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Group B Streptococcus-Induced Nitric Oxide Production in Murine Macrophages Is CR3 (CD11b/CD18) Dependent

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Nitric oxide (NO) is produced by murine macrophages in response to cytokines and/or gram-negative bacterial lipopolysaccharide. NO induction by gram-positive bacteria such as group B streptococci (GBS), the major etiologic agents of neonatal pneumonia and meningitis, has received little study. GBS as well as two other gram-positive bacterial species, *Staphylococcus aureus* and *Staphylococcus epidermidis*, were found to stimulate NO production in thioglycolate-elicited murine macrophages and in the mouse macrophage cell line J774A.1 in the presence of gamma interferon. Serotype Ia and III GBS were both stimulatory, as were asialo- and type antigen-deficient mutant strains of type III GBS. NO production was dose dependent, inhibitable by L-arginine analogs, and unaffected by polymyxin B. Since phagocytosis by murine and human phagocytes of GBS is dependent on complement receptor type 3 (CR3), the role of CR3 in the NO response to GBS was tested in the CR3-deficient myelomonocytic cell line WEHI-3. GBS did not induce NO, whereas *S. aureus* or lipopolysaccharide did induce NO in WEHI-3 cells. *S. epidermidis*, whose nonopsonic phagocytosis is also CR3 dependent, failed to induce NO in WEHI-3 cells. Monoclonal anti-CR3 (anti-CD11b or anti-CD18) in the presence of interferon also induced NO production in thioglycolate-elicited macrophages and in J774A.1 cells but not in WEHI-3 cells. This evidence suggests that ligated CR3 and gamma interferon act synergistically to induce NO production and that CR3 mediates the GBS-induced signal for NO production in interferon-treated macrophages.

Nitric oxide (NO) synthase is inducible in murine macrophages by cytokines and/or gram-negative bacterial lipopolysaccharide (LPS) (18). There is a strong synergy between agents which stimulate NO synthesis. Inducible NO synthase in macrophages generates large amounts of NO during inflammatory reactions and during infection (18). NO plays an important antimicrobial role in various bacterial (3, 30), fungal (9), and parasitic (18) infections, probably via inhibition of iron-containing enzymes and/or formation of additional toxic radicals by phagocytes following its combination with O_2^- radicals (18). In addition to LPS, other microbial components such as zymosan (36) and malarial antigens (12) can induce NO in murine macrophages.

The gram-positive pathogens *Staphylococcus aureus* and *Listeria monocytogenes* have each been demonstrated to act in synergy with gamma interferon to induce NO synthesis in macrophages (3, 36). NO production is implicated in antimicrobial defenses to both of these organisms (14, 36). How these bacteria trigger NO production is unknown; however, phagocytosis, even of inert latex beads, has been shown to signal NO production in interferon-stimulated mouse macrophages (6).

The ability of other gram-positive pathogens such as group B streptococci (GBS), the major etiologic agents of neonatal pneumonia and meningitis, to stimulate NO in vitro has not been examined; however, plasma nitrite is elevated in newborn infants with sepsis by either gram-positive or gram-negative bacteria (27). The specific aim of this study was to determine if nonopsonic phagocytosis of GBS could induce NO synthesis in gamma interferon-treated macrophages. These studies demonstrate GBS synergy with gamma interferon in the induction of NO in mouse macrophages and in the mouse macrophage cell line J774A.1.

Phagocytosis of GBS is dependent on complement receptor type 3 (CR3 or CD11b/CD18) present on the macrophage membrane (2, 29). The role of CR3 in phagocytosis-induced NO production has not been previously examined. These studies utilized a CR3-deficient cell line to address the role of CR3 in GBS-induced NO responses. GBS did not stimulate NO synthesis in the CR3-deficient cell line WEHI-3, which produces large amounts of NO in response to LPS or *S. aureus*. The evidence suggests that CR3 mediates the GBS-induced signal for NO production in interferon-treated macrophages. Such a function for CR3 is consistent with its role in mediating phagocytosis and subsequent killing of GBS (2, 29).

MATERIALS AND METHODS

Bacteria. GBS strains 2871 (serotype III) and 5015 (serotype Ia) were clinical isolates provided by Mario Marcon (Children's Hospital, Columbus, Ohio). The highly encapsulated serotype III GBS strain COH-1 and the isogenic mutants of COH-1, COH 1-13 (lacking type III polysaccharide) and COH 1-11 (lacking capsular sialic acid [7]) were provided by Craig E. Rubens (University of Washington School of Medicine, Seattle). *S. aureus* (protein A negative; ATCC 10832) and *Staphylococcus epidermidis* (ATCC 12220) were obtained from the American Type Culture Collection, Rockville, Md. Bacteria were grown in Todd-Hewitt broth (Difco, Detroit, Mich.) overnight at 37°C, harvested by centrifugation (10,000 × *g*, 15 min), and washed three times in phosphate-buffered saline (PBS). GBS suspensions were heat killed at 60°C (30 min). Staphylococcal suspensions were heat killed at 60°C for 45 to 60 min. Heat killing was confirmed by absence of growth on sheep blood agar culture plates inoculated with bacterial suspensions. Bacterial numbers in heat-killed suspensions were determined by direct microscopic count, using a Petroff-Hausser counting chamber.

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Macrophages. The mouse continuous cell lines J774A.1 (monocyte-macrophage, BALB/c [20]) and WEHI-3 (myelomonocyte, BALB/c [21, 33]) were obtained from the American Type Culture Collection. Cell lines were inoculated into 96-well culture dishes (5×10^4 viable cells per well) and cultured overnight prior to addition of bacterial treatments. Mouse macrophages were obtained by peritoneal lavage of CO_2-asphyxiated BALB/c mice (adult male or female, bred in-house) with 5 ml of Dulbecco calcium- and magnesium-free PBS plus 2 mg of glucose per ml. Elicited peritoneal macrophages were collected 4 days after intraperitoneal injection of 1 ml of thioglycolate broth (Difco). Peritoneal macrophage numbers were determined on the basis of total viable and differential cell counts. Peritoneal macrophages were purified by adherence to 96-well culture plates (10^5 macrophages per well) for 2 h followed by three washes with medium to remove nonadherent cells. Adherent peritoneal macrophages were cultured overnight and washed again three times prior to addition of bacterial treatments. Cell lines and macrophages were cultured in Dulbecco modified Eagle medium (DMEM) with 4,500 mg of glucose (GIBCO, Grand Island, N.Y.) per liter supplemented with 10% (vol/vol) fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.) and antibiotic-antimycotic (GIBCO) in a humidified atmosphere with 5% CO_2 at 37°C. DMEM contains 84 mg of L-arginine per liter as supplied by the manufacturer.

Antibodies. Rat anti-mouse CD11b (immunoglobulin G subclass 2b [IgG2b]; M1/70.15.11.5 cell line), rat anti-mouse CD18 (IgG2a; M18/2.a.8 cell line), and rat anti-mouse FcRII (rat IgG2b; 2.4G2 cell line) were purified by ammonium sulfate precipitation (10) from conditioned protein-free medium (Protein-Free Hybridoma Medium; GIBCO) of cultured hybridomas obtained from the American Type Culture Collection. Ammonium sulfate precipitates were dialyzed into PBS, concentrated, and filter sterilized. Purified rat IgG2b was obtained from Pharmingen (San Diego, Calif.).

Reagents. LPS (*Escherichia coli* O26:B6; trichloroacetic acid extract [Sigma]) stock solution (1 mg/ml) was suspended in PBS, sonicated, and filter sterilized. Stock solutions of recombinant mouse gamma interferon (Genzyme Corp., Cambridge, Mass.) were suspended in DMEM culture medium (2,000 U/ml), aliquoted, and stored at −135°C.

Gamma interferon was used at a final concentration of 10 U/ml. N^G-Monomethyl-L-arginine (Calbiochem, La Jolla, Calif.) was suspended in DMEM (5 mM) and used at a final concentration of 0.5 mM. N^G-Nitro-L-arginine methyl ester (Calbiochem) was suspended in DMEM (10 mM) and used at a final concentration of 2 mM. Polymyxin B sulfate (Sigma) was prepared freshly at 1 mg/ml in DMEM, filter sterilized, and used at a 10-μg/ml final concentration. Culture medium, PBS, and reagents (except LPS) at their final concentrations were free of endotoxin contamination at a sensitivity level of 10 pg/ml as determined by a *Limulus* amebocyte lysate assay (Pyrogent; BioWhittaker, Walkersville, Md.). As a control for unknown sources of endotoxin contamination, experiments (except those with added LPS) were duplicated in the presence of 10 μg of polymyxin B per ml. This level of polymyxin completely inhibited NO production in macrophages treated with 10 ng of LPS and 10 U of gamma interferon per ml.

EC3bi. EA (erythrocyte-antibody complexes) and EC3bi (erythrocyte-antibody complement complexes) were prepared by the sequential addition of anti-sheep erythrocytes (IgM) or anti-sheep erythrocytes and complement (C5-deficient mouse serum) to sheep erythrocytes (22). EC3bi preparations formed rosettes with mouse macrophages and were free of EC3b complexes as shown by the absence of rosette formation with human erythrocytes (22). EA and EC3bi were added to macrophages for NO experiments at 50 erythrocytes per macrophage.

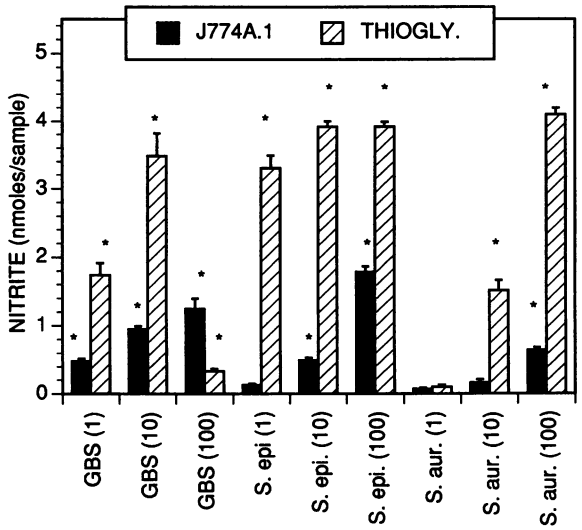

FIG. 1. NO induction by GBS, *S. epidermidis* (S. epi), and *S. aureus* (S. aur.) in gamma interferon-treated J774A.1 cells and thioglycolate-elicited (THIOGLY.) mouse macrophages. Macrophages were treated with 1, 10, or 100 CFU of heat-killed bacteria per cell. The GBS strain was serotype III 2871. Columns represent mean values ± standard deviations (eight samples per group) of accumulated nitrite in culture medium. Replicate experiments gave similar data. An asterisk (*) indicates a significant difference ($P < 0.05$) from untreated control or interferon-only treatment groups.

NO assay. Medium from overnight cultures of macrophage cell lines and mouse macrophages in 96-well culture plates was aspirated and replaced with medium (0.1 ml per well) containing bacterial treatments with or without gamma interferon. Macrophages were incubated in the presence of treatments at 37°C in humidified air with 5% CO_2 for 24 h. NO synthesis was determined colorimetrically as the accumulation of nitrite (NO_2^-) in macrophage culture medium. Briefly, 50-μl aliquots of conditioned medium were mixed with 50 μl of H_2O and 100 μl of Greiss reagent (1:1 [vol/vol] 0.02% *N*-[1-naphthyl]ethylenediamine dihydrochloride [Sigma] in H_2O–1% sulfanilamide [Sigma] in 3 N HCl) in flat-bottomed, 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.). Values shown in the figures represent nanomoles of accumulated nitrite per 0.05 ml of the medium from each sample well (0.1 ml of total medium per well). Nitrite concentration was determined from a standard curve generated with sodium nitrite ($NaNO_2$).

Statistics. Statistical differences between means of treatment groups were analyzed by either Student's t test or analysis of variance.

RESULTS

GBS-induced NO. Serotype III GBS strain 2871 was tested for the ability to induce NO in gamma interferon-stimulated macrophages. Thioglycolate-elicited macrophages and J774A.1 cells treated for 24 h with a combination of gamma interferon and heat-killed GBS at 1, 10, or 100 CFU per macrophage exhibited significant nitrite accumulation in the culture medium compared with untreated controls ($P < 0.05$) (Fig. 1). Nitrite levels increased with increasing doses of GBS

in gamma interferon-treated J774A.1 cells. Thioglycolate-elicited macrophages exhibited declining nitrite levels at 100 CFU of GBS per macrophage. Macrophages in all treatment groups appeared viable (>95% viable in all samples as shown by exclusion of trypan blue) at the time of nitrite assay. Similar induction of NO in gamma interferon-treated macrophages was found with *S. aureus* (Fig. 1) and 1 ng of LPS per ml (data not shown), stimulants previously shown to induce NO. *S. epidermidis* (Fig. 1) was also stimulatory. Type III GBS alone (100 CFU per macrophage) and *S. epidermidis* (100 CFU per macrophage) alone induced minimal but detectable levels of NO in both J774A.1 cells and thioglycolate-elicited macrophages (representative data of multiple experiments in thioglycolate cells: 0.048 ± 0.017 for untreated versus 0.205 ± 0.043 for GBS-treated versus 0.16 ± 0.025 nmol per sample for *S. epidermidis*-treated cultures, seven samples per group; $P < 0.05$ between treated and untreated groups). Lower GBS doses alone, *S. aureus* treatments alone, or LPS alone did not induce NO in macrophages. Macrophages treated with gamma interferon alone produced little (<0.1 nmol per sample in J774A.1 cells) or no nitrite above levels in untreated cells. Basal levels of nitrite in untreated cultures (representative of all experiments) were 0.031 ± 0.013 and 0.051 ± 0.019 nmol per sample for J774A.1 cells and thioglycolate-elicited macrophages, respectively. Stimulated thioglycolate-elicited macrophages produced significantly more ($P < 0.05$) nitrite than did stimulated J774A.1 cells for all treatments. Addition of polymyxin B (10 μg/ml) to macrophage cultures did not inhibit nitrite accumulation in GBS- or staphylococcus-treated cultures, indicating that these responses were not due to contaminating LPS. These results clearly demonstrate that GBS can synergize with gamma interferon to induce NO, as has been reported previously for other bacteria.

To determine if nitrite accumulation induced by GBS and gamma interferon is due to NO synthase, the arginine dependence of these responses was tested.

Inhibition of GBS-induced NO with L-arginine analogs. Addition of N^G-monomethyl-arginine to J774A.1 cultures treated with gamma interferon and GBS (strain 2871; 100 CFU per macrophage) inhibited nitrite accumulation by 76% (76% ± 4% for three samples). N^G-Monomethyl-L-arginine (0.5 mM) and N^G-nitro-L-arginine methyl ester (2 mM) added to thioglycolate-elicited macrophages treated with gamma interferon and GBS (50 CFU of COH-1 per cell) inhibited nitrite accumulation by 82% ± 1% (eight samples) and 77% ± 5% (eight samples), respectively. Similar results were obtained in replicate experiments. These data indicate that the NO responses to GBS and gamma interferon are L-arginine dependent.

Since capsular polysaccharide has been correlated with phagocytosis and virulence of GBS (capsular content of strain 2871 is unknown), strains of GBS with known capsular differences were next compared for induction of NO.

Comparison of GBS capsular mutants for NO induction. The role of type III capsular polysaccharide in induction of NO was tested by use of the heavily encapsulated type III strain COH-1 and its isogenic mutants COH 1-11 (deficient in terminal sialic acid of type III polysaccharide) and COH 1-13 (deficient in type III polysaccharide) (7). All three strains could act in synergy with gamma interferon to induce NO in thioglycolate-elicited macrophages and J774A.1 cells (Fig. 2). COH-1 induced approximately twice as much NO as either mutant at all doses. At 1 CFU per macrophage, COH 1-11 was significantly less stimulatory ($P < 0.05$) than the other two strains at the same dose. Doses were limited to 50 CFU per macrophage since strains COH-1 and COH 1-13 exhibited reduced NO at 100 CFU per macrophage in thioglycolate-elicited macrophages but not in J774A.1 cells. The difference in absolute nitrite produced in response to strain 2871 (Fig. 1) versus COH-1 (Fig. 2) is interesting but cannot be interpreted without comparative data on their capsular content. A role for capsular antigen and particularly sialic acid in modulating the macrophage NO response is indicated, but type antigen was not necessary for NO induction since the type antigen-deficient strain COH 1-13 did induce NO. Type Ia GBS also induced NO responses with nitrite levels similar to those induced in thioglycolate-elicited cells by type III strain 2871 GBS at each dose (one experiment; data not shown). This indicates as well that GBS type antigen is probably not the key trigger for NO responses.

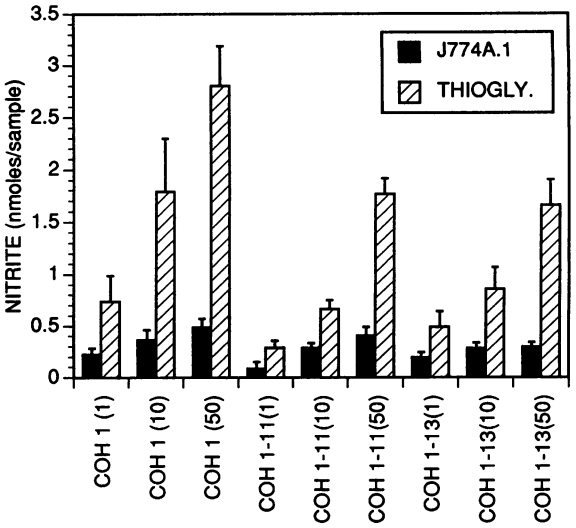

FIG. 2. NO induction by serotype III GBS strain COH-1 and its isogenic mutants COH 1-11 (sialic acid deficient) and COH 1-13 (type III antigen deficient) in gamma interferon-treated J774A.1 cells and thioglycolate-elicited (THIOGLY.) mouse macrophages. Macrophages were treated with 1, 10, or 50 CFU of heat-killed bacteria per cell. Columns represent mean values ± standard deviations (eight samples per group) of accumulated nitrite in culture medium. Replicate experiments gave similar data. All groups except COH 1-11 (1 CFU per macrophage) in J774A.1 cells were significantly different ($P < 0.05$) from untreated control or interferon-only treatment groups.

On the basis of our previous report that opsonin-independent phagocytosis of GBS is CR3 dependent (2), experiments were designed to test whether GBS-induced NO is also mediated via CR3.

Role of CR3 in GBS-induced NO response: effect of anti-CR3. Antibodies to CR3 (CD11b/CD18) inhibit macrophage or neutrophil phagocytosis of unopsonized GBS (2). CR3 is also necessary for efficient phagocytosis of GBS in the presence of opsonins (29). To investigate the role of CR3 in GBS-induced NO production, antibodies to murine CR3 were tested for inhibition of GBS-induced NO. M1/70 (anti-CD11b) and M18 (anti-CD18) at 100 μg/ml slightly, but significantly ($P < 0.05$), inhibited NO production in cultures receiving combined GBS and gamma interferon (Fig. 3). Control rat immunoglobulin (rat IgG2b, isotype matched with M1/70) was not inhibitory. The significance of this observation is difficult to interpret since either of the anti-CR3 antibodies (M1/70 or M18) and gamma interferon acted in synergy to induce NO without added GBS. M1/70 at 10 μg/ml or higher exhibited dose-dependent induction of NO in gamma interferon-treated macrophages. Control rat immunoglobulin (isotype matched with M1/70) also induced NO in gamma interferon-treated macro-

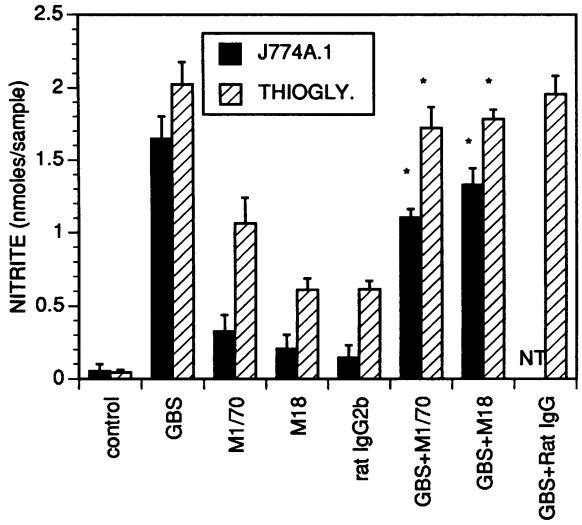

FIG. 3. Inhibition of GBS induction of NO in gamma interferon-treated J774A.1 cells and thioglycolate-elicited (THIOGLY.) mouse macrophages by monoclonal anti-CR3. Macrophages were treated with 100 CFU (J774A.1 cells) or 50 CFU (thioglycolate cells) of heat-killed serotype III GBS strain 2871 per cell. Monoclonal anti-CD11b (M1/70) or anti-CD18 (M18) was added to cultures at 100 μg/ml. Columns represent mean values ± standard deviations (eight samples per group) of accumulated nitrite in culture medium. Replicate experiments gave similar data. An asterisk (*) indicates a significant difference ($P < 0.05$) of antibody-treated groups from the respective control group without antibody. NT, not tested.

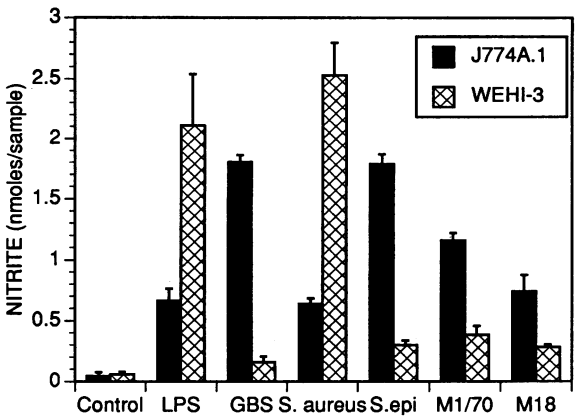

FIG. 4. Comparison of gamma interferon-treated J774A.1 and WEHI-3 cells for NO production in response to CR3-dependent and CR3-independent stimuli. Doses of treatments were as follows: LPS, 10 ng/ml; serotype III GBS strain 2871, 100 CFU per cell; *S. aureus*, 100 CFU per cell; *S. epidermidis* (S. epi.), 100 CFU per cell; M1/70 (anti-CD11b), 100 μg/ml; M18 (anti-CD18), 100 μg/ml. Columns represent mean values ± standard deviations (eight samples per group) of accumulated nitrite in culture medium. Replicate experiments gave similar data.

phages, but only at 100 μg/ml and at much lower levels than in M1/70-treated cultures (Fig. 3). The effects of isotype control antibody may be via interaction with macrophage FcR since the monoclonal anti-FcRII, 2.4G2 (rat isotype IgG2b), also synergized with gamma interferon to induce strong NO responses (data not shown). Macrophage treatment with anti-CR3 or control antibodies alone induced little or no detectable NO. Polymyxin B (10 μg/ml) included in cultures during the 24-h incubation did not alter NO responses to anti-CR3. These results imply that CR3 ligation, at least by antibody, may trigger NO production in synergy with gamma interferon in macrophages.

To further assess the role of CR3 in GBS-induced NO in macrophages, GBS were tested for the ability to induce NO in a CR3-deficient cell line.

Role of CR3 in GBS-induced NO response: NO induction in CR3-deficient WEHI-3 cells. WEHI-3 cells have been shown by this laboratory to be deficient in antigenic (shown by flow cytometry and immunoprecipitation) and functional (shown by erythrocyte-C3bi rosetting) CR3 and to exhibit markedly reduced nonopsonic phagocytosis of GBS (35; unpublished data). The mean percentages of phagocytosis (percentage of cells engulfing strain 2871 at 20 CFU per macrophage for 1 h under nonopsonic conditions) were 60% ± 14% for J774A.1 (mean of 10 separate experiments) and 2% ± 1% for WEHI-3 (mean of 3 separate experiments) cells. J774A.1 cells were compared for NO induction by LPS, GBS, M1/70, M18, *S. aureus*, and *S. epidermidis*, with and without added gamma interferon (Fig. 4). Both WEHI-3 and J774A.1 cells exhibited synergistic induction of NO by combination of gamma interferon with LPS or *S. aureus*. In contrast to J774A.1 cells, WEHI-3 cells did not exhibit synergistic induction of NO by combination of gamma interferon with GBS, M1/70, M18, or *S. epidermidis*. Isotype control antibody did not induce NO. Nonopsonic phagocytosis of *S. epidermidis*, but not of *S. aureus*, has been reported to be CR3 dependent (8, 24). The amounts of WEHI-3 cells engulfing *S. aureus* versus *S. epidermidis* were 28% ± 3% versus 4% ± 3%, respectively (four samples per group, representative of repeated experiments). WEHI-3 thus can produce large quantities of NO, much greater than J774A.1 cells, in response to gamma interferon and a costimulatory signal but fails to respond to known CR3-dependent stimuli. The inability of GBS to induce NO in WEHI-3 cells implies that CR3 ligation and/or CR3-mediated phagocytosis of GBS provides the signal for NO production.

To test the broad role of CR3 ligation versus CR3-mediated ingestion in NO responses, macrophages were next tested for their response to C3bi-coated erythrocytes. This experiment would also assess if ligation of CR3 in a manner which does not induce an oxidative burst would also not induce NO.

Macrophage response to EC3bi. Thioglycolate-elicited macrophages were challenged with C3bi-coated sheep erythrocytes (EC3bi), which do not trigger significant ingestion or a respiratory burst in phagocytes (23). EC3bi did not induce NO in interferon-treated macrophages (0.198 ± 0.054 nmol of nitrite per sample in the untreated control versus 0.165 ± 0.008 nmol of nitrite per sample in EC3bi-treated macrophages; four samples per group). Similar results were obtained in replicate experiments. These results show that CR3 ligation alone, at least via the C3bi site, does not provide signals necessary for NO production and that phagocytosis may also be required. These data also indicate that CR3 ligands which do not trigger a respiratory burst do not trigger NO responses and that not all CR3 ligands trigger NO.

DISCUSSION

GBS, the major etiologic agents of neonatal pneumonia and meningitis, were found to stimulate L-arginine-dependent NO production in thioglycolate-elicited murine macrophages and in the mouse macrophage cell line J774A.1 in the presence of gamma interferon. Serotype Ia and III GBS were both stimulatory. A sialic acid-deficient mutant and a type antigen-deficient mutant strain of type III GBS were active but less stimulatory than the fully encapsulated parent strain. Studies

of NO induction in macrophages treated with anti-CR3 and in a CR3-deficient macrophage cell line indicate that CR3-mediated phagocytosis of GBS signals NO production in interferon-treated macrophages.

GBS showed a pattern of NO induction similar to those of other previously described macrophage stimuli. A combination of GBS and gamma interferon produced a marked synergy with greatly enhanced NO production in macrophages. Synergy of several microbes with gamma interferon to induce NO has been attributed to induction of tumor necrosis factor alpha (TNF-α) in macrophages. Neutralizing antibodies to TNF-α will inhibit NO responses to gamma interferon in macrophages treated with *Leishmania* promastigotes (6), *Schistosoma mansoni* (19), and *Toxoplasma gondii* (13) but not with *L. monocytogenes* (3). Corradin et al. (6) suggest that phagocytosis enables gamma interferon to activate macrophages via the induction of TNF-α as an autocrine second signal. The role of TNF-α in GBS activation of macrophages was not examined in this study, but other authors have described TNF-α production in GBS-infected animals (31), in human infants with GBS sepsis or meningitis (34), and in GBS-treated cultured human monocytes (34).

NO production has been demonstrated to play an important antimicrobial role in murine defenses to many intracellular parasites and bacteria (18). In contrast, NO synthase is poorly or noninducible in human macrophages, indicating little or no role in antimicrobial defenses (18, 26). An antiparasitic function of NO may exist in human liver since human hepatic parenchymal cells exhibit strong NO production in response to cytokines and LPS (18). In addition to GBS, other gram-positive bacteria such as *L. monocytogenes* and *S. aureus* have been reported to act in synergy with gamma interferon to induce NO in murine macrophages (3, 36). A contribution of reactive nitrogen intermediates in the killing of *S. aureus* by human neutrophils has been suggested (14). The role of NO in animal or human immune responses to GBS infection is unknown; however, increased NO activity has been reported in GBS-infected piglets (25), and elevated plasma levels of nitrite present in infants with bacterial sepsis correlate with plasma TNF-α levels (27).

A signalling role for CR3 in NO induction by GBS was indicated by the failure of the CR3-deficient WEHI-3 cell line to respond to GBS stimulation. The lack of response to GBS was not a generalized defect, since gamma interferon-treated WEHI-3 cells were fully responsive to stimulation with LPS or nonopsonized *S. aureus*, whose interaction with macrophages is not CR3 dependent (8). Although LPS does interact with CR3 (CD11b/CD18), CD18 does not play a role in mediating LPS-induced transmembrane signalling (32). LPS stimulation of WEHI-3 cells must be via other macrophage receptors for LPS such as LPS-binding protein/CD14 (32). In support of the GBS data, WEHI-3 cells also failed to produce NO when exposed to nonopsonized *S. epidermidis*, whose uptake is dependent on CR3 (2, 24). In support of a role for phagocytosis in NO responses to bacteria, WEHI-3 cells engulf nonopsonized *S. aureus* but not nonopsonized GBS or *S. epidermidis*. Anti-CR3 (anti-CD11b or anti-CD18) could also act as one of two needed costimulatory signals for NO induction in CR3-expressing J774A.1 cells but not in CR3-deficient WEHI-3 cells. These data indicate that CR3 ligation by antibody or CR3 activation by GBS can trigger NO synthesis in interferon-treated macrophages. A similar signalling capacity of leukocyte integrins (lymphocyte function associated protein 1, CR3, and p150,95) has been reported for other phagocyte responses. TNF-α triggering of superoxide production in neutrophils can be primed by CD11/CD18-dependent adhesion of phagocytes (17).

The NO response of macrophages to GBS appears to be mediated through direct or indirect interaction of GBS with CR3 in a manner that triggers phagocytosis and primes macrophages for cytokine responses. Whereas phagocytosis and subsequent killing of GBS are dependent on CR3 (2, 29), direct binding of GBS to CR3 has not been proven. The ligand on GBS recognized by macrophages is also unknown, but studies by Sloan and Pistole (28) indicate that lectin-like recognition of capsular galactose by macrophages is not involved.

CR3-mediated phagocytosis rather than just CR3 binding appears to be required for the NO response to GBS; however, phagocyte responses mediated via CR3 may be more dependent on which site of CR3 is bound than on whether CR3 is bound (5). Cain et al. (5) report that for opsonized zymosan the C3bi site mediates binding, whereas ingestion and superoxide burst are triggered via a separate beta-glucan-binding site on CR3. In contrast to GBS, sheep erythrocytes coated with C3bi (EC3bi), which do not trigger significant ingestion or a respiratory burst in phagocytes (23), did not induce NO in macrophages. This indicates that CR3 ligands which do not trigger a respiratory burst also do not trigger NO responses and that only certain CR3 sites may trigger NO.

The ability of GBS to induce NO may simply reflect their relative phagocytosis by macrophages. The deposition of C3 on GBS, required for opsonic recognition by CR3, is regulated by the sialic acid-containing capsular polysaccharides of GBS (15). Phagocytosis of nonopsonized GBS is also thought to depend on recognition of capsular sugars possibly via the lectin-like site of CR3 (2, 29), and there was a correlation between relative phagocytosis of GBS strains and NO production (COH-1 > COH 1-13 > COH 1-11). This is an indication that capsular content influences the NO response, but since unencapsulated or encapsulated type III GBS, type Ia GBS, and *S. epidermidis* all induce CR3-dependent NO responses, GBS type-specific antigens are not essential to this response.

The pattern of phagocytosis we observed conflicts with the in vivo observation of Martin et al. (16) that neonatal rat alveolar macrophages more easily engulf nonencapsulated than encapsulated GBS. Several experimental differences may explain this conflict. Unknown in vivo opsonins as well as differential expression and use of complement receptors on alveolar macrophages (CR4 > CR3) versus peritoneal macrophages (CR3 > CR4) could markedly alter phagocytosis patterns in these two studies. Since TNF-α may be a necessary autocrine signal for NO responses, it is interesting to note as well that human monocytes exposed to encapsulated versus unencapsulated strains of type III GBS produce similar amounts of TNF-α (34).

Neonatal phagocytes exhibit reduced chemotactic activation which can be restored in the presence of gamma interferon (11). The full antimicrobial capacity of neonatal phagocytes for GBS may depend on the costimulatory activities of GBS (via CR3) and gamma interferon. Conditions of both reduced gamma interferon (4) and reduced antigenic and functional CR3 (1) in neonates would thus predispose neonates to GBS infection.

Recognition and engulfment of GBS are shown in this study to enhance responsiveness of phagocytes to secondary cytokine signals such as gamma interferon. Differential capacities of GBS strains to interact with CR3 and trigger costimulatory signals necessary for phagocyte activation by cytokines may distinguish virulent from avirulent organisms. Whereas the importance of NO in human defenses to GBS infection is unknown, these experiments reinforce the importance of CR3 in mediating phagocytic responses to GBS.

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