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Priming of Neutrophil Respiratory Burst Activity by Lipopolysaccharide from *Burkholderia cepacia*

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Neutrophil activation may play an important role in the pathogenesis of respiratory disease in *Burkholderia cepacia*-colonized cystic fibrosis (CF) patients. As bacterial lipopolysaccharides (LPS) are potent immunostimulatory molecules, we investigated the role of *B. cepacia* LPS in neutrophil activation processes. LPS extracted from a highly transmissible and virulent strain of *B. cepacia* (J2315) was found to increase neutrophil surface expression of the β_2 integrin, complement receptor 3, and to prime neutrophil respiratory burst responses to the neutrophil-activating agent fMet-Leu-Phe. By contrast, LPS extracted from a nonmucoid *Pseudomonas aeruginosa* strain isolated from a patient with CF showed little or no priming activity. As *B. cepacia* is currently being developed as a biocontrol agent for large-scale agricultural release, we compared LPS molecules from a range of bacterial strains for their proinflammatory ability. Priming activity was demonstrated in LPS extracts from all *B. cepacia* strains tested, with one environmental strain, J2552, showing the highest activity. These findings indicate (i) that *B. cepacia* LPS may contribute to the inflammatory nature of *B. cepacia* infection in CF patients, both by promoting increased neutrophil recruitment and by priming neutrophil respiratory burst responses, and (ii) that environmental strains of *B. cepacia* may have considerable inflammatory potential in susceptible individuals.

During the last 15 years, *Burkholderia cepacia* has emerged as a serious respiratory pathogen in cystic fibrosis (CF) (16). Epidemic spread of *B. cepacia* between CF patients has been associated with a subset of highly transmissible strains, including the Edinburgh/Toronto or electrophoretic type ET12 strain, which has been implicated in numerous outbreaks in both North American and United Kingdom CF centers and has been isolated from up to 40% of *B. cepacia*-colonized patients in the United Kingdom (23, 34). Recently, investigators have identified putative transmissibility markers, including adhesion to mucin and respiratory epithelium mediated via cable pili (39); enhanced adhesion to the cell surface glycolipid, globotriosylceramide (40); and a genomic marker termed the *Burkholderia cepacia* epidemic strain marker (30). In contrast, little is known of the pathogenic mechanisms responsible for severe *B. cepacia* infection. For example, it is not clear why some patients appear unaffected by *B. cepacia* colonization while others succumb to rapidly fatal necrotizing pneumonia and septicemia, the so-called cepacia syndrome.

There is increasing evidence that acute pulmonary deterioration associated with *B. cepacia* infection is due to a marked inflammatory response induced by this organism. In a case-controlled study, raised serum levels of the inflammatory markers C-reactive protein and neutrophil elastase were found during exacerbations with *B. cepacia* (11). In addition, anecdotal evidence has suggested that severe *B. cepacia* infection can be treated with anti-inflammatory therapy, in particular the use of hypergammaglobulin preparations (16). Shaw et al. (37) described the in vitro induction of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) from monocytes stimulated by lipopolysaccharides (LPS) extracted from clinical strains of *B. cepacia*, including a representative of the ET12 lineage (C1359). Interestingly, levels of TNF-α induced were over nine times greater than levels induced by LPS from *Pseudomonas aeruginosa*, the major respiratory pathogen in CF. Recently Palfreyman et al. (33) have described the induction of biologically active interleukin-8 from lung epithelial cells and human monocytes by a *B. cepacia* extracellular factor distinct from LPS.

Neutrophils have been implicated in immunologically mediated lung damage in CF through the release of reactive oxygen species (ROS) and proteolytic enzymes during activation processes (6, 9, 26). Chronic colonization with mucoid *P. aeruginosa* is believed to exacerbate neutrophil-mediated damage through "frustrated phagocytosis," as neutrophils attempt to engulf *P. aeruginosa* microcolonies embedded in a protective layer of alginate and release their granule contents in the process (14, 15). Similarly, bacterial extracellular products from *B. cepacia* or *P. aeruginosa* may also act directly on neutrophils either by inducing end stage activation with the release of granule contents or by priming responses to other immunostimulatory agents. Colonization with both *P. aeruginosa* and *B. cepacia* is associated with high bacterial counts in sputum, typically 10^9 CFU/ml, suggesting that high concentrations of LPS shed from both viable and nonviable bacteria will also be present. As LPS molecules from a number of bacterial species have been recognized as neutrophil-priming agents (1, 21, 24, 25, 32), we investigated the interaction of *B. cepacia* LPS with neutrophils. Our results indicate the potent priming activity of *B. cepacia* LPS and confirm the proinflammatory capabilities of this unusual and challenging pathogen.

MATERIALS AND METHODS

Materials and equipment. Bacterial strains used in this study are from the collection held in the Cystic Fibrosis Laboratory, Department of Medical Microbiology, Edinburgh University, and are described in Table 1. Strains were stored at −70°C in 5% skim milk (Oxoid, Basingstoke, United Kingdom) and were recovered onto nutrient agar (Oxoid) before use. Fluorescence-activated cell sorter lysing solution and CellFIX were supplied by Becton Dickinson UK

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TABLE 1. Bacterial strains used in this study

Strain	Comment	Reference(s)
B. cepacia		
J2315[a]	CF, genomovar III[b]	13,[c] 33
C1504	CF	37
J2540	Environment, genomovar I	7, 37
J2552	Environment, genomovar I	7, 33
J2505	Non-CF clinical, ATCC 17762	37
P. aeruginosa		
PAO1	Non-CF clinical isolate	29, 37
J1385	Nonmucoid, CF	17,[d] 41
C1250	Mucoid, CF	37
E. coli O18K−		31

[a] Representative strain of the transcontinental ET12 lineage (23).
[b] Based on polyacrylamide gel electrophoresis of whole-cell proteins and DNA-DNA hybridization of selected *B. cepacia* strains.
[c] Identified as CF5610 in this reference.
[d] Pyocin type 1/b (4, 8, b) s.

Ltd., Oxford, United Kingdom. Monoclonal antibodies (mouse anti-human CD11b and mouse isotype control antibody) conjugated with fluorescein isothiocyanate were obtained from Serotec, Oxford, United Kingdom. Dihydrorhodamine (DHR; Cambridge Biosciences, Cambridge, United Kingdom) was stored at −20°C as a 10-mg/ml stock solution in dimethyl sulfoxide and was diluted to a 200-μg/ml solution in 20% (vol/vol) dimethyl sulfoxide in Hanks balanced salt solution (HBSS) with 0.1% (wt/vol) glucose (mHBSS) immediately prior to use. All other reagents were provided by Sigma Chemical Corporation (Poole, United Kingdom). Flow cytometry was carried out on a Becton Dickinson FACSort, and results were analyzed by using CellQuest 1.2.2 (Becton Dickinson).

LPS extraction. Bacteria were grown for 48 h in an orbital incubator (140 rpm) at 37°C in nutrient broth no. 2 (Oxoid) containing 0.5% yeast extract (Difco Laboratories, Detroit, Mich.). LPS was extracted by a modification of the aqueous phenol method of Westphal and Luderitz (44) as described by Hancock and Poxton (19). LPS samples were resuspended in pyrogen-free water at a concentration of 2 mg/ml. Samples were sonicated at an amplitude of 10 μm for three to eight 30-s pulses to ensure adequate solubilization. Protein concentration was determined by using the Coomassie brilliant blue protein assay of Bradford (5), with bovine serum albumin as the standard. For all LPS samples, protein content was less than 0.5% of the total dry weight of LPS. LPS characterization was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the buffer system of Laemmli (28), and silver staining was done by a modification of the method of Tsai and Frasch (42) as described by Hancock and Poxton (19). Samples from *Escherichia coli* O18K−, *P. aeruginosa* PAO1, and *B. cepacia* J2540, J2552, and J2505 were found to be smooth in character, while samples from *P. aeruginosa* J1385 and C1250 and *B. cepacia* J2315 and C1504 were rough in character. All samples were stored at −20°C.

Preparation of plasma-free whole blood cells. Fresh heparinized blood was collected from healthy volunteers. As we have observed low levels of anti-*B. cepacia* LPS antibodies in healthy individuals (22a), plasma was removed from blood samples to prevent any opsonic neutrophil responses. Blood was transferred to Eppendorf tubes and spun at 6,500 rpm for 30 s in a Micro Centaur microcentrifuge (MSE, Loughborough, United Kingdom). Following the removal of plasma, the cell pellet was washed four times in HBSS. Finally, the cell pellet was resuspended in a volume of mHBSS equivalent to the initial volume of plasma removed. The resultant washed blood was kept on ice until use. All experiments were initiated within 1 h of blood collection. Polymorphonuclear cell viability was determined by trypan blue exclusion and was found to be greater than 95%. Comparison of washed whole blood neutrophils with untreated blood showed no differences in size, granularity, or baseline CD11b expression, indicating that little neutrophil activation had occurred during plasma removal.

Detection of surface CD11b following neutrophil exposure to LPS. All experiments were carried out with 100-μl aliquots of washed blood in 5-ml polystyrene tubes (Greiner, Stonehouse, Gloucestershire, United Kingdom), to which was added an equal volume of mHBSS or LPS diluted in mHBSS. The tubes were incubated for the desired time period at 37°C with gentle shaking. Reactions were stopped by transferring the sample tubes onto ice. After the addition of anti-CD11b-fluorescein isothiocyanate (5 μl) or control antibody (5 μl) to each sample, the tubes were mixed and incubated for 20 min on ice. Then erythrocytes were lysed by the addition of 2 ml of lysing solution for 30 min. Neutrophils were pelleted by centrifugation at 500 × *g*, washed once in phosphate-buffered saline and resuspended in 500 μl of CellFIX. Samples were stored at 4°C until flow cytometric analysis on the same day as the experiment. Neutrophils were gated on forward and side scatter characteristics by using CellQuest 1.2.2 software. CD11b expression was determined as the mean channel fluorescence (MCF) of the neutrophil population, using the green channel (FL1). As considerable interdonor variation in the magnitude of neutrophil responses was noted, MCF values were standardized by expression as a percentage of the MCF of neutrophils treated with buffer alone. Each complete experiment was conducted at least four times, using blood from a different donor on each occasion.

Intracellular H_2O_2 production following neutrophil priming and stimulation. All experiments were carried out with an initial aliquot of 100 μl of washed blood. Where required, samples were preincubated with mHBSS or LPS at the desired concentration at 37°C. Samples were then stimulated with mHBSS or fMet-Leu-Phe (FMLP; 5×10^{-7} M) for 15 min at 37°C in the presence of 30 mM DHR (4, 43). Reactions were stopped by transferring the sample tubes onto ice, erythrocytes were lysed by the addition of 2 ml of lysing solution for 30 min, and the remaining neutrophils were processed as described above. As H_2O_2 oxidizes DHR to fluorescent rhodamine, intracellular H_2O_2 production was determined as the MCF of the neutrophil population, using the red channel (FL2) (43). Results were analyzed by using CellQuest and expressed as the MCF of the total neutrophil population. Each complete experiment was conducted at least four times, using blood from a different donor on each occasion.

Statistical analysis. Statistical analysis was carried out by using Student's *t* test to compare the means of two populations. For comparison of *B. cepacia* and *P. aeruginosa* populations, data were transformed by conversion to logarithms, and analysis of variance was performed.

RESULTS

Induction of CD11b on neutrophils stimulated with LPS from representative strains. Neutrophils were exposed to increasing concentrations of LPS from representative strains of *B. cepacia*, *P. aeruginosa*, and *E. coli* for a 2-h period at 37°C. Both *E. coli* and *B. cepacia* LPS increased CD11b expression on neutrophils, although responses to *E. coli* LPS could be demonstrated at a lower concentration (1 ng/ml) than for *B. cepacia* LPS (10 ng/ml). The greatest effect was seen with *E. coli* LPS at 100 ng/ml, where CD11b expression reached 2.2 times levels on neutrophils incubated with buffer alone (Fig. 1). At all LPS concentrations, CD11b levels induced by *E. coli* tended to be greater than those induced by *B. cepacia*. However, for both organisms, raised CD11b levels above baseline became statistically significant only at concentrations of 100 ng/ml and above ($P < 0.02$). LPS from a nonmucoid CF strain of *P. aeruginosa*, J1385, had no effect on neutrophil CD11b expression at all concentrations tested.

Preliminary time course experiments indicated that at a low concentration (1 ng/ml), *B. cepacia* LPS was associated with a late rise in neutrophil CD11b expression, occurring after 3 h of incubation. By contrast, higher LPS concentrations induced a rise in CD11b expression to maximal levels in less than 2 h of incubation (data not shown). Subsequent experiments compar-

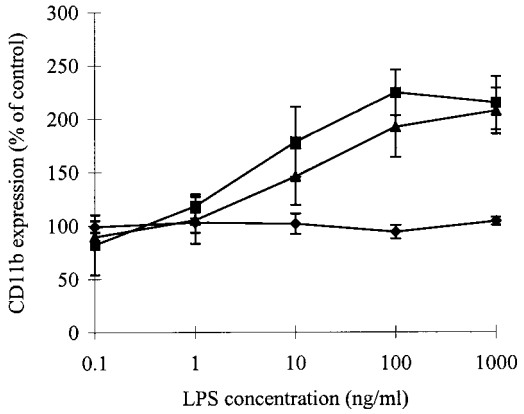

FIG. 1. Induction of CD11b on neutrophils following stimulation with various concentrations of LPS from *E. coli* O18K− (■), *B. cepacia* J2315 (▲), and *P. aeruginosa* J1385 (◆). Results are expressed as a percentage of the MCF of neutrophils treated with buffer alone for 2 h (mean of four experiments ± standard error of the mean).

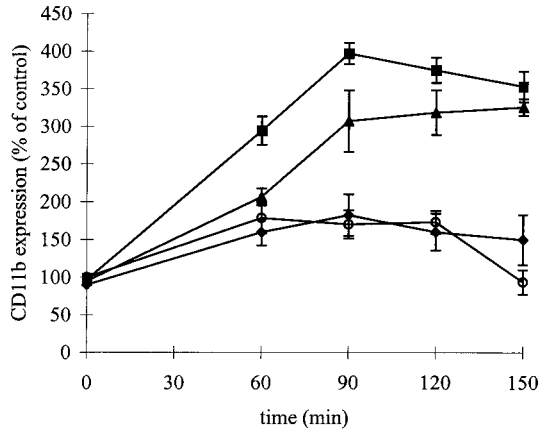

FIG. 2. Time course of induction of CD11b on neutrophils following stimulation with buffer alone (○) or LPS (100 ng/ml) from *E. coli* O18K− (■), *B. cepacia* J2315 (▲), and *P. aeruginosa* J1385 (◆) at 37°C. Results are expressed as a percentage of the MCF of neutrophils treated with buffer alone at time zero (mean of four experiments ± standard error of the mean).

ing LPS from *B. cepacia*, *P. aeruginosa*, and *E. coli* were carried out with an LPS concentration of 100 ng/ml (Fig. 2). Incubation with buffer alone was associated with a slight elevation in CD11b expression between 60 and 120 min, returning to baseline levels by 150 min. *E. coli* LPS was found to induce statistically significant levels of CD11b by 60 min ($P < 0.01$), peaking at 90 min ($P < 0.001$) with levels 2.3 times greater than those on neutrophils treated with buffer alone. Responses to *B. cepacia* LPS were slower to develop, rising to 1.8 times the levels on neutrophils treated with buffer alone by 90 min and reaching statistical significance after 120 min ($P < 0.01$). Incubation with *P. aeruginosa* LPS had little effect on CD11b expression over the entire incubation period.

Priming of FMLP-induced respiratory burst activity by LPS from *B. cepacia* and *E. coli*. DHR was used to detect the presence of intracellular H_2O_2 as a measure of neutrophil respiratory burst activity. Unstimulated neutrophils and unprimed neutrophils stimulated with FMLP fell within the R1 region (Fig. 3A) on the basis of size and granularity. Stimulation of LPS-primed neutrophils with FMLP was associated with changes in the forward and side scatter characteristics of a subpopulation of neutrophils (Fig. 3B and C, R2). By contrast, neither LPS priming alone nor stimulation of unprimed neutrophils with FMLP was associated with a shift in neutrophils from the R1 to the R2 population (Fig. 3A). In all instances, MCF for R2 populations was greater than MCF for R1 populations (Fig. 3D to F). However, as the patterns of change in MCF within each individual experiment were identical for both R1 and R2 neutrophils, respiratory burst activity was generally expressed as the MCF of the total neutrophil population (R1 plus R2).

Both *E. coli* O18K− and *B. cepacia* J2315 LPS primed for responses to FMLP in a dose-dependent manner. By contrast, *P. aeruginosa* J1385 LPS had no priming effect on neutrophils. Priming effects of *E. coli* LPS could be detected at lower concentrations (1 ng/ml) than for *B. cepacia* LPS (10 ng/ml) and were greater in magnitude at all concentrations tested (data not shown). Subsequent time course experiments were carried out with 100 ng of LPS per ml as standard. Neutrophils from individual donors varied considerably in both the magnitude and timing of priming responses (Fig. 4), but several general trends could be observed. None of the LPS preparations induced a respiratory burst in the absence of stimulation with FMLP (data not shown). Preincubation with buffer alone was associated with little or no priming of responses to FMLP. However, both *E. coli* LPS and *B. cepacia* LPS were potent priming agents of a respiratory burst response, with priming occurring after as little as 30 min of incubation. In assays using neutrophils from three of four donors, priming responses occurred more rapidly and displayed greater peak activity following stimulation with *E. coli* LPS rather than *B. cepacia* LPS (Fig. 4A to C). However, for one individual, little difference was observed between *E. coli* and *B. cepacia* LPS in both the speed and magnitude of priming responses (Fig. 4D).

In selected experiments, R1 and R2 populations were analyzed individually with respect to both MCF and the number of neutrophils in each population. In all instances, MCF activity was greatest within the R2 population, although considerable overlap in results for individual neutrophils occurred (Fig. 3D to F). Priming with both *E. coli* and *B. cepacia* LPS increased FMLP-induced respiratory burst responses in both R1 and R2 populations in a dose-dependent manner. Furthermore, LPS was associated with a shift of neutrophils from the R1 to the R2 population on stimulation with FMLP, so that the increase in MCF within R2 correlated in a linear fashion with an increase in the percentage of total neutrophils within the R2 population ($r^2 = 0.94$), while an increase of MCF within R1 correlated with a decrease in the percentage of neutrophils within the R1 population ($r^2 = 0.94$). Thus, LPS priming appeared to have a dual effect by first increasing the magnitude of responses in both R1 and R2 neutrophils and second shifting neutrophils into the highly responsive R2 population.

Neutrophil activation by LPS from a range of *B. cepacia* and *P. aeruginosa* strains. LPS from representative strains of *B. cepacia* and *P. aeruginosa* (Table 1) were investigated for the ability to induce CD11b and to prime FMLP-induced respiratory burst responses. LPS from *E. coli* O18K− was included as a positive control and induced maximal CD11b expression at 2.7 times the levels induced by buffer alone (Fig. 5). LPS from all *B. cepacia* strains, except the environmental strain J2540, induced levels of CD11b expression comparable to those induced by *E. coli* LPS. Furthermore, LPS from all *B. cepacia* strains, including J2540, induced CD11b levels which were higher than those induced by all *P. aeruginosa* LPS preparations. Of the *P. aeruginosa* strains tested, only LPS from strain PAO1, the classic laboratory strain, increased CD11b expression above that of unprimed neutrophils, while LPS from both a nonmucoid (J1385) and a mucoid (C1250) CF strain had no effect. Direct comparison of individual *P. aeruginosa* with *B. cepacia* strains by Student's *t* test found a significant difference ($P < 0.05$) for all combinations except PAO1 and J2540. Finally, analysis of variance between mean results for *P. aeruginosa* and *B. cepacia* strains indicated a significant difference between these populations for CD11b induction ($P < 0.001$).

Similar trends were observed for the priming effect of LPS on an FMLP-induced respiratory burst (Fig. 6). LPS from *B. cepacia* J2552 and *E. coli* O18K− primed intracellular H_2O_2 production to the greatest degree, with an approximately 10-fold increase in responses to FMLP following incubation with both LPS compared to neutrophils stimulated with buffer alone. LPS from all *B. cepacia* strains primed responses to levels greater than for all *P. aeruginosa* strains tested, and these observations were significant for all combinations of strains except PAO1 and J2540 (Student's *t* test, $P < 0.05$). Although slight neutrophil-priming effects were observed for LPS preparations from both *P. aeruginosa* PAO1 and *P. aeruginosa* C1250, these results were not statistically significant. LPS from *P. aeruginosa* J1385 had almost no effect on FMLP-induced respiratory burst activity compared to buffer controls. Again,

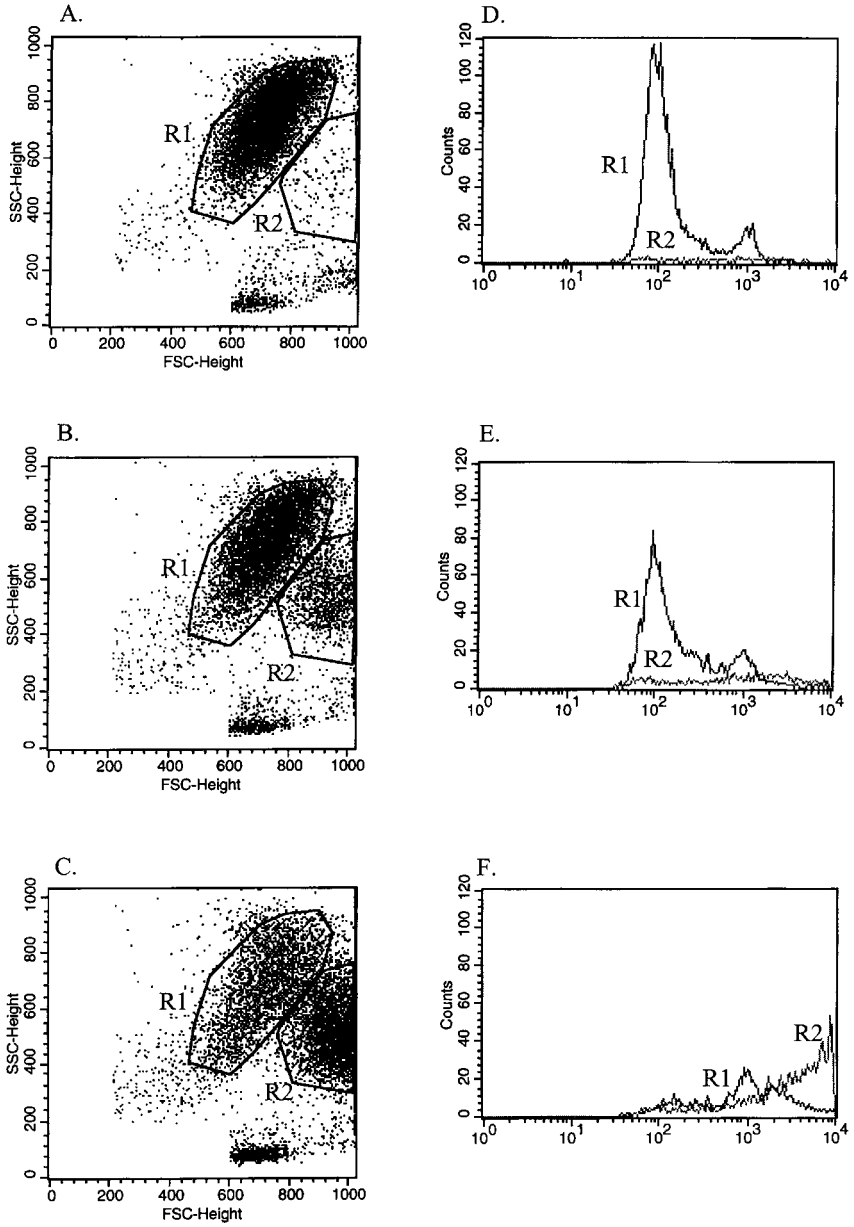

FIG. 3. Size, granularity, and intracellular H_2O_2 production of neutrophils stimulated with FMLP following preincubation with *E. coli* LPS. (A to C) In the dot plots of side versus forward scatter, neutrophils are gated as two populations, R1 and R2. (D to F) Histograms showing fluorescence of neutrophils in populations R1 and R2 in the presence of DHR. Neutrophils were preincubated for 90 min with buffer alone (A and D) or LPS at 1 (B and E) or 100 (C and F) ng/ml.

analysis of variance confirmed a significant difference between LPS from the *P. aeruginosa* and *B. cepacia* populations tested ($P < 0.001$).

DISCUSSION

Neutrophil activation in response to bacterial colonization has been implicated in the pathogenesis of CF lung disease by initiating and sustaining a cycle of increasing lung damage and bacterial colonization (9, 26). In this study, we investigated the role of *B. cepacia* LPS in two aspects of neutrophil activation: first, the up-regulation of surface CD11b; and second, the priming of neutrophil respiratory burst responses. We used flow cytometry to analyze neutrophils within a whole blood cell population from which plasma had been removed. Comparison of neutrophil size, granularity, and CD11b expression indicated that this procedure did not activate neutrophils to any significant degree.

CD11b associates with CD18 to form the β_2 integrin complement receptor 3 (CR3), which is expressed on the cell surface of granulocytes and macrophages/monocytes and is involved in numerous neutrophil functions, including adhesion, transmigration, phagocytosis, and activation (10). Increased surface expression of CR3 is a prerequisite for neutrophil transmigration from the pulmonary circulation to the alveolar spaces; thus, up-regulation is seen early in neutrophil activation processes (2, 38). In the present study, we have demonstrated that LPS from both clinical and environmental strains of *B. cepacia* induced a marked increase in neutrophil CR3 expression. By contrast, LPS from three representative *P.*

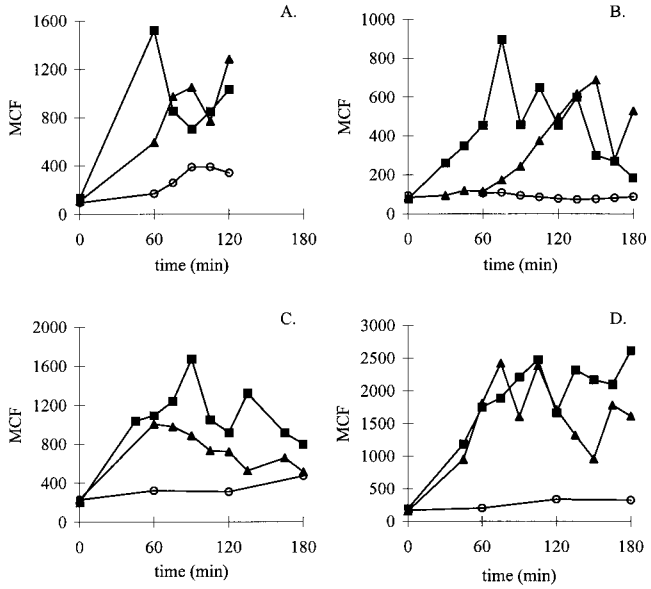

FIG. 4. Priming of FMLP-induced neutrophil respiratory burst responses by preincubation with buffer alone (○) or LPS (100 ng/ml) from *E. coli* O18K− (■) or *B. cepacia* J2315 (▲) for up to 3 h. Charts show results from studies on four individual donors. Intracellular H_2O_2 production is expressed as the MCF of the total neutrophil population stimulated with FMLP in the presence of DHR.

aeruginosa strains had little effect on CR3 expression. These results are consistent with those of Shaw et al. (37), who first reported that *B. cepacia* LPS induced a TNF-α response from circulating blood monocytes which was ninefold greater than that induced by *P. aeruginosa* LPS. TNF-α was detectable between 2.5 and 4.5 h after the addition of *B. cepacia* LPS, and levels peaked at approximately 3.5 h. As the assays in the present study were carried out on a whole blood cell population, it is possible that the increase in CR3 expression was due to secondary stimulation of neutrophils following the release of cytokines from the monocyte population. Indeed, at low LPS concentrations, an increase in neutrophil CD11b expression occurred after 3 h, possibly through stimulation by monocyte-derived TNF-α. However, the earlier timing of responses to

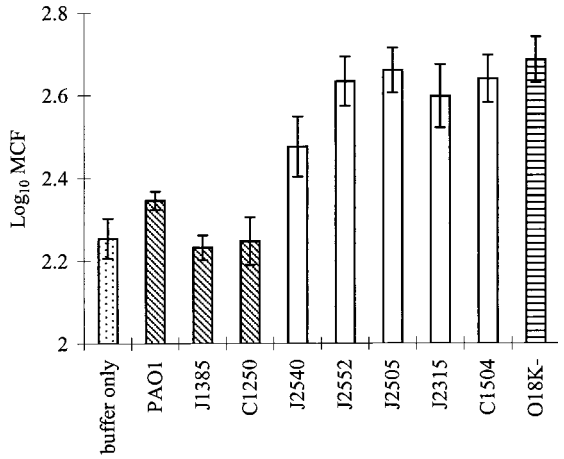

FIG. 5. Induction of CD11b on neutrophils following 90 min of incubation at 37°C with buffer alone (▨) or LPS (100 ng/ml) from strains of *P. aeruginosa* (▧), *B. cepacia* (□), and *E. coli* (▤). Results are shown as the $\log_{10}$ MCF of the neutrophil population (mean of six experiments ± standard error of the mean).

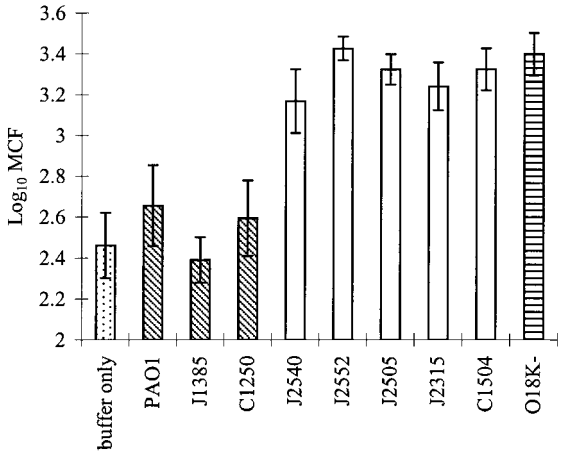

FIG. 6. Priming of FMLP-induced intracellular H_2O_2 production in neutrophils following 90 min of incubation at 37°C with buffer alone (▨) or LPS (100 ng/ml) from strains of *P. aeruginosa* (▧), *B. cepacia* (□), and *E. coli* (▤). Results are shown as the $\log_{10}$ MCF of the total neutrophil population in the presence of DHR (mean of five experiments ± standard error of the mean).

higher concentrations of LPS (<2 h) suggests a direct effect of LPS on neutrophils.

Respiratory burst activity occurs late in neutrophil activation processes and may be increased by prior exposure of neutrophils to priming agents, including LPS (1, 18, 21, 24, 25, 32). The bacterially derived peptide FMLP triggers respiratory burst activity in primed neutrophils. In the present study, little or no respiratory burst response was observed in unprimed neutrophils stimulated with FMLP. This is in agreement with the observations of previous investigators (8, 45) and confirms the low activation state of neutrophils in the whole blood cell preparations used. *E. coli* LPS has been demonstrated to prime FMLP-induced respiratory burst responses in neutrophils (24). Comparison of *B. cepacia* and *P. aeruginosa* LPS with *E. coli* LPS in the present study indicates that while *B. cepacia* LPS molecules were potent neutrophil-priming agents, little priming activity was induced by *P. aeruginosa* LPS. Of the *B. cepacia* strains tested, only J2540, an environmental strain, failed to induce priming significantly above the levels of all of the *P. aeruginosa* strains tested. In time course experiments using LPS from *B. cepacia* J2315 and *E. coli* O18K−, priming was observed after as little as 30 and 45 min, respectively, suggesting that neutrophil-priming responses were not secondary to LPS-induced cytokine release from circulating monocytes. LPS from two *P. aeruginosa* strains, PAO1, a well-characterized laboratory strain, and C1250, a mucoid strain isolated from a CF patient, primed neutrophils to a low degree. However, LPS from a nonmucoid CF strain, J1385, had no priming effect. Interestingly in a study of LPS extracted from five CF strains of *P. aeruginosa*, Kharazmi et al. (25) observed similar variation in neutrophil priming, with LPS from two mucoid strains showing the greatest overall activity.

Our observation of a high degree of correlation between mean CR3 surface expression and mean FMLP-induced respiratory burst activity agrees with recently published studies (8). Unlike increased CR3 expression, however, priming responses were not uniform throughout the neutrophil population, and a subpopulation of highly responsive neutrophils was identified by changes in size (forward scatter) and granularity (side scatter) on stimulation with FMLP (Fig. 3, R2). These results correspond to those of Yee and Christou (45), who observed that the FMLP-induced elevation of intracellular Ca^{2+} levels

in neutrophils primed with LPS was due to raised levels in a subpopulation of responsive cells. In the present study, highly responsive cells were virtually absent in unprimed populations but appeared in samples primed with LPS from both *B. cepacia* and *E. coli*. The observed changes in forward and side scatter for R2 neutrophils may simply reflect the activation of these cells following FMLP stimulation. However, since respiratory burst responses also increased among R1 neutrophils, and since a considerable overlap in individual responses for neutrophils in R1 and R2 populations was observed, it seems more probable that LPS is associated with a significant phenotypic change in R2 neutrophils prior to stimulation with FMLP. The nature of such a phenotypic change remains unclear but could involve changes in receptor expression. However, comparison of histogram distributions of CD11b expression and FMLP-induced intracellular H_2O_2 production indicated that the highly responsive subpopulation of neutrophils could not be identified on the basis of increased CR3 expression. FMLP receptors were not measured in the present study; however, previous investigators have found that both CR3 and FMLP receptors are up-regulated on LPS-treated neutrophils in a unimodal fashion, with no evidence of neutrophil subpopulations (45), suggesting that increased receptor expression is not the mechanism underlying the highly responsive phenotype.

The data presented here indicate that *B. cepacia* LPS may be an important virulence determinant in the development of inflammation in response to *B. cepacia* infection. The demonstration of neutrophil-priming activity even in the absence of plasma is particularly relevant to the CF lung, where concentrations of plasma factors are likely to be low, due both to poor diffusion into bronchiectatic airways and to the presence of high concentrations of proteolytic enzymes (9). The necrotizing pneumonitis and bacteremia which are characteristic of cepacia syndrome are unique to *B. cepacia* infection and have never been described in CF infection caused by *P. aeruginosa*. The up-regulation of CR3 expression in neutrophils exposed to *B. cepacia* LPS may be involved in the increased recruitment of neutrophils to the lung. At the same time, *B. cepacia* LPS may prime neutrophil responses to increase the release of tissue-damaging enzymes and reactive oxygen species from activated neutrophils. It is tempting to speculate that cepacia syndrome develops whenever the burden of activated neutrophils within the lung becomes too great for overstretched regulatory mechanisms, increasing the rate of inflammatory damage and permitting the bacteremic spread of *B. cepacia* beyond the lung parenchyma.

Environmental *B. cepacia* strains are currently being developed as biological control agents, both in the control of fungal plant pathogens and in the bioremediation of contaminated landfill sites (3, 12, 20, 22, 27, 36). Identification of strains which may represent a human hazard is therefore of paramount importance if *B. cepacia* is to be released on a large scale, particularly in agricultural programs. Recently, taxonomic analyses have revealed that bacterial strains currently identified as *B. cepacia* consist of at least four distinct species or "genomovars" (16, 35). Although numbers were small, investigation of a panel of CF, non-CF clinical, and non-CF environmental isolates suggested that strains belonging to genomovars II and III were associated with colonization and, in some cases, transmissibility in CF, while strains belonging to genomovar III were associated with severe disease and cepacia syndrome in CF patients (16). By contrast, most environmental isolates, including the *B. cepacia* phytopathogenic type strain ATCC 25416, belonged to genomovar I, which is less common in CF patients. A survey of Belgian CF patients identified genomovars II, III, and IV among *B. cepacia*-colonized patients but no genomovar I isolates (35). Comparison of a genomovar III strain (J2315) with two genomovar I strains (J2540 and J2552) in the present study has shown, however, the inflammatory potential of LPS from strains of both genomovars. Thus, the rarity of genomovar I isolates in CF may reflect the poor colonizing ability of these strains rather than any intrinsic lack of pathogenicity. As it is currently impossible to predict the effect of increased exposure on the risk of a CF patient acquiring a genomovar I strain, we believe that the use of environmental *B. cepacia* strains in large-scale agricultural release programs must be carefully reviewed and monitored.

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REFERENCES

1. **Aida, Y., K. Kusomoto, K. Nakatomi, H. Takada, M. J. Pabst, and K. Maeda.** 1995. An analogue of lipid A and LPS from *Rhodobacter sphaeroides* inhibits neutrophil responses to LPS by blocking receptor recognition of LPS and by depleting LPS-binding protein in plasma. J. Leukocyte Biol. **58:**675–682.
2. **Berger, M., J. O'Shea, A. S. Cross, T. M. Folks, T. M. Chused, E. J. Brown, and M. J. Frank.** 1984. Human neutrophils increase expression of C3bi as well as C3b receptors upon activation. J. Clin. Invest. **74:**1566–1571.
3. **Bhat, M. A., M. Tsuda, K. Horiike, M. Nozaki, C. S. Vaidyanathan, and T. Nakazawa.** 1994. Identification and characterization of a new plasmid carrying genes for degradation of 2,4-dichlorophenoxyacetate from *Pseudomonas cepacia* CSV90. Appl. Environ. Microbiol. **60:**307–312.
4. **Bjerknes, R., and D. Aarskog.** 1995. Priming of human polymorphonuclear neutrophilic leukocytes by insulin-like growth factor 1: increased phagocytic capacity, complement receptor expression, degranulation, and oxidative burst. J. Clin. Endocrinol. Metab. **80:**1948–1955.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brown, R. K., and F. J. Kelly.** 1994. Role of free radicals in the pathogenesis of cystic fibrosis. Thorax **49:**738–742.
7. **Butler, S. L., C. J. Doherty, J. E. Hughes, J. W. Nelson, and J. R. W. Govan.** 1995. *Burkholderia cepacia* and cystic fibrosis: do natural environments present a potential hazard? J. Clin. Microbiol. **33:**1001–1004.
8. **Condliffe, A. M., E. R. Chilvers, C. Haslett, and I. Dransfield.** 1996. Priming differentially regulates neutrophil adhesion molecule expression/function. Immunology **89:**105–111.
9. **Döring, G.** 1994. The role of neutrophil elastase in chronic inflammation. Am. J. Respir. Crit. Care Med. **150:**S114–S117.
10. **Edwards, S. W.** 1995. Cell signalling by integrins and immunoglobulin receptors in primed neutrophils. Trends Biol. Sci. **20:**362–367.
11. **Elborn, J. S., M. Dodd, J. Maddison, L. E. Nixon, J. Nelson, J. Govan, A. K. Webb, and D. Shale.** 1994. Clinical and inflammatory responses in CF patients infected with *Pseudomonas aeruginosa* and *Pseudomonas cepacia*. Pediatr. Pulmonol. Suppl. **10:**287.
12. **Fridlender, M., J. Inbar, and I. Chet.** 1993. Biological control of soilborne plant pathogens by a β-1,3-glucanase-producing *Pseudomonas cepacia*. Soil Biol. Biochem. **25:**1211–1221.
13. **Govan, J. R. W., P. H. Brown, J. Maddison, C. J. Doherty, J. W. Nelson, M. Dodd, A. P. Greening, and A. K. Webb.** 1993. Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. Lancet **342:**15–19.
14. **Govan, J. R. W., and V. Deretic.** 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. **60:**539–574.
15. **Govan, J. R. W., and S. Glass.** 1990. The microbiology and therapy of cystic fibrosis lung infections. Rev. Med. Microbiol. **1:**19–28.
16. **Govan, J. R. W., J. E. Hughes, and P. Vandamme.** 1996. *Burkholderia cepacia*: medical, taxonomic and ecological issues. J. Med. Microbiol. **45:**395–407.
17. **Govan, J. R. W., and J. W. Nelson.** 1992. Microbiology of lung infection in cystic fibrosis. Br. Med. Bull. **48:**912–930.
18. **Hallett, M. B., and D. Lloyds.** 1995. Neutrophil priming: the cellular signals that say 'amber' but not 'green.' Immunol. Today **16:**264–268.
19. **Hancock, I. C., and I. R. Poxton.** 1988. Bacterial cell surface techniques. John Wiley and Sons, Chichester, England.
20. **Havel, J., and W. Reineke.** 1993. Degradation of Aroclor 1221 in soil by a hybrid pseudomonad. FEMS Microbiol. Lett. **108:**211–218.
21. **Heiman, D. F., M. E. Astiz, E. C. Rackow, D. Rhein, Y. B. Kim, and M. H.**

Weil. 1990. Monophosphoryl lipid A inhibits neutrophil priming by lipopolysaccharide. J. Lab. Clin. Med. **116:**237–241.
22. **Homma, Y., Z. Sato, F. Hirayama, K. Konno, H. Shirahama, and T. Suzui.** 1989. Production of antibiotics by *Pseudomonas cepacia* as an agent for biological control of soilborne plant pathogens. Soil Biol. Biochem. **21:**723–728.
22a. **Hughes, J.** Personal observation.
23. **Johnson, W. M., S. D. Tyler, and K. R. Rozee.** 1994. Linkage analysis of geographic and clinical clusters in *Pseudomonas cepacia* infections by multilocus enzyme electrophoresis and ribotyping. J. Clin. Microbiol. **32:**924–930.
24. **Karlsson, A., M. Markfjall, N. Stromberg, and C. Dahlgren.** 1995. *Escherichia coli*-induced activation of neutrophil NADPH-oxidase: lipopolysaccharide and formylated peptides act synergistically to induce release of reactive oxygen metabolites. Infect. Immun. **63:**4606–4612.
25. **Kharazmi, A., A. Fomsgaard, R. S. Conrad, C. Galanos, and N. Hoiby.** 1991. Relationship between chemical composition and biological function of *Pseudomonas aeruginosa* lipopolysaccharide: effect on human neutrophil chemotaxis and oxidative burst. J. Leukocyte Biol. **49:**15–20.
26. **Konstan, M. W., K. A. Hilliard, T. M. Norvell, and M. Berger.** 1994. Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. Am. J. Respir. Crit. Care. Med. **150:**448–454.
27. **Krumme, M. L., K. N. Timmis, and D. F. Dwyer.** 1993. Degradation of trichloroethylene by *Pseudomonas cepacia* G4 and the constitutive mutant strain G4 5223 PR1 in aquifer microcosms. Appl. Environ. Microbiol. **59:** 2746–2749.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
29. **Liao, X., I. Charlebois, C. Quellet, M. J. Morency, K. Dewar, J. Lightfoot, J. Foster, R. Siehnel, H. Schweizer, J. S. Lam, R. E. Hancock, and R. C. Levesque.** 1996. Physical mapping of 32 genetic markers on the *Pseudomonas aeruginosa* PAO1 chromosome. Microbiology **142:**79–86.
30. **Mahenthiralingam, E., D. A. Simpson, and D. P. Speert.** 1997. Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. J. Clin. Microbiol. **35:**808–816.
31. **Nelson, D., T. E. S. Delahooke, and I. R. Poxton.** 1993. Influence of subinhibitory levels of antibiotics on expression of *Escherichia coli* lipopolysaccharide and binding of anti-lipopolysaccharide monoclonal antibodies. J. Med. Microbiol. **39:**100–106.
32. **Nielsen, H., S. Birkholz, L. P. Andersen, and A. P. Moran.** 1994. Neutrophil activation by *Helicobacter pylori* lipopolysaccharides. J. Infect. Dis. **170:**135–139.
33. **Palfreyman, R. W., M. L. Watson, C. Eden, and A. W. Smith.** 1997. Induction of biologically active interleukin-8 from lung epithelial cells by *Burkholderia* (*Pseudomonas*) *cepacia* products. Infect. Immun. **65:**617–622.
34. **Pitt, T. L., M. E. Kaufmann, P. S. Patel, L. C. A. Benge, S. Gaskin, and D. M. Livermore.** 1996. Type characterisation and antibiotic susceptibility of *Burkholderia* (*Pseudomonas*) *cepacia* isolates from patients with cystic fibrosis in the United Kingdom and the Republic of Ireland. J. Med. Microbiol. **44:** 203–210.
35. **Revets, H., P. Vandamme, A. Van Zeebroeck, K. De Boeck, M. J. Struelens, J. Verhaegen, J. P. Ursi, G. Verschraegen, H. Franckx, A. Malfroot, I. Dab, and S. Lauwers.** 1996. *Burkholderia* (*Pseudomonas*) *cepacia* and cystic fibrosis: the epidemiology in Belgium. Acta Clin. Belg. **51:**222–230.
36. **Rosales, A. M., L. Thomashow, R. J. Cook, and T. W. Mew.** 1995. Isolation and identification of antifungal metabolites produced by rice-associated antagonistic *Pseudomonas* spp. Phytopathology **85:**1028–1032.
37. **Shaw, D., I. R. Poxton, and J. R. W. Govan.** 1995. Biological activity of *Burkholderia* (*Pseudomonas*) *cepacia* lipopolysaccharide. FEMS Immunol. Med. Microbiol. **11:**99–106.
38. **Smith, C. W., S. D. Marlin, R. Rothlein, C. Toman, and D. C. Anderson.** 1989. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. J. Clin. Invest. **83:**2008–2017.
39. **Sun, L., R.-Z. Jiang, S. Steinbach, A. Holmes, C. Campanelli, J. Forstner, Y. Tan, M. Riley, and R. Goldstein.** 1996. The emergence of a highly transmissible lineage of *cbl*$^+$ *Pseudomonas* (*Burkholderia*) *cepacia* causing CF center epidemics in North America and Britain. Nat. Med. **1:**661–666.
40. **Sylvester, F. A., U. S. Sajjan, and J. F. Forstner.** 1996. *Burkholderia* (basonym *Pseudomonas*) *cepacia* binding to lipid receptors. Infect. Immun. **64:**1420–1425.
41. **Tatnell, P. J., N. J. Russell, J. R. W. Govan, and P. Gacesa.** 1996. Colonization of cystic fibrosis patients by non-mucoid *Pseudomonas aeruginosa*—characterisation of the alginate from mucoid variants. Biochem. Soc. Trans. **24:**406S.
42. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Anal. Biochem. **119:**115–119.
43. **van Pelt, L. J., R. van Zwieten, R. S. Weening, D. Roos, A. J. Verhoeven, and B. G. J. M. Bolscher.** 1996. Limitations on the use of dihydrorhodamine 123 for flow cytometric analysis of the neutrophil respiratory burst. J. Immunol. Methods **191:**187–196.
44. **Westphal, O., and O. Luderitz.** 1954. Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien. Angew. Chem. **66:**407–417.
45. **Yee, J., and N. V. Christou.** 1993. Neutrophil priming by lipopolysaccharide involves heterogeneity in calcium-mediated signal transduction. J. Immunol. **150:**1988–1997.

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