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Group B Streptococci (GBS) Injure Lung Endothelium In Vitro: GBS Invasion and GBS-Induced Eicosanoid Production Is Greater with Microvascular than with Pulmonary Artery Cells

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Neonatal group B streptococcal (GBS) sepsis and pneumonia cause lung endothelial cell injury. GBS invasion of the lung endothelium may be a mechanism for injury and the release of vasoactive eicosanoids. Pulmonary artery endothelial cells (PAEC) and lung microvascular endothelial cells (LMvEC) were isolated from neonatal piglets and were characterized as endothelial on the basis of morphology, uptake of acyl low-density lipoprotein, factor VIII staining, and formation of tube-like structures on Matrigel. PAEC and LMvEC monolayers were infected with COH-1 (parent GBS strain), isogenic mutants of COH-1 devoid of capsular sialic acid or all capsular polysaccharide, or a noninvasive *Escherichia coli* strain, DH5α. Intracellular GBS were assayed by plate counting of colony-forming units resistant to incubation with extracellular antibiotics. All GBS strains invaded LMvEC significantly more than PAEC, showing that the site of lung endothelial cell origin influences invasion. DH5α was not invasive in either cell type. Both isogenic mutants invaded PAEC and LMvEC more than COH-1 did, showing that GBS capsular polysaccharide attenuates invasion. Live GBS caused both LMvEC and PAEC injury as assessed by lactate dehydrogenase release; heat-killed GBS and DH5α caused no significant injury. Supernatants from PAEC and LMvEC were assayed by radioimmunoassay for prostaglandin E_2 (PGE_2), the stable metabolite of prostacyclin (6-keto-$PGF_{1\alpha}$), and the thromboxane metabolite thromoxane B_2. At 4 h, live COH-1 caused no significant increases in eicosanoids from both PAEC and LMvEC. At 16 h, live COH-1, but not heat-killed COH-1, caused a significant increase in 6-keto-$PGF_{1\alpha}$ greater than PGE_2 from LMvEC, but not PAEC. We conclude that live GBS injure and invade the lung microvascular endothelium and induce release of prostacyclin and PGE_2. We postulate that GBS invasion and injury of the lung microvasculature contribute to the pathogenesis of GBS disease.

Group B streptococci (GBS) are common gram-positive pathogens causing neonatal sepsis and pneumonia, with an incidence of 1.8 to 3.2 cases per 1,000 live births, and are associated with significant morbidity and mortality (2, 28, 44, 47). However, the pathogenesis of GBS disease is not completely defined. The primary portal of GBS entry into the fetus appears to be the lung by aspiration of infected amniotic fluid (2, 3, 28, 44, 47); multilobar pneumonia is observed in the majority of early-onset cases (21, 25). The GBS pneumonia in human newborns is predominantly localized to alveoli and alveolar ducts, with variable degrees of lung tissue invasion by GBS and invasion of the vessel walls of some intrapulmonary capillaries (3). GBS-induced lung endothelial cell injury is suggested by lung vessel wall invasion, intra-alveolar and interstitial hemorrhage, and protein-rich pulmonary edema in human infants (3, 21, 25) and by ultrastructural findings of lung capillary endothelial cell injury (i.e., basement membrane elevation or fragmentation and increased pinocytotic vesicles) in some animal models of GBS sepsis (30, 31, 33). These in vivo data suggest that the lung microvasculature is injured during the pathogenesis of early-onset GBS disease.

We previously reported that GBS can invade the lung epithelium, interstitial fibroblasts, and lung capillary endothelial cells in an in vivo newborn primate model of early-onset disease (7, 33). GBS can injure and invade human umbilical vein endothelial (HUVE) cells in vitro (17), but there are no in vitro data on the interaction of GBS with lung endothelial cells. The GBS virulence factors that promote endothelial cell invasion are uncertain (10, 17). Type III GBS capsular polysaccharide attenuates HUVE cell invasion, but its effect on the invasion of the lung endothelium is unknown (17). Both endotoxin infusion and GBS sepsis are associated with increased serum vasoactive eicosanoid levels in animal models (6, 16, 35). Endotoxin-induced endothelial cell injury is associated with release of vasoactive eicosanoids (6); the lung endothelial cell barrier may be a source of GBS-induced proinflammatory and vasoactive mediators in response to injury.

GBS-induced eicosanoid release and/or GBS invasion may be influenced by the vascular site of endothelial cell origin. Endotoxin causes a greater release of eicosanoids from bovine lung microvascular endothelial cells (LMvEC) than from pulmonary artery endothelial cells (PAEC) (27). Previous studies on the bacterial invasion of endothelial cells have used large-vessel endothelial cells (17, 18, 26, 36, 41). Our objectives included the study of GBS invasion into two large-vessel endothelial cell types, HUVE cells and piglet PAEC, and into piglet lung macrovascular endothelial cells and LMvEC.

We hypothesized (i) that live GBS would invade and injure piglet LMvEC and PAEC and that the vascular site of endothelial cell origin would affect invasion, (ii) that type III GBS capsular polysaccharide would attenuate GBS invasion into lung endothelial cells, and (iii) that live GBS would stimulate

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the release of more prostacyclin and prostaglandin E_2 (PGE_2) from LMvEC than from PAEC.

MATERIALS AND METHODS

Source of piglet tissue. Piglet PAEC and LMvEC were isolated from neonatal piglets (age 5 to 14 days). The tissue used for endothelial cell isolation was obtained from mixed-breed neonatal piglets that had served as control animals for various protocols within the Division of Neonatal and Respiratory Diseases at the University of Washington School of Medicine. Such piglets were anesthetized and mechanically ventilated, central venous and arterial catheters were placed, and sterile normal saline was infused for 1 to 4 h as described previously (16). The care of all piglets and the method of sacrifice (pentobarbital overdose, 100 mg/kg) had been approved by the Animal Care Committee, University of Washington.

Isolation of piglet PAEC. PAEC were harvested from neonatal piglet main pulmonary arteries by using collagenase and dispase, as previously described (38). The isolated cells were grown in a CS-1 serum-free medium system supplemented with 50 μg of CS-GF-1 endothelial growth factor (heparin-binding growth factor-1) per ml (Cell Systems Corporation). The cells were passaged with trypsin, and the cultures were used at passage 2 or 3 (<15 cumulative population doublings in vitro). The piglet pulmonary artery cells were characterized as endothelial on the basis of morphology, active incorporation of the fluorescent adduct of low-density lipoprotein Dil-acyl-LDL (Dil-Ac-LDL) (Biomedical Technologies, Staunton, Mass.) into membrane-bound cytoplasmic vesicles (42) (see Fig. 2), and indirect immunofluorescence staining for factor VIII (Sigma Chemical Company, St. Louis, Mo.).

Isolation of piglet peripheral LMvEC. We utilized a modification of the technique described by Meyrick et al. for the isolation and characterization of neonatal piglet LMvEC (27).

Preliminary experiments were performed to determine if an anti-ELAM-1 monoclonal antibody (MAb) (Becton-Dickinson, San Jose, Calif.; clone H18/7) would bind to piglet lung endothelium. Piglet lung tissue was initially fixed in methyl Carnoys solution and then embedded in paraffin. Deparaffinized lung sections were wet with phosphate-buffered saline (PBS)–0.01% azide and incubated for 30 min at room temperature with a 1:100 dilution (10 μg/ml) of ELAM-1 MAb or the immunoglobulin G2a (IgG2a) κ subclass isotype control (Becton-Dickinson; clone X39). The slides were washed twice with PBS for 5 min in a Coplin jar, and then the sections were incubated for 30 min at room temperature in the dark with a 1:320 dilution of Sigma $F(ab)_2$ fragment fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma). The sections were again washed twice with PBS in the dark, and the slides were mounted with PBS-glycerol (1:1, vol/vol). The indirect immunofluorescence staining showed significant staining of the lung endothelium with the ELAM-1 MAb but only faint background staining with the isotype control antibody (Fig. 1). We concluded that piglet lung tissue, prepared as described above, expressed ELAM-1 and that the ELAM-1 MAb could be used in piglet lung endothelial cell isolation.

The outer 1-cm rim of peripheral lung tissue was dissected free of visible vascular tissue, minced in Ca^{2+}-Mg^{2+}-free PBS–1% dialyzed fetal bovine serum, and digested by using collagenase and dispase (Boehringer Mannheim, Indianapolis, Ind.) and DNase I (Sigma) in 1% dialyzed fetal bovine serum at 37°C. The lung digest was filtered with a 70-μm cell strainer (Falcon, Lincoln Park, N.J.) to remove chunks of tissue and provided a pellet of approximately 10^7 cells. The lung digest was resuspended in 0.1 ml of biotinylated ELAM-1 MAb (50 mg/ml) and incubated at 4°C for 30 min. The ELAM-1 MAb was biotinylated by using a commercially available kit (Life Technologies, Gaithersburg, Md.). The antibody-tagged cells were separated from nonlabelled cells by panning on activated-surface AIS MicroCELLector flasks (Applied Immune Sciences) coated with streptavidin (Sigma). Antibody-labelled adherent cells were released, and the isolated cells were grown in the CS-1 serum-free medium system supplemented with CS-GF-1 (Cell Systems Corporation) and then passaged with trypsin. Piglet endothelial cell cultures were used in passage 2 or 3 (<15 cumulative population doublings in vitro). The piglet LMvEC were characterized as endothelial on the basis of morphology and active incorporation of the fluorescent adduct Dil-Ac-LDL into membrane-bound cytoplasmic vesicles (42) (Fig. 2). Piglet LMvEC indirect immunofluorescence staining was also positive for vimentin (Sigma), microfilaments (NBD-phallacidin; Molecular Probes, Eugene, Oreg.), and microtubules (Dako antibody) (data not shown). Primary cell cultures of LMvEC were further characterized for nonendothelial contaminating cell types by indirect immunofluorescence with a panel of MAbs (Sigma) to cell-type-specific cytoskeletal epitopes: smooth-muscle-specific actin (<1%), skeletal plus smooth muscle myosin (negative), vinculin (negative), desmin (negative), keratin and cytokeratin (negative), and neurofilament (negative) (data not shown). The LMvEC used for indirect immunofluorescence staining were fixed in ice-cold methanol for 10 min and permeabilized with 0.01% Triton X-100 in PBS for 5 min. After primary staining, the cells were incubated in a 1:320 dilution of Sigma $F(ab)_2$ fragment fluorescein isothiocyanate-conjugated goat anti-mouse IgG as described above.

Tube formation. The Matrigel used in the tube formation studies was preplated on 35-mm-diameter tissue culture dishes (Collaborative Biomedical, Bedford, Mass.). Piglet LMvEC were plated in CS-1 containing CS-GF-1 onto Matrigel (0.6 ml per dish) as described previously (7). After 5 to 7 days of culture, the cells were stained for active incorporation of the fluorescent adduct Dil-Ac-LDL into membrane-bound cytoplasmic vesicles (42). The cells were fixed in 2.0% paraformaldehyde in PBS (pH 7.4) for 10 min. Photography was performed with a Leitz high-pass rhodamine filter set.

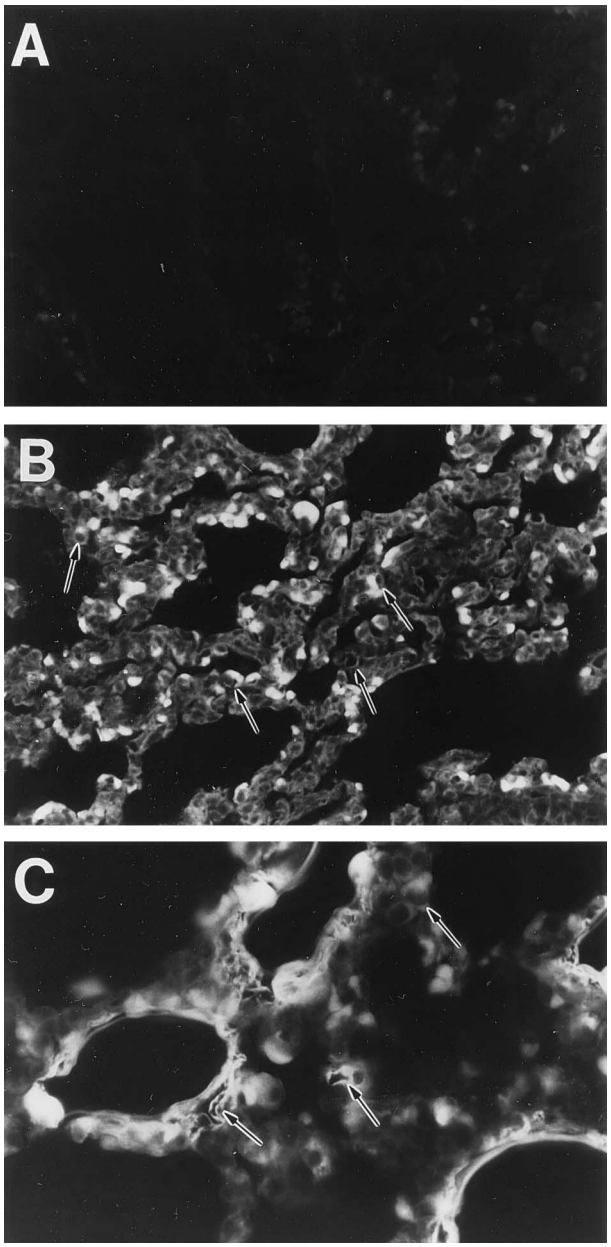

FIG. 1. Photomicrographs showing indirect immunofluorescence staining of piglet lung sections stained with ELAM-1 MAb (B and C) and IgG2a κ subclass isotype control MAb (A). Magnifications, ×100 (A and B) and ×400 (C). Arrows denote lung capillaries with endothelial cells stained with ELAM-1 MAb.

Bacteria. All bacterial strains were grown to stationary phase in Todd-Hewitt broth overnight. On the morning of experimentation, the broth culture was centrifuged and washed with RPMI, and the bacteria were resuspended in RPMI–1% fetal calf serum (FCS). A 0.4-ml aliquot of the resuspended bacteria was diluted into 10 ml of RPMI–1% FCS and grown to an optical density at 600 nm of 0.4 to provide an inoculum in mid-log phase. COH-1, a type III GBS clinical isolate (45), and isogenic transposon mutants of COH-1 have been described previously (32, 45). COH 1-11 is devoid of capsular sialic acid, and COH 1-13 is devoid of all type III capsular polysaccharide (26, 41). COH-1 was killed by boiling for 30 min, with the lack of viability confirmed by plate counting. A laboratory strain of *Escherichia coli*, DH5α, was used as a negative control for

the invasion assays (11) but is not a control for soluble gram-negative endotoxin. DH5α has previously been shown to be noninvasive and noninjurious to monolayers of Madin-Darby canine kidney cells (11). GBS inocula prepared as described above were previously shown to contain < 60 pg of endotoxin per ml as assessed by the *Limulus* amebocyte lysate assay (16).

Lung endothelial cell invasion assays. We utilized a bacterial invasion assay used to investigate the entry of GBS into HUVE cell monolayers (17). The assay is based upon the lack of intracellular accumulation of gentamicin and penicillin in eukaryotic cells (17, 24, 46). Therefore, this assay of cell lysates measures intracellular the number of colony-forming units resistant to extracellular antibiotics. LMvEC and PAEC (passage, <3) were grown to confluence in 24-well tissue culture plates (Costar, Cambridge, Mass.) in serum-free CS-1 medium with 50 μg of CS-GF-1 per ml (~10^5 cells per well). Twenty-four hours prior to inoculation, the cell cultures were washed and 2.0 ml of fresh RPMI–1% FCS was added; 1 h prior to inoculation, the cell monolayers were washed and fed with 0.5 ml of fresh RPMI–1% FCS (preliminary data showed better bacterial growth and invasion in RPMI than in CS-1 medium). Mid-log-phase bacteria were inoculated at a final ratio of 10 bacteria to 1 cell (10^6 CFU/10^5 cells) and then incubated at 37°C in 5% CO_2 for 6 h (five wells per experimental condition). The monolayers were washed three times with RPMI–1% FCS, and then RPMI–1% FCS with 5 μg of penicillin per ml and 100 μg of gentamicin per ml was added and the cells were incubated for 2 h at 37°C in 5% CO_2 to kill extracellular bacteria. The antibiotic-containing medium was removed, and the cells were washed as described above. Two wells per condition were used for measuring endothelial cell counts by a hemocytometer to determine cell loss compared with uninfected control monolayers and to determine if there were equivalent cell numbers for different endothelial cell types under baseline conditions. The monolayers were then treated with 200 μl of 0.25% trypsin without EDTA for 5 min, 0.025% Triton X-100 in distilled water was added to a final volume of 1.0 ml per well, and the mixture was incubated for an additional 5 min. The cell lysates from each well were pipetted up and down five times with a Pasteur pipette and then vortexed to ensure cell lysis and to disrupt streptococcal chains (17). Lysis of the cells was confirmed by light microscopy. The number of live intracellular bacteria was determined by plate counting on Todd-Hewitt agar. The percent invasion was calculated as number of intracellular colony-forming units/number of inoculum colony-forming units × 100 (17). Killing curves of Triton X-100 for GBS showed no effect on the organism at a concentration of up to 0.1% even after a 45-min exposure (17). A previous study with identical concentrations of bacteria inoculated onto 24-well plates without cells showed that penicillin plus gentamicin killed >99.99% of the extracellular bacteria by 2 h (17).

GBS-induced eicosanoid release from lung endothelial cells. The piglet lung endothelial cells (passage, <3) were plated and grown to confluence in serum-free CS-1 medium with 50 μg of CS-GF-1 per ml. Thirty-six hours prior to inoculation, the monolayers were washed and fed with 2.0 ml of fresh RPMI–1% FCS, since heparin-binding growth factor-1 is reported to reduce the expression of HUVE cell cyclooxygenase (22). On the morning of experimentation, the monolayers were washed three times with RPMI–1% FCS, and then 2.0 ml of fresh RPMI–1% FCS was added to each well 1 h prior to inoculation. Confluent endothelial monolayers on six-well plastic tissue culture plates (Costar) were inoculated with GBS or DH5α at a ratio of 10 bacteria per cell in RPMI–1% FCS (~10^6 cells per well). Heat-killed COH-1 cells were used to test the role of GBS viability in GBS-induced eicosanoid release from LMvEC. After 4 h of incubation, the supernatants from individual wells were harvested, and 2.0 ml of fresh RPMI–1% FCS was added to each well. The monolayers were then incubated to complete 16 h of stimulation, and the 16-h supernatants were harvested. All supernatants were centrifuged to remove cells and bacteria and then frozen at −70°C for later assay of the stable metabolite of thromboxane, the stable metabolite of prostacyclin (6-keto-$PGF_{1\alpha}$), and PGE_2. Wells incubated with medium in the absence of GBS served as negative controls, and wells incubated with the calcium ionophore A23187 (10 μM) (Sigma) served as positive controls.

The assay of endothelial cell supernatants for eicosanoids was performed by radioimmunoassay as described by Geissler et al., using antibodies (developed in rabbits) with high specificity and sensitivity against PGE_2 (14), the prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ (14), and the thromboxane metabolite thromboxane B_2 (13). Each assay was performed in duplicate as described previously (16). Fresh RPMI–1% FCS contained <10 pg of 6-keto-$PGF_{1\alpha}$ and PGE_2 per 0.1 ml and <5 pg of thromboxane B_2 per 0.1 ml.

GBS-induced release of LDH activity. Endothelial cell injury was assessed by the release of lactate dehydrogenase (LDH) activity into the culture medium (27). LMvEC and PAEC (passage, <3) were grown to confluence in 24-well tissue culture plates (Costar) in serum-free CS-1 medium with 50 μg of CS-GF-1 per ml (~10^5 cells per well). Twenty-four hours prior to inoculation, the cell cultures were washed and 2.0 ml of fresh RPMI–1% FCS was added; 1 h prior to inoculation, the cell monolayers were washed and fed with 1.0 ml of fresh RPMI–1% FCS. Mid-log-phase COH-1, COH 1-13, or DH5α bacteria were inoculated at a final ratio of 10 bacteria to 1 cell (10^6 CFU/10^5 cells; four wells per experimental condition). During the 6-h incubation, live bacteria grow to a final density of ~10^8 CFU/ml. Therefore, heat-killed COH-1 bacteria were inoculated at a dose of 10^8 bacteria per well to provide similar numbers of dead and live COH-1 bacteria at the end of the incubation period. The cultures were incubated at 37°C in 5% CO_2 for 4 h, and a 125-μl sample of supernatant was harvested from each well for same-day measurement of LDH activity. The same cultures were then incubated for a total of 16 h, and the remaining supernatant was harvested for same-day measurement of LDH activity (stored at 4°C).

The LDH activity in media and endothelial cell lysates was determined by a colorimetric assay for pyruvate, with the amount of residual pyruvate being inversely proportional to the amount of LDH activity (Sigma; diagnostic kit no. 500). The background amount of LDH activity in fresh RPMI–1% FCS was subtracted from the amount in the experimental supernatants. For each condition, the total LDH activity was measured in two sister wells by summing the LDH activities in the supernatants and from the monolayers lysed with 0.1% Triton X-100 in distilled water. The LDH release for each condition was expressed as LDH in the supernatant of experimental wells/LDH in the cell lysates and supernatants of sister wells × 100.

Electron microscopy. LMvEC monolayers in 24-well plates were inoculated with COH-1 and treated identically to monolayers used in the invasion assays described above. After 6 h of incubation in the absence of antibiotics, the monolayers were incubated for 2 h with penicillin and gentamicin. The LMvEC monolayers were then prepared for transmission electron microscopy with 45-degree angle sections as described previously (17). Specimens were examined with a JEOL-100B electron microscope.

Statistics. All data are expressed as means ± standard errors of the means. To compare intergroup mean values for invasion, LDH release, and eicosanoid release, the data were analyzed by analysis of variance followed by the Student-Neuman-Keuls correction for multiple comparisons (SPSS-PC+, version 6.0; SPSS, Inc., Chicago, Ill.). To compare intragroup mean values over time, paired *t* tests were performed. A P value of <0.05 was considered significant.

RESULTS

Characterization of piglet lung endothelial cells. Both piglet PAEC and peripheral LMvEC were characterized as endothelial by cobblestone morphology, their specific uptake (>95%) of the fluorescent adduct Dil-Ac-LDL (Fig. 2), and indirect immunofluorescence staining for factor VIII (data not shown). Endothelial cells characteristically can form tube-like structures when cultured on collagen-containing extracellular matrices, such as Matrigel (7). The culture of piglet LMvEC on a solid substratum of Matrigel resulted in the morphological differentiation of these cells into tube-like structures (Fig. 3). This provides further evidence for the endothelial cell origin of LMvEC (7). Primary cell cultures of PAEC and LMvEC also tested negative for nonendothelial contaminating cell types on the basis of indirect immunofluorescence staining for smooth muscle and fibroblast cell-type-specific cytoskeletal epitopes (see Materials and Methods). We conclude that the populations of piglet lung endothelial cells are free of significant contamination with fibroblasts and smooth muscle cells.

GBS invasion of piglet lung endothelial cells in vitro. We previously reported that GBS invade HUVE cells (17). In this study, we tested the ability of GBS to invade lung endothelial cells. LMvEC were compared with PAEC to test the effect of the lung vascular site of endothelial cell origin on GBS invasion. The cell counts from representative experimental wells were similar for PAEC ($1.0 \times 10^5 \pm 0.2 \times 10^5$ cells per well) and LMvEC ($0.9 \times 10^5 \pm 0.1 \times 10^5$ cells per well). There was >97% trypan blue exclusion for both cell types after 6 h of infection, with retraction of cells but no significant cell detachment (<5%). COH-1 invades LMvEC significantly more than PAEC (Fig. 4). To confirm that COH-1 invades LMvEC in vitro, transmission electron microscopy performed on equivalent monolayers showed intracellular GBS within membrane-bound vacuoles (Fig. 5). DH5α was noninvasive in both PAEC and LMvEC (<0.01%). The isogenic mutants COH 1-11, which is devoid of capsular sialic acid, and COH 1-13, which is devoid of type III capsular polysaccharide, invaded LMvEC significantly more than the parent COH-1 did (Fig. 4). These data suggest that (i) GBS can invade lung endothelium, (ii) LMvEC are more susceptible to GBS invasion, showing that the vessel size of lung endothelial cell origin influences the degree of GBS invasion, and (iii) type III capsular polysaccharide attenuates GBS entry into PAEC and LMvEC.

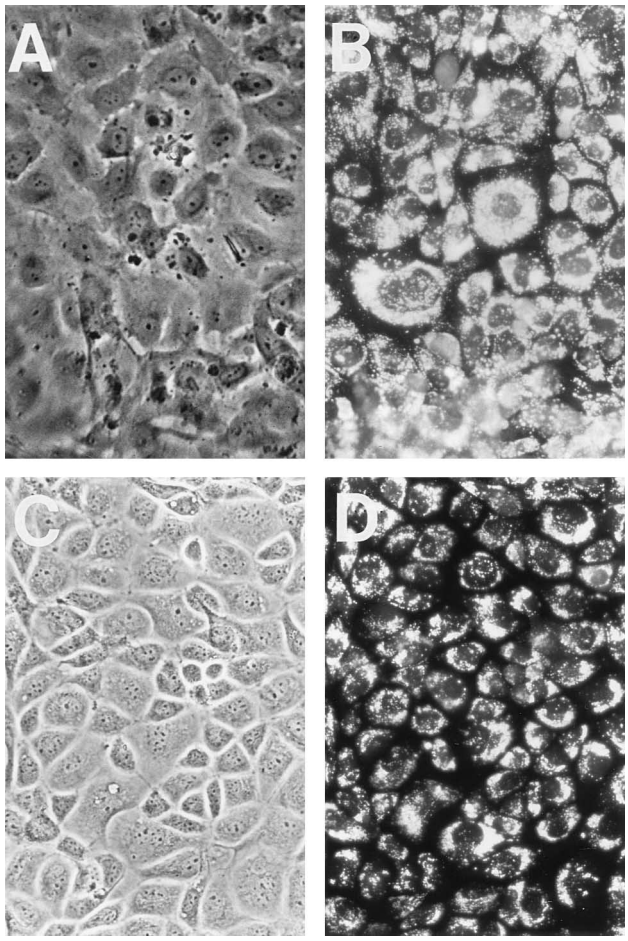

FIG. 2. Photomicrographs of piglet PAEC and LMvEC. (A) Unstained PAEC monolayer; (B) direct immunofluorescence for Dil-Ac-LDL uptake on PAEC monolayer; (C) unstained LMvEC monolayer; (D) direct immunofluorescence for Dil-Ac-LDL uptake on LMvEC monolayer. Both the PAEC and LMvEC monolayers were characterized as endothelial on the basis of morphology and specific staining for LDL uptake.

GBS-induced lung endothelial cell injury. Endothelial cell injury was assayed by the release of LDH into the culture supernatants (Table 1). After 4 h of infection, live COH-1 and COH 1-13 caused a minimal increase in LDH release from LMvEC and no significant increase above that of medium controls from PAEC. Both DH5α and heat-killed COH-1 caused no significant LDH release above medium control values at 4 h. At 16 h, there was increased release of LDH from unstimulated PAEC compared with unstimulated LMvEC. After 16 h of infection, both COH-1 and COH 1-13 caused similar and significant degrees of LDH release from both LMvEC and PAEC (Table 1). Both DH5α and heat-killed COH-1 caused no significant LDH release above medium control values at 16 h in LMvEC and PAEC.

These results show that live, but not heat-killed, GBS cause time-dependent lung endothelial cell injury that was similar for PAEC and LMvEC. The amount of GBS-induced LDH release was not proportional to the amount of GBS invasion when the PAEC and LMvEC data were compared. However, bacterial invasion or a toxic, heat-sensitive product produced by GBS may contribute to lung endothelial cell injury as judged by the lack of LDH release caused by DH5α and heat-killed GBS.

Effect of serum concentration on GBS-induced lung endothelial cell injury. Endotoxin-induced endothelial cell injury in vitro is influenced by the concentration of serum (6, 20). The influence of FCS concentration on GBS-induced lung endothelial cell injury was tested by infecting PAEC and LMvEC in RPMI–1% FCS, RPMI–3% FCS, or RPMI–10% FCS (three experiments with each condition in triplicate). To allow for potential increased injury at higher serum concentrations, all data for these studies were collected after 8 h of incubation, with the LDH assays performed as described above (Table 2). GBS-induced LDH release from both PAEC and LMvEC was increased during infection in the presence of 10% FCS compared with 1% FCS.

GBS-induced eicosanoid release from piglet lung endothelial cells. LMvEC and PAEC were compared in parallel experiments testing GBS-induced eicosanoid release. After 4 h of incubation, COH-1 did not cause significant increases in the release of any measured eicosanoid from either PAEC or LMvEC (Fig. 6). The calcium ionophore A23187 served as positive control for eicosanoid release and stimulated the release of 10- to 20-fold more 6-keto-$PGF_{1\alpha}$ and PGE_2 from LMvEC than from PAEC. The medium control levels of 6-keto-$PGF_{1\alpha}$ and PGE_2 at 4 h were higher in LMvEC than in PAEC.

After 16 h of incubation, COH-1 caused significant increases in the release of both 6-keto-$PGF_{1\alpha}$ and PGE_2 from LMvEC but no significantly increased production of any eicosanoid from PAEC (Fig. 6). COH-1 caused increased release of 6-keto-$PGF_{1\alpha}$ compared with PGE_2 from LMvEC. There was increased 6-keto-$PGF_{1\alpha}$ release into the medium of unstimulated LMvEC compared with unstimulated PAEC at 16 h (Fig. 6). The calcium ionophore A23187 also stimulated increased release of both 6-keto-$PGF_{1\alpha}$ and PGE_2 from LMvEC compared with PAEC cells at 16 h (Fig. 6). Heat-killed COH-1 (two experiments performed in quadruplicate) did not stimulate increased production of PGE_2 (45 ± 5 pg/0.1 ml) or 6-keto-$PGF_{1\alpha}$ (122 ± 22 pg/0.1 ml) from LMvEC above medium control values at 16 h. This suggests that live GBS are necessary for GBS-induced eicosanoid release. DH5α (two experiments performed in quadruplicate) failed to stimulate release of PGE_2 (23 ± 5 pg/0.1 ml) or 6-keto-$PGF_{1\alpha}$ (184 ± 32 pg/0.1 ml) from LMvEC at 16 h. These data suggest that a noninvasive bacterium does not induce eicosanoid release from LMvEC. In summary, our data suggest that infection with live GBS causes a time-dependent production of prostacyclin (greater than that of PGE_2) from LMvEC but not from PAEC.

DISCUSSION

We have shown that GBS can invade lung endothelial cells both in vitro and in vivo (17). Bacterial invasion of the endothelium is important in endovascular infections such as *Staphylococcus aureus* infections (18, 41), other streptococcal infections (26), and some rickettsial infections (43). The role of endothelial cell invasion in the pathogenesis of GBS pneumonia and sepsis has not been as well studied. Becroft et al. (3) reported GBS invasion into the vessel walls of intrapulmonary capillaries in human newborns with fatal cases of early-onset GBS disease, but there was no ultrastructural analysis to confirm endothelial cell invasion. In a primate model of early-onset neonatal GBS pneumonia and bacteremia, we observed intracellular GBS in lung alveolar epithelial cells, lung interstitial fibroblasts, and lung capillary endothelial cells with associated endothelial cell injury (17, 33). In animal models of sustained GBS sepsis, lung endothelial cell injury is evident (30, 31), but no ultrastructural studies for identification of

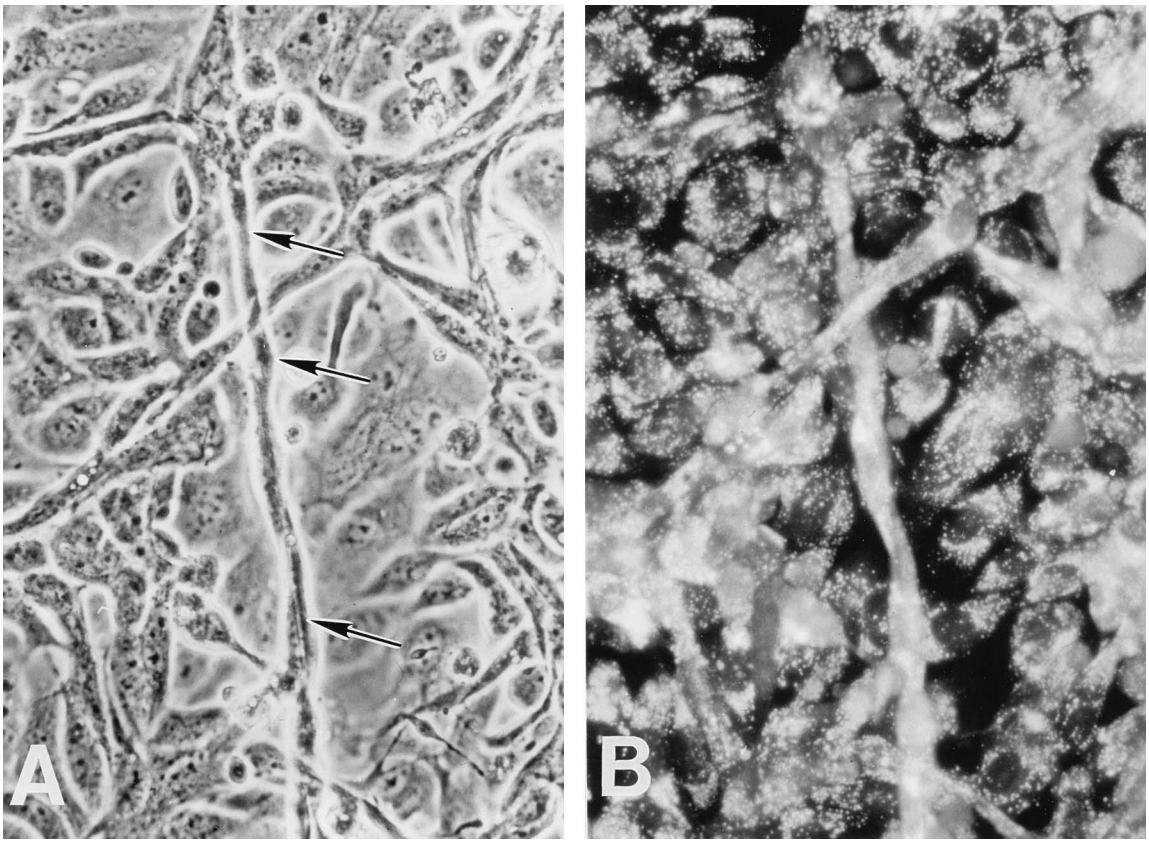

FIG. 3. Photomicrographs of LMvEC cultured on Matrigel. (A) Unstained LMvEC monolayer after 7 days of culture on Matrigel; (B) direct immunofluorescence for Dil-Ac-LDL uptake on LMvEC monolayer after 7 days of culture on Matrigel. Arrows denote tube-like structures with Dil-Ac-LDL staining. The data show that LMvEC can form tube-like structures and that the monolayer and tube-structures are endothelial on the basis of Dil-Ac-LDL staining.

intracellular GBS were performed. In studies of acute infection (1781 h) with ultrastructural analysis, the infusion of GBS into piglets resulted in the uptake and killing of GBS in pulmonary intravascular macrophages but no evidence of endothelial cell invasion (5). It is uncertain whether more-prolonged GBS infection in this model would lead to endothelial cell invasion. Our in vitro studies with HUVE cells showed minimal invasion at 0.5 h, with increased intracellular GBS at between 2 and 6 h of infection (17). GBS invasion contributes to endothelial cell injury in vitro (17), but it is uncertain whether GBS invasion significantly affects lung endothelial cell injury in vivo.

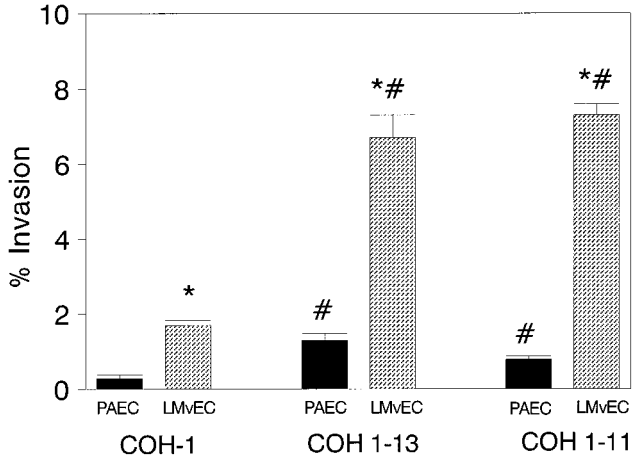

FIG. 4. Histogram plot of percent invasion for three isogenic GBS strains in LMvEC and PAEC (five experiments with five wells per condition per experiment). COH-1, parent type III GBS strain; COH 1-13, isogenic mutant devoid of all type III capsular polysaccharide; COH 1-11, isogenic mutant devoid of capsular sialic acid residues. *, $P < 0.05$ for LMvEC compared with PAEC for each strain; #, $P < 0.05$ for isogenic mutants compared with parent strain for the respective endothelial cell type.

GBS invasion into PAEC and LMvEC is attenuated by type III capsular polysaccharide. This finding is similar to the effect of capsule on *Salmonella minnesota* invasion of bovine PAEC (36). Unencapsulated and asialo isogenic GBS mutants also invade lung epithelial cells in culture more than the parent strain does (23). However, preincubation of lung epithelial cells with purified type III capsular polysaccharide did not alter epithelial cell invasion by the mutant strains (23). It is uncertain whether capsule bound to GBS is masking specific components important for adherence and invasion or is nonspecifically attenuating invasion by steric hindrance and surface charge (1). We speculate that in vivo modulation of type III GBS capsular polysaccharide may influence GBS pathogenesis by promoting intracellular invasion with reduced expression and by evading host phagocytic defenses with increased capsule expression (discussed in reference 17).

We observed that the vessel size of lung endothelial cell origin influences GBS invasion. Previous studies on bacterial invasion of the endothelium have used HUVE cells or large-vessel endothelial cells from the aorta or pulmonary artery (17, 18, 26, 36, 41). We isolated and characterized piglet PAEC and LMvEC for our study. All three isogenic GBS strains invaded

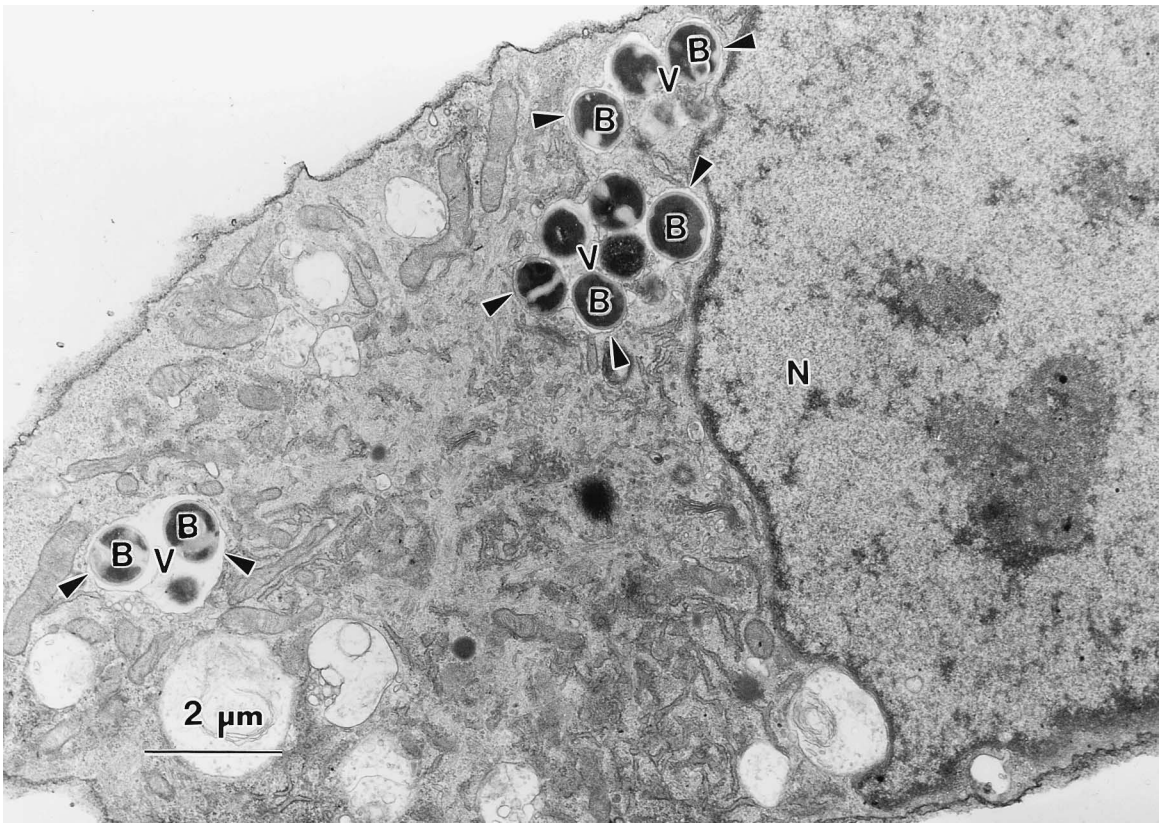

FIG. 5. Electron micrograph of GBS within an LMvEC in culture. In the LMvEC cytoplasm are phagocytic vacuoles (V) containing GBS bacteria (B). The vacuoles are enclosed by limiting membranes (arrowheads). The nucleus (N) of the cell appears normal.

LMvEC significantly more than PAEC. Previously, we reported that GBS remain viable within HUVE cells and respiratory epithelial cells but that there was no evidence of intracellular replication (17, 34). Increased intracellular GBS replication in LMvEC compared with PAEC could in part explain the observed differences in invasion. To address this possibility, we performed assays for intracellular replication in the lung endothelium as described previously (17). We observed no significant increase in the net number of intracellular colony-forming units at between 2 and 8 h of COH-1 infection in the presence of antibiotics in PAEC or LMvEC (two experiments performed in triplicate; data not shown). The combined data show that GBS invade LMvEC more than PAEC.

Endothelial cells are reported to have varied phenotypes depending upon both the organ and vessel size of origin (4, 37, 39). The microvasculature is reported to have increased numbers of basal and apical endocytotic vesicles (4) and increased secretory activity (37). Both bovine and human LMvEC are reported to have increased production of urokinase-type plasminogen activators (7, 39); bovine LMvEC released increased amounts of urokinase-type plasminogen activators compared with bovine liver and adrenal microvascular endothelial cells

TABLE 1. LDH release from piglet lung endothelial cells

Cell type and time (h)	% total LDH release (mean ± SEM)[a] with:				
	Medium	HK-COH-1	COH-1	COH 1-13	DH5α
PAEC					
4	6.0 ± 1.7	7.8 ± 1.7	11.0 ± 2.0	10.4 ± 2.0	7.7 ± 2.8
16	25.2 ± 5.2	24.2 ± 4.3	61.2 ± 11.3*	68.4 ± 9.2*	26.6 ± 1.7
LMvEC					
4	3.2 ± 2.8	4.1 ± 2.0	10.5 ± 2.7*	11.1 ± 2.1*	4.6 ± 2.2
16	10.8 ± 4.2	14.3 ± 3.2	54.1 ± 9.5*	56.0 ± 10.7*	13.8 ± 2.9

[a] LDH release into the supernatant compared with that in cell lysates plus supernatants of sister wells. Medium, RPMI–1% FCS control; HK-COH-1, heat-killed COH-1; COH-1, parent strain of GBS; COH 1-13, isogenic mutant devoid of type III capsular polysaccharide; DH5α, noninvasive *E. coli*. *, $P < 0.05$ compared with intragroup medium control value.

TABLE 2. Effect of serum concentration on GBS-induced lung endothelial cell LDH release

Cell type and FCS concn (%)[a]	% total LDH release (mean ± SEM)[b] with:	
	Medium	COH-1
PAEC		
1	16.6 ± 3.5	33.3 ± 7.8
3	25.8 ± 3.7	47.2 ± 6.2
10	27.3 ± 5.2*	60.6 ± 4.2*
LMvEC		
1	3.3 ± 2.5	21.6 ± 4.5
3	4.8 ± 2.4	29.3 ± 3.9
10	16.6 ± 4.4*	48.0 ± 5.1*

[a] Incubation was for 8 h.
[b] See Table 1, footnote *a*.

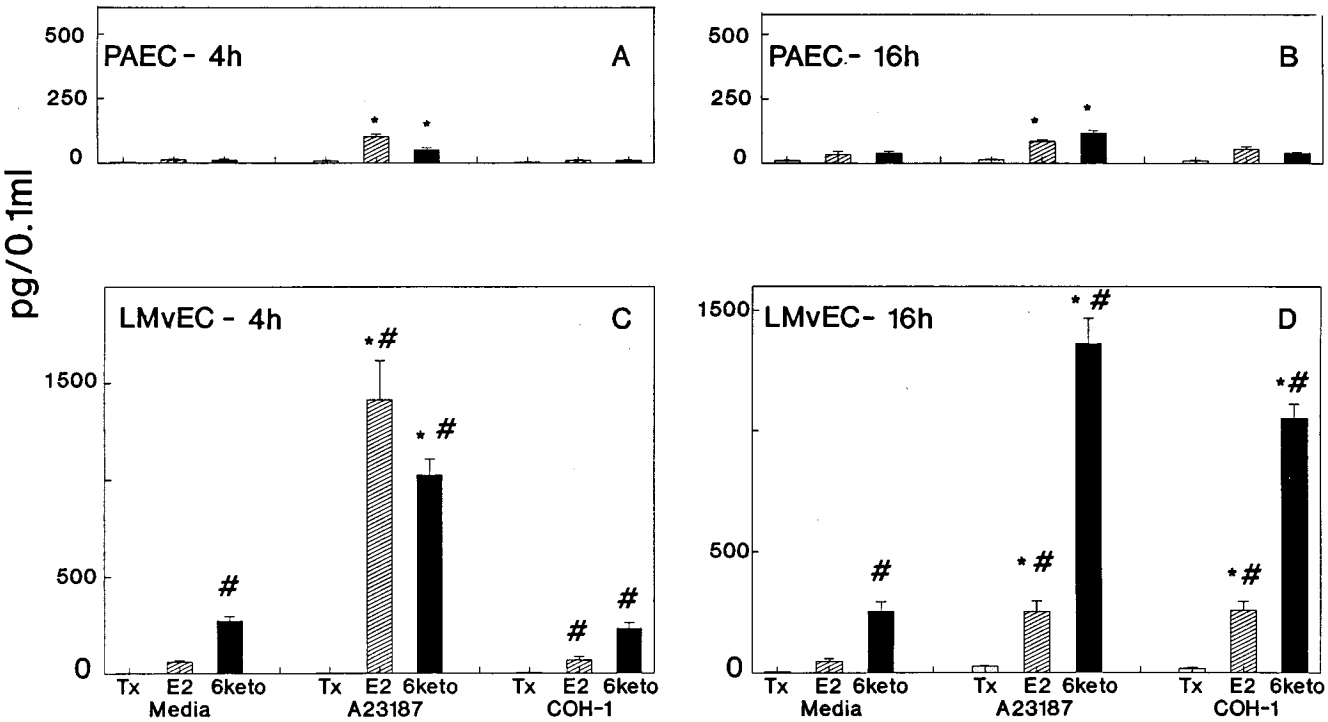

FIG. 6. GBS-induced eicosanoid release from PAEC and LMvEC (four experiments with each condition in triplicate per experiment). Tx, thromboxane B_2; 6keto, 6-keto-$PGF_{1\alpha}$; E2, PGE_2. *, $P < 0.05$ compared with intragroup medium control value; #, $P < 0.05$ compared with PAEC for the same eicosanoid.

(39). Combined with our observations, these data suggest that the extensive surface area of the lung microvascular endothelium has increased activity for the clearance of microthrombi and bacteria.

The data from prior studies and the current study show that GBS can injure the lung endothelium both in vitro and in vivo (17). Pulmonary microvascular endothelial cells are injured both in vivo and in vitro by gram-negative endotoxin (6). Both endotoxin- and GBS-induced endothelial cell injuries are augmented by the presence of increased concentrations of serum in vitro (20). The mechanisms for endothelial cell injury and this serum effect are unknown, but endotoxin-induced endothelial cell injury is complement independent (6, 20). Activation of protein kinase C in HUVE cells is reported to cause alterations in cell shape and the release of prostacyclin (12), but further studies are needed to determine the role of such signal transduction pathways in GBS-induced endothelial cell injury. Some reports suggest that both the species and tissue of endothelial cell origin can influence the response of large-vessel cells to endotoxin (6). Meyrick et al. reported that the vessel size of endothelial cell origin influences the response to endotoxin (27). Both sheep and bovine LMvEC were more resistant to endotoxin-induced injury than PAEC were; this assessment was based on reduced cell detachment and LDH release, reduced morphological changes, and reduced permeability changes for polar monolayers (27). The relationship between lung endothelial cell invasion and injury for GBS is not yet clear. Some of our data suggest that the extent of invasion is not proportional to the extent of injury: (i) PAEC and LMvEC were injured to a similar degree by live GBS despite the increased GBS invasion of LMvEC, and (ii) capsule-deficient isogenic GBS strains caused a similar degree of injury despite increased invasion in both PAEC and LMvEC. In contrast, some of our data suggest that bacterial invasion contributes to LMvEC injury: (i) a noninvasive bacterium, DH5α, did not cause LDH release from either PAEC or LMvEC, and (ii) the inhibition of GBS invasion by cytochalasin D attenuated HUVE cell injury in a previous study (17). We speculate that some threshold level of invasion is necessary for endothelial cell injury but that the extent of invasion is not directly proportional to the extent of injury. Previous studies have shown that ongoing synthesis of GBS protein, RNA, and DNA is important for GBS invasion of respiratory epithelial cells (34), and as COH-1 viability decreased, so did COH-1 invasion. Heat-killed GBS, which are nonviable with presumably no active metabolism, caused no significant LDH release from lung endothelial cells. These data suggest that GBS viability and metabolism contribute to GBS-induced endothelial cell injury. We speculate that factors other than invasion contribute to GBS-induced endothelial cell injury, such as an unidentified heat-sensitive GBS virulence factor (10).

The site of endothelial cell origin influences the profile of eicosanoids induced by inflammatory stimuli (7–9, 15, 27, 29). Human lung and foreskin microvascular endothelial cells (7, 8) and rabbit coronary, adipose, and renal microvascular endothelial cells release predominantly PGE_2 under basal and stimulated conditions (9, 15, 29). In contrast, bovine LMvEC release increased amounts of prostacyclin compared with PGE_2 under both unstimulated and endotoxin-treated conditions (27), and sheep LMvEC release equivalent amounts of prostacyclin and PGE_2 under basal and stimulated conditions (27). The combined data for piglet, bovine, and sheep LMvEC suggest that there is increased prostacyclin and/or PGE_2 production by these cells under basal and stimulated conditions compared with that by PAEC from the same species. The reason for the different eicosanoid profiles of human and nonhuman lung microvascular cells is uncertain but may reflect species differences, cell growth and assay conditions, and type of stim-

ulus (i.e., tumor necrosis factor for human LMvEC [7] and endotoxin or GBS for the nonhuman cells). In our studies, the type of stimulus applied to piglet LMvEC influenced the eicosanoid profile; i.e., GBS caused a greater ratio of 6-keto-$PGF_{1\alpha}$ to PGE_2 than did A23187. The absence of eicosanoid release from LMvEC after infection with heat-killed COH-1 suggests that a heat-sensitive GBS product is required. GBS invasion of lung endothelial cells may contribute to eicosanoid release, since (i) a noninvasive bacterium, DH5α, caused no significant eicosanoid release from LMvEC and (ii) the increased invasion of LMvEC compared with PAEC was associated with increased eicosanoid release from LMvEC. Like our findings for GBS, the extent of intracellular invasion of a rickettsial species in HUVE cells was associated with increased prostacyclin release (43). However, further studies with noninvasive isogenic GBS mutants are necessary to determine the relationship between endothelial cell invasion and eicosanoid release.

GBS sepsis in human newborns and animal models is associated with increased serum thromboxane and prostacyclin levels, pulmonary hypertension, and hypoxemia (16, 19, 35). There are no published reports on serum PGE_2 levels in human GBS disease. Multiple cell types may contribute to GBS-induced eicosanoid production, including platelets, neutrophils, mononuclear cells, and endothelial cells. The complex cascade of proinflammatory and vasoactive mediators during sepsis influences both pulmonary vascular tone and ventilation-perfusion matching (19, 40); this probably involves both paracrine and circulating factors. GBS-stimulated LMvEC did not release the potent vasoconstrictor thromboxane but released primarily the potent vasodilator prostacyclin. We speculate that the lung microvascular endothelium releases prostacyclin during GBS sepsis in response to multiple stimuli, and this may in part counteract multiple vasoconstrictive mediators, such as tumor necrosis factor and thromboxane produced by inflammatory cells (16). Local endothelial production of prostacyclin may increase regional pulmonary perfusion and the influx of inflammatory cells for bacterial clearance but also increase the risk of ventilation-perfusion mismatch and hypoxemia (40).

We postulate that GBS invasion of LMvEC contributes to lung injury during GBS pneumonia and sepsis. GBS presumably invade from the alveolar space through the alveolar epithelium, interstitium, and lung endothelium to enter the vascular space (18, 33). After the onset of bacteremia, GBS invasion of the apical surface of the endothelium may contribute to endothelial cell injury and eicosanoid release in multiple organs, including the lung. Our data show that GBS can invade the apical surface, but further studies are needed to investigate whether GBS can invade the basolateral surface of lung endothelial cells. This route of GBS invasion would provide a mechanism for transit from the alveolus into the circulation and a mechanism for acute lung endothelial cell injury and eicosanoid release.

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