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Lipopolysaccharide (LPS) from *Burkholderia cepacia* Is More Active than LPS from *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* in Stimulating Tumor Necrosis Factor Alpha from Human Monocytes

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Whole cells and lipopolysaccharides (LPSs) extracted from *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Escherichia coli* were compared in their ability to stimulate tumor necrosis factor alpha (TNF-α) from the human monocyte cell line MonoMac-6. *B. cepacia* LPS, on a weight-for-weight basis, was found to have TNF-α-inducing activity similar to that of LPS from *E. coli*, which was approximately four- and eightfold greater than the activity of LPSs from *P. aeruginosa* and *S. maltophilia*, respectively. The LPS-stimulated TNF-α production from monocytes was found to be CD14 dependent. These results suggest that *B. cepacia* LPS might play a role in the pathogenesis of inflammatory lung disease in cystic fibrosis, and in some patients it might be responsible, at least in part, for the sepsis-like cepacia syndrome.

In recent years, *Burkholderia cepacia* infection in cystic fibrosis (CF) patients has become a clinical issue of increasing concern. *B. cepacia* is highly transmissible from person to person (4, 9) and intrinsically resistant to many antibiotics (3, 10). Once acquired, *B. cepacia* infection is rarely eradicated from the lungs of CF patients. In addition, a proportion of patients who acquire *B. cepacia* infection develop a rapidly fatal pneumonia and sepsis with a high mortality rate, the so-called cepacia syndrome (6, 8). However, the pathogenic mechanisms involved in this syndrome remain unclear. Indeed, the nature of the virulence factors that contribute to the pathogenicity of *B. cepacia* in CF remains largely unknown. Due to its potent inflammatory activity and association with sepsis, lipopolysaccharide (LPS) might be an important virulence factor in *B. cepacia* infections. Elevated tumor necrosis factor alpha (TNF-α) levels have been implicated in both systemic and local inflammations (7) and may play a role in cepacia syndrome (5). In this report, we studied the activity of *B. cepacia* whole cells as well as purified LPS in stimulating human monocytes to release TNF-α and compared it with the activities of other gram-negative bacteria that also colonize the lungs of CF patients, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. Patients carrying these organisms may, over time, suffer a chronic deterioration of lung function, but there are no reports that acquisition of these organisms is followed by the acute pulmonary deterioration sometimes seen in *B. cepacia*-infected patients. *Escherichia coli* was included in this study since the LPS from this organism is often reported to represent the most biologically active form of LPS (11), and it is used as a reference standard for the measurement of LPS activity by the *Limulus* assay.

The human monocytic cell line MonoMac-6 (MM6) was obtained from the German collection of microorganisms and cell cultures (Deutsche Sammlung von Mikroorganismen),

Braunschweig, Germany. In this study, we used 13 *B. cepacia* clinical CF isolates (from 10 of which LPS was purified), 7 *B. cepacia* environmental isolates (from 4 of which LPS was purified), 8 *S. maltophilia* isolates (from all 8 of which LPS was purified), and 7 *P. aeruginosa* isolates (from all 7 of which LPS was purified). *E. coli* O111:B4 LPS was purchased from Sigma Chemical Co., Poole, United Kingdom, and LPS was also purified from *E. coli* NCTC 10418. LPSs were purified by the phenol-water extraction method (2) and characterized by silver staining on a sodium dodecyl sulfate–14% polyacrylamide gel. Coomassie blue staining of the gels did not reveal any visible protein bands in the purified LPSs. The activity of different doses of LPS to produce TNF-α was established by stimulating MM6 cells with LPS concentrations ranging from 0.1 to 1000 ng/ml. However, at an LPS concentration above 500 ng/ml, the linearity of the TNF-α response decreased, and thus 500 ng of LPS per ml was used as the optimum concentration for this study. MM6 cells suspended at 10^6/ml of RPMI 1640 medium containing 10% fetal calf serum (Gibco) and 5 ng of phorbol myristate acetate per ml (13) were stimulated with 500 ng of LPS or 10^6 CFU of whole bacteria (all bacterial isolates were grown under identical conditions). MM6 incubated with phorbol myristate acetate only (without LPS) did not produce any significant amounts of TNF-α. The cells were incubated overnight, and the supernatants were collected after centrifugation at $3,000 \times g$ for 3 min followed by filtration through a 0.2-μm-pore-size syringe filter (Acrodisc; Gelman Sciences, Ann Arbor, Mich.). Supernatants were stored at −80°C until assayed. The TNF-α enzyme-linked immunosorbent assay (ELISA) was performed with monoclonal anti-human TNF-α antibody and biotinylated anti-human TNF-α antibody (R & D, Minneapolis, Minn.) in accordance with the manufacturer's instructions.

Statistical significance was calculated by the nonparametric Mann-Whitney test by using Minitab release 10 software. The role of CD14 in the LPS-stimulated TNF-α production was investigated by using monoclonal anti-human CD14 antibodies MY4 (Coulter Corporation, Miami, Fla.) and UCHM-1 (Sigma). The monoclonal anti-human CD7 antibody 3A1 (Sigma) was used as an isotype control antibody for MY4 (immuno-

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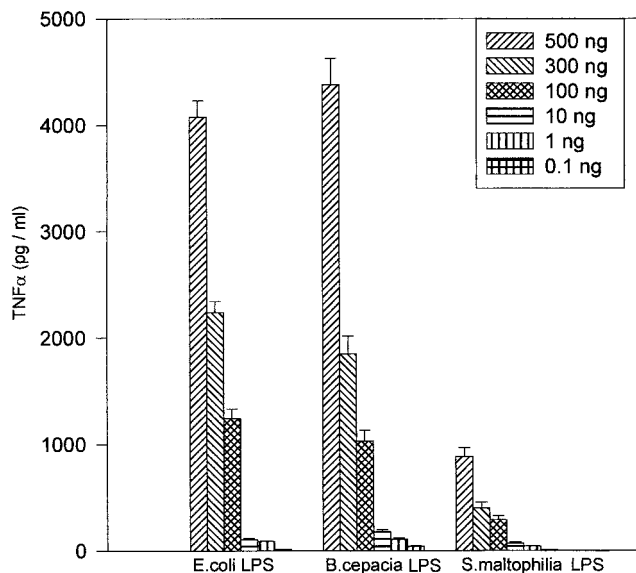
FIG. 1. Dose response of LPS-induced TNF-α production from stimulated MM6 cells. MM6 cells (10^6/ml) were incubated with 0.1 to 500 ng of LPS per ml, and TNF-α production was measured by an ELISA. The TNF-α produced was proportional to the LPS concentration up to an LPS dose of 500 ng/ml. Bars represent the mean (+ standard error) of four separate experiments.
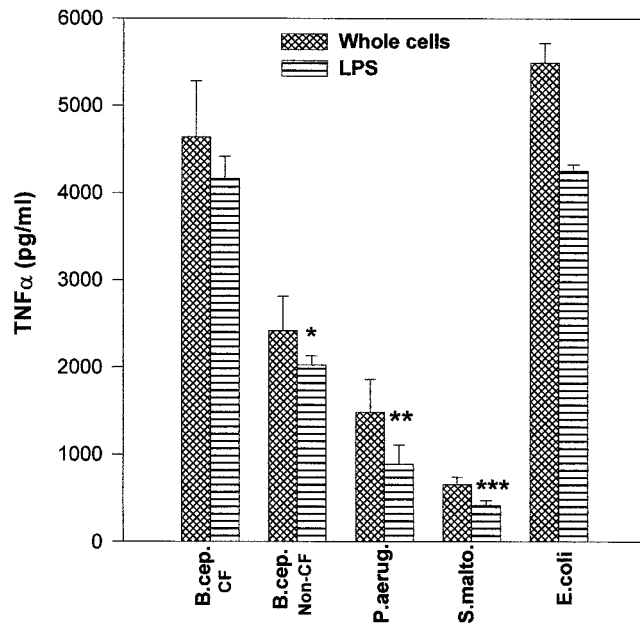

FIG. 2. TNF-α production by MM6 cells stimulated by whole bacteria and the extracted LPS. The bars show the mean (+ standard error) of TNF-α production in response to the different LPS types (number of different isolates = 4 to 10 in each group) from four separate experiments. *, $P = 0.021$; **, $P = 0.0054$; ***, $P = 0.00012$ (all versus results for B. cepacia [B.cep.] CF isolates). P.aerug, P. aeruginosa; S.malto, S. maltophila.

globulin G2b). Five microliters (2.5 μg) of monoclonal antibody was incubated with 10^6 MM6 cells at room temperature for 30 min, and the cells were then washed with RPMI 1640 medium to remove unbound antibody. The MM6 cells were then stimulated with 500 ng of LPS. The TNF-α produced was measured by an ELISA as described above.

Figure 1 shows the TNF-α production in MM6 cells stimulated with different doses of LPS. All of the LPS preparations used gave a dose response for TNF-α production, although there was variation in the total TNF-α stimulated by each type of LPS. A 500-ng/ml dose of LPS was found to be optimal for each LPS type for TNF-α production. Therefore, the different LPS types were compared at this dose. The ELISA results (Fig. 2) showed that B. cepacia LPS from CF patients induced a concentration of TNF-α similar to that induced by E. coli LPS (mean TNF-α concentration of 4,000 pg/ml). On the other hand, B. cepacia LPS from environmental isolates had half the activity (2,000 pg/ml) of that from CF isolates (Fig. 2). Moreover, LPS from B. cepacia CF isolates had fourfold the activity of P. aeruginosa LPS (1,000 pg/ml) and eightfold the activity of S. maltophilia LPS (500 pg/ml). TNF-α stimulated by the corresponding whole bacterial cells showed a similar pattern to that produced by the extracted LPS (Fig. 2). If LPS-mediated macrophage stimulation plays a role in the development of inflammatory lung pathology, the differences in the TNF-α stimulatory activity between LPSs from B. cepacia, P. aeruginosa, and S. maltophilia may in part explain the different pathogenic sequelae observed with lung infections due to these organisms in CF patients. S. maltophilia, in particular, is similar to B. cepacia in many aspects, such as high patient-to-patient infection rate, resistance to most antibiotics, and no significant exotoxin production. However, unlike the case with B. cepacia, despite numerous investigations, there have yet to be reports of acute pulmonary inflammation occurring following S. maltophilia acquisition (1).

To study the role of CD14 in B. cepacia LPS-mediated TNF-α stimulation, monoclonal anti-human CD14 antibodies

were used to block CD14 binding sites and consequently inhibit TNF-α production. When monoclonal antibodies were added, TNF-α production was significantly inhibited following B. cepacia LPS stimulation in comparison with TNF-α production in control cells or those cells incubated with the isotype
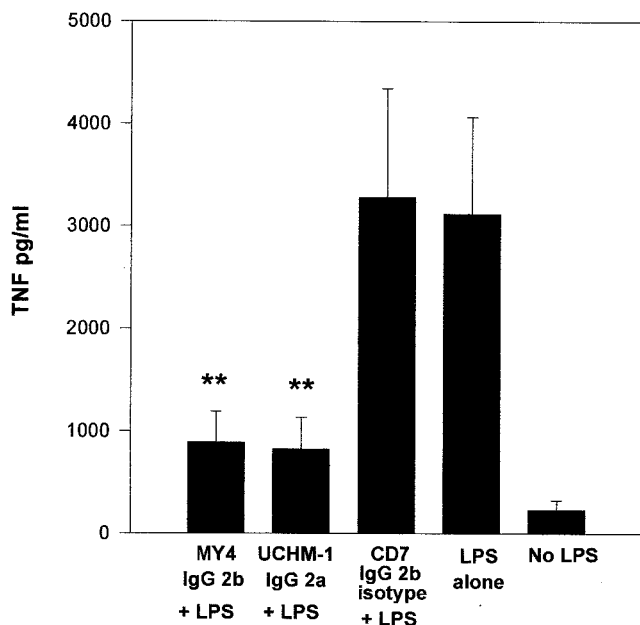

FIG. 3. Production of TNF-α from MM6 cells stimulated with B. cepacia LPS (500 ng/ml) and in the presence of the anti-CD14 monoclonal antibodies MY4 and UCHM-1 and the MY4 isotype control anti-CD7. **, $P = 0.0004$ versus results for LPS alone. IgG, immunoglobulin G.

antibody (Fig. 3). We found similar results with *S. maltophilia* and *P. aeruginosa*. Contrary to a previous report by Shaw et al. (12), our investigations suggest that *B. cepacia* LPS-mediated TNF-α stimulation is almost completely mediated by CD14.

We conclude that *B. cepacia* LPS is a potent immunostimulator and that chronic colonization may lead to LPS shedding and TNF-α production, resulting in tissue damage and lung inflammation.

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