Evidence for Lipopolysaccharide as the Predominant Proinflammatory Mediator in Supernatants of Antibiotic-Treated Bacteria

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Lipopolysaccharide (LPS), purified from gram-negative bacteria, is well known to induce proinflammatory responses in monocytes and macrophages, and release of LPS from the microbial surface has been suggested to be an important initiating event in the sepsis syndrome. However, numerous studies have documented that a variety of constituents present in the outer cell membrane of gram-negative bacteria have the capacity to activate cells of the immune system. Given that the majority of immunotherapeutic approaches designed to intervene in gram-negative sepsis have to date targeted the LPS molecule, it would be of value to assess the relative proinflammatory properties of LPS and other gram-negative structures. Experiments were therefore undertaken to assess stimulation of human monocytes by components released from Escherichia coli following bacteriolysis by the cell wall-active antibiotic ceftazidime. As assessed by both induction of procoagulant activity and release of tumor necrosis factor, bacterial culture supernatants contain significant proinflammatory activity. When culture supernatants are fractionated via either velocity sedimentation in sucrose gradients or isopycnic density gradient ultracentrifugation in cesium chloride, the predominant monocyte-stimulating activity is identified in LPS-containing fractions. Further, such activity can be readily abrogated by the addition of polymyxin B. These results provide support for the hypothesis that LPS may be responsible for the majority of the proinflammatory activity released from E. coli following bacteriolysis in vitro.

The systemic inflammatory response syndrome has recently been defined as a disease stemming from an uncontrolled proinflammatory response of the host immune system. While a variety of initiating stimuli have been associated with this syndrome, gram-negative microorganisms are often suggested as one of the leading causes of this disease. Cells of the monocyte/macrophage lineage have been strongly implicated as one of the major cellular effectors (11, 25). There exists good correlative experimental evidence to suggest that lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative microbes, may play an important role in the induction of the sepsis syndrome (22, 25). Perhaps one of the stronger lines of evidence to support this concept are experiments in which administration of purified LPS to human volunteers or experimental animals faithfully reproduces many of the pathophysiological events classically seen in patients with gram-negative sepsis (5, 34).

It has been established, however, that in addition to LPS, a variety of bacterial cell wall and/or outer membrane constituents, from both gram-negative and gram-positive bacteria, are also able to stimulate proinflammatory responses in cells of the immune system. In this respect, lipoprotein (10), Helicobacter pylori water extract (19), peptidoglycan (15), lipid A-associated protein (21, 30), and lipoteichoic acids (18) have all clearly been demonstrated to manifest proinflammatory activity in vitro culture with human or murine immunologically relevant cells. Additionally, it has been recognized that treatment of bacteria with cell wall-active antibiotics can potentiate the release of microbial constituents, including LPS (7, 9, 17, 27), which have the capacity to induce tumor necrosis factor (TNF) in whole blood ex vivo (4) and to cause lethality when administered to mice in vivo (7). While it is reasonable to postulate that the presence of LPS in culture supernatants of gram-negative bacterial lysates would be sufficient to account for the observed biological activity, the potential presence of alternative bacterial constituents in addition to LPS, which might also manifest biological activity, would preclude absolute conclusions regarding the role of LPS. We have therefore designed studies to assess the relative contribution of LPS versus other bacterial components following bacteriolysis with a β-lactam antibiotic, ceftazidime, in the induction of proinflammatory responses in human monocytes in vitro. For these studies, Escherichia coli O111:B4 was cultured in vitro with bactericidal concentrations of ceftazidime, and culture supernatant materials were then fractionated by two physical-chemical procedures designed to separate protein from LPS. Individual fractions were then examined for their capacity both to induce procoagulant activity (PCA) and to stimulate production of TNF in cultures of freshly isolated human monocytes. Polymyxin B, a well-characterized inhibitor of LPS biological activity (23), was used to discriminate between LPS-dependent and LPS-independent biological activities. The results of these studies support the conclusion that LPS may be the dominant biologically active constituent present in gram-negative bacterial lysates.

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MATERIALS AND METHODS

Generation of E. coli O111:B4 bacterial lysates. Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) was
inoculated with *E. coli* O111:B4 (provided by List Biological Laboratories, Inc., Campbell, Calif.) from agar plates and incubated for 16 to 18 h at 37°C with orbital shaking at 200 rpm. Fresh 50-ml volumes of Trypticase soy broth were then inoculated with 0.2 ml of the 16- to 18-h cultures; aliquots were placed in 50-ml conical centrifuge tubes and incubated for 1.5 to 2 h at 37°C with shaking at 200 rpm until bacterial counts reached 3 × 10^5 to 4 × 10^5 CFU/ml. At that time, ceftazidime in Trypticase soy broth was added to a final concentration of 20 μg/ml (80 times the MIC) for 1.5 h to allow filamentation of the organisms, followed by 600 μg/ml (2,400 times the MIC) for 2.5 h to induce lysis of the bacteria. The cultures were then centrifuged at 3,000 rpm for 10 min at 5°C to remove particulate bacterial debris, and the culture supernatants were used for all subsequent experiments.

**Colitose determination.** LPS present in the lysates was quantitated by measurement of colitose (3,6-dideoxy-l-galactose), as described by Morrison and Leive (24).

**Isopycnic density gradient ultracentrifugation in cesium chloride.** Cesium chloride density gradient ultracentrifugation was carried out essentially as described by Duncan and Morrison (8). Briefly, 4.8 ml of culture supernatants was added to 2.8 g of cesium chloride (Sigma Chemical Company, St. Louis, Mo.), and the solution was centrifuged in a SW 55 Ti swinging-bucket rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 194,400 × g at the maximal radius for 60 h at 4°C in a Beckman L8-M ultracentrifuge. Fractions of approximately 200 μl were collected from the bottom of the gradient.

**Velocity sedimentation in sucrose gradients.** One milliliter of the culture supernatant of the bacterial lysates was overlaid on a sucrose step gradient formed with 0.8 ml each of 50, 40, 30, 20, and 10% (wt/vol) sucrose in water. Centrifugation was carried out at 194,400 × g at the maximal radius for 18 h at 4°C with an SW 55 Ti rotor in a Beckman L8-M ultracentrifuge. Fractions of approximately 200 μl were collected from the bottom of the gradient.

**ELISAs.** Enzyme-linked immunosorbent assays (ELISAs) were used to detect the presence of LPS. These assays were carried out using experimental conditions previously established in our laboratory to provide optimal specific binding of antibody to LPS with minimal nonspecific binding (12). In brief, Immulon 1 polystyrene microtiter plates (Fisher Scientific, St. Louis, Mo.) were treated with poly-l-lysine (average molecular weight, 80,000; Sigma Chemical Company) for 30 min at room temperature. The poly-l-lysine was then removed by “flicking” the plates, which were then coated with cesium chloride or sucrose gradient fractions containing antibiotic-treated bacterial supernatants (see above) as the antigen at a dilution in phosphate-buffered saline (PBS, pH 7.4) of 1:5 for cesium chloride gradient fractions and 1:250 for sucrose gradient fractions. In preliminary experiments, these dilutions were found to provide optimal concentrations for antigen binding preparatory to addition of various antibodies. The plates were then incubated for 1 h at 4°C and washed three times with PBS. Blocking of nonspecific binding sites was accomplished by washing with 0.05% Tween 20 (PBS-Tween), a mouse immunoglobulin G2a (IgG2a) monoclonal antibody (MAb 5B10; generously provided by Peter Daddona, Centocor, Inc., Malvern, Pa.) specific for the O antigen of LPS from *E. coli* O111:B4 was added (1 μg/ml) and incubated for 2 h at 37°C. Plates were again rinsed three times with PBS-Tween and developed with a peroxidase-conjugated polyclonal goat anti-mouse IgG (Sigma Chemical Company) diluted 1:2,000 for 30 min at 37°C. Bound peroxidase was detected by 3,3’-5,5’-tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) for approximately 3 to 5 min, followed by the addition of 1.0 M H₂PO₄. The A₄₅₀ was determined with a Titertek Multiskan plate reader (Flow Laboratories, McLean, Va.).

**Protein assays.** Protein concentrations were determined by a colorimetric technique, using protein assay reagent 23200 or 23236 (Pierce Chemical, Rockford, Ill.), following the manufacturer’s instructions.

**Trypsin digestion of sucrose gradient fractions.** For trypsin digestion of fractionated components from bacterial lysates, 50 μl of gradient fractions was combined with 50 μl of 5-mg/ml porcine trypsin (JRH Biosciences) in Hanks’ balanced salt solution (HBSS) without Ca²⁺ or Mg²⁺ (HBSS⁺; JRH Biosciences), placed in microcentrifuge tubes, and incubated at 37°C overnight. Control samples were treated identically except that trypsin was omitted from the incubation mixture. For a trypsin control, a 1:1 solution of 5-mg/ml porcine trypsin and HBSS⁺ was also incubated as above.

**Monocytes.** Monocytes were isolated from heparinized blood (10 U of heparin per ml of blood) obtained from normal human donors who gave informed consent. Histopaque-1077 (Sigma Chemical Company) was used essentially according to the supplier’s instructions. Twelve milliliters of Histopaque-1077 was overlaid with 10 to 12 ml of whole heparinized blood and centrifuged at 400 × g for 30 min at room temperature with no brake in a Beckman model TJ-6 tabletop centrifuge (Beckman Instruments, Inc.). Mononuclear cells collected from the resulting interface were washed three times in HBSS⁺, resuspended in RPMI 1640 containing 15% untreated autologous human serum, 4 mM L-glutamine (JRH Biosciences), 200 μg of penicillin per ml, 200 μg of streptomycin (JRH Biosciences) per ml, and 5 × 10⁻⁵ M 2-mercaptoethanol (Sigma Chemical Company), and plated in 96-well cell culture plates (Costar, Cambridge, Mass.) with 1.5 × 10³ cells per well in a final volume of 100 μl. After 1 h at 37°C in a humidified 5% CO₂ atmosphere, nonadherent cells were removed by three rinses with 100 μl of 37°C RPMI 1640.

**Monocyte stimulation.** Fractionated or crude bacterial lysate samples were purified by centrifugation (E. coli O111:B4; List Biological Laboratories, Inc.) and diluted in RPMI medium supplemented as described above with the substitution of heat-inactivated (56°C, 1 h) autologous serum in place of the untreated serum and added to monocytes. Final dilutions of the fractions ranged from 100-fold to 7.8 × 10⁴-fold (fivefold serial dilutions) for induction of PCA, while final fraction dilutions for stimulation of TNF secretion ranged from 40-fold to 4.0 × 10²-fold. Some experiments included 5 μg of polymyxin B (Pfizer-Roerig, New York, N.Y.) per ml. The stimulation phase, carried out at 37°C with a humidified 5% CO₂ atmosphere, lasted for 4 h for the induction of PCA and approximately 24 h for TNF production.

**PCA determination.** PCA was determined by the method of Schreinemakers and Zuckerman (31). Briefly, after the 4-h stimulation phase, monocytes were rinsed twice with 37°C HBSS without phenol red (JRH Biosciences; 100 μl per well per rinse). Each well then received 100 μl of a solution containing 1.0 U of factor VII as proplot T, a mixture of human coagulation factors II, VII, IX, and X (Baxter-Hyland Division, Glendale, Calif.), per ml, with 200 μg of the factor X synthetic substrate S-2222 (Kabi Pharmacia, Franklin, Ohio) per ml in medium 199 (Gibco/BRL, Grand Island, N.Y.). The plates were then briefly centrifuged at 400 × g in the Beckman TJ-6 centrifuge to disrupt any existing air bubbles, and then the A₄₀₅ was measured using the Titertek Multiskan or the A₄₁₀ was
measured with a Dynatech MR 5000 plate reader (Dynatech Laboratories, Inc., Chantilly, Va.). After 2 h at 37°C with a humidified 5% CO₂ environment, absorbance measurements were repeated. The time zero absorbance values were subtracted from the 2-h values, and the net absorbance values were used to determine expression of PCA. The dilution of each fraction required to give a 50% absorbance value was determined graphically. The reciprocal of this dilution was defined to be the number of units of activity in 100 μl of the individual fractions tested.

TNF bioassay. TNF bioactivity was measured by modifications of the method of Ruff and Gifford (26). In short, L-929 cells were plated in 96-well flat-bottom plates with 3.5 × 10⁴ cells in 100 μl of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Intergen, Purchase, N.Y.), 4.0 mM L-glutamine, 200 U of penicillin per ml, and 200 μg of streptomycin per ml. After approximately 22 h at 37°C in a humidified 5% CO₂ environment, the medium was removed and replaced with RPMI 1640 supplemented as before but containing 5.0 μg of dactinomycin (Merck Sharp and Dohme, West Point, Pa.) per ml. The cultures were returned to the humidified, 37°C, 5% CO₂ environment for 2 to 4 h, and serial twofold dilutions of monocyte culture supernatants were added. After approximately 12 h at 37°C with a humidified 5% CO₂ atmosphere, the viability of the L-929 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method of Denizot and Lang (6). A₅₇₀ measurements were made with a Dynatech MR5000 plate reader. One unit of TNF activity was defined to be the amount required to cause 50% killing of the dactinomycin-sensitized L-929 cells.

Statistical analysis. Paired t tests were performed with SigmaPlot 5.0.

RESULTS

Induction of human monocytic proinflammatory responses by bacterial lysates. As an initial assessment of the ability of bacterial lysates to induce an LPS-dependent proinflammatory response in monocytes, antibiotic-treated bacterial lysates were prepared as described in Materials and Methods and compared with purified LPS for their ability to induce monocyte responses. The concentration of LPS in bacterial lysates was determined by a colitose assay, and equivalent concentrations of LPS, either in the lysate or in a chemically purified form, were assessed for the ability to induce PCA on monocytes. As shown by the data in Fig. 1, the response of monocytes to LPS-containing bacterial lysates was superimposable with that of purified LPS in terms of threshold dose of stimulus required for activity as well as responsiveness to concentrations of LPS up to 0.5 ng/ml. Interestingly, monocyte responsiveness to purified LPS appeared to reach maximal levels near 0.5 ng of LPS per ml, while the response to the bacterial lysate continued to increase, reaching maximal stimulation with roughly 2 ng of LPS per ml. While there was a trend toward higher maximal PCA induction with bacterial lysate material than with purified LPS, the maximal response to these different preparations did not achieve statistical significance, as assessed by paired t tests. As also shown by the data in Fig. 1, the addition of polymyxin B, an agent known to bind to the lipid A moiety of LPS and inhibit the biological effects of LPS (23), resulted in an approximately 10-fold reduction in PCA-inducing activity in the bacterial lysate, suggesting that the majority of the stimulatory activity could be attributed to the presence of LPS.

Isopycnic fractionation of bacterial lysates. In order to assess the relative stimulatory activity of LPS and other bacterial constituents present in bacterial lysates, samples were fractionated by ultracentrifugation in cesium chloride, which segregates individual constituents on the basis of partial specific volume, a procedure which readily separates protein and LPS. Following centrifugation, individual gradient fractions were assayed for the presence of LPS via ELISA and for protein via the Bradford assay. As shown by the data in Fig. 2, the LPS fractions contained a relatively broad band in the middle of the gradient, with an approximate isopycnic density of 1.46 g/cm³. In contrast, proteins present in the bacterial lysates equilibrium band at a density of approximately 1.37 g/cm³. In control experiments, bacteria not subjected to antibiotic-induced bacteriolysis showed minimal release of LPS and protein (data not shown).

To assess the relative biological activity contained in the various fractions, fivefold serial dilutions were assayed for the capacity to induce PCA on freshly isolated human monocytes, and the dilution factor required to stimulate half-maximal activity was then calculated. The results of this analysis, shown in Fig. 2, indicate that the majority of the monocyte-stimulatory activity is detected in those fractions with LPS, suggesting that LPS may be the dominant proinflammatory mediator found in bacterial lysates. Further evidence in support of this conclusion is provided by experiments with polymyxin B. As also shown by the data in Fig. 2, polymyxin B essentially abrogated the proinflammatory activity found in the LPS-containing fractions.

Velocity sedimentation fractionation of bacterial lysates. The studies summarized above were done with an isopycnic density gradient technique to effect fractionation of bacterial
culture supernatant components; while this technique is effective in separating macromolecular constituents with differing partial specific volumes, it would not differentiate components having similar partial specific volumes. Velocity sedimentation, an alternative fractionation procedure which separates constituents based on mass, was therefore also carried out to fractionate bacterial lysates. As shown by the data in Fig. 3, velocity sedimentation also effectively achieved separation of LPS and protein present in the bacterial lysates, with LPS sedimenting as a broad peak toward the bottom of the gradient. As was demonstrated earlier in the cesium chloride fractionation experiments, PCA-inducing activity was detected in those fractions containing the majority of the detectable LPS. Further, as shown by the data in Fig. 3, induction of PCA present in the gradient fractions was abolished by the addition of polymyxin B.

Experiments were also carried out to assess whether results obtained with PCA would extend to other important mediators of the LPS-initiated inflammatory response, specifically, secretion of TNF. Representative fractions from the LPS-enriched bottom of the gradient (fractions 3 and 4), the protein-enriched middle of the gradient (fractions 14 and 16), and the top of the gradient (fractions 22 and 24) were analyzed for their capacity to stimulate TNF release from human monocytes. As with PCA induction, various dilutions of these fractions were assayed, and the reciprocal of the dilution required to yield 400 U of TNF was then determined. The results of this experiment are summarized in Table 1 and indicate that the LPS-enriched gradient fractions were highly potent in eliciting TNF production by monocytes, and the bioactivity in these fractions was efficiently neutralized by polymyxin B. Less, but nevertheless significant, TNF-inducing activity was detected in the remaining fractions tested, and the activity in these fractions was less effectively neutralized by polymyxin B. Since previous studies have shown that certain LPS-protein

| TABLE 1. TNF-stimulatory activity of sucrose gradient fractions of culture supernatants of ceftazidime-treated E. coli |
|-----------------|-----------------|-----------------|
| Fraction nos.   | Relative TNF-  | % Reduction     |
|                 | stimulatory activity<sub>a</sub> |                 |
|                 | −PB             | +PB             |
| 3 and 4         | 33,000          | <40             | >99             |
| 14 and 16       | 5,000           | 250             | 95              |
| 22 and 24       | 3,300           | 110             | 97              |

<sup>a</sup> Dilutions of gradient fractions were used to stimulate monocytes for 24 h in the presence and absence of polymyxin B (PB). The reciprocal of the dilution required to generate 400 U of TNF per ml was used to determine relative TNF-stimulatory activity.
complexes do not readily bind to polymyxin B (21), it was possible that the presence of such LPS-protein complexes prevented polymyxin B-mediated neutralization of LPS activity. Alternatively, non-LPS components contained in these fractions might directly contribute to induction of TNF. To distinguish between these possibilities, the representative fractions 13 and 15 were first treated with trypsin and then reassessed for TNF-inducing activity as described above. Trypsin digestion resulted in an approximately threefold increase in the activity of these fractions, and the increased TNF-inducing activity in the trypsin-treated fractions was essentially abrogated by polymyxin B (data not shown), suggesting that proteins present in untreated fractions were blocking neutralization of the LPS by polymyxin B rather than contributing directly to monocyte activation per se. The increased TNF induction could not be attributed to contaminating LPS in the trypsin or some activity of the trypsin itself, since identical concentrations of trypsin did not induce TNF production by the monocytes (data not shown).

**DISCUSSION**

We have assessed stimulation of monocytes, a cell recognized to be of importance in the sepsis syndrome (25), using components contained in gram-negative bacterial cell lysates. Two independent systems for fractionation of the supernatants of bacterial lysates, specifically, isopycnic fractionation and velocity sedimentation, effectively separated LPS constituents from the majority of the protein present in such lysates; further, in both systems, the fractions which contained the majority of the LPS also contained most of the monocyte-stimulatory activity. Inhibition studies with polymyxin B support the conclusion that a substantial component of PCA- and TNF-inducing activity found in lysate supernatants is likely to be LPS.

Given that several microbial constituents have been shown to induce proinflammatory responses in cells of the immune system, it is of interest that LPS was the dominant microbial constituent present in *E. coli* bacterial lysates to induce PCA or TNF in human monocyte cultures. It should be pointed out, however, that these studies do not preclude the presence of biologically active non-LPS constituents. In this respect, if non-LPS components of significantly lower relative activity than LPS were present, they would not have been noted relative to the potent stimulation by LPS, recognizably among the most potent microbial stimuli for monocyte activation. Additionally, it is possible that induction of PCA is relatively restricted in proinflammatory responses, and in this respect, the ability of various bacterial constituents to induce PCA has not been thoroughly determined. It is established that gram-positive bacteria and mycoplasma lipopolysaccharides can induce PCA (13, 20), but induction of PCA by non-LPS derivatives of gram-negative bacteria is a less explored area. Further, our studies have not addressed the presence of proinflammatory components which may be present on *E. coli* but are retained by the bacterial fragments following bacteriolysis, since such fragments were removed prior to analysis of bacterial lysates. The possibility remains, therefore, that LPS is the major gram-negative bacterial component released in sufficient quantities and with sufficient potency to induce significant PCA in monocyte cultures via this form of bacterial lysis.

Numerous investigators have demonstrated the release of endotoxin into the microbial environment by in vitro treatment of gram-negative bacteria with cell wall-active antibiotics, including but not limited to ceftazidime (2, 7, 9, 17, 33). Hayes and Orr (16) have reported that relatively low concentrations of ceftazidime induce filamentation of *E. coli*, and the studies of Jackson and Kropp (17) correlate this with release of LPS. The initial addition of a subbactericidal concentration of ceftazidime to cultures in the experiments presented here was designed to allow such filamentation to occur. Clinically, suboptimal levels of antibiotics might be expected at the initiation of antibiotic therapy, because of the limitations of pharmacokinetics, and possibly during therapy, when trough levels occur between doses of the antibiotic. Hayes and Orr (16) have also shown that higher concentrations of ceftazidime result in rapid lysis of *E. coli*, and similar findings obtain in the studies reported in this paper, where higher concentrations of ceftazidime caused bacterial lysis and release of proteins and LPS. Other investigators have suggested the in vivo presence of such an LPS-releasing effect of antibiotics (28, 32, 33), and our results indicate that this released LPS appears to be a potent, and predominant, stimulus of a monocyte proinflammatory response.

The significance of this release of LPS, however, within the overall complex series of pathophysiologic events occurring during sepsis, although queried by many investigators over the past several decades, has yet to be established unequivocally (1, 4, 14). Thus, while pathophysiologic changes following administration of antibiotics to patients with gram-negative infections have been reported in the medical literature by many investigators (3, 29), there is no convincing evidence for a direct correlation between antibiotic-induced LPS release and adverse outcome. What is clear is that LPS released from the microbial surface is more biologically active than an equivalent amount of microbe-associated LPS (17a). Since our data reported here suggest that LPS contained in bacterial lysates is among the dominant microbial constituents capable of eliciting monocyte responses, these results would nevertheless be in accord with therapeutic strategies designed to target the LPS molecule in the treatment of gram-negative sepsis.

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