Survival of Group B *Streptococcus* Type III in Mononuclear Phagocytes: Differential Regulation of Bacterial Killing in Cord Macrophages by Human Recombinant Gamma Interferon and Granulocyte-Macrophage Colony-Stimulating Factor

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Phagocytic and killing capacities of resident and cytokine-activated human macrophages against group B *Streptococcus* (GBS) type III were studied. Evidence is presented that monocyte-derived macrophages from cord and adults ingest serum-opsonized GBS but that killing of bacteria was negligible in resident cells. Treatment of adult macrophages with recombinant human gamma interferon (rhIFN-γ; 100 U/ml) or recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; 200 U/ml) resulted in significant increases of killing of GBS ($P < 0.01$ for each). The killing capacity of cord macrophages treated with rhGM-CSF was also enhanced compared to that of untreated cells ($P < 0.01$). However, treatment with rhIFN-γ resulted in only a moderate increase in the capacity of cord macrophages to kill GBS ($P > 0.1$). These results mirrored the effect of rhIFN-γ on candidacidal capacities of cord and adult macrophages, reported earlier from our laboratory. These data indicate differential modulation of neonatal macrophages by rhGM-CSF and rhIFN-γ. We suggest that administration of rhGM-CSF to neonates with invasive GBS disease may enhance host resistance to these bacteria.

Group B streptococci (GBS) are the most common cause of deep-seated bacterial infection and sepsis in neonates (4, 20). Over the last decade, the burden of invasive GBS disease in immunocompromised children and adults has also been increasing (24). In adults, common predisposing conditions for severe GBS infection and sepsis include malignant neoplasms, diabetes mellitus, human immunodeficiency virus type 1 infection, trauma, and older age (6, 8, 18). The mortality of invasive GBS disease in both newborns and adults remains high despite advances in intensive care and the susceptibility of the pathogen to penicillin (4, 18, 20).

The propensity of GBS to cause invasive neonatal infections might be related to inappropriate opsonization due to the lack of maternally derived, type-specific antibodies (20). Polymorphonuclear neutrophil granulocytes play a key role in phagocytosis and eventual killing of streptococci and other extracellular pathogens. We reported earlier that a clinical isolate of GBS type III was rapidly ingested and killed by cord and adult granulocytes in the presence of serum opsonins (13). In contrast to what is found for granulocytes, the capacity of cord monocytes to kill GBS was decreased compared to that of adult blood monocytes (13). To gain more insight into the newborn's ability to resist infection by GBS, we studied various interactions between these bacteria and monocyte-derived macrophages isolated from the cord. Experiments were performed to study the effect of recombinant human gamma interferon (rhIFN-γ) and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) on phagocytosis

and the bactericidal capacities of cord and adult macrophages. We show here that cord macrophages efficiently phagocytose serum-opsonized GBS but that the ingested bacteria survive inside the cells. Bacterial killing was augmented by rhGM-CSF but not by rhIFN-γ, suggesting differential modulation of cord macrophages by these cytokines.

MATERIALS AND METHODS

Collection and preparation of sera. Whole blood was obtained from 15 healthy adults. Blood was allowed to clot at room temperature for 1 h. Next, blood was centrifuged at 4°C, and sera were removed and stored in aliquots at −70°C until use (12). Heat-inactivated serum was prepared by heating serum at 56°C for 30 min.

Monocyte-derived macrophages (MDM). Heparinized (10 U/ml) venous blood was obtained by venipuncture from healthy adult individuals. Cord blood (anticoagulated with 10 U of heparin/ml) was collected aseptically from the placental ends of the cut umbilical cords of healthy full-term neonates. Mononuclear cells were separated by differential centrifugation of 5 ml of heparinized blood on a gradient of lymphocyte separation medium (Organon Teknika, Durham, N.C.). After centrifugation and washes in Krebs-Ringer phosphate buffer containing 0.2% glucose (pH 7.34) (KRPD), the cell suspension contained <0.3% contaminating granulocytes. Viability of mononuclear cells before culture was >97% (trypan blue exclusion). The washed suspension of mononuclear cells was resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, N.Y.) with 2 mM L-glutamine and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated autologous serum. The suspension was adjusted to a final concentration of 2.5×10^6 cells/ml (11). Cells were incubated in Teflon beakers (Savillex, Minnetonka, Minn.) at 37°C and 5% CO_2 for 5 days. The percentage of monocytes in fresh or cultured suspensions was between 16 and 38% as determined by Giemsa stainings. The viability of cultured cells remained >96% (trypan blue exclusion).

Treatment of MDM with cytokines. rhGM-CSF (lot 89810; 5.9×10^6 U/mg) was generously provided by Ekke Liehl, Sandoz Forschungsinstitut, Vienna, Austria). rhIFN-γ was purchased from Boehringer Ingelheim (Vienna, Austria). At various concentrations, rhGM-CSF or rhIFN-γ was added to macrophages after 72 h of culture, and treatment was performed for an additional 48 h. Equivalent amounts of DMEM were added as control.

Granulocytes. Granulocytes were separated from heparinized (10 U/ml) venous blood as described previously (14). Cells were washed and resuspended to a concentration of 5×10^6/ml in KRPD. The granulocyte suspension contained

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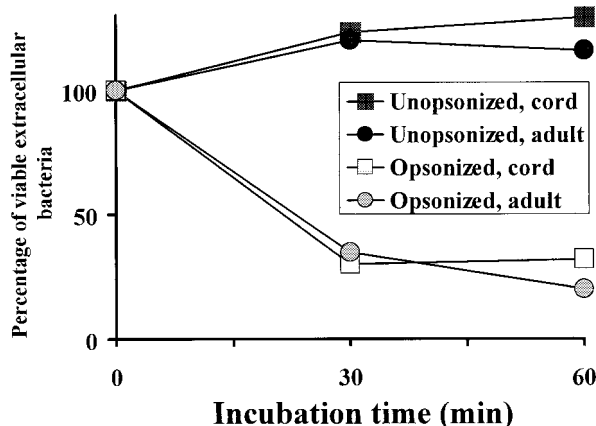
FIG. 1. Phagocytosis of unopsonized and opsonized GBS type III by cord and adult macrophages. Data represent means of at least six experiments with standard errors of maximally 7%.
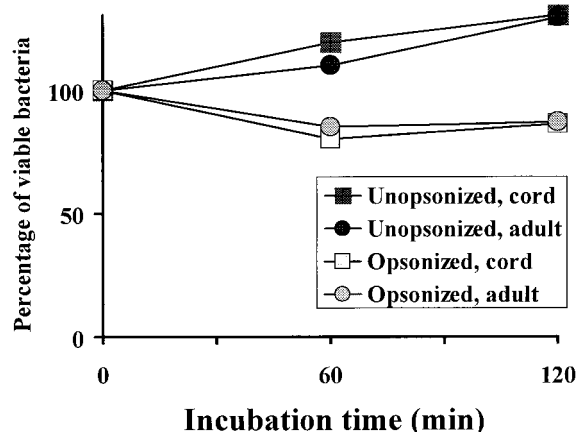

FIG. 2. Killing of unopsonized and opsonized GBS type III by cord and adult macrophages. Data represent means of at least six experiments with standard errors of maximally 9%.

more than 98% neutrophils (band form and segmented) as demonstrated by May Grünwald-Giemsa staining in cytocentrifuge preparations.

Bacteria. GBS type III (ATCC 31475) was cultured overnight at 37°C in nutrient broth (Oxoid, London, United Kingdom), harvested by centrifugation at 1,500 × g for 10 min, washed twice with KRPD, and finally resuspended in KRPD containing 0.1% gelatin to a concentration of 5×10^6 bacteria/ml.

Preopsonization. Preopsonization was performed by incubating 5×10^6 bacteria/ml with 10% pooled serum for 30 min at 37°C under rotation (4 rpm), followed by centrifugation and washes in KRPD at 4°C. The bacteria were finally resuspended to a concentration of 5×10^6/ml.

Phagocytosis assay. Phagocytosis of GBS was measured by incubating 100 μl of a phagocytic cell suspension containing 5×10^6 MDM/ml with an equal volume of a suspension of 5×10^6 preopsonized bacteria/ml for 60 min at 37°C under rotation (4 rpm). At 0, 30, and 60 min, 50-μl aliquots of the mixture were removed and added to 450 μl of ice-cold KRPD containing 0.01% human albumin (Sigma). Cells were centrifuged for 6 min at 75 × g, and the number of viable extracellular bacteria in the supernatant was determined by colony counts (12, 13). The percentage of phagocytosis of GBS by macrophages was determined as the decrease in the number of viable extracellular bacteria.

Killing assay. Equal volumes of cell suspension (5×10^6 macrophages/ml) and preopsonized or unopsonized GBS suspension (5×10^6 bacteria/ml) were mixed, and the mixture was incubated at 37°C under rotation (4 rpm). The total volume was 200 μl. The phagocytic mixture was incubated at 37°C under rotation (4 rpm) for 120 min. Aliquots of the suspension (50 μl) were removed at 0, 60, and 120 min of incubation and added to 450 μl of ice-cold distilled water containing 0.01% albumin. Lysis of mononuclear cells was achieved by three cycles of freezing and thawing in liquid nitrogen (10). Control experiments showed that the viability of GBS in buffer (5×10^6 bacteria/ml) remained >96% after freezing and thawing. Granulocytes were lysed in distilled water containing 0.01% bovine serum albumin. The percentage of bacteria that had been killed was determined by colony counting.

Data presentation. All results are means and standard errors of at least five experiments performed on different days. Statistical analysis to compare data was performed with a two-tailed Student t test for unpaired observations.

RESULTS

Opsonic capacity of pooled serum. Initial experiments were directed toward determining the opsonic activity of pooled serum against GBS. We found that the serum we used promoted 80% ± 7% ingestion and 70% ± 8% killing of GBS after 60 min of incubation of 5×10^6 granulocytes/ml and 5×10^6 bacteria/ml.

Phagocytosis of GBS by macrophages. We studied the phagocytosis of unopsonized and preopsonized GBS type III by cord and adult macrophages. In the presence of unopsonized bacteria, neither cord nor adult macrophages displayed ingestion (Fig. 1). Lysis of the cell pellet by freezing and thawing in liquid nitrogen suggested that the percentages of cell-associated bacteria at 30 and 60 min remained <5% with both cord and adult macrophages. In contrast to the results for

unopsonized bacteria, both cord and adult macrophages ingested a large number of preopsonized GBS (Fig. 1). Over a period of 60 min there was no significant difference in the levels of phagocytosis of bacteria by cord or adult macrophages in the presence of serum ($P > 0.2$ at both 30 and 60 min).

Killing of GBS by macrophages. We compared the killing of unopsonized and opsonized GBS type III by cord and adult macrophages over a period of 120 min. There was no detectable phagocytosis by macrophages of unopsonized GBS, and, as shown in Fig. 2, in the absence of opsonins no killing of bacteria could be detected. However, both cord and adult macrophages ingested approximately 70% of preopsonized GBS over a period of 60 min (Fig. 1) and killed less than 20% of the bacteria by 120 min (Fig. 2). No significant difference in the levels of killing of preopsonized GBS by cord and adult macrophages was observed ($P > 0.2$ at both 60 and 120 min).

Effects of rhIFN-γ and rhGM-CSF on phagocytosis and killing of GBS by macrophages. We studied earlier the effects of rhIFN-γ and rhGM-CSF on the killing of candida by MDM from healthy individuals (10, 15). Maximal activity of this function was achieved at concentrations of 100 U of rhIFN-γ/ml and 200 U of rhGM-CSF/ml, respectively, with no appreciable increases of this function by treatment of cells with higher concentrations of cytokines (10, 15). Accordingly, we compared the phagocytosis and killing of preopsonized GBS by resident macrophages and macrophages treated with 100 U of rhIFN-γ/ml or 200 U of rhGM-CSF/ml. Treatment of macrophages with either rhIFN-γ or rhGM-CSF resulted in a slightly, but not significantly, higher degree of ingestion of preopsonized GBS than that resulting from phagocytosis by resident cells over a period of 60 min (Fig. 3; $P > 0.1$). Treatment of adult macrophages with rhIFN-γ resulted in significantly higher degree of killing of GBS than that produced by untreated cells ($P < 0.01$; Fig. 3). In contrast, cord macrophages showed negligible response to activation by 100 U of rhIFN-γ/ml (Fig. 3). However, the extent of killing by both cord and adult cells was markedly increased by preincubation of macrophages for 48 h with rhGM-CSF ($P < 0.01$; Fig. 3).

DISCUSSION

The lack of type-specific opsonizing antibodies has been proposed to make newborns susceptible to invasive GBS disease (20). However, in a recent study of 321 healthy term
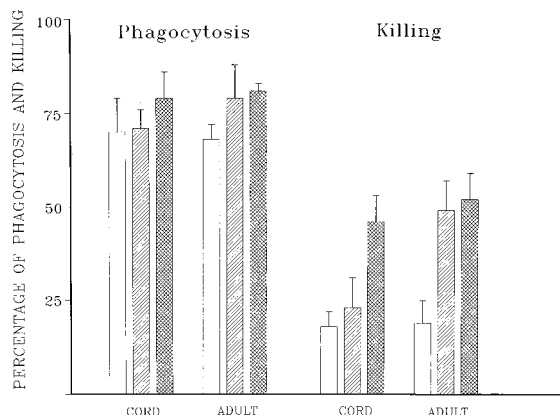
FIG. 3. Phagocytosis and killing of GBS type III by resident (open bars) or activated (rhIFN-γ, hatched bars; rhGM-CSF, cross-hatched bars) macrophages. Incubation was for 60 min. Data represent means ± standard errors of the means (n > 6).

newborns, immunoglobulin G (IgG) antibodies against capsular polysaccharides of GBS serotypes Ia, II, and III were present in 98 to 100% of cord sera (2). In addition, naturally occurring IgG antibodies with the capacity to opsonize GBS type III in a complement-dependent manner have been described (7). These data suggest that mechanisms other than insufficient opsonophagocytosis may, at least in part, be responsible for the increased susceptibility of neonates to severe GBS disease.

We show here that MDM do not display measurable ingestion of unopsonized GBS. Studies from other laboratories suggest that unopsonized GBS may be phagocytosed by mouse macrophages and macrophage-like cell lines (1, 17, 19, 21–23). Although the mechanisms of nonopsonic uptake of GBS by macrophages are not completely understood, lectin-like interactions might be involved (19, 21). Nonopsonic recognition of GBS mediated by complement receptor type 3 in mouse peritoneal macrophages and the macrophage cell line PU5-1.8 has been reported (19). We earlier published data showing that OKM1 and M1/70 monoclonal antibodies directed against the α subunit of complement receptor 3 inhibited phagocytosis and killing of GBS by human MDM (15). Although these in vitro data suggest an alternative mechanism of uptake of GBS by macrophages, it is difficult to estimate what the in vivo relevance of these findings might be. The number of viable exacellular bacteria and the total number of GBS in our phagocytosis and killing assay systems increased in the absence of serum (Fig. 1 and 2). We cannot rule out the possibility that some degree of ingestion in opsonin-free conditions occurred. However, based on data reported here, we believe that nonopsonic uptake plays a negligible role in the clearance of GBS in vivo.

In this study we provided evidence of vigorous phagocytosis of GBS by macrophages occurring in the presence of opsonizing serum. Most importantly, we report here that ingested GBS survived in macrophages over a period of 2 h and that the decrease of the total number of bacteria in the phagocytic mixture was limited compared to decreases in the presence of granulocytes (Fig. 2). These data clearly indicate that cord and adult macrophages phagocytose GBS equally but that these cells do not efficiently kill bacteria.

Survival strategies of GBS which interfere with macrophage bactericidal functions might exist. GBS types Ia and III may impair microbicidal systems in murine macrophages by inhib-

iting protein kinase C-dependent signal transduction pathways (5). Alternatively, macrophages may not kill GBS unless they are activated. To probe the latter hypothesis, we studied macrophage activation achieved by using rhIFN-γ, the most important macrophage-activating agent in vivo, and rhGM-CSF, a proinflammatory cytokine with the ability to augment the microbicidal capacity of monocyte-derived macrophages (10). We found that neonatal macrophages could not be fully activated with rhIFN-γ (16). Therefore, after phagocytosis these cells may become permissive for bacterial replication. Thus, ingestion by macrophages of specifically opsonized GBS may not enhance, and may even interfere with, elimination of these bacteria by granulocytes in tissue environments.

Recent studies of gene-targeted mice suggest that GM-CSF plays an important role in GBS clearance in vivo, mediated in part by its role in enhancing bacterial killing by macrophages (9). In pilot phase I and II human trials, GM-CSF was demonstrated to be safe and well tolerated by neonates (3). Further studies should define the clinical efficacy of this cytokine in newborns with bacterial infections caused by GBS or other bacteria and fungi.

We report here that rhGM-CSF displays significant macrophage-activating activity in vitro, with no difference between adult and cord cells. These data further support the concept that administration of rhGM-CSF to neonates with GBS sepsis may augment host defense against these bacteria by enhancing macrophage killing capacity.

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