Cytokine Responses to Group B Streptococci Induce Nitric Oxide Production in Respiratory Epithelial Cells

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Streptococcus agalactiae (group B streptococcus [GBS]) is a leading cause of neonatal pneumonia, sepsis, and meningitis. Early-onset GBS pneumonia is characterized by marked pulmonary epithelial and endothelial cell injury. Innate proinflammatory responses to GBS infection that may contribute to the respiratory pathology include the synthesis and release of cytokines, prostaglandins, and nitric oxide (NO). The hypothesis that NO is directly induced in lung epithelial cells by invading GBS or indirectly induced by cytokines released by GBS-infected mononuclear cells was tested. A549 cells expressing nitric oxide synthase (iNOS, NOS2) that is inducible by a mixture of cytokines (21) that should, based on in vitro data, be shown to induce NO production in cultured murine epithelial and endothelial cell cultures failed to induce NO, conditioned medium from GBS-infected mononuclear cells stimulated both NO secretion and iNOS expression by respiratory epithelial cells. Moreover, GBS interaction with human mononuclear cells, however, stimulated release of soluble factors that readily induced iNOS mRNA expression and NO secretion by A549 cells. Inflammatory mediator-induced nitric oxide (NO) production by alveolar epithelium may exceed that of other lung cell types such as macrophages, and induction during GBS infection may play a significant role in pulmonary defense or free-radical-mediated lung injury.

Streptococcus agalactiae (group B streptococcus [GBS]) is a leading cause of neonatal pneumonia, sepsis, and meningitis (26). GBS is also an important pathogen in maternal infections and in infections of nonpregnant adults with risk factors such as older age and underlying disorders (25). Early-onset GBS pneumonia is characterized by presence of numerous bacteria, an inflammatory exudate, and marked pulmonary epithelial and endothelial cell injury (1, 10). Innate proinflammatory responses to GBS infection which may contribute to the respiratory pathology include the synthesis and release of cytokines (12, 22), prostaglandins (16), and nitric oxide (NO) (6, 15).

In the lung, alveolar macrophages, airway epithelial cells, endothelial cells, and inflammatory cells may be sources for NO (24). Though GBS, with or without cytokines, has been shown to induce NO production in cultured murine macrophages (3, 6, 20), human macrophages produce far less NO (5- to 100-fold less) in response to cytokine or microbial stimuli than do murine macrophages (31). An alternate source of alveolar NO are human lung epithelial cells, which possess a nitric oxide synthase (iNOS, NOS2) that is inducible by a mixture of cytokines (21) that should, based on in vitro data, be released locally by GBS-infected alveolar macrophages and mononuclear cells (12). Alternatively, direct GBS invasion of respiratory epithelial cells (23) might also induce NO production.

In this report, human respiratory epithelial cells were directly cultured with GBS, cocultured with GBS-infected mononuclear cells, or exposed to conditioned culture medium from human mononuclear cells infected by GBS. The culture medium of epithelial cell cultures was assayed for nitrite, and the cell lysates were tested for induction of inducible nitric oxide synthase (iNOS) mRNA by reverse transcriptase PCR (RT-PCR). GBS-treated A549 cells neither secreted detectable NO nor expressed iNOS mRNA. GBS interaction with human mononuclear cells, however, stimulated release of soluble factors that readily induced iNOS mRNA expression and NO secretion by A549 cells. Inflammatory mediator-induced nitric oxide (NO) production by alveolar epithelium may exceed that of other lung cell types such as macrophages, and induction during GBS infection may play a significant role in pulmonary defense or free-radical-mediated lung injury.

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Nitrite assay. NO synthesis was determined colorimetrically as the accumula-
tion of nitrite (NO\(_2\)-) in culture medium. Briefly, 100- ul aliquots of conditioned medium were mixed with 100 \(\mu l\) of Greiss reagent (1:1 [vol/vol] 0.025% N-[1-
napthyl] ethylenediamine dihydrochloride [Sigma, St. Louis, Mo.] in H\(_2\)O-1%-sulfanilamide in 3 N HCl) in flat-bottom, 96-well immunosassays plates (Falcon; Becton-Dickinson, Oxnard, Calif.). After a 20-min incubation at room tempera-
ture, the \(A_{549}\) was measured on a microplate reader (Bio-Tek Instruments, Winooski, Vt.). The nitrite concentration was determined from a standard curve generated with sodium nitrite (NaNO\(_2\)). For samples with marginally detectable or undetectable nitrite levels, samples were incubated with nitrate reductase (from corn seedling [Sigma], 0.01 U/\(\mu l\)) plus NADH (0.2 mM final concen-
tration) to convert nitrate by-products to nitrite prior to the Greiss reaction.

iNOS mRNA expression. For RNA extraction, epithelial cells were cultured in six-well cluster plates or T25 culture flasks, and total RNA was extracted by using the RNAsena System (Promega Corp., Madison, Wis.) or RNA Stat-60 reagent (Tel-Test, Inc., Friendswood, Tex.). RNA pellets were suspended in RNase-free water and frozen at \(-135^\circ\)C. iNOS mRNA was detected by RT-PCR. A one-tube RT-PCR (Access RT-PCR System; Promega) was used as described by the manufacturer. To eliminate contaminating DNA, extracted RNA was DNase treated (1 U of RQI RNase-free DNase per \(\mu l\) of RNA, followed by DNase drop solution, as described by the manufacturer [Promega]). The DNase digestion reaction was followed by RT-PCR. RNA samples (1 \(\mu g\)) were used based on concentrations of extracted RNA estimated by optical density (260 nm/280 nm). Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers, IL-8 primers, and human iNOS primers were obtained from Clontech (Palo Alto, Calif.). As an additional control for variations in RNA quantity or quality, an Ambion (Austin, Tex.) human iNOS relative RT-PCR kit was also used. This multiplex PCR uses two primer sets in a single PCR (iNOS primers plus 18S rRNA primers and competitors as an invariant endogenous control). Optimal PCR products were obtained with 1.5 mM magnesium. The reaction incubation conditions were as follows: 1 cycle (48 \(^\circ\)C for 30 s, 60 \(^\circ\)C for 1 min, and 68 \(^\circ\)C for 2 min), 40 cycles (94 \(^\circ\)C for 30 s, 60 \(^\circ\)C for 1 min, and 68 \(^\circ\)C for 2 min), and 1 cycle (68 \(^\circ\)C for 7 min). Reaction cycles were controlled by a thermal cycler (Easy Cycler; Eri-

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\text{FIG. 1. Direct GBS infection of NHBE cells induces IL-8 but not iNOS mRNA. Total RNA was purified from cell lysates collected at 2, 8, and 24 h after initiation of a 2-h infection pulse with COH1-13 (100:1). RT-PCR products were resolved by electrophoresis on a 1.8%-agarose-eithidium bromide gel. (Gel A) G3PDH RT-PCR products (G3PDH PCR product = 983 bp). (Gel B) IL-8 RT-PCR products (IL-8 PCR product = 289 bp). (Gel C) iNOS RT-PCR products (iNOS PCR product = 259 bp). Lanes 1, 3, and 5, uninfected cells at 2, 8, and 24 h, respectively; lanes 2, 4, and 6, GBS-infected cells at 2, 8, and 24 h, respectively; lane 7, control RNA for respective genes; lane 8, DNA molecular weight markers.}
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for future assay of nitrite levels. The remainder of the medium was transferred to confluent A549 cell monolayers, which had been grown in supplemented medium in other six-well plates. After an additional 24 h of incubation at 37\(^\circ\)C, the culture medium was collected for nitrite assays, and the total RNA was extracted from the A549 cell monolayer. To generate larger samples of A549 RNA, for some experiments 5 \(ml\) of conditioned medium (from 1.4 \(\times\)10\(^6\) mononuclear cells in a T25 tissue culture flask) was collected and added to confluent A549 cells in a T25 flask. Background nitrate and/or nitrite levels in culture medium were accounted for by diluting nitrate standards for the Greiss assay in the stock culture medium and by using stock culture medium as a background control.

Statistics. Means of experimental groups were considered significant different if the \(P\) value was \(<0.05\). Statistical significance was analyzed by comparison of mean values by using the Student’s \(t\) test (unpaired, two tailed).

RESULTS

NO production by direct GBS infection of A549 cells or NHBE cells. Direct treatment of A549 cells or NHBE cells with living GBS (a 2-h infection pulse with doses of from 1 to 1,000 CFU of COH1 or COH1-13 per epithelial cell) did not induce detectable nitrite accumulation (\(<0.6 \mu M\) at 24 h in at least five experiments with each cell type) in the culture me-
dium, nor did GBS infection induce iNOS mRNA (at 2 or 24 h) in A549 cells as detected by RT-PCR. A barely visible iNOS PCR product was seen in both untreated and GBS-
treated (2 h) NHBE cells when NHBE cells (two lots) were tested in their first culture passage from receipt as frozen cells. NHBE cells may perhaps become transiently activated when collected from donors. In subsequent passages, GBS-treated NHBE cells did not exhibit iNOS expression (Fig. 1). Both NHBE and A549 cells were viable after GBS treatment and responsive to GBS, as shown by secretion and accumulation for 24 h in culture medium of immunoreactive IL-8 (data not

Cocultures. Mononuclear cell suspensions containing 9 \(\times\)10\(^4\) monocytes in 1.5 \(ml\) of supplemented RPMI were added for 1 h in 24-mm Transwell-Clear poly-
ester membrane inserts (0.4-\(\mu\)m pore size; Corning Costar, Cambridge, Mass.). The lower six-well culture chambers contained 2.6 \(ml\) of supplemented medium. Culture inserts with PBMC or with adherent culture-matured macrophages (see below) were placed in wells of confluent A549 cells. A549 cells were grown in DMEM and then switched to supplemented medium when inserts were added. Washed GBS at a ratio of 1:1 or 1:10 to the monocyte/macrophage count were added to the inserts. Cocultures were incubated for 24 h. A549 cell chamber medium and macrophage insert medium were collected and frozen at \(-20^\circ\)C for nitrite assays. Total RNA was extracted from A549 cells and stored at \(-135^\circ\)C.

To prepare inserts enriched for macrophages, nonadherent mononuclear cells were aspirated from culture inserts 1 h after addition of the washed mononuclear cell fraction, followed by two washes with PBS plus 2% glucose and the addition of fresh medium. Adherent monocytes were washed again after 24 h and cultured for 6 to 7 days at 37\(^\circ\)C in 5% \(CO_2\) for maturation into macrophages. The medium was changed every 72 h.

Collection of conditioned medium from mononuclear cells for treatment of A549 cells. A total of 5 \(\times\)10\(^4\) mononuclear cell/well in supplemented medium (2 \(ml\)well) was added to six-well multwell tissue culture plates. Washed GBS were added at a ratio of 1:1 or 10:1 as described above, and cultures were incubated at 37\(^\circ\)C. Conditioned medium was collected at 24 h and centrifuged to remove cells and bacteria. A small sample of the conditioned medium was frozen
shown; K. J. Goodrum, unpublished data) and induction of IL-8 mRNA (2 h, Fig. 1). As a control that epithelial cells could be induced to express iNOS under the conditions and timing of IL-1 and TNF-α, and IFN-γ/ml. Total RNA was extracted from cell lysates, and multiplex RT-PCR was conducted with two primer sets in a single PCR (iNOS primers plus 18S rRNA primers and competimers as an invariant endogenous control). PCR products were resolved by electrophoresis on a 1.8% agarose–ethidium bromide gel. Lane 1, DNA markers; lane 2, untreated (2 h) A549; lane 3 and 5, Cytomix-treated (2 h) A549; lane 4, same as lane 3 but without reverse transcriptase; lane 6, untreated (24 h) A549; lanes 7 and 9, Cytomix-treated (24 h) A549; lane 8, same as lane 7 but without reverse transcriptase; lane 10, 18S rRNA control (495 bp); lane 11, iNOS RNA control (349 bp).

FIG. 2. Cytomix induces iNOS mRNA in A549 cells. A549 cells were treated for 2 or 24 h with 10 ng each of IL-1, TNF-α, and IFN-γ/ml. Total RNA was extracted from cell lysates, and multiplex RT-PCR was conducted with two primer sets in a single PCR (iNOS primers plus 18S rRNA primers and competimers as an invariant endogenous control). PCR products were resolved by electrophoresis on a 1.8% agarose–ethidium bromide gel. Lane 1, DNA markers; lane 2, untreated (2 h) A549; lane 3 and 5, Cytomix-treated (2 h) A549; lane 4, same as lane 3 but without reverse transcriptase; lane 6, untreated (24 h) A549; lanes 7 and 9, Cytomix-treated (24 h) A549; lane 8, same as lane 7 but without reverse transcriptase; lane 10, 18S rRNA control (495 bp); lane 11, iNOS RNA control (349 bp).

FIG. 3. iNOS mRNA induction in A549 cells treated with conditioned medium from GBS-treated human mononuclear cells. We centrifuged 24-h-conditioned medium from GBS-treated (strain COH1, 1:1 or 10:1 ratio) human mononuclear cells to remove GBS and added it to monolayers of A549 cells for 24 h. Total RNA was extracted from cell lysates, and multiplex RT-PCR was conducted with two primer sets in a single PCR (iNOS primers plus 18S rRNA primers and competimers as an invariant endogenous control). PCR products were resolved by electrophoresis on a 1.8% agarose–ethidium bromide gel. Lane 1, DNA markers; lane 2, A549 cells treated with conditioned medium from control mononuclear cells (uninfected); lanes 3 and 5, A549 cells treated with conditioned medium from GBS (1:1)-infected mononuclear cells; lanes 4 and 6, same as lanes 3 and 5 but without reverse transcriptase; lane 7, A549 cells treated with conditioned medium from GBS (10:1)-infected mononuclear cells; lane 8, same as lane 7 but without reverse transcriptase; lane 9, 18S rRNA control (495 bp); lane 10, iNOS RNA control (349 bp).

Role of macrophages in cocultures. In an attempt to determine whether macrophages were the source of the soluble factors from PBMC, coculture experiments were conducted with adherent culture-derived macrophages. Neither NO secretion nor iNOS gene expression was found in A549 cells cocultured with GBS-treated PBMC or treated with supernatant fluid collected from GBS-treated human PBMC exhibited a marked morphologic change. A549 monolayers thus treated lost their cuboidal arrangement and became very elongated with dendritic processes (Fig. 5).
released in PBMC cocultures differs quantitatively or qualitatively from the Cytomix signal.

**DISCUSSION**

Innate immune responses to GBS within the respiratory tract may be initiated directly via invasion of respiratory epithelial cells and via phagocytosis by alveolar macrophages. In addition, the numerous proinflammatory factors released by infected epithelial and phagocytic cells may reciprocally modulate each other’s functions as well as recruit inflammatory cells and modulate the functions of other resident respiratory cells. GBS, a respiratory pathogen in neonates, is shown here to have no direct effect on iNOS expression when infecting human respiratory epithelial cells. GBS interaction with human mononuclear cells, however, stimulates the release of soluble factors that readily induce iNOS expression in human respiratory epithelial cells.

NO plays important roles in both physiological and pathological responses to infection (32). NO mediates proinflammatory actions by increasing blood flow and vascular permeability as well as mediating cell and tissue injury and potent antimicrobial activity (32). Coincident production of NO and superoxide (O$_2^-$) promotes formation of peroxynitrite (ONOO$^-$).

**FIG. 4.** iNOS mRNA induction in A549 cells cocultured with GBS-treated human mononuclear cells. A total of $5 \times 10^6$ mononuclear cells treated with GBS strain COH1 (1:1 ratio) were placed in 24-mm Costar Transwell culture inserts. Inserts were cocultured with monolayers of A549 cells on Transwell multiwell plates for 24 h. Total RNA was extracted from A549 cell lysates, and RT-PCR was conducted. PCR products were resolved by electrophoresis on a 1.8% agarose–ethidium bromide gel. (Gel A) RT-PCR products with Clontech iNOS primers. Lane 1, DNA markers; lane 2, A549 cocultured with uninfected mononuclear cells; lane 3, A549 cocultured with GBS in insert (without mononuclear cells); lane 4, A549 only (no coculture); lanes 5 and 6, A549 cocultured with GBS treated (1:1) mononuclear cells; lane 7, A549 cocultured with GBS-treated (10:1) mononuclear cells; lane 8, no RNA added; lane 9, iNOS RNA control (259 bp). (Gel B) The same samples as in gel A were reanalyzed with multiplex RT-PCR. Lanes 1 to 5 were as described above. Lane 6, same as lane 5 but without reverse transcriptase; lanes 7 and 8, duplicates of lanes 5 and 6; lane 9, A549 cocultured with GBS-treated (10:1) mononuclear cells; lane 10, same as lane 9 but without reverse transcriptase; lane 11, 18S rRNA control (495 bp); lane 12, iNOS RNA control (349 bp).

**FIG. 5.** Morphological change in A549 cells treated with conditioned medium from GBS-treated human mononuclear cells. We centrifuged 24-h-conditioned medium from GBS-treated (strain COH1, 1:1) human mononuclear cells to remove GBS and then added it to monolayers of A549 cells for 24 h. (A) A549 cells treated with conditioned medium from control untreated mononuclear cells. (B) A549 cells treated with conditioned medium from GBS-treated mononuclear cells. Both panels are at the same magnification.
an important factor in tissue injury at sites of inflammation (24). NO production in animal models of GBS infection has been shown to enhance meningeal inflammation (8), to mediate neuronal injury in brain cells (11), and to destroy lung surfactant (3). On the other hand, enhanced NO levels mediate beneficial hemodynamic effects in GBS infection by reducing pulmonary hypertension (2) and cerebral ischemia (15). The role of NO in septic shock and pulmonary hypertension in GBS infections of humans is controversial (5, 27).

Inducible NOS is present in a variety of cell types, including macrophages and respiratory epithelial cells (19), and transcription is the major step in regulation of iNOS mRNA (19, 32). Optimal expression of iNOS in human or rat respiratory epithelial cells requires synergistic signals from combinations of cytokines (14, 21) or lipopolysaccharide (LPS) plus cytokines (7). Whereas treatment of A549 cells with GBS alone or individual cytokines alone did not induce iNOS, GBS-induced soluble factors, presumably cytokines, from mononuclear cells did induce both iNOS and secreted NO in A549 cells. Since GBS alone does not induce iNOS mRNA in A549 cells, GBS passage between coculture chambers as reported in studies of GBS transcytosis of endothelial cell monolayers (18) would not explain the induction of iNOS mRNA. Possible synergy between GBS and individual cytokines or combinations of cytokines was not tested. Though bacterium-free culture supernatants from mononuclear cells were used to stimulate the A549 cells, it is possible that soluble or released bacterial factors acted in synergy with cytokines to induce iNOS. The GBS beta-hemolysin has been reported to induce iNOS in the RAW264.7 mouse macrophage cell line (20), as well as exhibit toxicity toward the A549 cell line (17). An investigation of epithelial responses to additional GBS serotypes and strains is necessary to fully resolve the direct versus indirect effects of GBS. Results similar to those reported here for A549 responses to GBS have been reported with other bacterial stimuli. Conditioned medium from tubercule bacillus-treated human mononuclear cells, more than conditioned medium from LPS-treated mononuclear cells, induced iNOS mRNA and NO production in A549 cells (13).

The GBS-induced soluble factor(s) involved in iNOS induction were not identified, but the known responsiveness of A549 cells to a mixture of IL-1, TNF-α, and IFN-γ would support a hypothesis for GBS induction of these cytokines. GBS-treated human monocytes can produce IL-1, IL-6, and TNF-α (30), whereas GBS-induced IL-12 from macrophages can innately induce IFN-γ from NK cells (4). The finding that GBS-treated macrophages failed to signal iNOS induction in A549 cocultures even when supplemented with IFN-γ argues against this hypothesis, but the relative concentrations of macrophage cytokines in these cocultures may be critical and were not specifically measured. Conditioned medium from GBS-treated human PBMC, which have been shown to produce IL-1, TNF-α, IFN-γ, and IL-12 (12), did induce iNOS in A549 cocultures. One or more of these or other unexamined factors secreted by GBS-treated PBMC must be modulating iNOS expression and/or cellular morphology in the A549 cells. Although many investigators have confirmed that NHBE cells and A549 cells (7, 14, 21) have parallel iNOS responses to Cytomix, the stimulatory effect of GBS-treated PBMC on iNOS induction in A549 cells was not examined in NHBE cells.

A549 cells cultured with conditioned medium from PBMC treated with the higher dose of GBS (10:1) consistently expressed iNOS mRNA but did not consistently show elevated nitrite in the medium. The higher GBS dose may have been toxic to the mononuclear cells, possibly inducing a set of factors quantitatively or qualitatively different from those induced at the lower dose (1:1) or generating conditioned medium with factors that induced iNOS but inhibited iNOS activity.

The cause of the morphologic changes seen in A549 cells treated with GBS-conditioned medium from PBMC is unknown. Transforming growth factor β1 (TGF-β1) has been reported to transform the morphologic phenotype of A549 cells in a fashion similar to that reported here (28). In addition, A549 cells cocultured with macrophages activated by LPS and IFN-γ are reported to exhibit a nitric oxide-mediated increase in synthesis and activation of latent TGF-β1 protein (29).

Experiments to determine specific cytokine levels in the conditioned medium from GBS-treated PBMC and experiments to test the effects of cytokine neutralization on A549 responses will be required to identify the factor or factors involved in the epithelial responses. Considering the importance of GBS infections in neonates and the reports of reduced cytokine responses to GBS in cord blood versus adult mononuclear cells (9), the studies described here should be repeated with human cord blood cells in order to assess the relevance of this pathway for iNOS induction in newborns. Production of NO via iNOS induction either in respiratory epithelial cells or in vascular endothelial cells in GBS infection may restrict intracellular survival and transcytosis of GBS, thus serving as an innate defense against sepsis and meningitis.

Inflammatory mediator-induced NO production by alveolar epithelium exceeds that of other lung cell types, such as macrophages (7), and induction during GBS infection may play a significant role in pulmonary defense or free-radical-mediated lung injury in GBS disease.

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