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Tumor Necrosis Factor Alpha Acts as an Autocrine Second Signal with Gamma Interferon To Induce Nitric Oxide in Group B Streptococcus-Treated Macrophages

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Nitric oxide production by mouse macrophages treated with group B streptococci and gamma interferon was inhibited by cytochalasin B or by antibody neutralization of macrophage-derived tumor necrosis factor alpha. Phagocytosis-induced tumor necrosis factor alpha is responsible for group B streptococcus-induced nitric oxide production in interferon-treated macrophages.

Group B streptococci (GBS) have been shown to stimulate nitric oxide (NO) production in murine macrophages in the presence of gamma interferon (7). CR3 (CD11b/CD18), required for opsonic and nonopsonic phagocytosis of GBS (1, 13), mediates the GBS-induced signal for NO production in interferon-treated macrophages (7); however, no direct evidence that phagocytosis is required for this response was provided in the study described in reference 7. Consistent with a general mechanism described by Corradin et al. (5), studies reported here show that phagocytosis of GBS enables gamma interferon to activate macrophages via the induction of tumor necrosis factor alpha (TNF-α) as an autocrine second signal. Demonstration of this mechanism for GBS indicates that autocrine priming of microbially stimulated macrophages for activation by interferon is not restricted to facultative intracellular parasites (2, 5, 8). The requirement for phagocytosis and the role of TNF-α in GBS activation of macrophages was examined in this study by testing the effects of cytochalasin B and neutralizing anti-TNF-α on GBS-induced NO production.

Thioglycolate-elicited mouse (BALB/c) peritoneal macrophages, J774A.1 and WEHI-3 macrophage cell lines (American Type Culture Collection), were cultured (200,000 cells added per well) in 96-well tissue culture plates 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) as previously described (7). Macrophage cultures were treated (per cell) with 10, 50, or 100 CFU of heat-killed serotype III GBS strain COH 1 (encapsulated) or its isogenic mutants COH 1-11 (sialic acid deficient) and COH 1-13 (type III polysaccharide deficient), provided by Craig E. Rubens (University of Washington School of Medicine, Seattle). NO synthesis was determined colorimetrically, by using Greiss reagent (7), as the accumulation of nitrite (NO_2^-) in macrophage culture medium after 24 h of treatment with GBS at 37°C in humidified air with 5% CO_2.

Similar to what has been reported for GBS-treated human monocytes (15), Fig. 1 shows that GBS-treated mouse macrophages also secrete TNF-α. TNF-α was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA) using anti-mouse TNF-α and biotin-labeled anti-mouse TNF-α in accordance with the manufacturer's protocol (Pharmingen, San Diego, Calif.). Purified recombinant mouse TNF-α (GIBCO BRL, Gaithersburg, Md.) was used as a standard. TNF-α responses were dependent on the dose of GBS and were detectable as early as 4 h after treatment with GBS (50 CFU per macrophage), with levels highest at 24 h and often declining at 48 h. Both living and heat-killed GBS, as well as capsule-deficient strains (COH 1-11 and COH 1-13), were active inducers of TNF-α (data not shown). Thioglycolate-elicited mouse macrophages produced 5- to 10-fold more TNF-α than reported for human monocytes at a similar dose of GBS (15). TNF-α production was not due to endotoxin contamination. Culture medium, phosphate-buffered saline, and reagents at their final concentrations were free of endotoxin contamination as determined by a *Limulus* amebocyte lysate assay (Pyrogent, BioWhittaker, Walkersville, Md.) with a sensitivity level of 10 pg/ml. As a control for unknown sources of endotoxin contamination, experiments were duplicated with mixtures containing 10 μg of polymyxin B per ml.

Table 1 demonstrates the phagocytosis dependence of both GBS-induced TNF-α and GBS-induced NO in interferon-treated macrophages (10 U of recombinant mouse gamma interferon per ml; Genzyme Corp., Cambridge, Mass.). NO production is dependent on the presence of both GBS and interferon (7), whereas GBS alone can induce TNF-α (Fig. 1). Cytochalasin B (Sigma) added simultaneously with other treatments inhibited both GBS-induced responses. Cytochalasin B at 5 and 10 μg/ml inhibited phagocytosis (assayed by direct microscopic counts of stained adherent macrophage cultures) of GBS by 76 and 97%, respectively, in replicate experiments. Partial inhibition by cytochalasin of NO responses (but not TNF-α) to a nonphagocytic signal, lipopolysaccharide (LPS) (*Escherichia coli* O26:B6, trichloroacetic acid extract; Sigma), indicates that cytochalasin inhibition of GBS effects is not solely related to inhibition of phagocytosis. Cytochalasin B binds glucose transporter proteins and inhibits macrophage transport of glucose and production of interleukin 1 in response to LPS (9). Inhibition of glucose transport and inhibition of cytoskeleton-dependent signals may both contribute to cytochalasin inhibition of NO responses to GBS plus interferon.

CD11b/CD18 (CR3) is required for phagocytosis of GBS, and macrophage cell lines lacking CR3 (WEHI-3) do not engulf GBS or produce NO in response to GBS and interferon (1, 7). The relationship between phagocytosis and TNF-α secretion in GBS-induced NO responses was further correlated by

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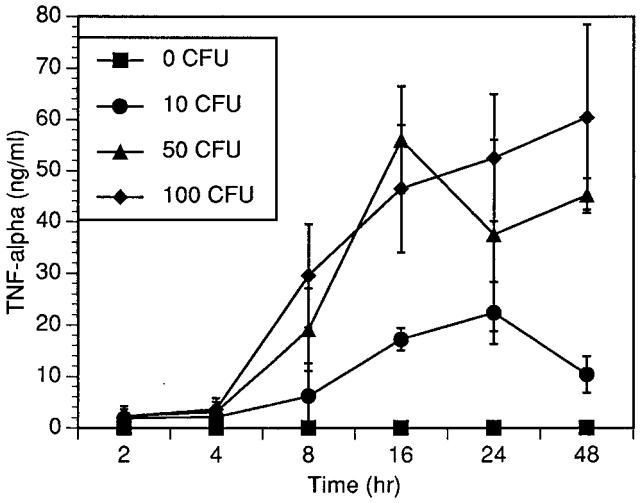

FIG. 1. Time course of TNF-α production by GBS-treated thioglycolate-elicited mouse peritoneal macrophages. TNF-α levels in conditioned medium from 24-h cultures of macrophages treated with heat-killed GBS strain COH 1 at 0, 10, 50, or 100 CFU per macrophage were measured by ELISA. Values represent means ± standard deviations (error bars) of results from duplicate experiments (two samples per time point in each experiment).

comparing the WEHI-3 and J774A.1 cell lines (expressing CD11b/CD18) for TNF-α secretion. GBS-treated WEHI-3 macrophages did not produce detectable TNF-α (assay sensitive to 400 pg of TNF-α per ml), whereas J774A.1 macrophages, which do engulf GBS and produce NO, secreted TNF-α in response to GBS but at a much lower level (844 ± 59 pg/ml at 24 h; four samples; representative experiment with 50 CFU of GBS per cell) than that in thioglycolate-elicited macrophages.

Synergy of several microbes with gamma interferon to induce NO has been attributed to induction of TNF-α in macrophages. Neutralizing antibodies to TNF-α will inhibit NO responses to gamma interferon in macrophages treated with *Leishmania* promastigotes (5), *Schistosoma mansoni* (10), and *Toxoplasma gondii* (8) but not in those treated with *Listeria monocytogenes* (3) or with LPS (5, 6). Table 2 demonstrates the requirement for secreted TNF-α in GBS-induced NO production by gamma interferon-treated macrophages. Increasing concentrations of neutralizing rabbit anti-mouse TNF-α (Genzyme), but not control normal rabbit serum, inhibited GBS-induced NO production. Consistent with reports by other investigators (5, 6), anti-TNF-α (5%, vol/vol) was found not to inhibit LPS (10 ng/ml)- and interferon (10 U/ml)-induced NO responses (anti-TNF-α-treated macrophages produced 97% ± 6% of the control-induced NO value [four samples, duplicate experiments]). Unlike the way it affected thioglycolate-elicited macrophages, anti-TNF-α did not inhibit the low-level NO responses of J774A.1 cells to GBS. This indicates that some signals of NO responses to GBS are not dependent on TNF-α. TNF-α-dependent high-level NO responses, as in thioglycolate-elicited macrophages, may reflect a differential state of macrophage activation or cytokine receptor expression in comparison with that in J774A.1 cells. Adding exogenous TNF-α to thioglycolate-elicited macrophages treated with cytochalasin plus GBS plus interferon partially restores NO production, indicating that separate phagocytic and TNF-α signals are not required (Table 3). The much stronger NO response to TNF-α plus interferon in the presence of GBS indicates that phagocytosis does provide additional signals or simply provides additional endogenous TNF-α. The effects of cytochalasin B and neutralizing anti-TNF-α on GBS-induced NO production in-

TABLE 1. Cytochalasin B inhibits GBS-induced NO and TNF-α production by thioglycolate-elicited mouse peritoneal macrophages

Treatment[a]	NO (nmol/sample)[b]	TNF-α (ng/ml)[b]
None	0	0
Cytochalasin at 5 μg/ml	0	0
Cytochalasin at 10 μg/ml	0	0
GBS + interferon	1.53 ± .37	11.5 ± 1.1
Cytochalasin at 5 μg/ml	0.11 ± .07[c]	0
Cytochalasin at 10 μg/ml	0.01 ± .04[c]	0
LPS + interferon	1.74 ± .29	7.9 ± 0.36
Cytochalasin at 5 μg/ml	1.01 ± .40[c]	9.7 ± 1.57
Cytochalasin at 10 μg/ml	1.09 ± .25[c]	8.4 ± 0.31

[a] Macrophages were treated with GBS strain COH 1 (10 CFU per cell), LPS (10 ng/ml), and gamma interferon (10 U/ml) for 24 h.
[b] Values represent means ± standard deviations for four samples per group. Results are representative of replicate experiments. GBS and interferon as single treatments induce little or no NO (0.033 ± 0.018 nmol for GBS only; 0.054 ± 0.032 nmol for interferon only).
[c] Significantly different from control value, $P < 0.05$, by Student's t test.

TABLE 2. Neutralizing anti-TNF-α inhibits GBS-induced NO production in interferon-treated thioglycolate-elicited mouse peritoneal macrophages but not in J774A.1 cells

Macrophage type and serum treatment (%, vol/vol)	Nitrite production (% control value)[a] in GBS + interferon-treated macrophages with:	
	Normal rabbit serum	Anti-TNF-α
Thioglycolate elicited		
0	100	100
1	99 ± 9	77 ± 9[b]
5	110 ± 7	47 ± 5[b]
10	105 ± 11	14 ± 1[b]
J774A.1		
0	100	100
10	100 ± 31	98 ± 14
20	82 ± 15	120 ± 7

[a] Values represent means ± standard deviations for nitrite production (nanomoles per sample, four samples per group) expressed as percentages of levels secreted by macrophages treated with GBS COH 1 (50 CFU per cell) and gamma interferon (10 U/ml). Similar results were obtained in a duplicate experiment.
[b] Significantly different ($P < 0.05$) from GBS + interferon control as calculated by Student's t test.

TABLE 3. Exogenous TNF-α partially restores NO production in cytochalasin B-inhibited macrophages treated with GBS and interferon

Treatment	Nitrite production (nmol/sample)[a] in macrophages treated with cytochalasin B at:		
	0 μg/ml	5 μg/ml	10 μg/ml
Medium only	0.03 ± 0.006	0.10 ± 0.03	0.06 ± 0.03
GBS + IFN	2.14 ± 0.49	0.10 ± 0.05[b]	0.08 ± 0.05[b]
TNF-α + IFN	0.40 ± 0.13	0.42 ± 0.2	0.35 ± 0.02
TNF-α + GBS + IFN	1.70 ± 0.6	0.49 ± 0.18[b]	0.42 ± 0.10[b]

[a] Values represent means ± standard deviations (four samples per group) for NO secreted by macrophages treated with GBS COH 1 (50 CFU per cell) and gamma interferon (IFN) (10 U/ml) or TNF-α (1,000 U/ml).
[b] Significantly different ($P < 0.05$) from value for non-cytochalasin-treated control group as calculated by Student's t test.

dicate that phagocytosis (versus only binding of GBS) and subsequent TNF-α production are required components of the NO response of macrophages to GBS.

The role of TNF-α and NO in responses to GBS infection is unknown; however, TNF-α is present in plasma of human infants with GBS sepsis or meningitis (15) and elevated levels of nitrite in plasma of infants with bacterial sepsis correlate with TNF-α levels (12). Increased NO activity has also been reported for GBS-infected piglets (11). TNF-α and interleukin 12 released from microbially stimulated macrophages have been shown to activate natural killer cell production of gamma interferon (2, 4), which works in synergy with TNF-α to activate macrophage killing of facultative intracellular parasites. Gamma interferon is present early after GBS infection of neonatal rats, and its induction requires TNF-α (14). Phagocytosis of GBS, as shown here, induces autocrine signals necessary for interferon induction of NO. Thus, in the response to GBS infection, as for infection with facultative intracellular parasites, if a T-cell-independent pathway of interferon generation exists, then synergistic cytokine activation of GBS-infected neonatal phagocytes may be an important innate defense mechanism. Although there are no studies that demonstrate a defense role of NO in humans or mice infected with GBS, NO was used here and in a previous study (7) as a marker of murine macrophage functional activation to show that GBS phagocytosis via CR3 triggers costimulatory signals necessary for phagocyte activation.

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