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Inhibition of Human Neutrophil Migration In Vitro by Low-Molecular-Mass Products of Nontypeable *Haemophilus influenzae*

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Nontypeable *Haemophilus influenzae* commonly causes infections in the lower and upper respiratory tract, although the mechanisms of its colonization and persistence in the airways are unclear. Culture filtrates from six clinical isolates of this bacterium were assessed for their abilities to influence neutrophil function in vitro. Each culture filtrate was assessed on six separate occasions with neutrophils obtained from six different donors. During the log and early stationary phases of growth (0 to 18 h), culture filtrates contained primarily neutrophil chemokinetic activity but no activity affecting neutrophil migration toward the chemotactic factors *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine and leukotriene B_4. In contrast, filtrates obtained after 24 h of culture contained factors which inhibited neutrophil migration toward both of these chemotactic factors. This chemotaxis-inhibitory activity persisted between 24 and 72 h of bacterial culture, and it was not associated with the presence of either chemotactic or chemokinetic activity as assessed by checkerboard analysis. Gel filtration of pooled 72-h filtrates yielded three major peaks of chemotaxis-inhibitory activity. Endotoxin was present together with two other low-molecular-mass hydrophobic factors of approximately 8 and 2 kDa. These low-molecular-mass factors are chloroform insoluble and heat stable, and they are inactivated by protease, periodate, and diborane reduction. Activity was completely retained on a wheat germ agglutinin column, and it could be eluted with *N*-acetyl-D-glucosamine. These data suggest that inhibitory activity is associated with *N*-acetyl-D-glucosamine-containing glycopeptides, possibly derived from the bacterial cell wall. The production of these compounds may contribute to the persistence of this bacterium in vivo by inhibiting neutrophil chemotaxis in the microenvironment of the respiratory mucosa.

Nontypeable *Haemophilus influenzae* (NTHi) is a normal nasopharyngeal commensal, and carriage rates as high as 80% have been found in some communities (19). The bacterium is also frequently associated with infections of mucosal surfaces, including middle-ear infections, sinusitis, and infective exacerbations of chronic bronchitis and bronchiectasis (19). These infections are associated with the rapid infiltration and subsequent activation of polymorphonuclear leukocytes (5). The most likely stimulus for this inflammatory response is the generation of endogenous and bacterially derived chemotactic factors (3, 13). In chronic diseases, these neutrophils, although activated, fail to clear the organism, which persists and spreads contiguously in the airways. The mechanism by which NTHi avoids elimination remains unknown, although several other gram-negative bacteria, including *Bordetella pertussis* (1), *Legionella micdadei* (7), and *Pseudomonas aeruginosa* (2, 10), all of which cause infections of the respiratory tract, have been previously shown to inhibit granulocyte functions. The mechanisms are varied, but they are thought to involve enzymic inhibition of G proteins, acid phosphatase-induced blockage of membrane signal transduction, and cytotoxic effects (1, 2, 7, 10).

In this study, we have investigated the effects of substances produced by clinical isolates of NTHi on the migration, surface C3bi receptor expression, and phagocytic capacity of human neutrophils, and we describe novel low-molecular-mass substances capable of inhibiting the neutrophil response to the chemotaxins *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) and leukotriene B_4 (LTB_4).

MATERIALS AND METHODS

Preparation of bacterial samples. Six strains of NTHi were isolated from the Sputasol-treated sputa of patients with chronic bronchitis, and they were identified by colonial morphology, Gram staining, and determining the requirement for factors V and X for growth according to standard microbiological techniques. Each of the six bacterial isolates was cultured in Trypticase soy broth (Unipath, Ltd., Basingstoke, United Kingdom) at 37°C (15) and sampled between 0 and 72 h. Samples of the medium were centrifuged (5,000 × *g*, 15 min) and filtered through 0.2-μm-pore-size filters (Sartorius, Ltd., Epsom, United Kingdom) to produce bacterium-free culture filtrates for each time point.

Neutrophil isolation. Human polymorphonuclear leukocytes were isolated from peripheral blood samples from human volunteers by dextran sedimentation and Ficoll-Isopaque separation (Pharmacia, Ltd., Milton Keynes, United Kingdom) according to the technique of Lee et al. (11). Neutrophils were washed (250 × *g*, 10 min) in Hanks

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balanced salt solution (HBSS) (Sigma Chemical Company, Poole, United Kingdom), buffered to pH 7.4 with 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and resuspended to a final concentration of 2×10^6 cells ml^{-1} in HBSS containing 0.2% ovalbumin (Sigma). Neutrophils accounted for >95% of cells and were >98% viable as assessed by trypan blue exclusion and lactate dehydrogenase estimation (Sigma). HBSS, HEPES, and dextran were all prepared with endotoxin-free water (Sigma).

Neutrophil migration. Bacterial culture filtrates were obtained from six different clinical isolates of NTHi cultured for up to 72 h. For each time point, the culture filtrate from each clinical isolate was assessed separately. Each culture filtrate was assessed on six separate occasions with neutrophils obtained from six different donors. Partially purified factors and the products of chemical and enzymatic activation were similarly addressed.

(i) Chemotaxis. Neutrophil migration was assessed over 90 min at 37°C in response to uninoculated culture medium, HBSS alone, and dilutions of bacterial culture filtrates (undiluted, 10^{-4}) obtained from the six strains of NTHi between 0 and 72 h by the leading front method in the modified Boyden technique (11). The positive control was FMLP (10^{-9} M). The level of chemoattractant in a culture filtrate was expressed as a percentage of the migratory response to FMLP. Checkerboard analysis was performed (20) with dilutions (10^{-1} to 10^{-4}) of the 7- and 72-h samples of bacterial culture filtrates obtained from each of the six clinical isolates.

(ii) Inhibition of migration. Neutrophils were pretreated either with dilutions (undiluted, 10^{-4}) of bacterial culture filtrates obtained at between 0 and 72 h of culture from the six strains of NTHi or with HBSS as the control for up to 90 min at 37°C. They were then washed twice in HBSS by centrifugation ($250 \times g$, 10 min) and resuspension, and their migratory responses to FMLP (10^{-9} M) were determined by the modified Boyden technique (11). In separate experiments, to investigate the reversibility of inhibition of migration, neutrophils were exposed to 72-h culture filtrates (10^{-2} dilution in HBSS for 20 min, 37°C) obtained from each of the six strains of NTHi, washed twice ($250 \times g$, 10 min), and allowed to stand at room temperature in HBSS for either 30 or 60 min prior to assessment of their migratory responses to FMLP (10^{-9} M). Inhibition was expressed as the percent reduction in migration toward the chemotaxin.

Neutrophil phagocytosis and C3bi receptor expression. Neutrophils were preincubated (20 min, 37°C) with uninoculated culture medium, dilutions of 7- and 72-h bacterial culture filtrates (10^{-1} to 10^{-4}) obtained from each of the six strains of NTHi, FMLP (10^{-3} M; Sigma), or HBSS alone. They were washed twice in HBSS by centrifugation ($250 \times g$, 10 min) and resuspension and were resuspended in HBSS-ovalbumin to a final concentration of 2×10^6 neutrophils per ml. Phagocytosis was assessed by light microscopy under oil immersion with an opsonized latex bead model of ingestion (with gentian violet staining to test for the presence of beads) according to a method previously described by Cline and Lehrer for macrophages (4). Opsonized beads (1.3 μm in diameter; Sigma) were prepared by incubation (overnight at 4°C) with fetal calf serum (ICN Flow, High Wycombe, United Kingdom) and were used at a concentration of 1.0 mg/ml following washing ($250 \times g$, 10 min) and resuspension in HBSS. The numbers of surface C3bi receptors on both treated and untreated neutrophils were assessed by fluorescence-activated cell sorter analysis with an EPICS C cell sorter (Coulter Electronics, Luton, United Kingdom) by an indirect immunofluorescence monoclonal antibody technique employing mouse anti-human C3bi and fluorescein-conjugated rabbit anti-mouse antibodies (DAKO, High Wycombe, United Kingdom) (17). Data were expressed as mean increases in fluorescence intensity above the baseline (HBSS values) ± standard errors of the means (SEMs).

Gel filtration. Bacterial culture filtrates (20 ml) obtained from each of the six strains of NTHi at 72 h (120 ml total) were mixed, lyophilized, and resuspended in 2 ml of endotoxin-free water for chromatography on a Sephadex G-50 gel column (81 by 1 cm, medium grade; Pharmacia). Elution was carried out with water at 51 ml h^{-1}, and 5-ml fractions were collected. The eluate was monitored by determination of UV A_{280} and also by bioassay for neutrophil chemotaxis-inhibitory activity at dilutions of between 10^{-1} and 10^{-4} in HBSS. Blue dextran, ribonuclease, vitamin B_{12}, and phenol red were used as molecular mass markers. Chromatography was performed on three separate occasions. Consecutive fractions containing biologically active material eluting from the column were pooled, yielding three peaks of activity, and these were further fractionated by passage through Detoxigel (Pierce & Warriner, Chester, United Kingdom) and C_{18} Sep-Paks (Waters Assoc., Cheshire, United Kingdom). The efficacy of the Detoxigel at removing endotoxin activity was confirmed with the *Limulus polyphemus* amebocyte lysate test (E-toxate kit; Sigma Poole). The biological activity in one of the peaks of pooled material was completely removed by this purification procedure, and the two remaining peaks of activity obtained from endotoxin-free pooled material were referred to as peaks of partially purified neutrophil chemotaxis-inhibitory activity.

Effect of partially purified neutrophil chemotaxis-inhibitory activity on neutrophil migration toward the chemotaxins FMLP and LTB_4. Neutrophils were treated (20 min, 37°C) with two pooled fractions of partially purified neutrophil chemotaxis-inhibitory activity at dilutions of between 10^{-1} and 10^{-4} in HBSS, and the cells were allowed to migrate toward either FMLP or LTB_4 in the range of 10^{-6} and 10^{-10} M, following centrifugation ($250 \times g$, 10 min) and resuspension in HBSS.

Chemical and enzymic inactivation. The two pooled fractions of partially purified neutrophil chemotaxis-inhibitory activity were treated with a number of reagents. All chemicals were obtained from either BDH or Sigma, and the reactions were carried out at room temperature unless otherwise stated. The treatments consisted of protease linked to Sepharose beads (*Staphylococcus griseus*, 0.1 U/ml, 37°C, 15 min), lipase linked to Sepharose beads (*Candida cylindracea*, 0.1 U/ml, 37°C, 15 min), a boiling water bath (15 min), 0.1 M HCl (56°C, 15 min), 0.1 M NaOH (56°C, 15 min), methanolic hydrogen chloride (prepared by bubbling hydrogen chloride through methanol for 2 min) for 3 h at room temperature, methanolic sodium borohydride (100 μg/ml, 3 h), methanol-acetic anhydride (3:1, vol/vol; 3 h), aqueous sodium periodate (10 mM, 3 h), and diborane in tetrahydrofuran (10 min). Controls consisted of both buffer alone which had been treated with reagents in a manner similar to that for the partially purified neutrophil chemotaxis-inhibitory activity samples and samples which had been treated with enzymes which had been inactivated by boiling. Solvents were removed under vacuum, and the samples were tested for neutrophil chemotaxis-inhibitory activity at a 10^{-2} dilution in HBSS.

Lectin adsorption. The two pooled fractions of partially purified neutrophil chemotaxis-inhibitory activity obtained from Sephadex G-50 were resuspended in endotoxin-free

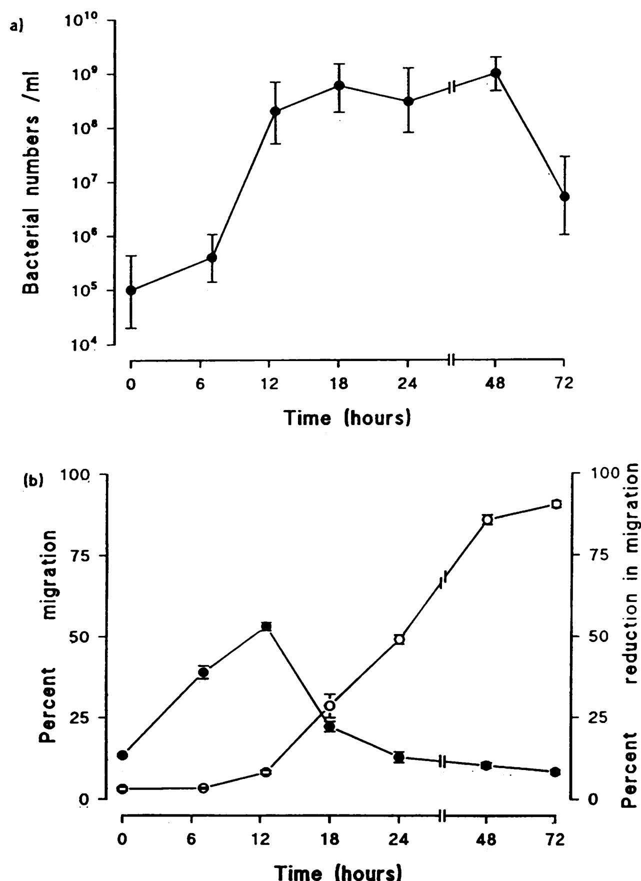

FIG. 1. Effects of culture filtrates of six clinical isolates of *H. influenzae* on neutrophil migration. (a) Bacterial growth curves of six clinical isolates of NTHi. (b) Chemotactic (●) and chemotaxis-inhibitory (○) activities of culture filtrates of six clinical isolates of NTHi toward neutrophils. Chemotactic activity is expressed as a percentage (mean ± SEM) of that caused by FMLP (10^{-9} M) used as a standard. Maximum activity was observed at 12.5 h and returned to basal levels by 24 h. Inhibitory activity is expressed as a percent (mean ± SEM) reduction of that caused by FMLP (10^{-9} M). Inhibitory activity was first observed at 18 h and had not reached a maximum by 72 h, by which time an appreciable drop in bacterial numbers had occurred.

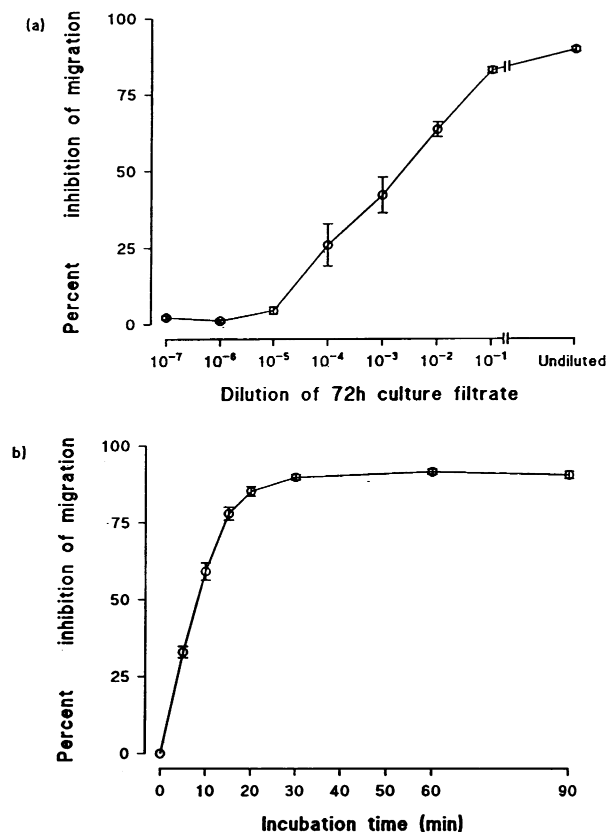

FIG. 2. Effects of filtrate dilution and time of preincubation of neutrophils with culture filtrates of six clinical isolates of NTHi on inhibition of neutrophil migration toward FMLP. (a) Neutrophil chemotaxis-inhibitory activity of 72-h culture filtrates is still present after a 10^4-fold dilution. (b) Preincubation of neutrophils with a 10^{-2} dilution of 72-h culture filtrates for 20 min resulted in maximal chemotaxis-inhibitory activity.

water and passed through separate columns (1 ml) of *Triticum vulgaris* wheat germ agglutinin coupled to Sepharose (Sigma). The column was washed with water (4 ml) followed by aqueous *N*-acetyl-D-glucosamine (4 ml, 0.5 M; Sigma). Fractions were assayed at a 10^{-2} dilution in HBSS with *N*-acetyl-D-glucosamine (0.5 M) as the negative control.

Statistical analysis. Control and test results were compared by nonparametric statistics with the Wilcoxon signed rank test.

RESULTS

Time course of appearance of activity influencing neutrophil functions in culture filtrates from NTHi. Six clinical isolates of NTHi were cultured for up to 72 h in Trypticase soy broth. Bacterial numbers increased logarithmically for the first 12 h, they were stationary for a further 36 h, and they thereafter declined (Fig. 1a). Neutrophil chemoattractant activity was present in each of the culture filtrates obtained from the six clinical isolates of NTHi, and it rose during the log phase of bacterial growth; maximal levels were achieved at around 12.5 h, and activity declined to basal levels by 24 h (Fig. 1b). In addition to chemoattractant activity, filtrates obtained following 12 h of culture of the six isolates of NTHi were found to possess activity (which was not present at earlier time points) that inhibited human neutrophil migration toward FMLP without affecting cell viability. Chemotaxis-inhibitory activity increased during bacterial culture and was maximal at 72 h (Fig. 1b), even though the bacterial count had fallen by 2 log units (Fig. 1a).

Effects of dilution and time of preincubation with NTHi 72-h culture filtrates on inhibition of neutrophil migration toward FMLP; the reversibility of this effect and influence of the filtrates on neutrophil viability. Activity was still present in filtrates diluted 10^4-fold, and a 10^{-2} dilution was used in all further experiments on the effects of 72-h culture filtrates (Fig. 2a). The maximum reduction in the response of the neutrophils to FMLP required preincubation with a 10^{-2} dilution of the 72-h filtrates for 15 to 20 min (Fig. 2b), and this time was used for all further incubations. The inhibitory action of the culture filtrate was irreversible: there was no difference in the migratory responses to FMLP for culture filtrate-treated neutrophils which had been washed and allowed to stand at room temperature in HBSS for 30 min (79.2% ± 3.1% reduction in FMLP response) or 60 min

TABLE 1. Effects of culture filtrates (7 and 72 h) obtained from six clinical isolates of NTHi on neutrophil phagocytosis and C3bi receptor expression

Treatment	Result after phagocytosis[a]		C3bi receptor expression[b]
	% of cells containing beads	No. of beads/cell	
HBSS (control)	59.9 ± 2.2	4.5 ± 2.2	1.0 ± 0.1
7-h culture filtrate	83.8 ± 4.0[c]	9.5 ± 2.4[c]	1.5 ± 0.3[c]
72-h culture filtrate	57.8 ± 4.9	4.7 ± 1.8	0.7 ± 0.4[c]
FMLP (10^{-3} M)	94.5 ± 3.7[c]	15.5 ± 5.3[c]	2.9 ± 0.8[c]

[a] Percentages of cells containing beads and numbers of beads per cell were determined at 30 min. Data are expressed as means ± SEMs of values obtained with the six clinical isolates.

[b] Expressed as ratio of increase in fluorescence intensity above the baseline (HBSS values). Data are expressed as means ± SEMs of the increase produced with the six clinical isolates.

[c] $P < 0.01$ versus control by Wilcoxon signed rank test.

(80.4% ± 2.3%) and those for treated neutrophils tested immediately (82.2% ± 2.7%). Incubation with culture filtrates for up to 90 min did not affect the viability of the neutrophils as assessed by trypan blue exclusion (all cells were >96% viable) or lactate dehydrogenase release (<5% of total lactate dehydrogenase was released by 90 min).

Effects of 7- and 72-h NTHi culture filtrates on neutrophil chemotaxis and chemokinesis. Seven-hour culture filtrates obtained from the six isolates of NTHi contained significant levels of chemoattractant (mean percentage of FMLP migration = 45.9% ± 1.0%) compared with those in controls (2.2% ± 0.9%) at all concentrations (dilutions of 10^{-1} to 10^{-4}; $P < 0.01$). By checkerboard analysis, this activity was found to be primarily chemokinetic, with some associated chemotactic activity. In contrast, 72-h culture filtrates did not produce chemotaxis or chemokinesis at any concentration studied (mean percentage FMLP migration = 3.1% ± 1.2%).

Effects of 7- and 72-h NTHi culture filtrates on neutrophil phagocytosis and C3bi receptor expression. Following incubation of neutrophils with the 7-h filtrates, there was a significant ($P < 0.01$) increase in their phagocytic capacity and a 22.8% increase in their C3bi surface receptors (Table 1). This is in agreement with the presence of a chemoattractant in these filtrates leading to cellular activation (15). The 72-h filtrates had no effect on neutrophil phagocytosis, although a small but significant decrease in C3bi receptor expression was observed, indicating that the inhibitory effects on migration did not appear to be due to cellular activation (11.6% ± 3.1%; $P < 0.01$) (Table 1). The biological significance of the small decrease in neutrophil C3bi receptors is not clear.

Partial purification of activity inhibiting FMLP-induced neutrophil migration (neutrophil chemotaxis inhibitory activity) from 72-h NTHi culture filtrates. A lyophilized sample of the pooled 72-h culture filtrates was chromatographed on Sephadex G-50, and three peaks of chemotactic inhibitory activity were observed (Fig. 3). The activity associated with peak I ($V/V_0 = 1.1$) was completely removed by passage through Detoxigel, suggesting that it was endotoxin, which is known to affect neutrophil function. This is consistent with previous studies on endotoxin and neutrophil priming (9). In contrast, Detoxigel treatment of peaks II ($V/V_0 = 2.8$) and III ($V/V_0 = 3.5$) did not remove the inhibitory activity. Inhibitory activity present in peaks II and III was retained on a C_{18} Sep-Pak column and could be eluted in methanol (fractions SPIIb and SPIII). Fractions SPIIb and SPIII reduced the

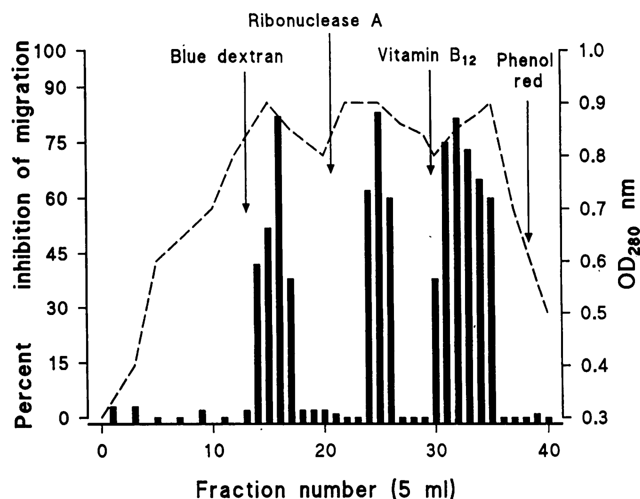

FIG. 3. Gel filtration on Sephadex G-50 of pooled 72-h culture filtrates from six clinical isolates of NTHi resulting in three peaks of chemotaxis-inhibitory activity. Fraction I is associated with endotoxin. Blue dextran (2×10^3 kDa), ribonuclease (13.7 kDa), vitamin B_{12} (1.355 kDa), and phenol red (0.354 kDa) were used as molecular mass markers. Optical density at 280 nm (OD_{280}) is shown as a broken line.

chemotactic responses of neutrophils to both FMLP and LTB_4 (10^{-6} to 10^{-10} M) at all dilutions tested (10^{-1} to 10^{-4}), with maximal effects being observed at a dilution between 10^{-1} and 10^{-2} in HBSS (data shown for 10^{-2} dilution in HBSS in Fig. 4). Peak II also contained substances which did not bind to the solid-phase column (fraction SPIIa). It is not known whether this represents weak binding of the major bioactive species to the Sep-Pak or there are other, more polar substances present. This fraction (SPIIa) was not examined further.

Biochemical analysis of partially purified neutrophil chemotaxis-inhibitory activity (after Sephadex G-50, Detoxigel, and C_{18} Sep-Pak). The active species present in fractions SPIIb and SPIII could not be extracted into chloroform, suggesting that the neutrophil chemotaxis-inhibitory substances were not associated with simple lipids. Boiling, acid and base treatment, and borohydride reduction had no effect on the activity of either fraction SPIIb or fraction SPIII (Table 2). The activities of both fractions were destroyed (>90%) by proteolytic digestion, indicating the presence of a peptide or protein, by treatment with sodium periodate (which attacks *cis* glycols such as sugars), and by diborane reduction (which attacks double bonds) (Table 2). Partial inactivation (>55%) was achieved by lipase treatment (which attacks lipids) and acetylation (which attacks hydroxyl and amine groups) (Table 2). These data are consistent with the bioactive factors containing peptides or proteins, lipids, sugars, double bonds, and hydroxyl, carboxyl, and amine groups. Activity was completely retained on a wheat germ agglutinin column and could be eluted with *N*-acetyl-D-glucosamine. These data are consistent with the inhibitory substances being *N*-acetyl-D-glucosamine-containing glycopeptides, perhaps linked through an acyl group to a lipid. The higher molecular mass of fraction SPII (as determined by gel filtration) and the reduced recovery of activity on a C_{18} Sep-Pak would be consistent with increased glycosylation.

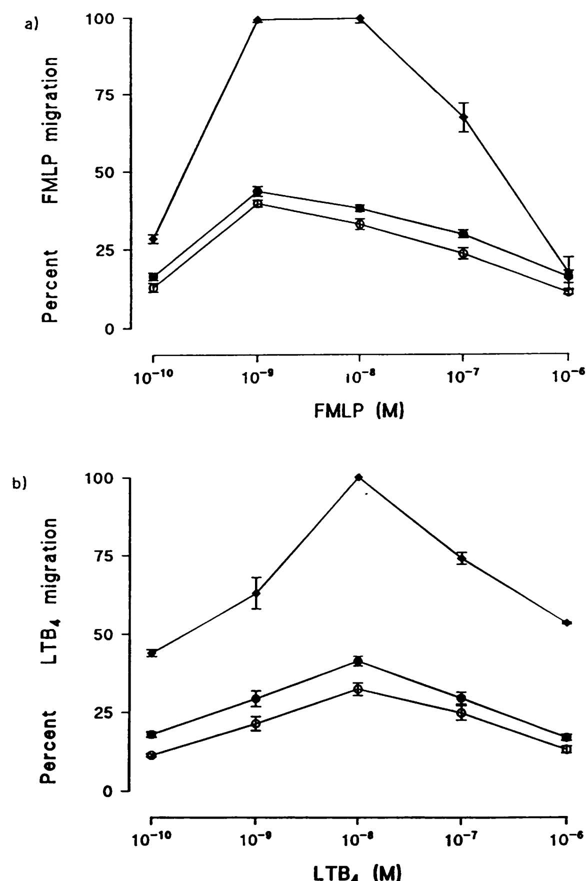

FIG. 4. Fractions SPIIb and SPIII (after Sephadex G-50, Detoxigel, and a C_{18} Sep-Pak) at a 10^{-2} dilution in HBSS suppressed the chemotactic dose-response curve to both FMLP (a) and LTB_4 (b). Data are expressed as means ± SEMs (n = 6) for the chemotaxin control (◆), fraction SPIIb (○), and fraction SPIII (●).

TABLE 2. Biochemical analysis of partially purified neutrophil chemotaxis-inhibitory activity from 72-h culture filtrates of clinical isolates of NTHi

Treatment or substance used in treatment	Result[a] (%) for fraction:	
	SPIIb	SPIII
None	0	0
Freeze-thawing	5.0 ± 2.6	1.6 ± 0.9
pH stabilization (acid)	1.4 ± 0.9	0.5 ± 0.4
pH stabilization (alkali)	0.3 ± 0.2	4.9 ± 2.4
Boiling	4.2 ± 1.0	0.3 ± 0.1
Protease	96.0 ± 2.7	94.4 ± 3.7
Lipase	67.3 ± 1.8	13.5 ± 2.9
Sodium borohydride	0.8 ± 0.7	2.3 ± 1.5
Sodium periodate	93.4 ± 2.9	97.2 ± 2.3
Pyridine-acetic anhydride	63.3 ± 1.1	65.4 ± 1.7
Methanol-acetic anhydride	88.9 ± 2.9	57.9 ± 3.4
Methanol-hydrochloric acid	22.1 ± 3.2	13.7 ± 1.7
Diborane	83.2 ± 1.7	94.7 ± 1.3

[a] Activity was obtained by Detoxigel and C_{18} Sep-Pak fractionation of Sephadex G-50 gel filtration peaks II and III. Values given in the table are percent falls in the levels of neutrophil chemotaxis inhibitory activity from untreated values. Activity is the percent reduction in migratory response to FMLP (10^{-9} M). Background migration has been subtracted from all values shown, which are means ± SEMs (n = 3).

DISCUSSION

Polymorphonuclear leukocytes are recruited to sites of infection in response to the production of endogenous and bacterium-derived chemotaxins (12). These cells play a key role in the defense against bacterial infections of mucosal surfaces (5, 13). Several pathogenic species, including *B. pertussis* (1), *L. micdadei* (7), and *P. aeruginosa* (2, 10), release substances which either are directly cytotoxic to neutrophils or act through inhibiting signal transduction, and this may be an important factor in their survival and proliferation in the lung.

We have shown that six clinical isolates of the common respiratory pathogen NTHi produce factors which adversely affect neutrophil function. As do many other respiratory tract bacteria, NTHi generated neutrophil chemoattractants during the log and stationary phases (0 to 18 h) of growth (13). After 12 h of culture, this attractant activity diminished and was replaced by factors which inhibited neutrophil migration toward two unrelated chemotaxins (FMLP and LTB_4). Inhibitory activity increased with time and was maximal at 72 h, even though the bacterial viable count had fallen. This inhibitory effect on migration was not reversible, although the cells remained viable for up to 90 min.

The mechanism of action of the *Haemophilus*-derived inhibitory factor(s) is not known. The *Haemophilus* factors described in this study are not directly cytotoxic and do not increase neutrophil movement (either chemotactic or chemokinetic), phagocytosis, or C3bi receptor expression, suggesting that this is not simply a generalized effect on the neutrophil cell wall that produces neutrophil cellular activation. The dose-response curves to both chemotaxins are flattened by the factors, which suggests that the effects on neutrophil migration toward the chemotactic factors FMLP and LTB_4 are not related to an alteration in receptor number. A similar flattening of the dose-response curve to FMLP also occurs in response to treatment of neutrophils with pertussis toxin, which similarly produces its effects without loss of cellular viability (1). Pertussis toxin is known to inhibit neutrophil chemotaxis and enzyme secretion and is believed to produce these effects by blocking the α subunit of G_i, the inhibitory GTP-binding regulatory protein.

The inhibitory factors have not yet been characterized. Gel filtration of a pooled 72-h filtrate yielded two major peaks of low-molecular-mass (<15-kDa) chemotaxis-inhibitory activity which were not associated with endotoxin. Some of the activity associated with one of the peaks (peak II) was not bound to a C_{18} Sep-Pak column, and it is not clear whether this represents weak binding of the major bioactive species to the Sep-Pak or there are other, more polar substances present. This is currently being investigated. The studies presented in this paper have concentrated on the activity bound to C_{18} Sep-Pak columns which could be eluted by methanol (fractions SPIIb and SPIII). Chemical and enzymic inactivation studies of these two fractions are consistent with the presence of *N*-acetyl-D-glucosamine-containing glycopeptides, perhaps linked to lipids. As bioactivity was not retained on the endotoxin-affinity matrix, Detoxigel, it is unlikely that these factors contain lipid A. The presence of *N*-acetyl-D-glucosamine residues suggests that the neutrophil chemotaxis-inhibitory activity detected may derive from the bacterial cell wall. Several gram-negative bacteria, including *Escherichia coli*, *Salmonella typhimurium*, and *Neisseria gonorrhoeae*, release peptidoglycans during cell division (8, 14), and some fragments have molecular masses similar to those of fractions SPIIb

and SPIII (14). Although the majority of the peptidoglycan is reincorporated by the bacteria, in the case of *E. coli*, up to 8% per generation is lost (8), and for *N. gonorrhoeae*, as much as 35% per generation is lost (14). Peptidoglycans have been previously shown to have numerous activities, including inhibition of macrophage chemotaxis, activation of complement (13), and the induction of pulmonary inflammation in a rabbit model (18). Large fragments (35 to 40 kDa) of peptidoglycan (30 to 45 kDa) may also stimulate human mononuclear cells to produce factors which inhibit neutrophil chemotaxis toward FMLP without affecting either cellular viability or phagocytosis (6), although their direct effects on human neutrophil migration remain unknown.

NTHi organisms very commonly colonize the lower respiratory tracts of patients with chronic airway diseases, and they are associated with infective exacerbations. Infections of the lower airways are associated with neutrophil infiltration, which results from the interaction of neutrophils with a number of locally derived endogenous and bacterially derived chemotactic factors, which in turn leads to their activation. Host-derived chemotactic factors include the complement component C5a, LTB_4, platelet-activating factor, and leukocyte-derived cytokines (13). Our data show that while some products released earlier in the growth cycle are chemoattractants, other products released later inhibit chemotaxis. If these inhibitory factors are released into the microenvironment of the bronchial mucosa, they might interfere with bacterial clearance by neutrophils. This might help to explain why these bacteria are not eradicated in chronic bronchial infections despite there being a normal neutrophil response (16).

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