Effect of bacterial products on neutrophil migration in vitro

Gerhard Ras, Robert Wilson, Howard Todd, Graham Taylor, Peter Cole

Abstract

Chronic bronchial inflammation is associated with migration of large numbers of granulocytes into the bronchial tree. A study was designed to find out whether products of bacteria commonly isolated in chronic bronchial infection stimulate neutrophil migration in vitro. Neutrophils from healthy donors were studied by a modified Boyden chamber technique. Bacterial culture filtrates stimulated neutrophil migration over a wide dilution range and the chemotactic activity was heat stable. Culture filtrates derived from *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* were significantly chemokinetic and directionally chemotactic, whereas filtrates from *Staphylococcus aureus* were only chemotactic. Gel filtration of *S aureus* and *P aeruginosa* culture filtrates yielded high, medium, and low molecular weight fractions showing chemotactic activity. *S pneumoniae* and *H influenzae* yielded fractions with only low molecular weight chemotactic activity. Neutrophil chemotactic responses, occurring in response to all bacterial species tested, appear to represent a defence mechanism by the host. Chemoattractant activity may, however, contribute to bronchial damage mediated by products released from continuously attracted, activated neutrophils.

**Host Defence Unit,
Department of
Thoracic Medicine,
National Heart and
Lung Institute,
London**
G Ras
R Wilson
H Todd
P J Cole

**Department of
Clinical
Pharmacology, Royal
Postgraduate Medical
School, London**
G Taylor

Address for reprint requests:
Professor P J Cole,
Department of Thoracic
Medicine, National Heart
and Lung Institute, London
SW3 6LY.

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Migration of inflammatory cells from the intravascular compartment into the lung is an essential pulmonary defence mechanism. Endogenous and bacteria derived chemotactic factors can be generated in the airways or alveoli and initiate an inflammatory reaction.[1] Such inflammation is usually controlled and useful and may be clinically undetectable.[2] In patients with chronic bronchial infection, however, persistent production of neutrophil attractants by respiratory pathogens could stimulate a chronic inflammatory process with host mediated damage to the bronchial tree.[3 4]

Chronic bronchial infection is associated with the movement of numerous granulocytes to the lungs.[5] In patients with severe bronchiectasis, half the circulating granulocytes may be lost via the sputum[5]—similar to the loss of granulocytes in the faeces in patients with severe inflammatory bowel disease.[6] This large neutrophil influx results in the generation of toxic oxygen radicals and release of enzymes that cause tissue injury.[7] The protease activity of sputum from patients with cystic fibrosis has been directly correlated with the numbers of neutrophils in sputum specimens,[8] though the stimulus for this inflammatory response is largely unknown.

In this study we investigated whether products of bacteria commonly isolated in chronic bronchial infection affect neutrophil migration in vitro and, if so, whether that migration is chemokinetic (random) or chemotactic (directional), or both. We also examined the chemolocomotion stimulating properties of low, middle, and high molecular weight fractions obtained by gel filtration of bacterial culture filtrates.

Methods

ISOLATION OF BACTERIA

Early morning sputum specimens were collected from patients with clinical exacerbations of chronic infective respiratory disease. Sputum was cultured by standard and selective bacteriological techniques.[9] Bacterial species were purified by repeated subculture, and identified by standard methods.[10] This yielded three isolates each of *Pseudomonas aeruginosa* (mucoid and non-mucoid), *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. Isolates of *Pseudomonas cepacia* were obtained from patients with cystic fibrosis.

PREPARATION OF BACTERIAL CULTURE FILTRATES

A single colony of each isolate was inoculated into 10 ml Medium 199 tissue culture fluid (Flow Laboratories, Irvine) and incubated for 18 hours at 37°C. For culture of *Haemophilus* spp Medium 199 was supplemented by 0·1% (w/v) Lab-Lemco (Oxoid), haemin 5 μg/ml and nicotinamide 10 μg/ml. For culture of *S pneumoniae* Medium 199 was supplemented by the addition of 10% Levinthal base consisting of defibrinated horse blood (one part) and brain heart infusion (two parts) (Oxoid), which had been centrifuged at 5000 g for 10 minutes at 4°C and filtered through a 0·2 μm Acrodisc (Gelman Sciences Inc, Ann Arbor, Michigan). A viability count (colony forming units/ml) was performed by dilution and overnight incubation on appropriate agar. The culture was centrifuged (5000 g) for 20 minutes at 4°C and the supernatant fluids filtered (0·2 μm Acrodisc, Gelman) to yield bacteria free fil-

trates. The sterility of filtrates was confirmed by overnight subculture. Filtrates were divided into aliquots and stored at −70°C until they were used in chemolocomotion experiments. To assess the heat stability of any chemolocomotory activity present, one culture filtrate from each species was heated in a water bath at 100°C for 30 minutes before experiments.

GEL FILTRATION

To determine the approximate molecular weight of factors contributing to chemolocomotory activity, fresh clinical isolates of *P aeruginosa*, *H influenzae*, *S aureus*, and *S pneumoniae* (one of each species) were grown overnight as described. Aliquots (20 ml) of bacterial culture filtrate were freeze dried, redissolved in 2 ml sterile distilled Milli Q water (Millipore, Harrow), and applied to 500 × 26 mm columns of Biogel P6 (Biorad Laboratories) and Sephadex G100 (Pharmacia). The eluant was distilled water and the eluate was collected at a flow rate of 40 ml/h at 4°C. The ultraviolet absorbance of the eluate was monitored continuously at 206 nm, and 10 ml fractions were collected. A new column was prepared for each culture filtrate separation. Elution fractions were pooled according to molecular weight: high (>50 000 daltons), medium (10 000–50 000 daltons), and low (<10 000 and <6000 daltons). Pooled fractions were frozen and freeze dried before storage at −70°C. Each column was calibrated with molecular weight markers (Blue Dextran (200 000 MW), bovine serum albumin (67 000 MW), ovalbumin (43 000 MW), ribonuclease A (13 700 MW), insulin (5734 MW), glucagon (3485 MW), and hydroxycobalamine (1380 MW)). The osmolality of each of the fractions after reconstitution was checked by lack of crenation of human red blood cells and was corrected if required by dropwise addition of distilled water.

MEASUREMENT OF NEUTROPHIL MIGRATION IN VITRO

Peripheral venous blood was obtained from healthy donors. Neutrophils were prepared by dextran sedimentation of the heparinised blood followed by Ficoll gradient separation. After lysis of residual erythrocytes with 0·84% ammonium chloride, the cells were washed, resuspended to 3 × 10⁶/ml in HEPES (*N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid) buffered Medium 199 with Earl's salts and supplemented with 0·4% egg albumin. Neutrophil migration was measured with a modified Boyden chamber technique by means of a 48 well microchemotaxis assembly (Neuro Probe Inc, Cabin John, Maryland) and nitrocellulose filters (Sartorius membrane filters, 8 μm pore size, Gottingen, West Germany).[11] Twenty five microlitres of test chemoattractant and controls were placed in the lower compartment of the chemotaxis chamber. Fifty microlitres of neutrophil suspension, adjusted to 3 × 10⁶/ml, were placed in the upper compartment. The chambers were incubated for 90 minutes at 37°C, after which the filters were removed and

the neutrophils fixed in industrial methylated spirit and stained with haematoxylin. The filters were then mounted in reverse position (the face that had been adjacent to the lower chamber mounted uppermost) on microscope glass slides and stored overnight at 4°C. Random motility was measured in the absence of leucoattractant but in the presence of Medium 199 tissue culture fluid (supplemented with growth factors in the case of *H influenzae* and *S pneumoniae*) in the lower chamber only. The synthetic chemotactic tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP; Miles Laboratories Inc, Elkhard, Indiana) at a final concentration of 1 × 10⁻⁸ mmol/l (previously determined as optimal) was used as a positive control.

Results were expressed as the number of polymorphonuclear neutrophil leucocytes that completely traversed the filters per microscope high power field (PMNL/HPF). Data were pooled for three isolates of the same microorganism, each of which was assayed nine times on separate occasions (n = 27). Ten fields were counted per filter to obtain a mean value. The coefficient of variation for this technique was less than 15%. The chemotactic index for gel filtrates was calculated as:

$$\frac{\text{(PMNL directed migration per HPF)} - \text{(PMNL random migration per HPF)}}{\text{PMNL random migration per HPF}}.$$

CHEMOKINESIS AND CHEMOTAXIS

To determine the effect of culture filtrates on chemokinesis (undirected movement) and chemotaxis (directed movement), neutrophil migration was assessed with fixed identical concentrations of bacterial culture filtrates diluted with Medium 199 above and below the micropore filter in the Boyden chamber (a chemokinesis system) or with positive gradients of the leucoattractant placed in the lower chamber alone (a chemotaxis system).[12 13] Single clinical isolates of *P aeruginosa*, *H influenzae*, *S aureus*, and *S pneumoniae* were examined.

EXPRESSION OF RESULTS

Results were expressed as means and standard errors of the mean for each series of experiments. Statistical analysis of data was performed by means of the two sample *t* test.

Results

NEUTROPHIL MIGRATION

Results of neutrophil migration experiments for the different respiratory pathogens are shown in the table. In the culture filtrates neutrophil migration over a wide range of dilutions was substantially greater than random migration. Optimal stimulation of migration was observed with 1:10 and 1:100 dilutions of all bacteria studied (p < 0·01). All isolates studied had heat stabile chemotactic activity.

CHEMOTAXIS AND CHEMOKINESIS

Culture filtrates from *P aeruginosa* (1:10 and 1:100), *S pneumoniae* (1:100 and 1:1000), and *H influenzae* (1:10) were chemotactic and chemokinetic (p < 0·01) (fig 1). Culture filtrate

In vitro effect of bacterial culture filtrates on neutrophil migration (mean (SEM) numbers of neutrophils per high power field)*

Bacteria	Neat		10^{-1}		10^{-2}		10^{-3}		M199		FMLP 10^{-8}	
Haemophilus influenzae	45	(14)**	70	(16)**	63	(24)**	25	(14)	25	(8)	67	(12)
Haemophilus parainfluenzae	41	(19)	72	(19)**	75	(18)**	29	(9)	22	(7)	95	(18)
Streptococcus pneumoniae	41	(10)**	47	(7)**	70	(10)	57	(8)**	9	(4)	59	(4)
Staphylococcus aureus	21	(5)	62	(13)**	65	(15)**	35	(6)	21	(7)	109	(10)
Pseudomonas aeruginosa												
Mucoid	42	(9)**	93	(12)**	70	(15)**	50	(15)**	24	(4)	101	(11)
Non-mucoid	84	(13)**	112	(19)**	93	(19)**	52	(10)	25	(11)	119	(23)
Pseudomonas cepacia	33	(15)	95	(15)**	91	(17)**	50	(12)**	14	(21)	97	(6)

*Data pooled for three isolates of the same microorganism, each of which was assayed nine times on separate occasions (n = 27).
**$p < 0.01$ v M199 alone.

dilutions derived from *S aureus* (1:10 and 1:100), *H influenzae* (1:100 and 1:1000), and *P aeruginosa* (1:1000) stimulated only chemotaxis significantly ($p < 0.01$).

GEL FILTRATION
High, medium, and low molecular weight chemotactic activity was seen with both *S aureus* and *P aeruginosa* culture filtrates (fig 2). Low molecular weight chemotactic activity only occurred with *S pneumoniae* and *H influenzae* culture filtrates (fig 2).

Discussion
Chronic infection of the bronchial tree often occurs against a background of previous pulmonary damage—caused, for example, by underlying genetic disease such as cystic fibrosis or an acute damaging event such as whooping cough or pneumonia in childhood. Affected areas of the respiratory tract are compromised with respect to first line defence mechanisms, such as mucociliary clearance. A few bacterial species exploit this, colonise the respiratory tract, and subvert defence mechanisms.[14] An inflammatory response develops, including traffic of neutrophils into the bronchial tree,[5] and, if the microorganisms are not thereby eliminated, this inflammatory response becomes chronic and may itself damage "innocent bystander" normal lung. For example, potentially damaging enzymes such as neutrophil elastase are released and may overwhelm the capacity of the lung to neutralise them,[15] leaving free enzyme in the secretions bathing the epithelial surface.[16] Such host mediated damage supplements that done by bacterial products themselves[17] and may lead to increased bacterial colonisation. The result is a self perpetuating "vicious circle" of bronchial damage, which may progress to cardiorespiratory failure.[3 4]

A central feature of this "vicious circle" hypothesis[3] is the recruitment of granulocytes to the site of the chronic infection. Sol phase sputum from such patients contains some chemoattractant activities that have not yet been identified as bacterial or host derived.[18] Little is known about the role of host chemoattractants in chronic bronchial infection but in various inflammatory conditions complement fragments (for example, C5a), leukotrienes (for
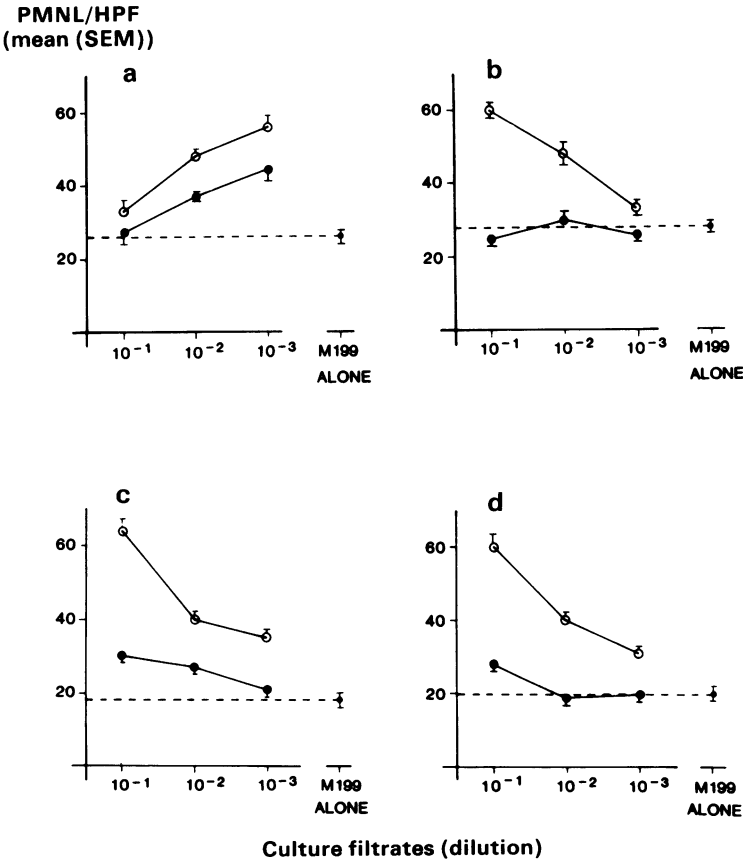
PMNL/HPF
(mean (SEM))

Figure 1 In vitro chemokinetic and chemotactic effects of culture filtrates from (a) Streptococcus pneumoniae, (b) Staphylococcus aureus, (c) Pseudomonas aeruginosa, and (d) Haemophilus influenzae. Results are expressed as the mean (SEM of triplicate experiments) number of neutrophils per high power field (PMNL/HPF) stimulated to move by the culture filtrate of one clinical bacterial isolate. Combined chemotactic and chemokinetic response (O——O); pure chemokinetic response (●——●). Pure chemotaxis is represented by the difference between these responses at each dilution of culture filtrate. - - - The negative control of Medium 199 alone.
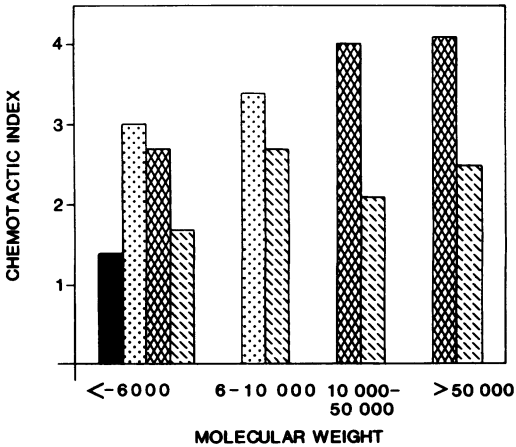
Culture filtrates (dilution)

Figure 2 In vitro effect on neutrophil locomotion of pooled fractions of bacterial culture filtrates obtained by gel filtration. The chemotactic index = (directed migration minus random migration)/random migration per high power field. ■ Streptococcus pneumoniae; ▨ Haemophilus influenzae; ▩ Pseudomonas aeruginosa; ▧ Staphylococcus aureus.

example, LTB₄) secreted by alveolar macrophages, and platelet activating factor (released by stimulated neutrophils, alveolar macrophages, and endothelial cells) are known to be present and will attract neutrophils from blood into the tissues and increase microvascular permeability. In addition, most microorganisms are capable of generating complement mediated chemotactic activity. Endotoxins of Gram negative bacteria are an example of powerful activators of complement, causing the liberation of the powerful chemotactic peptide fragment C5a,[19 20] by either the classical or the alternative pathway.[21] Direct proteolytic attack on C5 by bacterial proteases may also generate chemotactic products.[22] Similarly, little is known about the role of bacterial chemoattractants in chronic respiratory infection, but the results of this in vitro study would suggest that they may also make a potent contribution to the chemotactic activity of infected sputum sol phase.

In patients with chronic bronchial infection chemotactic factors may be released actively or passively from microbial or host cells and may also be generated enzymatically from components of plasma and connective tissue.[23] In our study the bacterial cultures were studied during the stationary growth phase and the chemotactic activity associated with a secreted product of the microorganism. All the culture filtrates produced in this study were highly chemotactic but certain bacterial species produce factors with negative chemotactic properties. *Pseudomonas* heat stable haemolysin (rhamnolipid) stimulates both chemotaxis and chemokinesis but can also impair a chemotactic response to FMLP.[24] A cytotoxic protein active against leucocytes is produced by *P aeruginosa* and *S aureus* and impairs granulocyte motility.[25 26] Injection of glycolipoprotein, the toxic slime antigen produced by *P aeruginosa*, into mice causes leucopenia and death by entering the blood stream and becoming associated with neutrophils. The neutrophil-glycolipoprotein complex is deposited in the liver, leading to leucopenia.[27] In vivo leucocyte function in any particular patient may depend on the balance between these factors.[19]

We have shown the products of *P aeruginosa*, *H influenzae*, and *S pneumoniae* to be chemokinetic and chemotactic. *S aureus* culture filtrates gave rise to products that were solely chemotactic for neutrophils. Previous studies showed the major chemotactic effect of staphylococci on neutrophils to be indirect, operating via complement activation.[28] Direct chemotactic activity on monocytes, however, was found in numerous staphylococcal fractions.[28] A high molecular weight fraction produced by staphylococci was found to be directly chemotactic for neutrophils.[29] *Staphylococcal* protein A has been reported to generate cytotoxigenic activity by binding to Fc fragments of IgG and thus fixing complement.[30] The occurrence of low molecular weight chemotactic activity in *S pneumoniae* culture filtrates is consistent with other findings.[31] The absence of a high molecular weight

chemotactic activity in the gel filtrate from *H influenzae* culture filtrates could be due to binding on the column or could be a true dose dependent effect.

The neutrophil chemotactic response seen with the products of all the bacterial species tested would appear to be an appropriate defence mechanism by the host. In the absence of effective clearance of the bacteria it may, however, persist and contribute to damage by persistently attracting activated neutrophils into the bronchial tree. Bacterial colonisation of the respiratory tree would therefore create chronic bronchial inflammation and possibly contribute to the pathogenesis of bronchiectasis.

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