilibrated with 2 mM Tris pH 8.0. Elution with 0.1 M NaCl in 2 mM Tris resulted in the emergence of two sharp peaks of red protein (containing bound phenol red) which easily separated from each other. Both peaks represented less than 10% of the applied protein. The remainder of the bound protein was eluted with 1 M NaCl. It contained no phenol red or chemotactic activity, FIGURE 2. All three protein fractions were

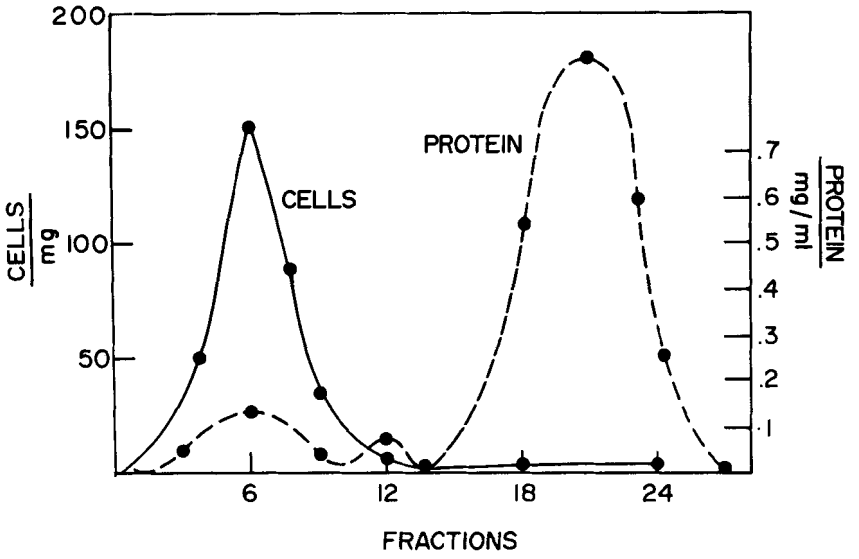

FIGURE 2. Anion exchange chromatography of dialyzed secreted protein from *E. coli*. *E. coli* (a human colon isolate) were grown for 48 to 72 hours in absence of added protein. The secreted soluble proteins, (in supernate after centrifugation at 40,000 × g av for 30 min) were adsorbed to DE52 column (2 × 20 cm) equilibrated with 5 mm Tris pH 8.2 and eluted with 0.09 M NaCl. Two peaks (1 and 2) both of which were red at pH 8.2 and yellow at pH 6.0 emerged as indicated. Fraction volume=4 ml. At fraction 14, 1.0 m NaCl was added as elutant. Chemotactic assays were run as indicated using 0.3 mg of eluted protein, (see METHODS).

subjected to electrophoresis containing dodecyl sulfate and mercaptoethanol as above in duplicate and stained with Coomassie or periodic fuchsin.

RESULTS

Two Types of Chemotaxis

Two broad types of chemotaxins for PMN's appear to exist. One type is those materials, such as the crude casein or *E. coli* extracts, which cause massive migration and a more or less linear does response. The other group, such as the cotton fluorescent material and concanavalin A are more weakly chemotactic, exhibit an initial delay and the dose response curve is more logarithmic. Neither type of dose response curves are saturated under these conditions, FIGURE 3 and FIGURE 4.

Both types are completely abolished by cytochalasin B, 2 μg/ml and digitonin, 5 μg/ml. Other lipids at comparable concentrations, such as dipalmitoyl lecithin, palmitic acid, or triton-X-100 are without effect, TABLE 1. Unless stated, all agents were added to the side opposite to the cell side. Normal

human serum inhibits both types of chemotaxis, especially so when added to the same side as the cells. In fact, serum on the same side as the cells almost completely abolishes the attachment of polymorphs, but not the macrophages, to the cell-side of the filter, TABLE 1.

To derive further insight about the specificity or mechanism of these two types of chemotaxis, other suspect agents have been tested. Since casein could be acting by serving as substrate for membrane protein phosphokinases, other known substrate proteins (histones, protamine, and bovine cytochrome c) were assayed both as activators and as inhibitors of chemotaxis. None had any effect. Other inactive proteins tested included bovine serum albumin and γ-globulins, human serum albumin and γ-globulins, *E. coli* iron and Mn^{++} superoxide dismutases, and human complement, Clq, (prepared by method of Stroud and co-workers)[11], TABLE 1.

Since cotton extracts contain many flavanoids and many polyphenols and since these agents are quite chemotactic *in vivo*,[1] several of these polyphenols were also tested *in vitro*. Quercitin, quercitrin, ellagic acid, resorcinol, crude phenolic cotton duct extracts, phloroglucinol and piperonylic acid were ineffective at concentrations of up to 200 μg/ml or less.

Reductants (mercaptoethanol, and glutathione) were also ineffective.

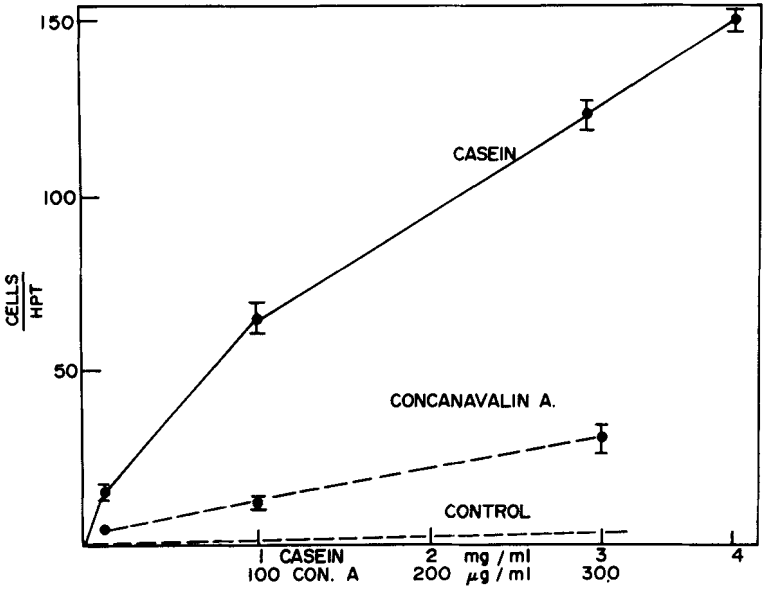

FIGURE 3. Concentration curve for crude casein and concanavalin A on chemotaxis. Guinea pig polymorphonuclear leukocytes (2×10^5) were exposed in Borden chambers to varying concentrations of the two chemotaxins, as indicated, for 2½ hours. The number of cells which migrated through the filter were counted and expressed per high power field. The bars at each point represent total variation of counts in triplicate experiments.

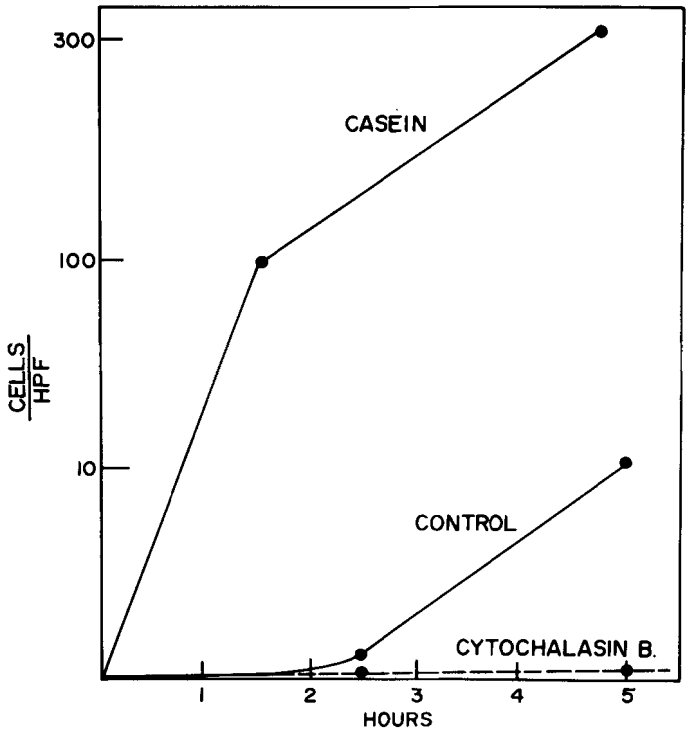

FIGURE 4. Effect of time, crude casein, and cytochalasin B on chemotaxis. PMN were incubated for varying times as in FIGURE 3. Crude casein 3 mg/ml, or casein plus cytochalasin B (3 μg/ml) were added to the side opposite to the cells. Migration is expressed as cells per high power field.

Isolation of Chemotaxins: Attempts to Identify the Active Agent(s) in Crude Casein, E. coli *Broth, and Cotton Dust*

Crude Casein

Alpha-casein (Worthington, CASA 21A) at concentrations of 5 mg/ml was not chemotactic. Nor did it inhibit the chemotactic activity of crude casein. Lipid extraction of this casein failed to produce any lipid. However most fractions of dialyzed crude casein are chemotactic, including the fraction which is soluble in ethanol, TABLE 1. The hexane soluble fraction is not chemotactic. The lipid material from crude casein has not been characterized further. It probably represents oxidized lipids from plasma membranes which are secreted in bovine milk.[12, 13] The possibility of the presence of bacterial products in casein is also good.[4, 14] Removal of the brown lipid contaminants from casein by organic solvents is not complete since part of the brown color in the casein persists even after extensive extraction. It appears therefore, that

crude casein contains chemotactic water insoluble contaminants which bind tightly to casein monomers and resist removal by organic solvents or by ion exchange chromatography.

E. coli *Chemotaxis*

Accumulation of the secreted protein which is associated with chemotaxis in the media of growing *E. coli* is logarithmic with time and parallels growth rates. This material is not dialyzable. As indicated in FIGURE 2, it is bound by DE52 and elutes as a nearly homogenous protein to which phenol red is very tightly bound. This fraction contains one peptide by SDS gel electrophoresis of monomeric molecular weight 78,000 (90%) and a smaller amount of a smaller protein of 70,000 M.W. (10%). Relative amounts of each peptide were estimated with a densitometer using Coomassie as the stain. The smaller peptide is a glycopeptide, i.e., it is stained permanently by periodic fuchsin, as well as by Coomassie. As assayed by the phenol-sulfuric assay,[15] the *E. coli* active fraction contains about 1% carbohydrate by weight. It is not clear which of the peptides binds phenol red. As indicated in FIGURE 2, these two peptides represent about 15% of the total secreted protein, but 100% of the chemotactic activity.

Extraction of this active lyophilized fraction with chloroform-methanol failed to release any chemotactic lipid, nor did the organic solvent extraction alter the chemotactic potency of the purified peptide, TABLE 1.

TABLE 1

CHEMOTACTIC AGENTS FOR POLYMORPHONUCLEAR LEUKOCYTES

Chemotaxins	Chemotactic Inhibitors	Ineffective Agents	
Fluorescent cotton unknown, 10 μg/ml	human serum diluted 1/10	dithiothretol 25 μg/ml	quercetin
Casein lipids 10 μg/ml	cytochalasin B 4 μg/ml	histones, protamine, RNAse	ellagic acid
E. coli purified protein, 20 μg/ml	digitonin 5 μg/ml	phloridzin colchicine	KCN fatty acids
Concanavalin A 20 μg/ml		silica (1.2 μ)	*E. coli* superoxide dismutase
Delipidated casein		phospholipids	hexane soluble lipids from crude casein
Delipidated *E. coli* protein		macrophages	heated sera 56° for 30 min

Cotton Chemotaxis

Aqueous suspensions or steam volatile distillates of cotton mill respirable dust or of cotton bracts are chemotactic for polymorphonuclear cells. Purification of this activity by silicic acid and alumina column chromatography and preparative gas chromatography (see METHODS) indicates that the active agent is a fluorescent substance with mass of 260. High resolution mass spectroscopy

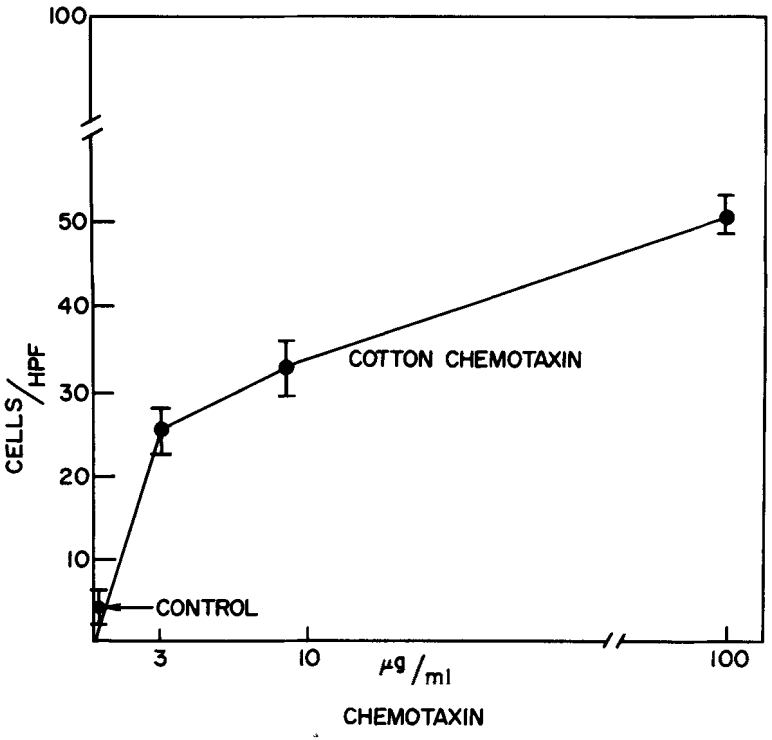

FIGURE 5. Concentration curve for the fluorescent oxygen heterocycle obtained from cotton bracts. Migration of polymorphonuclear cells through the sartorius filters was expressed as cells/high power field after 3 hours of incubation, as in FIGURE 3. The cotton material was obtained from the gas chromatogram and consisted of two isomers whose mass is 260, (see METHODS).

is most consistent with the structure C_{16} H_{20} O_3. Infra red spectroscopy reveals the presence of two carbonyls (1715 and 1741 nm) as the only two intense chromophores. The 1741 nm chromophore may be a lactone, since there is no evidence for an ester in the mass spectral data. This cotton material accounts for most of the slow type of chemotactic activity of crude cotton dust extracts. Similar extractions of dried tobacco leaves or of dry pine bark (loblolly) failed to yield any chemotactic material. This material accounts for less than 3% (dry weight) of the steam distillate of cotton bracts. Essentially all chemotactic

activity is removed from aqueous suspensions of respirable dust by repeated steam distillation.

The lipid-like chemotaxin isolated from crude casein also remains unknown, but since it is neither steam volatile nor fluorescent nor soluble in hexane, it is probably not the material in cotton dust.

Discussion

A lipid material extracted from cotton dust accounts for the slow type of chemotactic response of polymorphonuclear cells seen in the absence of serum or complement. This chemotactic response is inhibited by human or bovine serum,[3, 16, 17] but not by serum albumin; thus it is not mediated by the complement system of serum. It is somewhat specific, i.e., phospholipids, dipalmitoyl lecithin, fatty acids, polyphenols or many other lipids and detergents are not chemotactic. In fact, digitonin and cytochalasin B are, at quite low concentrations, potent inhibitors of chemotaxis.

Lipid extracts of crude casein, probably containing bovine mammary epithelial plasma membranes,[12] are also quite chemotactic for neutrophiles. However, delipidated crude casein (chloroform/methanol), retains half of its original activity. This is probably due to failure of the organic solvent to remove all lipid.

Attempts to remove a chemotactic lipid from the purified *E. coli* chemotactic protein were unsuccessful. Nor did organic solvent extraction alter the activity of the protein fraction. Since bacterial chemotaxin binds very tightly the polyphenol, phenol red, it is possible that the organic solvents did not remove a bound chemotaxin. The bacterial chemotaxin also contains some carbohydrate (periodic-fuchsin and phenol-sulfuric acid positive) which carbohydrate appears on gel electrophoresis to be bound to a peptide of 70,000 m.w. This carbohydrate may represent a fraction of the lipopolysaccharide pyrogenic endotoxin, previously noted to be chemotactic.[6, 16] However, a purified delipidated preparation of lipopolysaccharide (LPS) prepared from *proteus vulgaris* by Dr. Robert Wheat of Duke University is not chemotactic under our conditions. A cruder preparation of the LPS, containing non-covalent lipid is chemotactic.

The data in this report, thus, indicate that several types of biological materials, notably a water-insoluble heterocycle from cotton dust, is quite chemotactic for polymorphonuclear cells in the absence of complement. In fact, sera from human or calf strongly inhibits chemotactic activity.

Preliminary data indicate that guinea pig or rabbit peritoneal or rabbit alveolar macrophages do not respond chemotactically to the cotton lipid or to casein lipids; however, macrophage migration is accelerated by crude casein.

Previously, studies on polymorphonuclear chemotaxis have implicated lipid-containing substances. Bacterial and plant extracts containing lipo-saccharides are chemotaxins.[6, 16] Baum and co-workers [6] have shown that serum both increases and decreases chemotaxis of human blood polymorphonuclear leukocytes, depending on conditions, and also that *E. coli* lipopolysaccharide endotoxin (Difco) stimulates chemotaxis at low concentrations, but not at high concentrations.

Human serum markedly inhibits both the slow type and fast type of chemotaxis. This effect of serum is not specific, for it inhibits the chemotaxins from *E. coli*, casein, or cotton. The inhibitory material in serum is not known, but

it is not albumin. Nor is it dialyzable. It is inactivated by heat (60° for 30 min), (unpublished data). The inhibitory function of serum appears to be to inhibit the ability of polymorphs, but not macrophages, to bind to the filters. Migration through this type of matted filter apparently must be accompanied by binding of cells to the filter. Cytochalasin B, in contrast, inhibits migration but not binding. In the experiments of Baum and co-workers,[6] the effect of serum is not as great, probably because the cells were concentrated on the filter by centrifugation before addition of the chemotoxin. Characterization of this serum inhibitor is the subject of another report.

Whether this uncharacterized cotton chemotaxin is related to byssinotic symptomatology or not awaits isolation of sufficient amounts of this material for assays on whole animals or humans. The polyphenols which have been shown to be active *in vivo* on chemotaxis[1] may all work by complement activation,[16] since they are inactive *in vitro* in the absence of serum.

Conclusion

A steam-volatile fluorescent material of mass 260, isolated from cotton mill dust or cotton bracts, is an *in vitro* chemotaxin of the slow type for peritoneal polymorphonuclear cells in the absence of serum.

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