

Induction of Biologically Active Interleukin-8 from Lung Epithelial Cells by *Burkholderia (Pseudomonas) cepacia* Products

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The frequency of isolation of *Burkholderia cepacia* from the sputum of cystic fibrosis (CF) patients is increasing. Using the human A549 lung epithelial cell line, we have investigated the ability of *B. cepacia* exoproducts to stimulate interleukin-8 (IL-8) release. Cell-free supernatants from a panel of CF clinical, non-CF clinical, and nonclinical *B. cepacia* isolates were found to stimulate IL-8 release, with levels ranging from 11.8 ± 2.8 to 80.0 ± 3.5 ng/ml. A similar pattern was seen at the level of the IL-8 mRNA. The bioactivity of the IL-8 was confirmed by examining its effect on the intracellular free calcium in neutrophils and inhibition by a neutralizing anti-IL-8 antibody. *B. cepacia* lipopolysaccharide, which was able to stimulate IL-8 release from monocytes, did not release IL-8 from the A549 cells. Furthermore, the stimulating ability of the bacterial cell-free supernatant was not diminished by polymyxin B, was markedly reduced by boiling, and appeared unrelated to *N*-acylhomoserine lactones. The ability of *B. cepacia* to elicit IL-8 release from epithelial cells may be important in the pathology of CF.

Human infections with the phytopathogen *Burkholderia (Pseudomonas) cepacia* occur relatively infrequently and are usually nosocomial and affect immunocompromised patients. However, the frequency of isolation of *B. cepacia* from the sputum of cystic fibrosis (CF) patients is increasing. The proposed methods of acquisition and transmission of the organism remain controversial, but several studies have indicated case clustering, person-to-person transmission, and emergence of epidemic strains in several centers in both North America and the United Kingdom (5, 25). The clinical course following *B. cepacia* colonization progresses in one of three ways: (i) chronic asymptomatic carriage, (ii) progressive deterioration over many months, and (iii) rapidly fatal decline in lung function, sometimes with septicemia. The latter case, termed cepacia syndrome, occurs in approximately 20% of colonized patients (6, 9). The predisposing factors for *B. cepacia* colonization are not well defined, but young adult CF patients, many of whom may have been colonized with *Pseudomonas aeruginosa* for many years, seem to be at particular risk. Epidemiological data indicate that patients colonized with the same strain can have dramatically different clinical outcomes (5), suggesting that cepacia syndrome is not solely strain dependent but also includes a component of host response to colonization.

Airway inflammation, characterized by massive influx and activation of neutrophils, plays a major role in the pathology of CF lung disease (6, 8), and attention has been focused on the generation of neutrophil chemoattractants. Interleukin-8 (IL-8) is a neutrophil-selective stimulus for chemoattraction, adhesion, and elastase release (1, 26), and there is growing evidence of its importance in CF. It has been detected in CF sputum and bronchial lavage fluid (14, 20), and a significant negative correlation between IL-8 levels and Shwachman scores of clinical status has been reported (3). Potential sources in CF include bronchial and alveolar epithelial cells (13, 17, 21) and phagocytes (10). Several agents have been

proposed to stimulate IL-8 release, including neutrophil elastase (17), leading Nakamura et al. (17) to propose a cycle of inflammation in which neutrophils, attracted to the lung, release elastase, which results in further neutrophil accumulation. Ruff et al. (21) have found that neutrophil elastase or IL-1 β elicits IL-8 release from a CF airway epithelial cell line homozygous for the phenylalanine deletion at position 508. While *P. aeruginosa* lipopolysaccharide (LPS) was inactive in this study, other workers have characterized release of IL-8 from epithelial cells in response to *P. aeruginosa* products, including *rpoN*-controlled pilin and flagellin and a homoserine lactone autoinducer molecule (4, 13).

Less is known of the inflammatory response against *B. cepacia* in CF, although Shaw et al. (22) have shown that LPS from clinical and environmental sources was more endotoxic than *P. aeruginosa* LPS and elicited high levels of tumor necrosis factor alpha from human monocytes. In this work we demonstrate that an extracellular factor from *B. cepacia* elicits high levels of bioactive IL-8 from lung epithelial cells and peripheral blood monocytes. This activity is heat labile and unrelated to LPS or homoserine lactones.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. cepacia* J2315 and J2552 were obtained from J. R. W. Govan (University of Edinburgh, Edinburgh, United Kingdom). All other strains were from either the Birmingham (United Kingdom) CF clinic or our culture collection. Strains were grown in succinate minimal medium (16) supplemented with 1% (wt/vol) Casamino Acids (Difco Laboratories, Detroit, Mich.) at 37°C until late logarithmic phase (optical density at 470 nm of 0.8) was reached. Culture supernatants were collected by centrifugation at $6,000 \times g$ for 15 min, filtered through a 0.2- μ m-pore-size cellulose acetate filter, and stored in aliquots at -80°C.

Preparation of LPS. LPS was prepared by the hot phenol method of Westphal and Jann (27) as described by Lacy et al. (12). *Escherichia coli* LPS was purchased from Sigma Chemical Co. (Poole, United Kingdom).

Measurement of IL-8 production by ELISA. The type II pneumocyte A549 cell line (ATCC CCL185) was maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and penicillin (100 U/ml)-streptomycin (100 μ g/ml) (Gibco, Paisley, United Kingdom) and passaged by harvest with trypsin and EDTA. For experimental use, cells were grown until confluent (approximately 5×10^5 cells/well) in 12-well plates and the medium was replaced with Dulbecco's modified Eagle's medium-5% FBS. Test agents (*B. cepacia* supernatant and purified LPS) were added to replicate wells, and the cultures were incubated for 1 to 48 h before collection of A549 supernatants. IL-8 levels were assessed by a specific enzyme-linked immunosorbent assay (ELISA) using a

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murine monoclonal anti-IL-8 capture antibody and a biotin-conjugated goat polyclonal secondary antibody (2) and were detected with streptavidin-peroxidase-*o*-phenylenediamine substrate. Levels were determined from the means of duplicate determinations for each supernatant sample and compared with a standard curve for human recombinant IL-8. The assay routinely detected IL-8 levels from 0.2 to 4 ng/ml.

Preparation of monocytes. Monocytes were prepared from heparinized normal donor blood by density centrifugation (Lymphoprep separation medium; Nycomed PharmaAS, Oslo, Norway) and 2 h of adhesion to plastic. Cells were harvested with a cell scraper and plated at 0.5×10^6 per well in 0.5 ml of RPMI 1640–5% FBS (Gibco).

Neutrophil stimulation by A549 epithelial cell supernatants. The presence of IL-8-like bioactivity was assessed by the ability of supernatants to elevate the concentration of intracellular free calcium ($[Ca^{2+}]_i$) in fura-2-loaded human neutrophils, essentially as previously described (26). Neutrophils (>90% pure) were recovered from the Lymphoprep erythrocyte fraction by dextran sedimentation and labelled by incubation (30 min, 37°C) with fura-2 acetoxyethyl ester (2.5 μ M; Calbiochem, La Jolla, Calif.). Cells were then washed twice in Ca^{2+} - and Mg^{2+} -free Hanks buffered salt solution (Gibco) and suspended in Hanks buffered salt solution supplemented with 0.1% bovine serum albumin at 10^6 neutrophils/ml. Extracellular calcium and magnesium were restored to 1 mM, and $[Ca^{2+}]_i$ was monitored in stirred 2-ml aliquots in a fluorimeter (PTI Inc., Surbiton, United Kingdom) with dual excitation wavelengths (340 and 380 nm) and the emission wavelength at 510 nm. $[Ca^{2+}]_i$ was calculated by the ratio method (7). In neutralization studies, A549 supernatants were incubated with goat anti-IL-8 antibody (5 μ g per sample; R & D Systems, Abingdon, United Kingdom) for 30 min before the assay.

Northern blot analysis. A549 cells were grown as described above. Test agents were added, and the cells were incubated for 1 to 48 h. The cell monolayer was lysed into RNazol B (Tel-Test, Friendswood, Tex.) and stored at -80°C . Total cellular RNA was prepared in accordance with the manufacturer's instructions. RNA concentration was assessed by absorbance at 260 nm, and 10 μ g per lane was loaded and separated by electrophoresis through a 1% agarose-formaldehyde gel. Separated RNA was blotted onto nylon membranes (Boehringer, Mannheim, Germany) and fixed by baking at 120°C for 20 min. Blots were hybridized with a digoxigenin-labelled IL-8 probe cocktail (10 ng/ml; R & D Systems) and detected with alkaline phosphatase-conjugated antidigoxigenin Fab fragments and chemiluminescence substrate, as described by the kit supplier (Boehringer). X-ray film was exposed to the blots for 1 to 2 h, and mRNA levels were quantitated by scanning densitometry (GS 670 imaging densitometer and Molecular Analyst software; Bio-Rad, Richmond, Calif.). Adjustments for small differences in loading were made by using densitometry of the ethidium bromide fluorescence of the 18S band of the gel photographed prior to blotting.

RESULTS

Induction of IL-8. Our initial studies focused on strain J2315 associated with *B. cepacia* colonization in a number of centers in the United Kingdom. Bacterial culture supernatants, from late logarithmic-phase cultures grown in an iron-deficient succinate minimal medium, were added to the medium of confluent A549 epithelial cells. Without bacterial stimulation, A549 cells released small amounts of IL-8 (approximately 5 ± 3 ng/ml) after 24 h. In marked contrast, cells stimulated with 1-in-5 to 1-in-200 dilutions of *B. cepacia* J2315 supernatant released large amounts of IL-8 (Fig. 1). Bacterial supernatant at a dilution of 1 in 10 of the A549 cell culture medium volume was selected for subsequent studies. Time course studies revealed that A549-derived IL-8 was detected 2 h after incubation with bacterial culture supernatant and continued to accumulate over the 48-h period studied (Fig. 2A). This accumulation of IL-8 protein was consistent with measurements of IL-8 mRNA (Fig. 2B), for which maximal expression was detected after 2 h. mRNA levels then decreased, but a second phase of expression was consistently seen at 32 h. This may suggest induction of secondary cytokine signals. Culture supernatants from a panel of CF clinical, non-CF clinical, and non-clinical isolates at 1-in-10 and 1-in-100 dilutions were examined for their ability to elicit IL-8 release (Table 1). The CF isolates had a wide range of IL-8-inducing activity from approximately 10 to 80 ng/ml. The IL-8-inducing activity in the culture supernatants of most isolates was reduced to near control levels (>75% reduction) by boiling, although 4 out of the 17 isolates retained some activity after boiling. In addition,

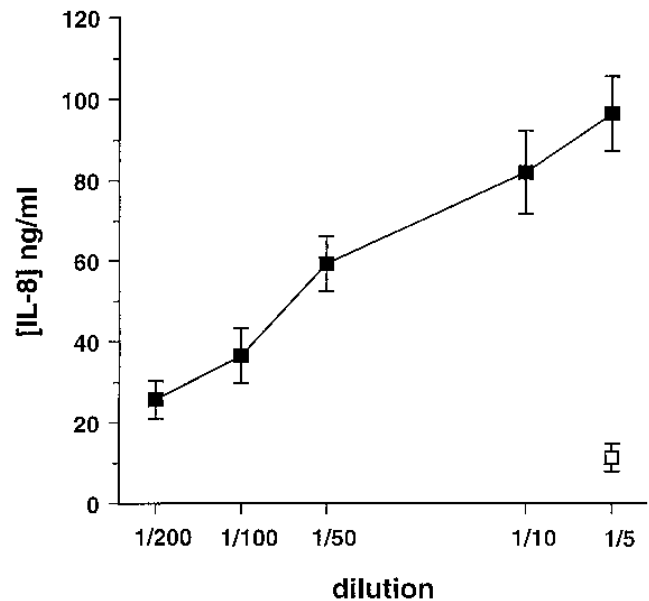


FIG. 1. IL-8 release by A549 cells. Cells were stimulated for 24 h with *B. cepacia* J2315 cell-free supernatant at dilutions from 1 in 5 to 1 in 200 (■) or a 1-in-5 dilution of bacterial growth medium (□), and IL-8 release was measured by ELISA. Values are means \pm standard errors of the means (error bars) ($n = 5$).

supernatants from these four isolates showed little or no reduction in IL-8-inducing activity on dilution from 1 in 10 to 1 in 100. The levels of IL-8 induction achieved by the different strains were also reflected by the mRNA levels (Fig. 3). Interestingly, the three non-CF clinical isolates all elicited high IL-8 levels.

Bioactivity of IL-8. Elevation of $[Ca^{2+}]_i$ is a well-characterized response of neutrophils to IL-8 (26). *B. cepacia* J2315 supernatant alone did not increase the $[Ca^{2+}]_i$ (data not shown); however, medium from *B. cepacia*-treated A549 cells caused a rapid and transient increase in $[Ca^{2+}]_i$ (Fig. 4). Preincubation of this A549 supernatant with anti-IL-8 antibody resulted in a marked reduction in this response (Fig. 4). In addition, studies in which neutrophils were pretreated with a high concentration of IL-8 (10 nM) indicated complete desensitization of the response to the supernatant (data not shown).

Role of *B. cepacia* LPS. We sought to determine whether LPS, perhaps sheared off during cell growth, had a role in the extracellular IL-8-inducing activity. LPS was prepared from *B. cepacia* J2315, a CF isolate, and *B. cepacia* J2552, a botanical isolate, and added to A549 cultures at 10 μ g per ml. In no case was the level of IL-8, measured after 24 h of incubation, greater than levels found with controls stimulated with bacterial growth medium alone (Fig. 5). In order to confirm the bioactivity of our LPS preparations, we measured IL-8 production from monocytes after 24 h of incubation with commercially prepared LPS from *E. coli* and from the two *B. cepacia* strains (Fig. 6). In each case, high levels of IL-8, which were completely abolished by coinubation with polymyxin B, were measured. In support of these data, we found that heating the bacterial supernatant at 100°C for 20 min reduced IL-8-inducing activity by $73 \pm 8\%$ ($n = 3$).

Role of *N*-acylhomoserine lactones. To examine the possible involvement of *N*-acylhomoserine lactones in our experimental system, we measured IL-8 production after 24 h of incubation with 100 μ M *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butyryl-L-homoserine lactone (BHL). Nei-

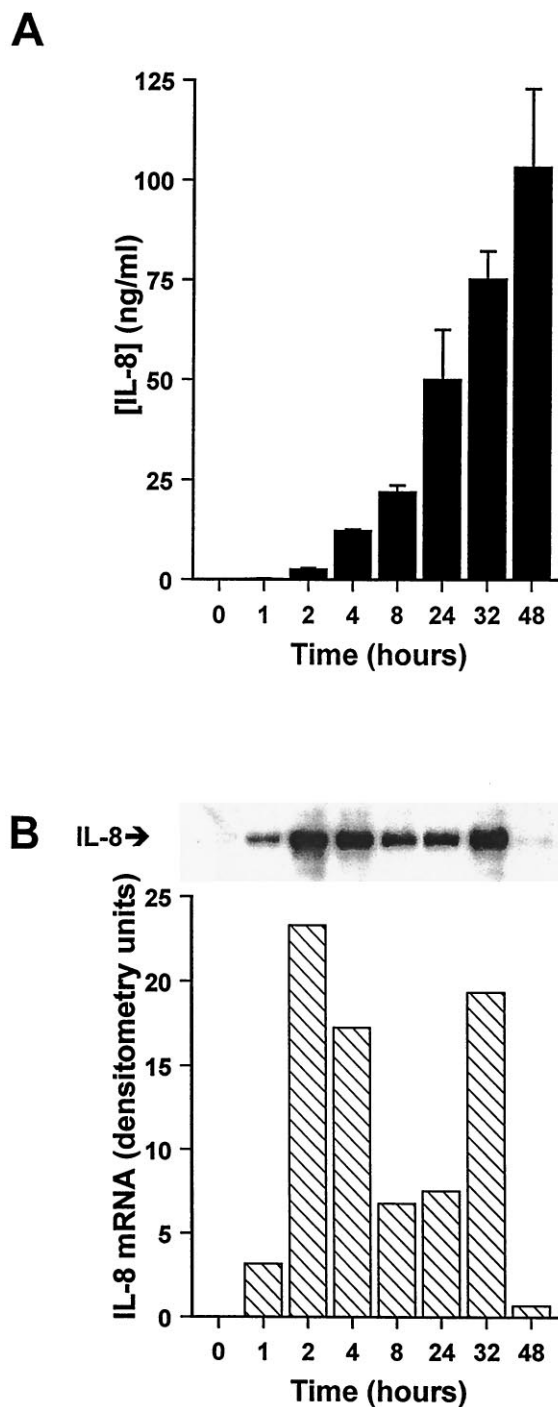


FIG. 2. Time course of IL-8 production by A549 cells. A 1-in-10 dilution of *B. cepacia* J2315 cell-free supernatant was used to stimulate A549 cells for up to 48 h. (A) IL-8 protein measured by ELISA. Values are means \pm standard errors of the means (error bars) ($n = 3$). (B) IL-8 mRNA measured by Northern blotting.

ther compound induced release of IL-8 compared to the control (Table 2).

DISCUSSION

The influx of neutrophils into the airways is one of the defining features of CF lung infections. In this work we provide

evidence of a heat-labile component from *B. cepacia* which is likely to induce neutrophil accumulation via stimulation of epithelial cells. All of the isolates tested could upregulate IL-8 mRNA and elicit immunoreactive IL-8 from human A549 lung epithelial cells in culture. The *B. cepacia*-treated A549 cells released a neutrophil-stimulating bioactivity which was substantially reduced by a neutralizing anti-IL-8 antibody. In an attempt to characterize the nature of the *B. cepacia* product(s) responsible for inducing IL-8, we found that lung epithelial cells were unresponsive to purified LPS. Similar findings have been reported with these cells using LPS from other species (23). In contrast with these epithelial cells, *B. cepacia* LPS did elicit high levels of IL-8 from peripheral blood monocytes. This effect was inhibited by polymyxin. Bacterial cell-free supernatants were also able to elicit IL-8 release from monocytes. Further support for our hypothesis that the inducing component is not related to LPS arises from the finding that it is heat labile. In this respect, it differs from the *P. aeruginosa* IL-8-inducing exoproduct, which was not affected by boiling or freezing (13).

A number of candidate cellular components and exoproducts from *P. aeruginosa* have been examined for their ability to elicit IL-8 induction, including the mucoid exopolysaccharide alginate, which appeared to have little effect (10), and phospholipase C, which gave a dose-dependent decrease in IL-8 production by monocytes (11). DiMango et al. (4) noted that *rpoN* mutants lacking pilin and flagellin did not bind well to respiratory epithelial cells or elicit an IL-8 response. They also found that *Pseudomonas* autoinducer OdDHL, one of several diffusible *N*-acylhomoserine lactone molecules associated with cell density-dependent activation of virulence factor genes in *P.*

TABLE 1. Effect of *B. cepacia* strains on IL-8 production by A549 cells and reduction by boiling^a

<i>B. cepacia</i> strain	Source	Mean IL-8 concn \pm SEM (ng/ml) at dilution:		% Loss of IL-8-inducing activity on boiling
		1/10	1/100	
JL21	CF clinical isolate (J2315)	76.7 \pm 5.4	37.7 \pm 11.8	96
JL22	Environmental isolate (J2552)	29.2 \pm 0.9	11.9 \pm 3.8	85
JL25	CF clinical isolate	59.5 \pm 7.4	68.5 \pm 0.9	26
JL26	NCTC 10661	78.5 \pm 4.3	57.9 \pm 0.2	79
JL27	CF clinical isolate	55.8 \pm 8.3	79.9 \pm 4.4	14
JL29	CF clinical isolate	20.1 \pm 2.9	12.1 \pm 4.0	76
JL30	Non-CF clinical isolate	73.4 \pm 6.4	46.4 \pm 3.3	87
JL31	Non-CF clinical isolate	76.0 \pm 3.3	25.1 \pm 4.5	87
JL32	Non-CF clinical isolate	73.8 \pm 3.8	61.1 \pm 8.7	56
JL33	CF clinical isolate	30.7 \pm 2.1	12.9 \pm 3.7	88
JL35	CF clinical isolate	11.8 \pm 2.8	11.2 \pm 3.9	ND ^b
JL36	CF clinical isolate	57.3 \pm 1.7	13.6 \pm 5.1	95
JL37	CF clinical isolate	12.6 \pm 1.9	14.4 \pm 5.5	ND
JL38	CF clinical isolate	12.1 \pm 1.8	12.6 \pm 4.7	ND
JL39	CF clinical isolate	44.8 \pm 2.1	15.6 \pm 4.9	88
JL40	CF clinical isolate	12.4 \pm 2.8	13.6 \pm 5.3	ND
JL41	CF clinical isolate	80.0 \pm 3.5	72.2 \pm 2.3	37

^a A549 epithelial cells were incubated with 1-in-10 and 1-in-100 dilutions of the *B. cepacia* cell-free supernatant for 24 h before the medium was assayed for IL-8 by ELISA ($n = 3$). To determine the heat stability of supernatant, 1-in-10 dilutions were boiled for 10 min prior to addition to A549 cells. A549 cells incubated with 1-in-10 and 1-in-100 dilutions of succinate minimal medium plus Casamino Acids were used as a control. The resulting values for IL-8 (mean concentrations \pm standard error of the means [in nanograms per milliliter]) were 5.4 \pm 3.0 and 9.3 \pm 5.5 when 1-in-10 and 1-in-100 dilutions, respectively, were used.

^b ND, not determined.

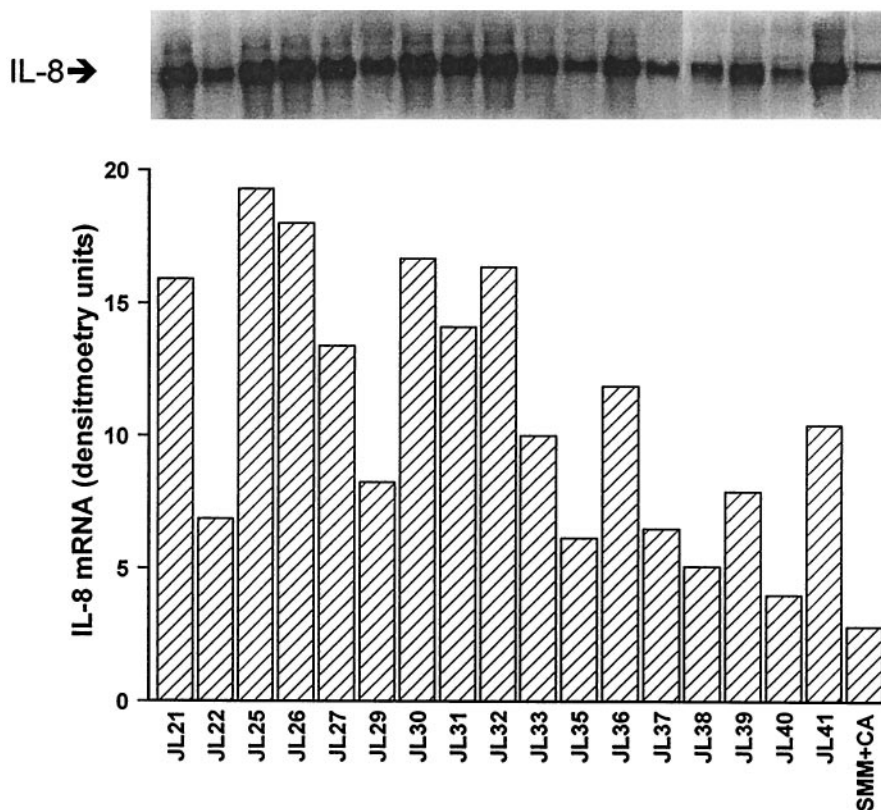


FIG. 3. IL-8 mRNA levels in A549 cells following stimulation for 2 h with a 1-in-10 dilution of cell-free supernatant from different *B. cepacia* isolates, described in Table 1. IL-8 mRNA levels were assessed by Northern blotting and quantified by densitometry. SMM+CA, succinate minimal medium plus Casamino Acids.

aeruginosa (19, 28), also induced low levels of IL-8. Relatively little is known of the contribution of *B. cepacia* extracellular products, such as hemolysin, lipase, protease, exopolysaccharide, and siderophores, to virulence (18) or of the mechanisms of regulation, although recent evidence suggests that the latter

may be influenced by homoserine lactones (15). Expression could be increased by addition of *N*-acylhomoserine lactones from the culture supernatant from *P. aeruginosa* but not by supernatant from a mutant of *P. aeruginosa* producing 1,000- and 20-fold-reduced levels of OddHL and BHL, respectively.

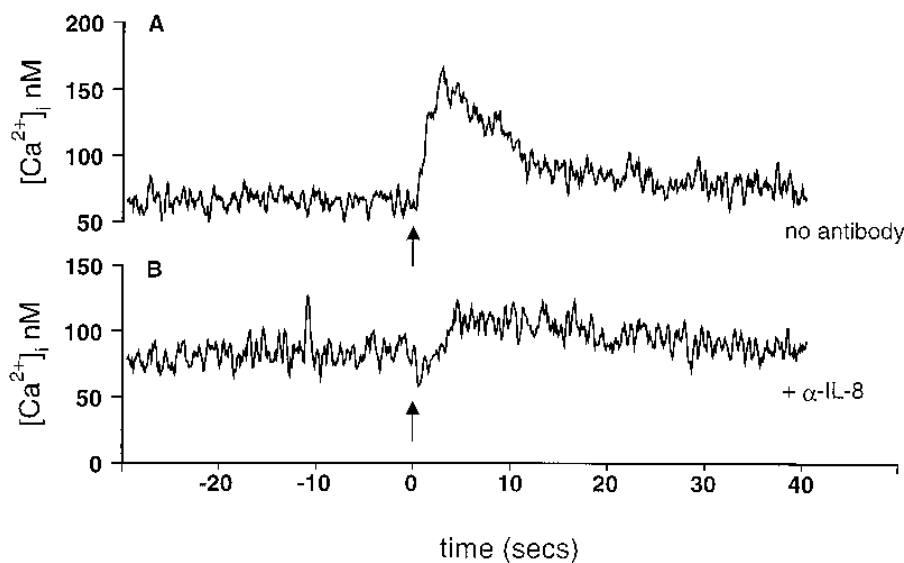


FIG. 4. Elevation of human neutrophil $[Ca^{2+}]_i$ by *B. cepacia*-treated A549 medium. (A) A549 cells were treated for 24 h with a 1-in-10 dilution of *B. cepacia* J2315 supernatant before assessment of A549 medium bioactivity at a 1-in-100 final dilution on 2×10^6 fura-2-loaded neutrophils in stirred cuvettes. (B) Elevation of neutrophil $[Ca^{2+}]_i$ by the same A549 medium after treatment with 5 μ g of anti-IL-8 antibody.

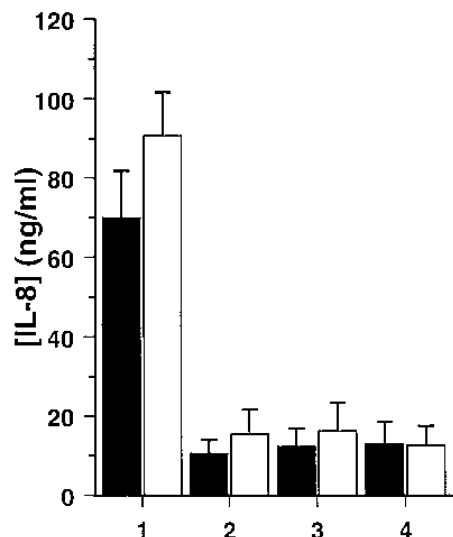


FIG. 5. Effect of *B. cepacia* LPS on IL-8 release by A549 cells. The level of released IL-8 was measured by ELISA after a 24-h stimulation with a 1-in-10 dilution of J2315 cell-free supernatant (bars 1), J2315 LPS (10 µg/ml) (bars 2), J2552 LPS (10 µg/ml) (bars 3), or a 1-in-10 dilution of the bacterial growth medium (bars 4) in the absence (■) or presence (□) of polymyxin B (100 µg/ml). Values are means \pm standard errors of the means (error bars) ($n = 3$).

In addition, McKenney et al. (15) noted the presence of *N*-(3-oxohexanoyl)-*L*-homoserine lactone, *N*-hexanoyl-*L*-homoserine lactone, and BHL in the supernatants of two *B. cepacia* isolates. Two findings lead us to believe that *N*-acylhomoserine lactones do not have a major role in eliciting IL-8 release in our system. Firstly, we were unable to detect IL-8 production in response to OdDHL or BHL, and secondly, examination of *N*-acylhomoserine lactone production by four *B. cepacia* isolates showed no correlation with the isolates' ability to elicit

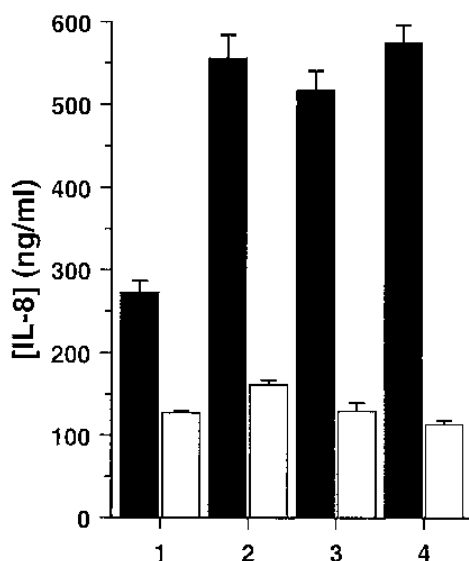


FIG. 6. Effect of *B. cepacia* LPS on IL-8 release by peripheral blood mononuclear cells. The level of released IL-8 was measured by ELISA after a 24-h stimulation with no additions ($n = 2$) (bars 1), *E. coli* LPS (10 µg/ml) ($n = 2$) (bars 2), J2315 LPS (10 µg/ml) ($n = 3$) (bars 3), or J2552 LPS (10 µg/ml) ($n = 3$) (bars 4) in the absence (■) or presence (□) of polymyxin B (100 µg/ml). Values are means \pm standard errors of the means (error bars) from replicate experiments.

TABLE 2. Effect of *N*-acylhomoserine lactones on IL-8 release by A549 cells^a

<i>N</i> -acylhomoserine lactone (concn [µM])	Mean IL-8 concn \pm SEM (ng/ml)
BHL (10)	1.5 \pm 0.5
BHL (30)	1.5 \pm 0.5
BHL (100)	1.4 \pm 0.5
OdDHL (10)	1.3 \pm 0.3
OdDHL (30)	1.5 \pm 0.5
OdDHL (100)	1.9 \pm 0.4
Control	1.5 \pm 0.5

^a A549 cells were incubated with *N*-acylhomoserine lactone for 24 h before the medium was assayed for IL-8 by ELISA ($n = 4$).

IL-8 release. J2552 and JL39 (Table 1) were strongly positive in *Chromobacterium*- and LuxR-based reporter assays, whereas J2315 and JL40 (Table 1) were negative (27a). Our OdDHL data are in contrast to the finding reported by DiMango et al. (4), and while we accept that the two experimental systems are different, it is noteworthy that the maximum IL-8 release measured by those workers approximated the control levels in our system.

Our analysis of 17 *B. cepacia* isolates demonstrated wide variation in their ability to elicit IL-8 release. *B. cepacia* strains exhibit marked phenotypic variability, but we were unable to relate this to their ability to elicit IL-8 release. More detailed studies would be required to establish whether any clonal relationship among these strains exists. Conflicting reports of the epidemiology and transmissibility of *B. cepacia* isolates have appeared in the literature (5, 24), although one recent study, combining chromosomal restriction fragment length polymorphism and DNA sequence data, suggests that there is at least one significantly divergent, highly transmissible clonal lineage plus numerous moderately heterogeneous lineages of negligible transmissibility (25).

In this work we have shown that *B. cepacia* isolates are capable of eliciting high levels of bioactive IL-8 from lung epithelial cells in vitro. While it is clear that IL-8 plays a major role in neutrophil-dominated epithelial inflammation and damage in CF patients, there is no simple correlation between the ability of *B. cepacia* to elicit IL-8 release in vitro and the fatal cepacia syndrome occurring in 20% of *B. cepacia*-colonized patients. Cepacia syndrome most likely results from a complex interaction between host and bacterial factors. Our current studies are focused on the effect of *B. cepacia* on the migration of neutrophils across the epithelium and on epithelial barrier integrity.

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