CONCISE COMMUNICATION

Synergistic Action of Nitric Oxide Release from Murine Macrophages Caused by Group B Streptococcal Cell Wall and β-Hemolysin/Cytolysin

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Group B streptococcus (GBS) is the leading cause of sepsis in neonates. Nitric oxide (NO) release plays a role in the hypotension that characterizes septic shock. It has been shown that GBS β-hemolysin/cytolysin (β-h/c) stimulates the transcription of inducible NO synthase (iNOS) in murine macrophages via intracellular pathways similar to those that mediate lipopolysaccharide-induced iNOS activation. Here, it is demonstrated that the GBS cell wall and β-h/c act synergistically to induce iNOS in interferon (IFN)–α–primed RAW 264.7 murine macrophages. In nonprimed macrophages, combined activation by the GBS cell wall plus β-h/c is necessary to induce an NO response, which indicates that both virulence factors cooperate to substitute for the priming signal typically provided by IFN-α.

Group B streptococcus (GBS) is the leading cause of pneumonia, meningitis, and septic shock in newborns, particularly those born prematurely. All clinical isolates are encapsulated, and 98%–99% are β-hemolytic [1]. GBS β-hemolytic activity is mediated by a pore-forming cytotoxin, the genetic basis of which has recently been elucidated [2]. In vitro, the GBS β-hemolysin/cytolysin (β-h/c) is associated with injury to lung epithelial, lung endothelial, and brain endothelial cells and is thus speculated to contribute to GBS penetration of host cellular barriers. Very recently, we reported that β-h/c is responsible for high mortality and liver injury in a rabbit model of GBS septic shock [3]. The hypotension of septic shock is thought to be due, in part, to an excess production of nitric oxide (NO), because elevated NO levels are found in patients with sepsis. Three isoforms of NO synthases (NOS) are present in mammals, the high-output inducible NOS (iNOS) and 2 constitutive isoforms, one originally identified in neurons and the other in endothelial cells. Activated macrophages constitute a major source of NO production. The inflammatory activation of macrophages is thought to involve 2 sequential steps, priming and triggering [4]. Priming is initiated by binding of interferon (IFN)–α to its specific receptor, which results in a number of biochemical and functional alterations that render the macrophages sensitive to triggering agents such as proinflammatory cytokines, lipopolysaccharide (LPS) [4], or gram-positive bacterial cell-wall components [5]. Large, constant amounts of NO are released by activated macrophages after iNOS induction, which accounts in part for the antimicrobial properties of these cells. In animal models of septic shock, circulatory failure has been associated with enhanced NO production, most conspicuously via iNOS [6]. We have reported elsewhere that β-hemolysin is chiefly responsible for GBS-mediated transcriptional induction of iNOS in murine macrophages [7], which suggests that β-h/c may exhibit proinflammatory properties relevant in the sepsis cascade. Because cell-wall components from other gram-positive organisms, peptidoglycan and lipoteichoic acid, are known to induce the release of NO from iNOS in murine macrophages [5, 8], we sought to investigate whether cell-wall preparations derived from a nonhemolytic GBS mutant might synergize with β-h/c to induce NO in murine macrophages.

Materials and Methods

Materials. Dulbecco's minimal essential medium, L-glutamine, fetal bovine serum containing <0.1 endotoxin units (EU)/mL, and the Limulus amebocyte lysate assay were purchased from BioWhittaker. Recombinant mouse IFN-α, aprotinin, sodium vanadate, leupeptin, Triton-X 100, phenylmethylsulfonyl fluoride, the assay kit for lactate dehydrogenase (LDH), the components of the Griess reagent, and the casein plus yeast medium constituents [7] were purchased from Sigma Chemical.

Bacterial strains. COH1-20 (nonhemolytic) and IN40 (hyperhemolytic) isogenic derivatives of wild-type GBS strain COH1 have been described elsewhere, with each of them containing a single insertion of Tn916ΔE into their chromosome [3, 9]. The transposon insertions in these mutants have been mapped [3] and lie in chromosomal loci distinct from the operon encoding the putative GBS hemolysin structural gene, *cylE* [2]. The loss of in vitro cytolytic

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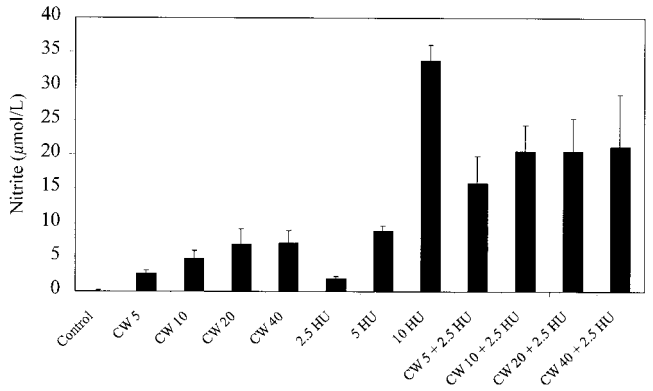

Figure 1. Nitrite formation in RAW 264.7 murine macrophages primed with 2.5 U/mL interferon (IFN)–α for 4 h prior to treatment with group B streptococcus (GBS) β-hemolysin/cytolysin (β-h/c) extracts, purified cell wall (CW) from the nonhemolytic GBS mutant COH1-20, or both for 42 h. The activity of β-h/c ranged from 2.5 to 10 hemolytic units (HU), determined as described in Materials and Methods. The nos. after CW in the figure indicate concentration of CW (5–40 μg/mL).

activity and in vivo virulence potential in the nonhemolytic transposon mutant COH1-20 and a targeted COH1 *cyl*E knockout are comparable [2, 3]. Bacteria were grown as described elsewhere [3].

Cultures and assays. Murine macrophage cell cultures, nitrite assay, LDH assay, Western blot analysis, preparation of stabilized hemolysin extracts, and assay for hemolytic activity were performed as described elsewhere [7].

Preparation of GBS cell wall. GBS cell wall was prepared using a modification of the method reported by Tuomanen et al. [10]. Strain COH1-20 was grown in 1-L batches of casein and yeast medium [7] to a concentration of $\sim 2.5 \times 10^8$ cells/mL. After centrifugation at 3000 *g,* the bacteria were resuspended in 40 mL of ice-cold 50 mmol/L Tris-HCl (pH, 7.0). This suspension was slowly added to 120 mL of a 5% SDS solution and boiled for 15 min. The suspension was then centrifuged at 10,000 *g* for 10 min at ambient temperature and cooled to 4°C overnight. The pellets were washed twice in 1 mmol/L NaCl and 3 times in distilled water and were mixed with an equal volume of 100-μm glass beads. This mixture was vortexed for 1 min, followed by 1 min of sonicating. This treatment was repeated 10 times before the glass beads were separated by sedimentation. Next, the suspension was centrifuged twice at 3000 *g* for 5 min at room temperature to remove unbroken cells, and both supernatants were combined and centrifuged at 10,000 *g* for 15 min to pellet the cell-wall material. The pellet was resuspended in 100 mmol/L Tris-HCl (pH, 7.5) plus 20 mmol/L $MgSO_4$, 10 μg/mL DNAse I, and 50 μg/mL RNAse and was incubated for 2 h at 37°C. Subsequently, trypsin at a final concentration of 100 μg/mL and $CaCl_2$ at 10 mmol/L were added for further incubation for 10–12 h at 37°C. All enzymes were from Worthington Biochemicals. Next, the cell walls were centrifuged at 10,000 *g* for 30 min, resuspended in 20 mL of 1% SDS, and treated at 60°C–80°C for 15 min. Detergent was removed by 8 cycles of washing. After centrifugation at 10,000 *g* for 30 min, the pellet was dried, weighed, and stored in 100 μL PBS at −70°C. The preparation did not contain any detectable contaminating endotoxin (<0.05 EU/mL), as assessed by the Limulus amebocyte lysate test.

Results

Cell wall and β-hemolysin extracts dose-dependently induce NO release from IFN-α–primed RAW 264.7 cells. RAW 264.7 cells were primed with 2.5 U/mL IFN-α for 4 h prior to incubation for 42 h with purified cell wall obtained from the β-h/c–negative GBS mutant COH1-20. Figure 1 shows that cell-wall preparations induced NO release in a dose-dependent manner, as assessed by the Griess reaction. The highest nitrite concentration in the supernatant was measured after incubation with 40 μg/mL cell wall. However, the absolute amount of NO release induced by cell wall even at these high concentrations was moderate (nitrite accumulation, ≤8.1 μmol/L) (figure 1). There was no evidence of cytotoxic effects from GBS cell-wall preparations at any time point or concentration, as determined by the LDH release assay (data not shown).

Starch-stabilized β-h/c extracts, obtained from the hyperhemolytic mutant IN40, dose-dependently induced NO release from IFN-α–primed RAW 264.7 cells up to a hemolytic activity of 10 hemolytic units (HU) (figure 1). β-h/c preparations containing >10 HU displayed cytotoxic properties, resulting in diminished NO production (not shown). The peak amount of nitrite, induced by β-hemolysin at 10 HU, was 4-fold higher than that induced after maximal activation with β-h/c–free cell wall, indicating that the secreted β-h/c toxin is a highly potent and the cell wall only a moderately potent inducer of NO in murine macrophages.

Cell wall and β-h/c act synergistically to induce NO production in IFN-α–primed RAW 264.7 macrophages. The induction of NO by cell wall after 42 h was significantly potentiated by simultaneous incubation with β-h/c preparations, even at concentrations as low as 2.5 HU, which individually induced only small amounts of NO (figure 1). This synergistic effect resulted in nitrite accumulation greater than twice the calculated additive amounts, indicating that cell wall and β-h/c at submaximal concentrations synergistically induce maximum NO release.

Induction of NO release from IFN-α–primed RAW 264.7 cells is due to transcriptional activation of iNOS. Primed macrophages were treated with 10 μg/mL β-h/c–free cell wall, β-h/c preparations containing 2.5 HU, or both for 42 h before they were lysed, separated on a 10% SDS gel, and blotted to nitrocellulose for detection of iNOS using monoclonal antibodies. The blots show that the synergistic induction of NO by cell wall and β-h/c is reflected in a similar increase of cellular iNOS protein, compared with cells treated with either of these virulence factors alone (figure 2).

Synergism of cell wall and β-h/c is required to induce NO in nonprimed RAW 264.7 cells. In contrast to IFN-α–primed RAW 264.7 macrophages, cell wall, and β-h/c individually did not induce NO release to any significant degree in naive, nonprimed macrophages after 42 h of incubation (40 μg/mL cell wall, 0.59 ± 0.2 μmol/L; 10 HU, 0.78 ± 0.58 μmol/L). However, when both virulence factors at these maximum concentrations were combined to treat naive RAW 264.7 macrophages

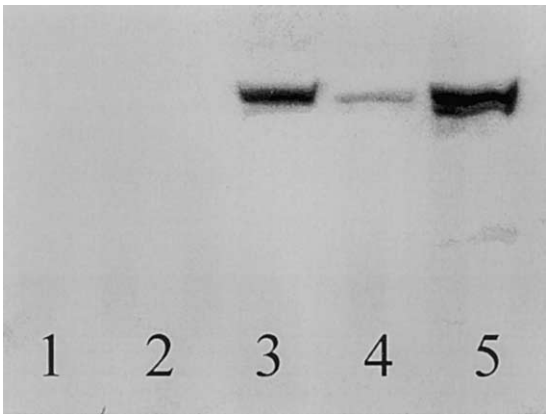

Figure 2. Expression of inducible nitric oxide synthase in RAW 264.7 cells after the following treatments: *lane 1,* control, measured after 46 h; *lane 2,* priming with 2.5 U/mL interferon (IFN)–α for 4 h, measured after additional 42 h of incubation; *lane 3,* priming with 2.5 U/mL IFN-α for 4 h followed by treatment with 5 hemolytic units (HU) of β-hemolysin/cytolysin (β-h/c) for 42 h; *lane 4,* priming with 2.5 U/mL IFN-α for 4 h followed by treatment with 10 μg/mL of cell wall for 42 h; and *lane 5,* priming with 2.5 U/mL IFN-α for 4 h followed by treatment with 10 μg/mL of cell wall plus 5 HU of β-h/c for 42 h.

for 42 h, a moderate concentration of nitrite was detectable in the supernatant (17.6 $\pm$ 4.6 μmol/L). This amount is equal to 52.1% of what β-hemolysin (10 HU) and 250% of what cell wall (40 μg/mL) induce in IFN-α–primed RAW 264.7 cells, which indicates that β-h/c and cell walls can synergize to substitute for the priming signal typically provided by IFN-α.

Discussion

Our study demonstrates that cell walls and β-h/c from GBS synergize to induce iNOS protein and the release of NO in IFN-α–primed and naive RAW 264.7 murine macrophages. In the absence of IFN-α priming, treatment with either cell wall or β-h/c alone did not result in any significant NO production. Our findings with the GBS cell wall corroborate recent reports that have shown that pneumococcal cell wall [5] and the purified cell-wall compound, lipoteichoic acid, of *Staphylococcus aureus, Bacillus subtilis,* and *Streptococcus sanguis* [8] require priming by IFN-α before they induce iNOS in macrophages. In our study, priming of RAW 264.7 macrophages was achieved by a relatively low concentration of IFN-α, which did not itself induce any expression of iNOS. However, the cells could be fully activated—that is, induced to express iNOS and release high amounts of NO—by exposure to the GBS β-h/c at subcytolytic concentrations. In contrast, the purified cell wall obtained from the β-h/c–negative mutant COH1-20 was a 4-fold less potent inducer of NO release in IFN-α–primed RAW 264.7 macrophages. This difference of the proinflammatory potency confirms our previous observations that β-h/c determines the propensity of GBS to cause NO release from macrophages [7] and high mortality from GBS sepsis in vivo [3]. The precise contribution of β-h/c-induced NO production to GBS virulence in the mouse model will be the focus of future studies. It is known that β-h/c contributes significantly to mortality in adult mice in association with the production of proinflammatory cytokines such as IL-1α and IL-6 [11]. Expression of these 2 cytokines typically reflects activation of NF-κB pathways, and NF-κB activation has recently been shown to be an early step in the induction of iNOS gene expression by GBS [12].

Our study shows for the first time that the GBS cell wall is indispensable as a cofactor of β-h/c for the induction of iNOS in naive macrophages and might therefore play a significant pathophysiological role in pathogenesis of the sepsis syndrome. Because neither the cell wall nor β-h/c individually induced any significant NO release from nonprimed RAW 264.7 cells, the cooperative action of both GBS virulence factors appears to involve both priming and triggering mechanisms. The molecular basis of the cell wall's action has not been delineated; however, binding to the complement receptor 3 (CR3) might play a role, because both phagocytosis of GBS and GBS-mediated NO release in murine macrophages depend on CR3 [13], and opsonized streptococcal cell wall has been shown to bind this member of the integrin receptor family [14].

Wild-type GBS strains (with cell walls and producing β-h/c) induce iNOS in RAW 264.7 cells via intracellular pathways similar to those that mediate LPS-induced iNOS activation, including tyrosine kinases and p38 [7]. LPS has recently been described to synergize with *S. aureus* or *B. subtilis* cell wall to induce NO, TNF-α, organ injury, and septic shock in vivo [15]. This suggests that GBS β-h/c might use the same signal transduction pathways as LPS to cooperate with the cell wall as a proinflammatory agonist.

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