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Release of Toll-Like Receptor-2-Activating Bacterial Lipoproteins in *Shigella flexneri* Culture Supernatants

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***Shigella* spp. cause dysentery, a severe form of bloody diarrhea. Apoptosis, or programmed cell death, is induced during *Shigella* infections and has been proposed to be a key event in the pathogenesis of dysentery. Here, we describe a novel cytotoxic activity in the sterile-culture supernatants of *Shigella flexneri*. An identical activity was identified in purified *S. flexneri* endotoxin, defined here as a mixture of lipopolysaccharide (LPS) and endotoxin-associated proteins (EP). Separation of endotoxin into EP and LPS revealed the activity to partition exclusively to the EP fraction. Biochemical characterization of *S. flexneri* EP and culture supernatants, including enzymatic deactivation, reverse-phase high-pressure liquid chromatography analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and a Toll-like receptor-2 (TLR2) activation assay, indicates that the cytotoxic component is a mixture of bacterial lipoproteins (BLP). We show that biologically active BLP are liberated into culture supernatants of actively growing *S. flexneri*. In addition, our data indicate that BLP, and not LPS, are the component of endotoxin of gram-negative organisms responsible for activating TLR2. The activation of apoptosis by BLP shed from *S. flexneri* is discussed as a novel aspect of the interaction of bacteria with the host.**

The gram-negative bacterium *Shigella* is the etiological agent of dysentery, a severe form of bloody diarrhea that causes approximately 1.1 million deaths annually (13). Dysentery is characterized by fever, painful abdominal cramps, and frequent stools containing blood and mucus. These symptoms result from invasion of the colonic mucosa by *Shigella* and the intense inflammatory response of the host to the bacteria (16).

The initial site of tissue invasion by *Shigella* occurs at epithelia overlying lymphoid follicles of the gut-associated lymphatic tissue (GALT) (22). After invasion, the bacteria localize exclusively to the follicular dome, within and directly below the epithelial layer (23). *Shigella flexneri* has been shown to induce apoptosis in macrophages in vitro, through a pathway dependent on the secreted virulence protein called invasion plasmid antigen B (IpaB). To kill macrophages, IpaB must be released directly into the cytoplasm of *S. flexneri*-infected cells (6). In vivo, however, the distribution of cell death induced by *S. flexneri* in the lymphoid follicle is not restricted to the location of the bacteria (40). These observations suggest that a second diffusible effector of cell death is generated during *S. flexneri* infections. We proposed that this toxin is produced either by *S. flexneri*-infected cells or by the bacteria themselves.

Here, using a macrophage cytotoxicity assay, we identify a novel cytotoxic activity in the sterile-culture supernatants of *S. flexneri*. Biochemical characterization of the cytotoxic activity, including enzymatic deactivation, high-pressure liquid chromatography (HPLC), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a Toll-like receptor-2 (TLR2) activation assay, indicates that it is a mixture of bacterial lipoproteins (BLP). BLP are made by all bacteria and are characterized by a unique N-terminal lipo-amino acid, *N*-acyl-*S*-diacylglyceryl cysteine (34). These molecules are capable of activating numerous host immunologic responses, as well as cell death. These cellular responses are mediated by the cell surface receptor, TLR2 (1, 5). BLP are localized only to the periplasmic leaflets of the inner and outer membranes in gram-negative bacteria (34). Given this topology, it is not clear whether BLP are available to engage TLR2 on host cells. Our results demonstrate that biologically active BLP are liberated from culture supernatants of *S. flexneri*.

MATERIALS AND METHODS

Reagents and cell culture. Synthetic lipopeptides $Pam_3CysSerLys_4$ (sBLP) and 47L, the negative-control compounds Pam_3Cys and 47, and *Escherichia coli* murein lipoprotein have been described (1, 25). Lipopeptide stocks were made at 1 mg/ml in endotoxin-free water with 0.05% human serum albumin (Grifols, Miami, Fla.). Silver staining and colloidal-gold staining were performed with the Bio-Rad Silver Stain kit and Bio-Rad Colloidal Gold Total Protein Stain (Bio-Rad Laboratories, Hercules, Calif.). All other reagents were from Sigma.

J774, THP-1, and 293 cell lines were maintained as previously described (1). 293 cells stably expressing human Toll-like receptor-2 (293TLR2), or human CD14 (293CD14), or both (293TLR2/CD14) were previously described (1, 36).

Preparation of culture supernatants of *S. flexneri* and *S. flexneri*-infected macrophages. Unless otherwise indicated, bacteria were grown in tryptic soy broth at 37°C with agitation. An overnight culture of *S. flexneri* (serotype V [37] or serotype 1A [ATCC 9199]) was subcultured at a dilution of 1:100 and grown for 2 h. The bacteria were collected by centrifugation, washed with serum-free RPMI 1640, and suspended to the appropriate concentration in serum-free RPMI 1640. J774 cells were infected at a multiplicity of infection of 100 as described previously (38) and incubated at 37°C for 2 to 3 h. The culture supernatants were collected, sterile filtered (0.2-μm-pore-size filter), and concentrated approximately 20-fold using a 10-kDa Centriprep concentrator (Amicon, Beverly, Mass.). Supernatants were stored at −20°C until used in cytotoxicity assays. Culture supernatants of bacteria only and uninfected macrophages were prepared similarly in parallel.

Cytotoxicity assays. Cytotoxicity assays were performed essentially as described previously (1). Cells were plated in triplicate wells of a 96-well plate and

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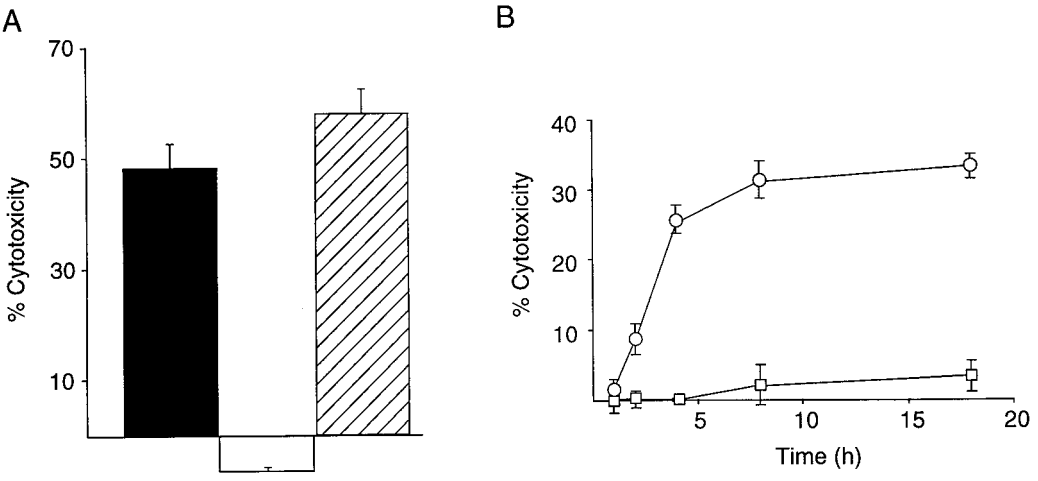

FIG. 1. Cytotoxic activity in *S. flexneri* culture supernatants and infected macrophages. (A) Culture supernatants of *S. flexneri* type 1A-infected J774 macrophages (black bar) and *S. flexneri* type 1A alone (striped bar) contain similar levels of cytotoxic activity. The culture supernatants of macrophages alone (white bar) were noncytotoxic. (B) Time course of the cytotoxic activity in commercial *S. flexneri* type 1A LPS preparations. THP-1 cells were incubated with 10 μg of *S. flexneri* type 1A PW-LPS (open squares) or TCA-endotoxin (open circles) per ml and assayed for cytotoxicity at the indicated time points. Percent cytotoxicity was determined by an LDH release assay.

allowed to adhere for 6 h, and then the components to be tested were added. Between 1×10^4 and 2.5×10^4 cells were used per well. Cytotoxicity was determined 3 to 6 h after the addition of test components by a lactate dehydrogenase (LDH) release assay (Cytotox96 kit; Promega, Madison, Wis.). Phorbol myristate acetate-stimulated THP-1 cells or, where indicated, cycloheximide-treated THP-1 cells were used as described previously (1). Cell culture supernatants were diluted to a final concentration of 3× with serum-free tissue culture media.

Preparation of TCA-endotoxin, EP, and PW-LPS. The preparation of trichloroacetic acid (TCA)-endotoxin was performed as previously described (27). The preparation of endotoxin-associated proteins (EP) and phenol-water (PW)-lipopolysaccharide (LPS) from TCA-endotoxin was performed as previously described (28).

Preparation of phenol extracts of bacterial supernatants. Forty milliliters of an overnight culture of *S. flexneri* type 1A was added to 4 liters of tryptic soy broth and grown to an optical density at 600 nm of 0.8. The bacteria were centrifuged, washed with serum-free RPMI 1640, resuspended in 5,500 ml of serum-free RPMI 1640, and grown for 2.5 h. The bacteria were removed by centrifugation, and the supernatant was sterile filtered and concentrated to 5.5 ml using an Amicon Series 8400 Stirred Cell Concentrator and a 10-kDa cutoff ultrafiltration membrane (Millipore, Bedford, Mass.). The concentrated supernatant was extracted with hot phenol as described above for the preparation of EP and PW-LPS from TCA-endotoxin. Ethanol precipitates of the phenol and aqueous phases were resuspended in 5.5 ml of endotoxin-free 1× phosphate-buffered saline (PBS).

RP-HPLC and mass spectrometry. Reverse-phase (RP)-HPLC was performed on a Beckman HPLC using a C_4 or diphenyl RP-HPLC column (Vydac, Hesperia, Calif.). All solvents were HPLC grade from Fisher Chemicals and were degassed for 10 min under vacuum prior to use. HPLC conditions are indicated in the legend to Fig. 4. Fractions were tested for cytotoxic activity by serial dilution directly into tissue culture media or by lyophilizing the fraction, resuspending in 1× PBS and diluting serially into tissue culture media. Both methods yielded similar results. Samples for mass spectrometry were lyophilized, resuspended in 80% acetonitrile (ACN)–0.1% formic acid, and analyzed by electrospray-ionization mass spectrometry at the William M. Keck Foundation Biotechnology Resource Laboratory, Yale University.

Measurement of reactive oxygen species production and NF-κB activation. Measurement of reactive oxygen species production by peripheral blood leukocytes by lucigenin-enhanced chemiluminescence and the NF-κB luciferase reporter gene assay were performed as previously described (1) or as indicated in the figure legends.

Extraction of cytotoxic activity from Tris-Tricine gels. Five hundred microliters of a 10-mg/ml solution of *S. flexneri* EP was incubated with 0.2 U of proteinase K (PK) agarose beads or an equivalent amount of uncoupled agarose beads as a control for 4 h at 37°C. The beads were removed by centrifugation, and the supernatant was collected, mixed with 2 volumes of Tris-Tricine SDS-PAGE sample buffer (Bio-Rad), and heated at 95°C for 5 min. Fifty microliters of each was loaded on nonadjacent lanes of a 10-well 16.5% Tris-Tricine Ready Gel (Bio-Rad). An additional gel was loaded identically, except that a peptide molecular weight (MW) marker (Bio-Rad) was included, and run in parallel. Individual lanes were isolated and cut into 11 5-mm sections. Individual gel sections were extracted with 200 μl of 25 mM octyl glucoside (Anatrace, Maumee, Ohio) for 5 min at 100°C as previously described (19). Supernatants were collected and tested for cytotoxic activity. The second gel containing the peptide MW marker was silver stained.

Purification of the cytotoxic activity from *S. flexneri* EP by SDS-PAGE and RP-HPLC for the TLR2 stimulation assay. A total of 2.5 mg of *S. flexneri* EP was mixed with 500 μl of Tris-Tricine SDS-PAGE sample buffer, heated for 5 min at 95°C, and resolved on a Bio-Rad 16.5% Tris-Tricine Preparative Ready Gel. The portion of the gel between 1.5 and 3 cm from the gel front was collected, minced with a clean razor blade, and extracted with 4 ml of 25 mM octyl glucoside for 10 min at 100°C. One milliliter of this extract was mixed with 4 ml of 62.5% ACN–0.125% trifluoroacetic acid and loaded onto a diphenyl RP-HPLC column (Vydac) and resolved under the following conditions. Buffer A consisted of 52.4% ACN and 0.1% TFA, buffer B consisted of 95% ACN and 0.1% TFA, and the flow rate was 1 ml/min. The gradient was as follows: 0 to 5 min, 100% buffer A; 5 to 15 min, 0 to 100% buffer B (linear gradient); 15 to 17 min, 100% buffer B; 17 to 19 min, 100 to 0% buffer B. Under these conditions, the octyl glucoside eluted in the flowthrough. One-minute fractions (1 ml each) were collected and assayed for cytotoxic activity on THP-1 cells. The activity completely eluted between min 8 and 9. Control gels loaded with an equal amount of Tris-Tricine SDS-PAGE sample buffer did not produce cytotoxic activity in this HPLC fraction.

RESULTS

The culture supernatants of *S. flexneri* contain a cytotoxic activity. As indicated in the introduction, *S. flexneri* induces a widespread pattern of cell death in vivo that does not completely colocalize with the distribution of intratissular bacteria (40). We proposed that a diffusible cytotoxin is active in *S. flexneri* infections. Since *S. flexneri* encounters resident tissue macrophages early in the pathogenesis of dysentery (39), we hypothesized that the diffusible, apoptosis-inducing factor may be produced by *S. flexneri*-infected macrophages. Alternatively, the cytotoxin may be produced by the bacteria themselves. Supernatant preparations from *S. flexneri* type 1A-infected macrophages and *S. flexneri* type 1A alone were found to contain similar levels of cytotoxic activity as assayed on phorbol myris-

tate acetate-stimulated THP-1 cells (Fig. 1A). The culture supernatants of uninfected macrophages were not cytotoxic. Supernatant preparations from macrophages infected with either a wild-type *S. flexneri* type V strain (M90T) or an isogenic avirulent derivative cured of the *Shigella* virulence plasmid (BS176) (37) were equally cytotoxic (data not shown), indicating that production of the cytotoxic activity is not dependent on the *Shigella* virulence plasmid (24). The cytotoxic activity was stable during proteinase K treatment and boiling for 10 min and did not pass through a 10,000-MW-cutoff filter, indicating that it is greater than 10 kDa or aggregated (data not shown).

LPS, like the cytotoxic activity described above, is heat stable, protease resistant, and released into bacterial culture supernatants (11). Polymyxin B (PB) is an amphipathic LPS binding molecule (17). Pretreatment of *S. flexneri* type 1A culture supernatants with PB-agarose beads depleted the cytotoxic activity (data not shown). These data suggested that the cytotoxin was either LPS (1), a factor associated with LPS (2), or a factor capable of independently binding to PB (3).

TCA-endotoxin, but not PW-LPS, is cytotoxic to THP-1 cells. Endotoxin of gram-negative organisms contains LPS, an outer membrane glycolipid with potent immunostimulatory properties, proteins, and phospholipids (8), and in composition resembles the bacterial outer membrane (11, 21). The proteins in endotoxin, EP, are a mixture of porins, BLP, and other outer membrane proteins (7). LPS can be isolated from smooth gram-negative enteric bacteria by either a TCA precipitation method, originally outlined by Boivin (27) (TCA-endotoxin), or a hot-phenol extraction method, described by Westphal and Jann (32) (PW-LPS). In the Boivin procedure, precipitation of bacteria with TCA releases complexes of LPS and EP that can be isolated by precipitation with cold ethanol. For clarity, we call this heterogeneous preparation TCA-endotoxin. In the Westphal hot-phenol extraction method, LPS partitions to the aqueous layer due to the hydrophilic nature of the core and O-antigen carbohydrate side chains. EP, however, are extracted into the phenol phase. Therefore, LPS isolated by the Westphal phenol extraction method is purer than preparations isolated by the Boivin procedure.

Commercial preparations of *S. flexneri* type 1A TCA-endotoxin, but not PW-LPS, induced cell death in THP-1 cells within 4 h of exposure (Fig. 1B). However, as little as 625 pg of PW-LPS per ml induced reactive oxygen species production in human peripheral blood leukocytes (data not shown), indicating that this preparation has potent biologic activity. PW-LPS was also capable of stimulating tumor necrosis factor alpha and pro-interleukin-1β production in murine peritoneal macrophages (data not shown).

The cytotoxic activity of TCA-endotoxin partitions to the organic phase in a hot-phenol extraction. TCA-endotoxin was prepared in our laboratory from *S. flexneri* type 1A by the Boivin procedure and fractionated into PW-LPS and EP by a Westphal hot-phenol extraction (28). Five micrograms of TCA-endotoxin per milliliter induced an appreciable cytotoxic response. However, only 50 ng of EP/ml was necessary to kill THP-1 cells (Fig. 2A), indicating that the cytotoxic activity is enriched in the EP fraction of TCA-endotoxin. In contrast, doses as high as 50 μg of PW-LPS per ml were noncytotoxic.

The compositions of TCA-endotoxin, PW-LPS, and EP preparations were analyzed by SDS-PAGE (Fig. 2B). Five micrograms of each preparation was resolved on duplicate gels. One gel was silver stained to reveal LPS, and the other was stained with colloidal gold to visualize protein (15). TCA-endotoxin contained both LPS (Fig. 2B, lane 1, silver stain) and proteins (Fig. 2B, lane 1, colloidal-gold stain). PW-LPS looked identical to TCA-endotoxin on the silver-stained gel (Fig. 2B, lane 2, silver stain) but did not contain proteins (Fig. 2B, lane 2, colloidal-gold stain). EP contained no detectable LPS (Fig. 2B, lane 3, silver stain) but was highly enriched for proteins (Fig. 2B, lane 3, colloidal-gold stain).

The cytotoxic activity in EP had the same properties as the cytotoxic activity identified in bacterial supernatants. The cytotoxic activity in EP bound to PB (Fig. 2C, lane 3). Surprisingly, almost all the proteins were specifically depleted from the EP preparation by PB (Fig. 2D, compare lanes 1, 2, and 3), suggesting that EP can interact with PB. Interestingly, although the cytotoxic activity in EP was insensitive to PK (Fig. 2C, lane 4), the protease treatment degraded all the proteins in EP (Fig. 2D, compare lanes 1, 2, and 4). The increased staining at the gel front in PK-treated EP (Fig. 2D, lane 4) likely represents degraded protein fragments running at the gel front. The cytotoxic activity in EP was also stable when boiled for 10 min (data not shown).

The phenol extraction method used to isolate EP from TCA-endotoxin was applied to *S. flexneri* culture supernatants. Ethanol precipitates of the phenol and aqueous phases were tested for cytotoxic activity on THP-1 cells (Fig. 2E). As with the *S. flexneri* EP, the cytotoxic activity in *S. flexneri* culture supernatants partitioned exclusively to the phenol phase. Phenol extractions of media alone performed in parallel as a control did not yield cytotoxic activity in either the phenol or aqueous phases (data not shown). Taken together, the data in Fig. 2 indicate that the cytotoxic activities in *S. flexneri* EP and in culture supernatants have similar properties.

BLP are cytotoxic to THP-1 cells. BLP are produced by all bacteria, including gram-negative and gram-positive bacteria, as well as spirochetes and *Mycoplasma* spp. (8). BLP are characterized by a unique, N-terminal lipo-amino acid, *N*-acyl-*S*-diacylglyceryl cysteine ($Acyl_3Cys$) (34), which activates host defense mechanisms (12, 26). It has recently been shown that BLP also induce apoptosis through TLR2 (1, 2). The immunomodulatory properties of BLP are stable during heat and protease treatment (20, 31, 33), since the N-terminal lipo-amino acid is unaffected by either treatment. In addition, BLP contain proteinaceous and lipid components and would be expected to be soluble in phenol. Therefore, it was hypothesized that BLP were the cytotoxic factors in *S. flexneri* EP and culture supernatants.

Synthetic BLP analogs consisting of a palmitylated version of *N*-acyl-*S*-diacylglyceryl cysteine (Pam_3Cys) and a few C-terminal amino acids can mimic the immunomodulatory effects of BLP (12). Interestingly, the lipo-amino acid Pam_3Cys alone is insufficient for immunostimulation (1). The synthetic lipopeptide 47L, which corresponds to the N terminus of the *Treponema pallidum* 47-kDa lipoprotein (25), and $Pam_3CysSerLys_4$ (sBLP) both induced cytotoxicity in THP-1 cells (Fig. 3). Pam_3Cys alone or an unlipidated 47L peptide did not induce cytotoxicity (Fig. 3).

RP-HPLC analysis of sBLP, EP, and the phenol extracts of *S. flexneri* culture supernatants. Phenol extracts of *S. flexneri* culture supernatants and a preparation of *S. flexneri* EP were

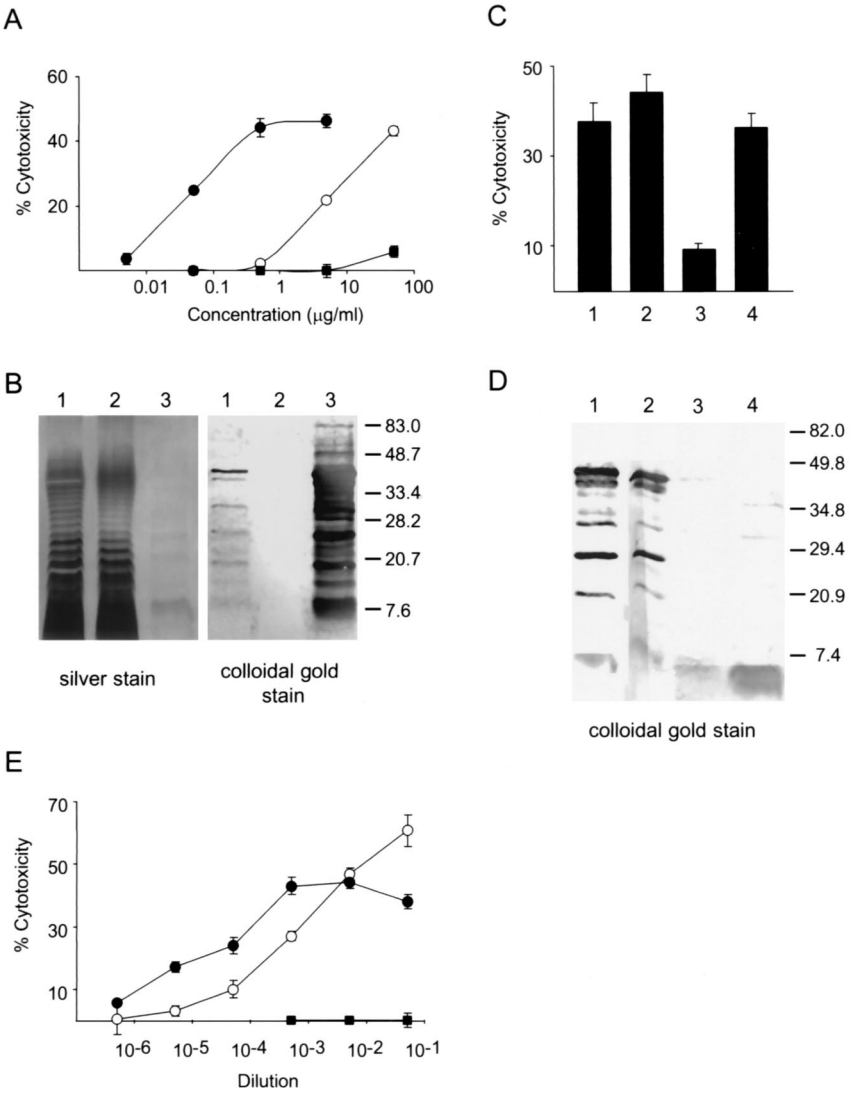

FIG. 2. The cytotoxic activity of TCA-endotoxin partitions to the phenol phase in a phenol-water extraction. (A) PW-LPS (closed squares) and EP (closed circles) were prepared from *S. flexneri* type 1A TCA-endotoxin (open circles). These preparations were tested for cytotoxic activity at the indicated concentrations on THP-1 cells. Percent cytotoxicity in panels A, C, and E was determined by an LDH release assay. (B) Silver and colloidal-gold protein stain of *S. flexneri* type 1A TCA-endotoxin (lane 1), PW-LPS (lane 2), and EP (lane 3) resolved by SDS-PAGE (15% polyacrylamide). On the silver-stained gel, note the characteristic LPS ladders in both TCA-endotoxin and PW-LPS, indicative of repeating O-antigen units. The colloidal-gold protein stain illustrates that the proteins in TCA-endotoxin partition exclusively to the phenol phase (EP). (C) The cytotoxic activity in EP is stable when subjected to PK treatment but is depleted by PB-coated agarose beads. A 1-mg/ml solution of EP was either mock treated (1) or incubated with 50 μl of uncoupled agarose beads (2), 50 μl of PB-agarose beads (3), or 0.2 U of PK-agarose beads (4) for 4 h at 37°C. The beads were removed by centrifugation, and supernatants were tested for cytotoxic activity at a dilution of 1:500. (D) SDS–15% PAGE of samples shown in panel C. The gel was stained with the colloidal-gold protein stain. (E) The cytotoxic activity in *S. flexneri* type 1A culture supernatants is extracted by phenol. *S. flexneri* type 1A culture supernatants were prepared, concentrated 1,000-fold, and extracted with hot phenol. Ethanol precipitates of the phenol (closed circles) and aqueous phases (closed squares) were resuspended in equivalent volumes of PBS and tested for cytotoxicity on THP-1 cells at the indicated dilutions. As a reference, unextracted 1,000-fold-concentrated *S. flexneri* type 1A culture supernatant was also tested (open circles).

loaded on a RP-HPLC column and resolved with a linear gradient of increasing ACN concentration. The cytotoxic activity in both *S. flexneri* culture supernatants and *S. flexneri* EP eluted at a high concentration of organic solvent (between 85 and 95% ACN) (Fig. 4A and B). This indicates that the toxin is extremely lipophilic. Equivalent fractions collected from a blank HPLC run did not contain cytotoxic activity (data not shown).

Identical RP-HPLC conditions were used to resolve the commercial preparation of sBLP. A major peak and a minor peak of cytotoxic activity were obtained (Fig. 4C, peaks a and b). Electrospray-ionization mass spectrometry identified the compound in the minor peak (peak a) as $Pam_2CysSerLys_4$ (molecular mass, 1,272.9 Da) and the compound in the major peak (peak b) as $Pam_3CysSerLys_4$ (molecular mass, 1,510.7 Da). This elution profile indicates that, as expected, the retention of lipopeptides on a C_4 RP-HPLC column is proportional to the hydrophobic character of the N-terminal lipo-amino acid. Interestingly, $Pam_3CysSerLys_4$ (the

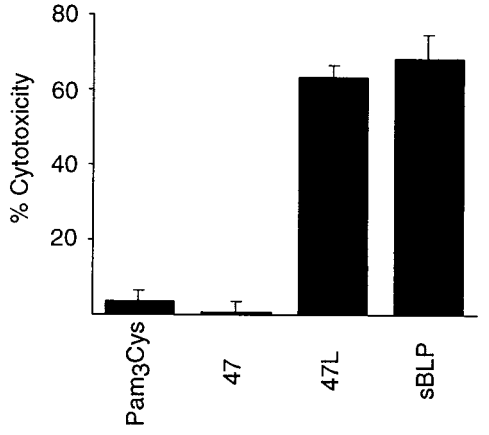

FIG. 3. BLP are cytotoxic to THP-1 cells. THP-1 cells were incubated with the lipopeptides sBLP or 47L at a final concentration of 10 nM and assayed for cytotoxicity. The negative-control compounds, 47 and Pam_3Cys, were tested at 1,000 nM. Percent cytotoxicity was determined by an LDH release assay.

triacylated compound) eluted at the same organic solvent concentrations as the cytotoxic activities in phenol extracts of culture supernatants (Fig. 4A) and in *S. flexneri* EP (Fig. 4B). A less hydrophobic diphenyl RP-HPLC column was also used to resolve the cytotoxic activities in phenol extracts of bacterial culture supernatants, *S. flexneri* EP and sBLP. Using a linear elution gradient similar to the one above, the cytotoxic activity in each preparation eluted between 60 and 70% of ACN (data not shown). Taken together, these data suggest that the cytotoxic factor in *S. flexneri* EP and culture supernatants contain the N-terminal lipo-amino acid $Acyl_3Cys$, which determines its retention on an RP-HPLC column. These data also show that the commercial preparation of sBLP is a mixture of diacylated and triacylated compounds. Interestingly, both compounds are cytotoxic (Fig. 4C). This result is consistent with studies showing that *Mycoplasma* spp. make diacylated BLP with immunomodulatory activity (18, 19).

The cytotoxic activity in EP contains lipid and protein components. sBLP and *S. flexneri* EP were treated with agarose beads coupled to a triacylglycerol lipase and tested for the ability to kill THP-1 cells. After treatment with the lipase beads, the EP and sBLP solutions were 100-fold less active in the cell death assay than control treated samples (Fig. 5A and B). This finding supports the hypothesis that the cytotoxic activity in EP is carried out by BLP. Lipase beads similarly inhibited the cytotoxic activity in phenol extracts of bacterial supernatants (data not shown).

BLP can be extracted from polyacrylamide gels by boiling in octyl glucoside solutions. This treatment does not affect the immunomodulatory properties of BLP (19) and is a powerful tool for the purification of these molecules. *S. flexneri* EP was resolved by Tris-Tricine PAGE. Gel lanes were isolated and cut in 11 5-mm sections. Each gel slice was extracted with hot octyl glucoside, and the extracts were tested for cytotoxic activity on THP-1 cells (Fig. 5C). The cytotoxic activity in *S. flexneri* EP eluted over a range of six consecutive fractions (1 to 6), spanning approximate molecular masses of 1.4 to 20 kDa. If the cytotoxic activity in EP were a single molecule, a more focused peak of activity would be expected. No cytotoxic activity was found in sections from gels loaded with PBS (EP diluent) mixed with an identical amount of Tris-Tricine sample buffer (data not shown). Pretreatment of EP with PK prior to SDS-PAGE resulted in an increase in the electrophoretic mobility of the cytotoxic activity. Importantly, the same total amounts of cytotoxic activity were recovered from the gel lane loaded with untreated EP and from the gel lane loaded with

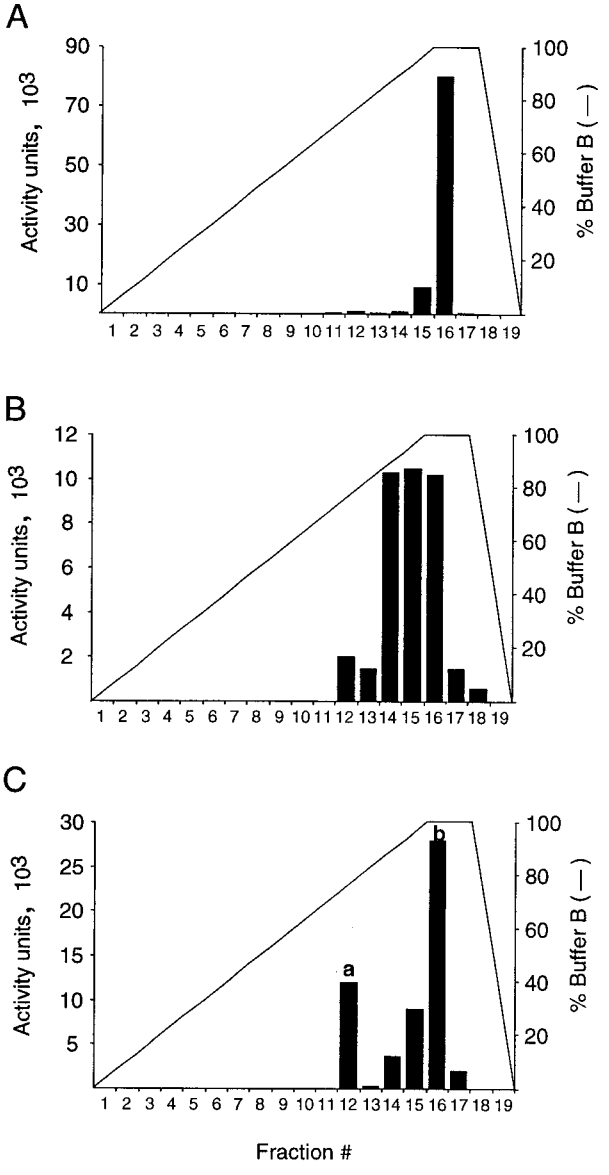

FIG. 4. The cytotoxic activities in *S. flexneri* culture supernatants. *S. flexneri* EP and sBLP coelute from an RP-HPLC column. Eight hundred microliters of a phenol extract of *S. flexneri* culture supernatants (A), 200 μg of EP (B), or 200 μg of sBLP (C) was loaded onto a C_4 RP-HPLC column and eluted with a linear gradient of increasing ACN concentration. One-milliliter fractions were collected and assayed for cytotoxic activity by an LDH release assay on THP-1 cells by serial dilution directly into tissue culture media. Activity is defined as 1/(dilution yielding 50% maximal cytotoxic activity). HPLC conditions were as follows. Buffer A contained dH_2O and 0.1% TFA, buffer B contained 95% ACN and 0.1% TFA, and the flow rate was 1 ml/min. The gradient was as follows: 0 to 15 min, 0 to 100% buffer B (linear gradient); 15 to 17 min, 100% buffer B; 17 to 19 min, 100 to 0% buffer B.

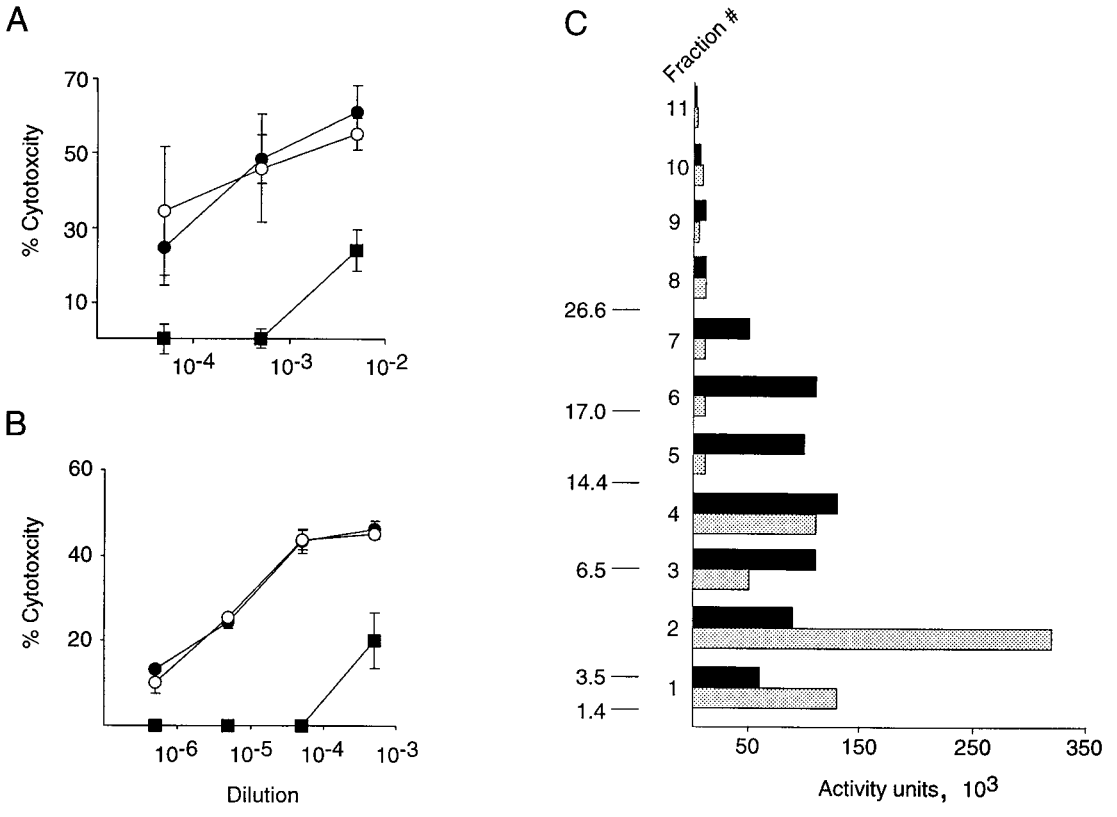

FIG. 5. The cytotoxic activity in EP contains lipase and protease sensitive components. (A) A 50-μg/ml *S. flexneri* EP solution was incubated for 4 h at 37°C either alone (closed circles), with uncoupled agarose beads (open circles), or with 22 U of wheat germ type 1A triacylglycerol lipase-agarose beads per ml (closed squares). Beads were removed by centrifugation, and the supernatants were tested for cytotoxicity on THP-1 cells at the indicated dilutions. Percent cytotoxicity in panels A and B was determined by an LDH release assay. (B) A 200-μg/ml sBLP solution was treated and assayed as described for panel A. (C) SDS-PAGE analysis of the cytotoxic activity in *S. flexneri* EP and PK-treated *S. flexneri* EP. One hundred sixty-six micrograms of *S. flexneri* EP (black bars) or 166 μg of PK-treated *S. flexneri* EP (gray bars) was resolved by Tris-Tricine SDS–16.5% PAGE. Eleven 5-mm gel sections (fractions) were made per lane, extracted with octyl glucoside, and tested for cytotoxic activity by an LDH release assay on cycloheximide-treated THP-1 cells by serial dilution directly into tissue culture media. Activity is defined as 1/(dilution yielding 50% maximal cytotoxic activity). Peptide molecular mass markers (in kilodaltons) are shown on the left.

PK-treated EP (680,000 and 670,000 U, respectively). Taken together, the data in Fig. 5 indicate that the cytotoxic factor in *S. flexneri* EP is a mixture of molecules that contain protein and lipid components. As expected for BLP, the protein component does not appear necessary to exert the cytotoxic activity, but it does influence its mobility on polyacrylamide gels.

An RP-HPLC fraction containing the cytotoxic activity in EP activates TLR2 signaling. BLP activate apoptosis and NF-κB-dependent transcription through TLR2 (1, 5, 9, 14). NF-κB is a transcriptional regulator of numerous host defense genes (3). We reasoned that if the cytotoxic activity elements purified from *S. flexneri* TCA-endotoxin are BLP, then they should also stimulate TLR2 signaling.

293 human kidney epithelial cells, which do not express TLR2 (1), were transiently transfected with expression plasmids encoding TLR2 and a luciferase reporter gene under the control of the NF-κB-dependent, E-selectin promoter (36). Stimulation of these cells with as little as 100 ng of *S. flexneri* type 1A TCA-endotoxin per ml activated the reporter gene (Fig 6A). Similarly to the cytotoxic activity, the TLR2 stimulatory activity in TCA-endotoxin was recovered in the EP component. Moreover, PW-LPS was inactive in this assay.

The cytotoxic activity in EP was purified sequentially by SDS-PAGE and RP-HPLC (see Materials and Methods). A population of 293 cells (293TLR2/CD14) stably transfected with TLR2 and CD14, an accessory protein involved in TLR signaling (30), were transiently transfected with the NF-κB-dependent luciferase reporter gene. An HPLC fraction containing the cytotoxic activity in EP, but not an equivalent fraction from a blank HPLC run, activated the reporter gene (Fig. 6B). sBLP was used as a positive control and stimulated NF-κB-dependent transcription. Approximately 100 pg of sBLP per ml induced the reporter gene equivalently to a 1:2,000 dilution of the active HPLC fraction. Signaling was dependent on the expression of TLR2, as parental 293 cells, or cells stably transfected with CD14 only (36), did not respond to the active HPLC fraction (Fig. 6C). Since BLP, but not LPS, activate TLR2 signaling, these data support the proposition that the cytotoxic component in *S. flexneri* EP consists of BLP.

DISCUSSION

In vivo, *Shigella* induces a pattern of apoptosis that does not colocalize with intratissular bacteria (23, 40). These data suggested that a diffusible toxin might be involved during infections. Here, we identify a cytotoxic activity in the sterile cul-

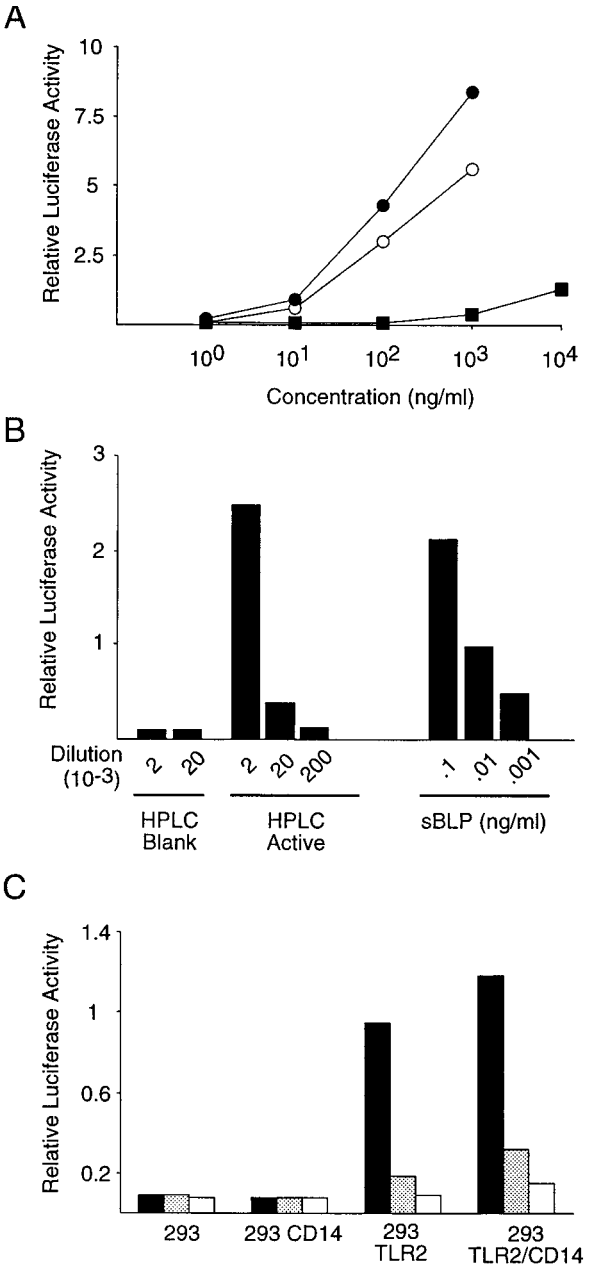

FIG. 6. An HPLC fraction containing the cytotoxic activity in EP stimulates an NF-κB-regulated reporter gene through TLR2. (A) *S. flexneri* TCA-endotoxin and EP, but not PW-LPS, activate TLR2 signaling. 293 cells were transiently transfected with expression plasmids encoding TLR2 and an NF-κB-regulated luciferase reporter gene. The cells were stimulated with *S. flexneri* TCA-endotoxin (open circles), EP (closed circles), or PW-LPS (closed squares), and reporter gene activity was assayed. (B) The cytotoxic activity in EP was purified sequentially by preparative SDS-PAGE and RP-HPLC. RP-HPLC fractions were assayed for cytotoxic activity on THP-1 cells, and an active fraction was identified as described in Materials and Methods. 293TLR2/CD14 cells were transiently transfected with the NF-κB-regulated luciferase reporter construct as described previously (1) and stimulated with the indicated dilutions of the cytotoxic HPLC fraction (HPLC Active) or an identical HPLC fraction collected from a blank HPLC run (HPLC Blank). As a positive control for TLR2 activation, cells were stimulated with sBLP. (C) The indicated cell lines were transiently transfected with an NF-κB-regulated luciferase reporter construct and incubated with 1:2,000 (black bars), 1:20,000 (gray bars), or 1:200,000 (white bars) dilutions of the cytotoxic HPLC fraction.

ture supernatants of *S. flexneri*. The following data support the proposition that the cytotoxic factors in *S. flexneri* EP and bacterial culture supernatants are BLP: (i) a triacylglycerol lipase abolishes the cytotoxic activity (Fig. 5A), (ii) the factors contain a protein component that is not essential for biologic activity (Fig. 5C), (iii) the cytotoxic activity displays an elution profile on an RP-HPLC column similar to that of a synthetic bacterial lipopeptide (Fig. 4), (iv) an RP-HPLC fraction containing the cytotoxic activity stimulates TLR2 signaling (Fig. 6), and (v) the cytotoxic activity can be reproduced by a purified BLP (murein lipoprotein) (data not shown) and two synthetic lipopeptides (Fig. 3).

There is likely to be a high degree of heterogeneity among the BLP present in *S. flexneri* EP. *E. coli* is genetically very similar to *Shigella* spp. and contains at least 20 putative BLP (34). In fact, we found that when EP was resolved by SDS-PAGE, the cytotoxic activity migrated over a broad MW range (Fig. 5C). This suggests that numerous BLP of various MW are responsible for the cytotoxic activity in EP. Additional diversity is likely generated by variability among the three acyl chains of the amino-terminal, lipid-modified cysteine residue (4, 34). Mass spectrometry was used to analyze HPLC fractions containing the cytotoxic activity from *S. flexneri* EP and phenol extracts of bacterial supernatants (data not shown). Unfortunately, we were unable to consistently detect single mass species. This is likely due to the cumulative heterogeneity among the lipid and protein components of *S. flexneri* BLP. We conclude that the cytotoxic activity in *S. flexneri* EP and culture supernatants is due to a mixture of BLP.

It has recently been shown that repurification of LPS from preparations contaminated with EP eliminates its ability to activate TLR2 (10, 29). Our data support this finding and extend this observation by showing that the TLR2-stimulatory properties of endotoxin can be quantitatively isolated in the EP fraction (Fig. 6A). Furthermore, HPLC analysis of EP shows that the TLR2-activating component copurifies with a cytotoxic activity likely to be BLP.

BLP have been previously purified from bacteria using macrophage stimulation assays (18, 31). However, in each case the initial source of activity was bacterial lysates. To our knowledge, the data presented here represent the first purification of a BLP immunomodulatory activity from bacterial culture supernatants. In gram-negative bacteria, BLP localize to the periplasmic side of either the inner or the outer membrane (34, 35). With this topology, it was previously unclear whether macrophages actually have access to the BLP of gram-negative bacteria. Our data indicate that BLP are recognized by macrophages in a native form released from the bacteria into culture supernatants.

It has been shown that macrophages infected with *S. flexneri* undergo cell death through a pathway dependent on the secreted virulence factor, IpaB (6). We suggest that the release of BLP is a second mechanism by which *S. flexneri* engages the host apoptotic machinery. We propose the following model for the pathogenesis of shigellosis: (i) translocation of *S. flexneri* from the lumen of the intestine into the GALT by M cells; (ii) infection of subepithelial macrophages; (iii) induction of rapid macrophage apoptosis by IpaB with concomitant release of interleukin-1β, which initiates an acute inflammatory response; (iv) destruction of the follicular associated epithelium, allowing the invasion of more *S. flexneri*; (v) exposure of cells

in the GALT to BLP released from both luminal and invading bacteria; and (vi) the induction of a widespread pattern of cell death in the lymphoid follicles by BLP through TLR2. In addition to inducing cell death, BLP would stimulate the production of proinflammatory cytokines and thus further aggravate the inflammation. Mitigation of the host response to BLP might alleviate the tissue destruction and symptoms of shigellosis. Moreover, since all bacteria produce BLP, the results presented here have implications for how the immune system recognizes and responds to pathogens and suggests new avenues for therapeutic exploration.

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REFERENCES

1. **Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky.** 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science **285:**736–739.
2. **Aliprantis, A. O., R. B. Yang, D. S. Weiss, P. Godowski, and A. Zychlinsky.** 2000. The apoptotic signaling pathway activated by Toll-like receptor-2. EMBO J. **19:**3325–3336.
3. **Baeuerle, P., and T. Henkel.** 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. **12:**141–179.
4. **Belisle, J. T., M. E. Brandt, J. D. Radolf, and M. V. Norgard.** 1994. Fatty acids of *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins. J. Bacteriol. **176:**2151–2157.
5. **Brightbill, H. D., D. H. Libraty, S. R. Krutzik, J. T. Belisle, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, P. J. Brennan, B. R. Bloom, P. J. Godowski, and R. L. Modlin.** 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science **285:**732–736.
6. **Chen, Y., M. R. Smith, K. Thirumalai, and A. Zychlinsky.** 1996. A bacterial invasin induces macrophage apoptosis by directly binding ICE. EMBO J. **15:**3853–3860.
7. **Goldman, R. C., D. White, and L. Leive.** 1981. Identification of outer membrane proteins, including known lymphocyte mitogens, as the endotoxin protein of Escherichia coli 0111. J. Immunol. **127:**1290–1294.
8. **Henderson, B., S. Poole, and M. Wilson.** 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60:**316–341.
9. **Hirschfeld, M., C. J. Kirschning, R. Schwandner, H. Wesche, J. H. Weis, R. M. Wooten, and J. J. Weis.** 1999. Inflammatory signaling by Borrelia burgdorferi lipoproteins is mediated by toll-like receptor 2. J. Immunol. **163:**2382–2386.
10. **Hirschfeld, M., Y. Ma, J. H. Weis, S. N. Vogel, and J. J. Weis.** 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J. Immunol. **165:**618–622.
11. **Hoekstra, D., J. W. van der Laan, L. de Leij, and B. Witholt.** 1976. Release of outer membrane fragments from normally growing Escherichia coli. Biochim. Biophys. Acta **455:**889–899.
12. **Hoffmann, P., S. Heinle, U. F. Schade, H. Loppnow, A. J. Ulmer, H. D. Flad, G. Jung, and W. G. Bessler.** 1988. Stimulation of human and murine adherent cells by bacterial lipoprotein and synthetic lipopeptide analogues. Immunobiology **177:**158–170.
13. **Kotloff, K. L., J. P. Winickoff, B. Ivanoff, J. D. Clemens, D. L. Swerdlow, P. J. Sansonetti, G. K. Adak, and M. M. Levine.** 1999. Global burden of Shigella infections: implications for vaccine development and implementation of control strategies. Bull. W. H. O. **77:**651–666.
14. **Lien, E., T. J. Sellati, A. Yoshimura, T. H. Flo, G. Rawadi, R. W. Finberg, J. D. Carroll, T. Espevik, R. R. Ingalls, J. D. Radolf, and D. T. Golenbock.** 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J. Biol. Chem. **274:**33419–33425.
15. **Manthey, C. L., P. Y. Perera, B. E. Henricson, T. A. Hamilton, N. Qureshi, and S. N. Vogel.** 1994. Endotoxin-induced early gene expression in C3H/HeJ (Lpsd) macrophages. J. Immunol. **153:**2653–2663.
16. **Maurelli, A. T., and P. J. Sansonetti.** 1988. Genetic determinants of *Shigella* pathogenicity. Annu. Rev. Microbiol. **42:**127–150.
17. **Morrison, D. C., and D. M. Jacobs.** 1976. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry **13:**813–818.
18. **Muhlradt, P. F., M. Kiess, H. Meyer, R. Sussmuth, and G. Jung.** 1997. Isolation, structure elucidation, and synthesis of a macrophage stimulatory lipopeptide from Mycoplasma fermentans acting at picomolar concentration. J. Exp. Med. **185:**1951–1958.
19. **Muhlradt, P. F., M. Kiess, H. Meyer, R. Sussmuth, and G. Jung.** 1998. Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis*. Infect. Immun. **66:**4804–4810.
20. **Radolf, J. D., M. V. Norgard, M. E. Brandt, R. D. Isaacs, P. A. Thompson, and B. Beutler.** 1991. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis. Analysis using a CAT reporter construct. J. Immunol. **147:**1968–1974.
21. **Rothfield, L., and M. Pearlman-Kothencz.** 1969. Synthesis and assembly of bacterial membrane components. A lipopolysaccharide-phospholipid-protein complex excreted by living bacteria. J. Mol. Biol. **44:**477–492.
22. **Sansonetti, P. I.** 1999. *Shigella* plays dangerous games. ASM News **65:**611–617.
23. **Sansonetti, P. J., J. Arondel, R. Cantey, M. C. Prevost, and M. Huerre.** 1996. Infection of rabbit Peyer's patches by *Shigella flexneri*: effect of adhesive or invasive bacterial phenotypes on follicle-associated epithelium. Infect. Immun. **64:**2752–2764.
24. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1981. *Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence. Infect. Immun. **34:**75–83.
25. **Sellati, T. J., D. A. Bouis, R. L. Kitchens, R. P. Darveau, J. Pugin, R. J. Ulevitch, S. C. Gangloff, S. M. Goyert, M. V. Norgard, and J. D. Radolf.** 1998. Treponema pallidum and Borrelia burgdorferi lipoproteins and synthetic lipopeptides activate monocytic cells via a CD14-dependent pathway distinct from that used by lipopolysaccharide. J. Immunol. **160:**5455–5464.
26. **Sellati, T. J., M. J. Burns, M. A. Ficazzola, and M. B. Furie.** 1995. Borrelia burgdorferi upregulates expression of adhesion molecules on endothelial cells and promotes transendothelial migration of neutrophils in vitro. Infect. Immun. **63:**4439–4447.
27. **Staub, A. M.** 1965. Bacterial lipido-proteino-polysaccharides ('O' somatic antigens) extraction with trichloroacetic acid, vol. 5. Academic Press, New York, N.Y.
28. **Sultzer, B. M., and G. W. Goodman.** 1976. Endotoxin protein: a B-cell mitogen and polyclonal activator of C3H/HeJ lymphocytes. J. Exp. Med. **144:**821–827.
29. **Tapping, R. I., S. Akashi, K. Miyake, P. J. Godowski, and P. S. Tobias.** 2000. Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharides. J. Immunol. **165:**5780–5787.
30. **Ulevitch, R. J., and P. S. Tobias.** 1999. Recognition of gram-negative bacteria and endotoxin by the innate immune system. Curr. Opin. Immunol. **11:**19–22.
31. **Vidal, V., I. G. Scragg, S. J. Cutler, K. A. Rockett, D. Fekade, D. A. Warrell, D. J. Wright, and D. Kwiatkowski.** 1998. Variable major lipoprotein is a principal TNF-inducing factor of louse-borne relapsing fever. Nat. Med. **4:**1416–1420.
32. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further application of the procedure, vol. 5. Academic Press, New York, N.Y.
33. **Wooten, R. M., T. B. Morrison, J. H. Weis, S. D. Wright, R. Thieringer, and J. J. Weis.** 1998. The role of CD14 in signaling mediated by outer membrane lipoproteins of Borrelia burgdorferi. J. Immunol. **160:**5485–5492.
34. **Wu, H.** 1996. Biosynthesis of lipoproteins, p. 1005–1014. *In* F. C. Neidhardt et al. (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
35. **Yakushi, T., K. Masuda, S. Narita, S. Matsuyama, and H. Tokuda.** 2000. A new ABC transporter mediating the detachment of lipid-modified proteins from membranes. Nat. Cell Biol. **2:**212–218.
36. **Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski.** 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature **395:**284–288.
37. **Zychlinsky, A., B. Kenny, R. Ménard, M. C. Prévost, I. B. Holland, and P. J. Sansonetti.** 1994. IpaB mediates macrophage apoptosis induced by *Shigella flexneri*. Mol. Microbiol. **11:**619–627.
38. **Zychlinsky, A., M. C. Prévost, and P. J. Sansonetti.** 1992. *Shigella flexneri* induces apoptosis in infected macrophages. Nature **358:**167–168.
39. **Zychlinsky, A., and P. Sansonetti.** 1997. Perspectives series: host/pathogen interactions. apoptosis in bacterial pathogenesis. J. Clin. Investig. **100:**493–495.
40. **Zychlinsky, A., K. Thirumalai, J. Arondel, J. R. Cantey, A. Aliprantis, and P. J. Sansonetti.** 1996. In vivo apoptosis in *Shigella flexneri* infections. Infect. Immun. **64:**5357–5365.

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