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Role of Gamma Interferon In the Pathogenesis of Bacteremic Pneumococcal Pneumonia

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Gamma interferon (IFN-γ) concentrations in blood, but not in lungs, rose significantly at 24 to 48 h after murine pulmonary infection with virulent pneumococci. In contrast, infection with avirulent pneumococcal strains produced minimal rises in serum IFN-γ concentrations. Compared with that of immunocompetent mice, mortality was appreciably increased after pulmonary infection of IFN-γ gene knockout mice, suggesting a protective role for IFN-γ in host response to pneumococcal disease.

The early mortality from bacteremic *Streptococcus pneumoniae* pneumonia remains high despite current antibiotic therapy and aggressive intensive care (8, 11, 13), and the emergence of antibiotic-resistant *S. pneumoniae* threatens to further limit the effectiveness of available therapies (1). Consequently, modulation of the initial inflammatory responses to *S. pneumoniae* infection has been considered as an alternative treatment strategy to reduce this early mortality (6). Gamma interferon (IFN-γ) has been identified in studies over the past decade as a critical immunomodulator in early host defense against a variety of infections (5). As a component of early host defense against infection, IFN-γ is a key activator of macrophage killing activity and NK cell cytotoxicity and also recruits circulating neutrophils and lymphocytes to sites of infection. Although the role of IFN-γ in host defenses against intracellular pathogens has been well characterized, its function in natural immunity to *S. pneumoniae* infection is poorly defined. Consequently, we sought to better define IFN-γ responses in experimental *S. pneumoniae* pneumonia and to determine whether specific deletion of IFN-γ production affected early survival in invasive *S. pneumoniae* infection.

For these studies, bacterial strains from the American Type Culture Collection were stored at −70°C on glass beads in Trypticase soy broth supplemented with 10% glycerol and were passed through mice immediately before experiments to ensure virulence. After isolation from mouse spleens, bacteria were grown to mid-log phase under microaerobic conditions in brain heart infusion broth (Difco Laboratories, Detroit, Mich.), washed, and then resuspended in Dulbecco's phosphate-buffered saline for inoculation. Inoculum size was confirmed by quantitative culture on blood agar plates.

Specific-pathogen-free white female National Institutes of Health Swiss outbred mice (20 to 30 g) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, Ind.). IFN-γ knockout (IFN-GKO; C57BL6/J-Ifg^{tml}) breeder mice and C57BL6/J control mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Animals were housed in a pathogen-free barrier facility fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Animal studies were performed in accordance with the established guidelines (15a), and the research was approved by the Animal Study Subcommittee of the Veterans Affairs Medical Center (Minneapolis, Minn.).

For murine experimental pneumonia studies, mice were anesthetized by intraperitoneal (i.p.) injection of 50 mg of sodium pentobarbital per kg, and 50 μl of bacterial suspension was injected intranasally (i.n.). During pneumonia, clinical severity of infection was measured by change in individual animal weights and by scoring clinical symptoms as (i) no symptoms, (ii) mild piloerection, (iii) marked piloerection and listlessness, (iv) minimal response to stimuli, and (v) dead. At selected times after infection, mice were sacrificed by i.p. injection of 50 to 100 μl of Beuthanasia (Schering-Plough Animal Health, Kenilworth, N.J.). After cardiac puncture to obtain blood for culture and cytokine assays, the lungs were dissected from major vessels and bronchi, rinsed, and homogenized. The numbers of viable bacteria in samples of blood and lung homogenate were determined by quantitative culture of serial dilutions on blood agar plates. Bacteria were identified as *S. pneumoniae* by colony morphology of α-hemolytic organisms and by Optochin sensitivity.

IFN-γ concentrations in samples of serum and lung homogenates were assayed for each animal in duplicate by enzyme-linked immunosorbent assay using a commercially available kit (Genzyme, Cambridge, Mass.) with a detection limit of 5 pg/ml. Cytokine concentrations are expressed as nanograms per milliliter in serum and as nanograms per lung in homogenates. Each datum point in figures and tables represents the mean ± standard deviation for five or six animals, and results of each experiment were confirmed by at least one repeat experiment. Where indicated, statistical significance was calculated by comparison of multiple geometric means by one-way analysis of variance and Bonferroni's multiple comparison tests or by one-way analysis of variance on ranks and Dunn's multiple comparison tests for skewed data, using Sigmastat for Windows (Jandel Scientific, San Rafael, Calif.).

For survival studies, groups of eight IFN-GKO or C57blk control mice were infected i.n. with defined concentrations of type 2 *S. pneumoniae*, and survival was recorded at 8-h intervals for 84 h. The 50% lethal dose was calculated by the method of Reed and Muench (16).

During experimental pneumonia with *S. pneumoniae* serotypes virulent for mice (types 1, 2, 3, and 4), 10^5 to 10^6 CFU were recovered from mouse lungs at 6 h after infection, and bacterial numbers per lung gradually increased over the next

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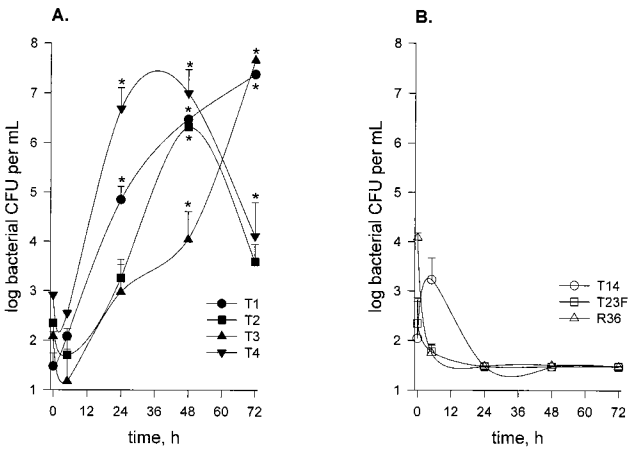

FIG. 1. Bacteremia after infection of mice with virulent and avirulent *S. pneumoniae* serotypes. Bacteremia at indicated times after i.n. infection with virulent strains of serotypes (T) 1, 2, 3, and 4 (A) and avirulent strains of serotypes 14, 23F, and R36 (B). Data points represent geometric means ± standard errors for five mice. $^*P < 0.05$ compared with zero time value.

48 h. Concomitantly, significant bacteremia was evident at 24 h after i.n. infection with these strains and increased over the next 24 h (Fig. 1A). Depending on the infecting strains, bacteremia either progressed between 48 and 72 h after infection and was associated with a moribund clinical state (or death before the scheduled sacrifice) or regressed and was associated with clinical recovery.

Concentrations of IFN-γ in serum rose significantly at 24 to 48 h after i.n. infection with these virulent strains (Fig. 2A). In contrast, despite high bacterial numbers in mouse lungs (approximately 10^6 CFU per lung), IFN-γ concentrations in lung homogenates did not change significantly during *S. pneumoniae* pneumonia (data not shown). Interestingly, among mice surviving to the scheduled sacrifice at 72 h after infection, concentrations of IFN-γ in serum were consistently decreased despite the persistence of high-grade bacteremia in some mice. Despite the appreciable interanimal variability in the severity of invasive *S. pneumoniae* infection, serum IFN-γ concentrations correlated with the degree of bacteremia and the severity of illness of individual mice at 24 to 48 h after infection with virulent strains ($R^2 = 0.56$, $p < 0.0001$).

In contrast to infection with virulent strains, i.n. infection with *S. pneumoniae* serotypes lacking virulence for mice (types 14, 23, and R36) produced significantly lower numbers of bacterial CFU in mouse lungs at 24 and 48 h and did not produce detectable bacteremia at 24 to 72 h after infection (Fig. 1B). Accordingly, concentrations of IFN-γ in serum were approximately 10 to 100-fold lower at 24 to 48 h after infection with these strains compared with those after infection with virulent strains (Fig. 2B).

In order to determine whether these serum IFN-γ responses during murine *S. pneumoniae* bacteremia were associated with the animal's ability to survive invasive infection, we compared the survival of IFN-GKO mice that had specific deficiency of IFN-γ production with matched immunocompetent mice after i.n. infection with a virulent type 2 strain. IFN-GKO mice were appreciably more susceptible to *S. pneumoniae* infection, with a calculated 50% lethal dose at 48 h after i.n. infection that was 20-fold lower than that for control mice (2.5×10^6 and 5×10^7 CFU, respectively). Of note, none of the control immunocompetent mice succumbed to i.n. infection with inocula lower than 10^8 CFU, whereas over 50% of IFN-GKO mice infected with inocula of 10^7 CFU or lower died.

In summary, we have shown that concentrations of serum, but not lung, IFN-γ increase during invasive *S. pneumoniae* infection in mice, that the magnitude of the rise correlated with the virulence of the infecting organisms, and that interanimal variations in disease severity correlated with serum IFN-γ concentrations. Furthermore, the functional significance of the observed IFN-γ responses is supported by the finding of markedly increased susceptibility of IFN-γ-deficient mice to *S. pneumoniae* infection. These results are consistent with earlier limited studies indicating a protective role for IFN-γ in host defense against *S. pneumoniae*. Weigent et al. (21) showed decreased early mortality of mice injected with IFN-γ before i.p. infection with type 25 *S. pneumoniae* compared with mice injected with control diluent. More recently, van der Poll et al. (20) provided indirect evidence for the importance of IFN-γ in host defense against *S. pneumoniae* by demonstrating that administration of interleukin-10 (IL-10), which decreased lung concentrations of IFN-γ and tumor necrosis factor, increased bacterial counts in lungs and blood and resulted in early lethality from *S. pneumoniae* pneumonia.

Although IFN-γ is critical to host defense against many intracellular bacteria, fungi, protozoa, and viruses, it is now clear that IFN-γ regulates inflammatory responses to extracellular bacteria as well, with adverse or beneficial consequences depending upon the infecting organism. The synergy of IFN-γ with lipopolysaccharide contributes to the detrimental pathophysiology of gram-negative sepsis (5, 10, 18, 19). In contrast, IFN-γ appears to play a more protective role in defense against bacteremia with gram-positive organisms, such as *Staphylococcus aureus* and streptococci (3, 17, 20–22). Our current results lend further support to the concept that IFN-γ has important protective effects in host defense against gram-positive bacteria. The site of infection may also be important, as suggested by recent studies in which IL-10 (and presumably decreased IFN-γ) produces more severe *Klebsiella* pneumonia, despite the protective effects of IL-10 in *Klebsiella* bacteremia (7).

Our studies have important implications for the development and use of cytokine/anticytokine therapies in patients. Deficiencies in IFN-γ production have been found in a number of immunocompromising conditions associated with increased

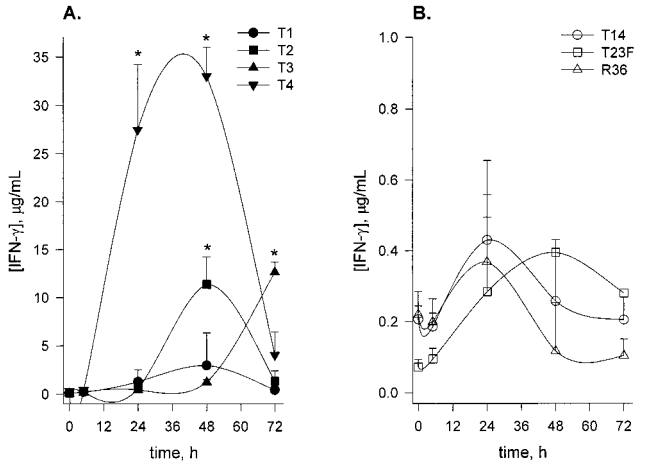

FIG. 2. Serum IFN-γ concentrations after infection of mice with virulent and avirulent *S. pneumoniae* serotypes. IFN-γ was measured for serum collected at indicated times after i.n. infection with virulent strains (A) and avirulent strains (B). Data points and statistics are explained in the legend to Fig. 1.

rates of invasive *S. pneumoniae* infection, including human immunodeficiency virus infection (15), immunoglobulin A deficiency (4), immunoglobulin G2 deficiency (9), hypogammaglobulinemia, common variable immunodeficiency (12), and chronic granulomatous disease (2). In addition, IFN-γ deficiencies may explain in part the higher rates of invasive *S. pneumoniae* infection seen in medical illnesses such as collagen vascular diseases and hematologic malignancies (14). In the face of such epidemiologic associations, our studies support future investigations into the use of IFN-γ-based immunomodulatory therapies (use of recombinant IFN-γ or possibly IL-12) as adjuncts to currently available treatments for severe *S. pneumoniae* infections.

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