The role of gamma interferon in the neonatal mouse model of group B streptococcal disease

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The aim of this study was to assess the role of gamma interferon (IFN-γ) in a neonatal mouse model of group B streptococcal (GBS) sepsis. IFN-γ was produced by spleen cells at 24, 48, and 72 h after GBS challenge. Treatment with anti-IFN-γ at 6 h before challenge totally abrogated the IFN-γ response but did not affect survival. Subcutaneous administration of recombinant IFN-γ (2,500 IU per pup) at 18 h after challenge resulted in increased survival time and reduced blood colony counts at 48 and 72 h. In addition, IFN-γ affected TNF-α, selected serum samples before assay. No change in cytotoxicity occurred, making it unlikely that the cycle of IFN-γ in response to common mitogens or specific antigens (35). The cellular basis of this defect is thought to reside in T lymphocytes (19) and macrophages (24), decreased proliferation of myeloid progenitors (6), or defects in the ability of mononuclear phagocytes to recruit PMNL through secretion of chemokines (28).

IFN-γ is produced by natural killer (NK) cells and T lymphocytes and has multiple effects, including enhancement of the antimicrobial activities of macrophages and PMNL (10). There appears to be a developmental immaturity of IFN-γ production at birth (18, 33, 35). Circulating mononuclear cells from human neonates produce considerably less IFN-γ in response to common mitogens or specific antigens (35). The cellular basis of this defect is thought to reside both in T lymphocytes (19) and macrophages (31), which are necessary accessory cells in IFN-γ production. In vitro, preincubation with recombinant IFN-γ (rIFN-γ) restored impaired chemotaxis of human neonatal PMNL, apparently by increasing intracellular calcium influx (15, 16).

Little is known about the role of IFN-γ in neonatal sepsis. This cytokine is produced in the spleens of neonatal rats infected with GBS (32). Theoretically, IFN-γ may exert both beneficial and detrimental effects. By enhancing chemotaxis and phagocytic killing, IFN-γ would be expected to contribute to bacterial clearance. On the other hand, a number of observations in adult septic shock models indicate that IFN-γ may directly mediate pathological changes and lethality, possibly in conjunction with other proinflammatory mediators (13, 14, 17).

Indeed, one of such mediators, tumor necrosis factor alpha (TNF-α), was previously shown to induce IFN-γ and to play a pathophysiological role in experimental neonatal GBS sepsis (32). The present study was undertaken to assess the role of IFN-γ in a murine model of GBS disease.

MATERIALS AND METHODS

Neonatal mice. Neonatal (24-h-old) BALB/c mice were used. Parental mice were obtained from Harlan Nossan (Milan, Italy). Pups from each litter were randomly assigned to control or experimental groups, marked, and kept with the mother.

Bacteria. GBS strain COH1, a highly virulent strain originally isolated from a septic neonate, was kindly provided by Craig Rubens, University of Washington, Seattle. Bacteria were grown to the late logarithmic phase in Todd-Hewitt broth (Difco, Diagnostic International Distribution, Milan, Italy) and adjusted to the appropriate concentration in phosphate-buffered saline (PBS; 0.01 M phosphate, 0.15 M NaCl [pH 7.2]) before inoculation into neonatal mice. Bacterial numbers were calculated by using a previously constructed curve in which CFUs were plotted against ΔA600.

Neonatal sepsis model. Mouse pups were infected subcutaneously with viable GBS (150 CFU) resuspended in 25 μl of PBS. This inoculum corresponded to a 90% lethal dose. Mortality was assessed every 12 h.

Treatments. Mouse IFN-γ (10 μg), hamster anti-mouse IFN-γ monoclonal antibody (MAb), and rabbit anti-mouse TNF-α serum were purchased from Genzyme (Cinisello Balsamo, Italy). All treatments were given subcutaneously in 25 μl. Anti-IFN-γ MAb and rIFN-γ were given neat and diluted in PBS supplemented with 0.1% bovine serum albumin, respectively. Anti-IFN-γ was always given at 6 h before challenge. Anti-TNF-α and rIFN-γ were given at 18 h after challenge unless otherwise noted.

Cytokine measurements. Mice were killed by decapitation under ether anesthesia at the indicated times after GBS challenge. Mixed venous-arterial blood was collected by cardiac puncture at birth (less IFN-γ in response to common mitogens or specific antigens (35). The cellular basis of this defect is thought to reside in T lymphocytes (19) and macrophages (24), decreased proliferation of myeloid progenitors (6), or defects in the ability of mononuclear phagocytes to recruit PMNL through secretion of chemokines (28).

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human neonates (16), we investigated whether administration of rIFN-γ would be beneficial in our model. Neonatal mice were inoculated with rIFN-γ (2,500 IU per pup) at 18 h after challenge with GBS. This treatment increased survival time, as evidenced by decreased (P < 0.05) lethality at 48 and 72 h (Table 1). However, this effect was no longer significant after 96 h. Table 1 shows that 1,250 IU had no effect on survival. A dose of 5,000 IU did not confer further protection over that afforded by 2,500 IU. In addition, the administration of two 2,500 IU doses of rIFN-γ per pup at 24 and 48 after challenge did not increase survival time compared with a single administration (not shown).

**Effects of rIFN-γ on blood colony counts.** To investigate whether the protective effect of rIFN-γ might have been due to augmented antibacterial defenses, the numbers of CFU in the blood of GBS-infected mouse pups were determined at different times after challenge. CFU were significantly lower in rIFN-γ-treated pups at 48 and 72 h after challenge compared with vehicle controls (Fig. 2).

**Effects of rIFN-γ on plasma TNF-α levels.** Since TNF-α was previously shown to play a pathophysiologic role in this sepsis model (21, 32), we sought to determine if rIFN-γ administration was associated with changes in plasma TNF-α levels. Mouse pups were treated with 2,500 IU of rIFN-γ or vehicle at 18 h, and plasma was obtained at 48 and 72 h after GBS challenge. TNF-α levels measured in five plasma pools did not

**RESULTS**

**Effects of IFN-γ blockade.** IFN-γ was measured in plasma samples of neonatal mice at different times after infection with GBS. As previously found in neonatal rats (32), plasma IFN-γ was below the limit of detection of the assay at various times after infection, except in a minority of plasma pools (one and two of six pools each obtained at 48 and 72 h after infection, respectively; data not shown). IFN-γ responses were then measured in cultures of spleen cells explanted at various times after infection. This method was previously found to detect ongoing IFN-γ responses that may be too low to be detectable in the circulation (11). Figure 1 shows that IFN-γ production could be detected at 24, 48, and 72 h after infection. Treatment with anti-IFN-γ MAb (25 μg per pup) 6 h before GBS challenge totally abrogated the IFN-γ response (Fig. 1). In further experiments, the effects on lethality of the same anti-IFN-γ treatment were assessed. Survival was not significantly different in anti-IFN-γ-treated pups and vehicle controls (14 animals in both groups [data not shown]).

**Effects of rIFN-γ on lethality.** Since rIFN-γ enhanced functional activities and intracellular calcium influx of PMNL from

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**TABLE 1. Effects of rIFN-γ on lethality induced by GBS in neonatal mice**

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>IFN-γ dose (IU/mouse)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 (vehicle)</td>
<td>1(7)</td>
<td>4(29)</td>
<td>9(64)</td>
<td>12(86)</td>
</tr>
<tr>
<td></td>
<td>2,500</td>
<td>2(14)</td>
<td>2(14)</td>
<td>2(14)</td>
<td>8(57)</td>
</tr>
<tr>
<td></td>
<td>5,000</td>
<td>1(7)</td>
<td>1(7)</td>
<td>2(14)</td>
<td>9(64)</td>
</tr>
<tr>
<td>2</td>
<td>0 (vehicle)</td>
<td>2(14)</td>
<td>5(35)</td>
<td>10(71)</td>
<td>12(86)</td>
</tr>
<tr>
<td></td>
<td>1,250</td>
<td>1(7)</td>
<td>4(29)</td>
<td>9(64)</td>
<td>11(79)</td>
</tr>
<tr>
<td></td>
<td>2,500</td>
<td>2(14)</td>
<td>2(14)</td>
<td>3(21)</td>
<td>7(50)</td>
</tr>
</tbody>
</table>

*Mouse pups were challenged with 150 CFU of strain COH1. Each group contained 14 pups.

*P < 0.05 by Fisher exact test compared with the respective vehicle controls.
differ in rIFN-γ and vehicle-treated animals at either 48 h or 96 h (232 ± 56 and 305 ± 66 at 48 h and 164 ± 50 and 190 ± 33 at 72 h for treatment and control groups, respectively). Since IFN-γ is known to synergize with the lethal effects of TNF-α in adult shock models (25), we sought to determine if the beneficial effects of IFN-γ could be further augmented by the simultaneous blockade of TNF-α activity. For this reason, anti-TNF-α rabbit serum (25 μl) was given simultaneously with IFN-γ (2,500 IU per pup) at 18 h after challenge (Table 2). Combined treatment with these agents did not afford any further protection over that observed with rIFN-γ or anti-TNF-α alone.

**Effects of rIFN-γ on PMNL infiltration.** Although GBS can be detected in large numbers within the organs of infected human and murine neonates, there is often little inflammatory response (27). Since rIFN-γ was previously found to enhance PMNL chemotaxis, it was of interest to ascertain whether PMNL infiltration was augmented in the organs of IFN-γ-treated animals. No differences in PMNL numbers were found by either histopathology or assay of myeloperoxidase activity in lungs, kidneys, livers, or brains of treated pups relative to vehicle controls (not shown).

**In vitro effects of rIFN-γ on bacterial growth.** Since the increased clearance of GBS in rIFN-γ-treated animals was not apparently due to stimulation of chemotaxis, we hypothesized that the effects of rIFN-γ were due to a direct activation of the microbicidal activities of leukocytes. When GBS were mixed with whole blood obtained from uninfected pups, rapid growth occurred over the 1-h incubation time, resulting in 13- to 18-fold increases in CFU (Fig. 3). When blood cells were pretreated with IFN-γ for 1 h before the addition of bacteria, however, a dose-dependent restriction in bacterial growth was observed (Fig. 3).

Addition of rIFN-γ (50 IU/ml) did not affect GBS growth in Todd-Hewitt broth, thus excluding a direct effect of the cytokine on bacteria (not shown).

**DISCUSSION**

In the first part of this study, we measured endogenous IFN-γ levels in neonatal GBS sepsis. IFN-γ production was detected in the spleens, but not the circulation, of infected mice, confirming the results of a previous rat study (32). Whether these IFN-γ elevations play a role in the pathogenesis of GBS disease remains to be clarified. Neutralization of endogenous IFN-γ with specific antibodies had no effect on lethality. This is in contrast with a number of observations in adult sepsis models showing mediation of pathophysiologic changes and lethality by endogenous IFN-γ. Anti-IFN-γ antibodies protected adult mice from the generalized Shwartzman reaction (14, 25). IFN-γ neutralization also protected adult mice from intravascular challenge with endotoxin (7, 13, 26) or live bacteria (29) or from focal gram-negative peritonitis spreading into the circulation (17). It is remarkable that similar protective effects of IFN-γ blockade were observed in different sepsis models, since the response to anticytokine agents, specifically anti-TNF-α, is affected by the route of inoculation and the virulence of infecting bacteria (2).

It is unlikely that differences between this and previous studies in the effects of IFN-γ neutralization reflect the use of gram-positive bacteria rather than gram-negative bacteria or endotoxin. Indeed, a similar lack of protection of anti-IFN-γ antibodies was observed in a neonatal model of endotoxemic shock (19a). Moreover, bidirectional cross-tolerance between GBS and endotoxin suggests that these stimuli share common transduction and activation pathways in host cells (20, 23).

It is likely that the lack of effects of IFN-γ blockade that we observed is accounted for by production of low levels of this cytokine in the neonatal period. Recent studies indicate that IFN-γ responses of neonatal mice to endotoxin are 80 to 90% lower than those of adults (6a). Similarly, human newborns produce low levels of IFN-γ in response to common mitogens or specific antigens (15, 18, 33, 35).

In the second part of our study, we showed that exogenously administered IFN-γ can significantly increase survival time in this model of GBS disease. Since blood colony counts were lower in rIFN-γ-treated animals, it is possible that the beneficial effects of this cytokine are at least in part mediated by an enhancement of neonatal defenses. Accordingly, in vitro pretreatment of whole blood with rIFN-γ before the addition GBS resulted in significant restriction of bacterial growth. These data are in agreement with the notion that IFN-γ is a potent activator of microbicidal activities of PMNL and monocytes through enhanced production of oxygen metabolites (10). In experimental neonatal infections with *Listeria monocytogenes*, a facultative intracellular bacterium, treatment with rIFN-γ improved survival and reduced spleen and liver colony counts (5). Since rIFN-γ was previously found to enhance chemotaxis of human neonatal PMNL (15), it was of interest to ascertain whether PMNL infiltration was increased in the organs of rIFN-γ treated animals. Histopathologic analysis showed that this was not the case, ruling out improved chemotaxis as a mechanism underlying the beneficial effects of rIFN-γ administration. These data, however, do not exclude that impaired local production of IFN-γ may play a role in the reduced chemotactic response of neonatal PMNL (15, 16).
Septic shock is known to be the end result of complex interactions. TNF-α, an important pathophysiologic mediator in experimental GBS disease, induces IFN-γ production (32). In turn, IFN-γ can modulate the release of proinflammatory mediators of shock, including TNF-α and interleukin-1. It has been suggested that endogenous IFN-γ may participate in a negative feedback loop down-regulating TNF-α levels (13). On the other hand, treatment with IFN-γ before, but not after, endotoxin challenge increases TNF-α production (13). Similarly, in the present study, rIFN-γ administration 18 h after GBS challenge did not affect TNF-α levels.

Previous work has shown protective effects of anti-TNF-α antibodies in neonatal models of GBS disease (22, 32). Therefore, we sought to determine if the beneficial effects of rIFN-γ could be further augmented by the simultaneous blockade of TNF-α activity. Our data, however, indicated that this was not the case. Since IFN-γ treatment can decrease the expression of macrophage TNF receptors (8, 30), and endogenous TNF-α is a mediator of lethality in our model, it is possible that the beneficial effects of rIFN-γ that we observed are in part mediated by reduced expression of TNF receptors. This would explain the lack of synergy of rIFN-γ and anti-TNF-α administration. Further investigations on TNF receptor expression in septic neonates are needed to clarify this point.

In previous studies, we have shown that administration of recombinant interleukin-6 and recombinant interleukin-10 can increase survival time of GBS infected pups, apparently by decreasing circulating TNF-α (19a, 25). However, these agents were not effective when given after challenge, in contrast with results obtained here with IFN-γ. Collectively, our data indicate that rIFN-γ may be a potential candidate for treatment of neonatal sepsis.

ACKNOWLEDGMENTS

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