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

Kenneth J. Goodrum* and Jane Poulson-Dunlap

Department of Biomedical Sciences, College of Osteopathic Medicine, Ohio University, Athens, Ohio 45701-2979

Received 4 June 2001/Returned for modification 10 September 2001/Accepted 11 October 2001

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, 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 transformed human respiratory epithelial cells were directly cultured with GBS, cocultured with GBS-infected human mononuclear cells or purified macrophages, or exposed to conditioned culture medium from human mononuclear cells infected by GBS. The culture medium of A549 cultures was assayed for NO secretion, and the cell lysates were tested for presence of inducible nitric oxide synthase (iNOS) mRNA by reverse transcriptase PCR (RT-PCR). Whereas direct GBS infection of epithelial cells failed to induce NO, conditioned medium from GBS-treated mononuclear cells stimulated both NO secretion and iNOS expression by respiratory epithelial cells.

MATERIALS AND METHODS

Bacteria. The highly encapsulated serotype III GBS strain COH1 and the isogenic mutant of COH1, COH1-13 (lacking type III polysaccharide), were provided by Craig E. Rubens (University of Washington School of Medicine, Seattle). GBS were harvested by centrifugation from log-phase cultures in Todd-Hewitt broth and washed in phosphate-buffered saline (PBS). GBS were enumerated by viable colony counts on blood agar, and equivalent doses of GBS strains were adjusted by optical density comparisons of bacterial suspensions.

Reagents. Polymyxin B, gentamicin, and penicillin G were obtained from Sigma Chemical Co. (St. Louis, Mo.). Recombinant human interleukin-1-β (IL-1), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) were purchased from Biosource International (Camarillo, Calif.).

Respiratory epithelial cells. Primary cultures of normal human bronchial epithelial (NHBE) cells were obtained from Clonetics Cell Systems (BioWhittaker, Walkersville, Md.) and cultured in serum-free medium provided by Clonetics. The A549 human lung carcinoma cell line (ATCC, CCL185) was routinely cultured in Dulbecco modified Eagle medium (DMEM) with 4,500 mg of glucose/liter (HyClone, Logan, Utah) with or without 10% (vol/vol) fetal bovine serum (Atlanta Biologicals, Norcross, Ga.), plus 100 U of penicillin G and 100 μg of streptomycin/ml.

GBS infection of epithelial cells. Epithelial cells were cultured in antibiotic-free medium 24 h prior to infection. Bacterial invasion of epithelial cell cultures was initiated as described by Rubens et al. (23) by centrifugation (800 × g, 10 min) of viable washed log-phase GBS (doses of 1 to 1,000 CFU of GBS/epithelial cell) directly onto adherent epithelial cells in multiwell culture plates. After a 2-h invasion period at 37°C, culture wells were washed four times with PBS to remove excess extracellular bacteria, and medium with antibiotics (100 μg of gentamicin and 5 μg of penicillin G/ml) was added to kill the remaining extracellular bacteria. Invasion periods of up to 4 h were tested but resulted in a loss of epithelial cell viability. Culture medium and/or cell lysates were collected at timed intervals from replicate culture wells for NO assay or RNA extraction.

* Corresponding author. Mailing address: Department of Biomedical Sciences, College of Osteopathic Medicine, Ohio University, Athens, OH 45701-2979. Phone: (740) 593-2390. Fax: (740) 597-2778. E-mail: goodrum@ohio.edu.
Nitrite assay. NO synthesis was determined colorimetrically as the accumulation of nitrite (NO$_2^-$) in culture medium. Briefly, 100-μl aliquots of conditioned medium were mixed with 100 μl of Greiss reagent (1:1 [vol:vol] 0.025% N-[1-naphthyl]ethylendiamine dihydrochloride [Sigma, St. Louis, Mo.] in H$_2$O-1% sulfuric acid in 3 N HCl) in flat-bottom, 96-well immunoassay plates (Falcon, Becton-Dickinson, Oxnard, Calif.). After a 20-min incubation at room temperature, the A$_{540}$ 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/sample) plus NADH (0.2 mM final concentration) 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 RNAgent 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°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 RNase-free DNase per μg of RNA, followed by DNase stop solution, as described by the manufacturer [Promega]). The DNase digestion reaction was followed by RT-PCR. RNA samples (1 μg) 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 IL-8 mRNA primers and competitors as an invariant endogenous control). Optimal PCR products were obtained with 1.5 mM magnesium. The reaction incubation cycles were as follows: 1 cycle (48°C for 45 min), 1 cycle (94°C for 2 min), 40 cycles (94°C for 30 s, 60°C for 1 min, and 68°C for 2 min), and 1 cycle (68°C for 7 min). Reaction cycles were controlled by a thermal cycler (Easy Cycler; Ericom, San Diego, Calif.). PCR products were resolved by electrophoresis on a 1.8% agarose-ethidium bromide gel.

PBMNC. Human peripheral blood mononuclear cells (PBMNC) were purified from venous blood from healthy adult volunteers. The use of human subjects was reviewed by the Ohio University Institutional Review Board and complied with all relevant federal guidelines and institutional policies. Blood samples (50 ml in EDTA-containing blood collection tubes) were layered over equal volumes of 1.8% agarose-ethidium bromide gel. (Gel A) G3PDH RT-PCR products (657 bp) and human iNOS PCR product (983 bp). (Gel B) IL-8 RT-PCR products (135 bp). 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-ethidium bromide gel. For samples with marginally detectable or undetectable nitrite levels, samples were incubated with nitrate reductase (from corn seedling [Sigma], 0.01 U/sample) plus NADH (0.2 mM final concentration) to convert nitrate by-products to nitrite prior to the Greiss reaction.

Cocultures. Mononuclear cell suspensions containing 9 × 10$^4$ monocytes in 1.5 ml of supplemented RPMI were added for 1 h in 24-mm Transwell-Clear polycarbonate inserts (0.4-μm pore size; Corning Costar, Cambridge, Mass.). The lower six-well culture chambers contained 2.6 ml of supplemented medium. Culture inserts with PBMNC 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 10:1 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°C for nitrite assays. Total RNA was extracted from A549 cells and stored at −135°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°C in 5% CO$_2$ for maturation into macrophages. The medium was changed every 72 h.

Conditioned medium from mononuclear cells for treatment of A549 cells. A total of 5 × 10$^5$ mononuclear cells/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°C. Conditioned medium was collected at 24 h and centrifuged to remove cells and bacteria. A small sample of the conditioned medium was frozen 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°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 × 10$^5$ 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 nitrite 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 significantly 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 μM at 24 h in at least five experiments with each cell type) in the culture medium, 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
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 the cultures, A549 cells were treated for 24 h with a mixture (Cytomix) of IL-1, TNF-α, and IFN-γ (10 ng/ml each). Cytomix-treated A549 cells secreted NO (a mean nitrite level in 24-h culture medium of 6.4 ± 4.3 μM in four experiments) and expressed iNOS mRNA detectable by RT-PCR at 2 h post-treatment, with higher expression detectable at 24 h (Fig. 2).

iNOS gene expression in A549 cells cocultured with GBS-treated PBMC. A549 cells are known to express iNOS when treated with a mixture of IL-1, TNF-α, and IFN-γ (Cytomix) but poorly (14) or not at all (21) in response to the doses of any single cytokine in this mixture. Since GBS-treated PBMC are known to release all of these cytokines (12), indirect induction of iNOS was examined in A549 cells cocultured (using culture inserts) with human PBMC or culture-derived human macrophages. In addition to testing cocultures of A549 and PBMC, separate experiments tested the iNOS response of A549 cells when cultured in the presence of conditioned medium from separately cultured PBMC. Based on the timing of detectable induced NO levels and iNOS mRNA in Cytomix-treated A549 cells, samples from coculture experiments were collected at 24 h.

Secreted NO in the culture medium and iNOS gene expression were undetectable in untreated A549 cultures, A549 cultures treated directly with GBS, A549 cocultures with uninfected macrophage or PBMC, and A549 cocultures with only GBS-inoculated culture medium in the culture insert. Nitrite was not detectable in conditioned medium from GBS-treated PBMC or GBS-treated macrophages. Only when A549 cells were cocultured with GBS-treated PBMC or were treated with supernatant fluid collected from separately cultured GBS-treated PBMC was nitrite accumulation in the medium (mean ± the standard deviation of five experiments = 2.8 ± 1.9 μM at a GBS [strain COH1]/PBMC treatment ratio of 1:1) and iNOS gene expression readily detectable (Fig. 3 and 4). Culture medium supernates from PBMC treated at a GBS/mononuclear cell treatment ratio of 1:1 reproducibly induced NO and iNOS in A549 cells in five experiments with mononuclear cells from five different donors. Induction of iNOS mRNA with a GBS (strain COH1)/mononuclear cell treatment ratio of 10:1 was found in four of four experiments; however, NO production (nitrite accumulation in the medium) was detected in only two of four experiments at this dose. Conditioned medium generated with either COH1 or with the isogenic nonencapsulated mutant strain COH1-13 (1:1 treatment ratio of GBS/mononuclear cell) was effective at inducing NO production in A549 cells (mean value from combined experiments with two donors [duplicate cultures of each] of 4.4 ± 1.7 and 3.7 ± 1.2 μM nitrite with COH1 versus COH1-13, respectively).

Changes in epithelial cell morphology. Whereas the relationship to iNOS induction is unknown, 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).

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 cocultured with GBS-treated adherent culture-derived human macrophages, indicating that macrophages alone do not produce the factor or combination of factors responsible. Since GBS-stimulated human monocytes/macrophages are known to produce two (IL-1 and TNF-α) of the three components in Cytomix, the A549-macrophage coculture experiment was repeated with the addition of the third Cytomix component (10 ng of human IFN-γ/ml). IFN-treated A549-macrophage cocultures also failed to release NO or express iNOS in response to GBS treatment (three experiments). This indicates that the factor(s)
FIG. 4. iNOS mRNA induction in A549 cells cocultured with GBS-treated human mononuclear cells. A total of 5 × 10⁶ 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.

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 (O₂⁻) promotes formation of peroxynitrite (ONOO⁻),...
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 tubercle 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 fail 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.

ACKNOWLEDGMENTS

We are grateful for the technical assistance of John Price in performing the RT-PCR procedures. This work was supported by the Ohio University College of Osteopathic Medicine.

REFERENCES


Editor: E. I. Tuomanen