Induction of Interleukin (IL)-1 β and IL-8 mRNA Expression in Porcine Macrophages by Lipopolysaccharide from *Serpulina hyodysenteriae*

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Lipopolysaccharide (LPS) is a classic inducer of inflammatory cytokines and is a key virulence factor for most gram-negative pathogens. The effect of phenol-water (LPS) and butanol-water (endotoxin) extracts from *Serpulina hyodysenteriae* on inflammatory cytokine mRNA expression from porcine alveolar macrophages was investigated. The LPS and endotoxin extracts from *S. hyodysenteriae* induced a dose-dependent expression of interleukin 1 β (IL-1 β) and IL-8 which was weak compared with the responses induced by *Escherichia coli* LPS. In addition, the spirochetal extracts induced no detectable upregulation of mRNA expression for either IL-6 or tumor necrosis factor alpha.

Serpulina hyodysenteriae, a gram-negative anaerobic spirochete, induces a mucohemorrhagic diarrheal disease of growing swine (9). Putative virulence determinants of *S. hyodysenteriae* include a β -hemolysin(s) (11, 17, 18) and a lipopolysaccharide (LPS) or LPS-like molecule (7, 15, 16). This LPS-like molecule isolated by phenol-water extraction possesses various in vitro biologic activities, including toxicity for murine peritoneal macrophages, enhancement of C3 and Fc γ receptor-mediated phagocytosis, splenocyte mitogenesis, and stimulation of leukocyte chemotaxis (14). In addition, *S. hyodysenteriae* endotoxin extracted with butanol-water induces production of tumor necrosis factor (TNF) and interleukin-1 (IL-1) activity by murine peritoneal exudate cells in vitro (8). The present experiments were undertaken to extend this previous research on the stimulation of inflammatory cytokines in murine macrophages to the stimulation of macrophages from swine, the host species of swine dysentery. With porcine alveolar macrophages used as a model system, the objective of the current study was to determine whether stimulation of porcine macrophages with *S. hyodysenteriae* LPS (protein-free, phenol-water extract) or endotoxin (protein-containing, butanol-water extract) in vitro induces TNF- α , IL-1 β , IL-6, or IL-8 mRNA expression. The relative levels of mRNA detected for these cytokines and the dose of LPS required were compared with the levels of mRNA induced by *Escherichia coli* LPS.

LPS from *E. coli* K235 and from *Salmonella typhimurium* was obtained from Sigma Chemical Co., St. Louis, Mo. LPS (phenol-water extract) and endotoxin (butanol-water extract) from *S. hyodysenteriae* B204 and B234 were prepared as previously described (7). It has been shown that the LPS and endotoxin preparations differ in the percentage of protein, hexose, and 2-keto-3-deoxyoctanate (7). Preparations of endotoxin or LPS were dissolved in pyrogen-free saline (2 mg/ml on a dry weight

basis), sterilized by being heated at 100°C for 10 min, and stored at 4°C until use. The serpulinal LPS and endotoxin preparations had comparable amounts of endotoxin activity compared with *E. coli* LPS in the *Limulus* amebocyte lysate assay, as has been previously reported (7).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 14% separating gel with a 4% stacking gel. Gels were electrophoresed at 75 V for 1.5 h per gel (Bio-Rad Mini Electrophoresis System, Richmond, Calif.). The LPS (5 to 20 μ g) and endotoxin (20 μ g) samples were visualized by the modified silver staining technique of Fomsgaard et al. (6).

Alveolar macrophages were collected via lung lavage from crossbred pigs obtained from local commercial swine producers and cultured as described previously (2). Briefly, 10⁷ cells were cultured for 2 h at 37°C in 7% CO₂ and washed twice with phosphate-buffered saline to remove nonadherent cells. Porcine alveolar macrophages have been used in previous studies of the induction of porcine inflammatory cytokine mRNA expression in response to *E. coli* LPS and provide a basis for comparison with serpulinal LPS or endotoxin preparations (1, 2, 13). The adherent population was cultured overnight in Dulbecco's modified Eagle's medium supplemented with 2% normal swine serum, L-glutamine, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), penicillin, streptomycin, and gentamicin sulfate. To activate the macrophages, cells were stimulated for 4 h with LPS from *E. coli* (1 or 10 μ g/ml), LPS from *S. hyodysenteriae* B204 (1, 10, or 50 μ g/ml), or endotoxin from *S. hyodysenteriae* B204 (1, 10, or 50 μ g/ml). It was previously shown that stimulation of porcine alveolar macrophages for 4 h with *E. coli* LPS induces an upregulation of inflammatory cytokine mRNA expression (2, 13). After incubation with LPS or endotoxin, supernatants were removed, and total RNA was isolated from the macrophages.

RNA isolation was performed as previously described (3). RNA samples (10 μ g per lane) were electrophoresed in 1% agarose-formaldehyde gels and transferred to Magna nylon membranes (Micron Separations, Inc., Westboro, Mass.) in 10 $\times$ SSC buffer (1 $\times$ SSC contains 150 mM NaCl and 15 mM sodium citrate [pH 7.0]). Nucleic acids were fixed to the nylon

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FIG. 1. Silver-stained SDS-PAGE gel of *S. hyodysenteriae* butanol-water and phenol-water extracts. Lanes: A, *S. typhimurium* LPS (5 μ g); B, *E. coli* K235 LPS (5 μ g); C, *S. hyodysenteriae* B204 butanol-water extract (20 μ g); D, *S. hyodysenteriae* B204 phenol-water extract (20 μ g); E, *S. hyodysenteriae* B234 butanol-water extract (20 μ g); F, *S. hyodysenteriae* B234 phenol-water extract (20 μ g). The LPS and endotoxin preparations were electrophoresed in a 14% polyacrylamide gel and stained with silver as indicated in the text.

membranes by UV cross-linking. Prehybridization was carried out at 42°C for at least 2 h in Church buffer (0.25 M phosphate buffer, 1.0 mM EDTA, 7.0% SDS [pH 7.4]) containing 50% formamide (4). DNA probes were a full-length cDNA for porcine TNF- α (EMBL accession no. X57321), a 743-bp fragment of porcine IL-1 β cDNA (GenBank accession no. M86725), a 667-bp fragment of porcine IL-6 cDNA (GenBank accession no. M86722), a 1,370-bp fragment of porcine IL-8 (GenBank accession no. M86923), and a 1.25-kb fragment of a human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA clone. GAPDH is a constitutively expressed housekeeping enzyme, and a probe for GAPDH was included to ensure equivalent loading of RNA samples in each lane. Probes were random primed with [32 P]dCTP (Amersham, Arlington Heights, Ill.) and hybridized overnight at 42°C in Church buffer. Membranes were washed twice for 20 min in 2 $\times$ SSC–0.1% SDS at 42°C and once for 30 min in 0.1 $\times$ SSC–0.1% SDS at 50°C. Autoradiography was performed by exposing the membranes to Fuji X-ray film overnight. Autoradiograms were quantified by scanning densitometry.

The endotoxin and LPS preparations used in this study to stimulate porcine macrophages were analyzed by SDS-PAGE. Gels stained with the modified silver staining technique specific for LPS showed distinct banding patterns of endotoxin and LPS preparations from *S. hyodysenteriae* strains B204 and B234 compared with LPS from *S. typhimurium* and *E. coli* (Fig. 1) and confirmed that the banding patterns of the endotoxin and LPS preparations from *S. hyodysenteriae* used for stimulation of porcine macrophages were similar to banding patterns previously described (7).

Stimulation of porcine macrophages with *E. coli* LPS has been shown to induce the expression of several inflammatory cytokines, including TNF- α , IL-1 β , and IL-8 mRNA (2, 13, 19). To determine whether LPS or endotoxin preparations from *S. hyodysenteriae* induce an upregulation in the expression of inflammatory cytokines similar to that observed for *E. coli* LPS, porcine alveolar macrophages were stimulated with various concentrations of *S. hyodysenteriae* B204 LPS or endotoxin (1, 10, or 50 μ g/ml) for 4 h followed by RNA isolation and Northern (RNA) blot analysis. Stimulation of porcine macrophages with *E. coli* LPS (1 or 10 μ g/ml) was included for

FIG. 2. Northern blot analysis of mRNA expression for IL-1 β (A) and GAPDH (B) in porcine alveolar macrophages stimulated with bacterial LPS. Total cellular RNA was isolated from the macrophages, and 10 μ g of total RNA was electrophoresed in a 1% agarose gel. The RNA was then transferred to a Magna nylon membrane and cross-linked with UV light. Lanes: 1, RNA from unstimulated macrophages; 2 and 3, RNA from macrophages stimulated with 1 or 10 μ g of *E. coli* LPS (E-LPS) per ml, respectively; 4, 5, and 6, RNA from macrophages stimulated with 1, 10, or 50 μ g of the phenol-water extract of *S. hyodysenteriae* B204 (S-LPS) per ml, respectively. The membrane was hybridized sequentially with 32 P-labeled cDNA probes specific for porcine IL-1 β or human GAPDH. Autoradiograms were generated by overnight exposure to X-ray film at –70°C. Scanning densitometry was utilized to quantify differences in IL-1 β mRNA expression, and the scan units relative to GAPDH are depicted in panel C.

comparison. Blots were hybridized sequentially with 32 P-labeled probes for TNF- α , IL-1 β , GAPDH, IL-6, and IL-8.

S. hyodysenteriae LPS induced low levels of IL-1 β mRNA expression in a dose-dependent manner compared with GAPDH mRNA levels, which were unchanged (Fig. 2). However, the level of IL-1 β mRNA expression observed after stimulation with 50 μ g of *S. hyodysenteriae* LPS per ml was approximately threefold lower than that observed for *E. coli* LPS at 1 μ g/ml on the basis of data obtained from scanning densitometry, which were normalized to GAPDH mRNA (Fig. 2C). Similar results were obtained with *S. hyodysenteriae* endotoxin (data not shown).

Stimulation of porcine macrophages with *S. hyodysenteriae* LPS also induced a dose-dependent expression of porcine IL-8 mRNA similar to that observed for the induction of IL-1 β mRNA (Fig. 3). Furthermore, as observed for IL-1 β mRNA expression, the level of IL-8 mRNA expression induced by stimulation with 50 μ g of *S. hyodysenteriae* LPS per ml was severalfold less than that observed after stimulation with *E. coli* LPS at 1 μ g/ml.

As shown in Fig. 4, TNF- α mRNA expression was readily induced in porcine macrophages after stimulation with *E. coli* LPS. However, TNF- α mRNA expression was not upregulated after stimulation with *S. hyodysenteriae* LPS. The *S. hyodysenteriae* LPS or endotoxin preparations did not upregulate TNF- α mRNA expression even at a concentration of 50 μ g/ml (data not shown). These results were confirmed in three independent experiments. Differences in amounts of RNA loaded

FIG. 3. Northern blot analysis of mRNA expression for IL-8 (A) and GAPDH (B) in porcine alveolar macrophages stimulated with bacterial LPS. Total cellular RNA was isolated from the macrophages, and 10 μ g of total RNA was electrophoresed in a 1% agarose gel. The RNA was then transferred to a Magna nylon membrane and cross-linked with UV light. Lanes: 1, RNA from unstimulated macrophages; 2 and 3, RNA from macrophages stimulated with 1 or 10 μ g of *E. coli* LPS per ml, respectively; 4, 5, and 6, RNA from macrophages stimulated with 1, 10, or 50 μ g of the phenol-water extract of *S. hyodysenteriae* B204 per ml, respectively. The membrane was hybridized sequentially with 32 P-labeled cDNA probes specific for human GAPDH or porcine IL-8. Autoradiograms were generated by exposure to X-ray film at -70°C .

in the gel did not account for the lack of TNF- α mRNA expression in *S. hyodysenteriae*-stimulated samples, as determined by hybridization of the blots with GAPDH-specific cDNA probe (Fig. 4). Stimulation of porcine macrophages with either *E. coli* LPS or *S. hyodysenteriae* LPS also did not result in an upregulation of IL-6 mRNA expression (data not shown). Similar observations that IL-6 mRNA is not readily induced after stimulation of porcine alveolar macrophages with *E. coli* LPS have been reported (13).

Swine dysentery is an inflammatory, mucohemorrhagic diarrheal disease of the porcine large intestine. Swine infected with *S. hyodysenteriae* often exhibit signs of fever, anorexia, and depression, and the affected tissue is hyperemic, edematous, and hemorrhagic, with associated fibrin deposits and excess mucus. One of the putative virulence determinants of *S. hyodysenteriae*-induced dysentery is an LPS-like molecule (16).

After exposure to LPS isolated from members of the family *Enterobacteriaceae*, macrophages release inflammatory mediators, such as TNF- α , IL-1, IL-6, and IL-8, with IL-1 having been shown to be one of the key mediators (5). TNF has been shown to contribute to hemorrhagic tissue necrosis and to the development of vascular thrombi. IL-1 and IL-6 elicit systemic inflammatory responses, as in the induction of fever and the production of acute-phase reactants. In addition, IL-1 can elicit local inflammatory responses such as increased vascular

FIG. 4. Northern blot analysis of mRNA expression for TNF- α (A) and GAPDH (B) in porcine alveolar macrophages stimulated with bacterial LPS. Total cellular RNA was isolated from the macrophages, and 10 μ g of total RNA was electrophoresed in a 1% agarose gel. The RNA was then transferred to a Magna nylon membrane and cross-linked with UV light. Lanes: 1, RNA from unstimulated macrophages; 2 and 3, RNA from macrophages stimulated with 1 or 10 μ g of *E. coli* LPS per ml, respectively; 4 and 5, RNA from macrophages stimulated with 1 or 10 μ g of the phenol-water extract of *S. hyodysenteriae* B204 per ml, respectively. The membrane was hybridized sequentially with 32 P-labeled cDNA probes specific for porcine TNF- α or human GAPDH. Autoradiograms were generated by overnight exposure to X-ray film at -70°C .

permeability and edema (5) and may synergize with TNF- α to potentiate the effects of prostaglandins in altering intestinal epithelial cell ion transport (10). IL-8 is a neutrophil chemotactic peptide, which has also been shown to induce migratory responses by T lymphocytes and basophils (12). Given the inflammatory nature of swine dysentery, it might be speculated that these proinflammatory cytokines play a role in the pathogenesis of swine dysentery.

In the present study, stimulation of porcine macrophages with *S. hyodysenteriae* LPS-like or endotoxin-like extracts induced the expression of IL-1 β and IL-8 mRNA, albeit less effectively, than *E. coli* LPS. Previously, it was shown that in vitro stimulation of murine peritoneal exudate cells with *S. hyodysenteriae* endotoxin induced inflammatory cytokines such as TNF- α and IL-1 but required doses 5- to 50-fold higher than that of *E. coli* LPS (8). Thus, the experiments herein confirm in swine, the host species for *S. hyodysenteriae* infection, that serpulinal LPS preparations can induce the expression of specific proinflammatory cytokines in macrophages, but that severalfold higher levels of these preparations are required compared with the level of *E. coli* LPS to induce measurable amounts of a given cytokine. Moreover, these experiments have shown that serpulinal LPS or endotoxin preparations are not able to induce TNF- α expression in swine alveolar macrophages, which is in contrast to previous experiments showing an induction of TNF- α in murine peritoneal exudate cells after stimulation with serpulinal endotoxin. The observations that IL-1 β and IL-8 responses were weak and that TNF- α and IL-6 mRNA expression in porcine macrophages was not readily upregulated in vitro by spirochetal preparations appear paradoxical, given the inflammatory nature of swine dysentery. However, it is plausible that in the natural infection, other phlogistic components (e.g., cell wall components of intestinal microflora) or host factors (e.g., prostaglandins) play a role in the induction of these cytokines or that cells other than macrophages produce these inflammatory cytokines (1).

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