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Role of Gamma Interferon in Natural Clearance of *Bordetella pertussis* Infection†

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Using a mouse model of *Bordetella pertussis* infection, we have analyzed the role of gamma interferon (IFN-γ) in bacterial clearance from the respiratory tract. Adult BALB/c mice began to clear a respiratory infection within 3 weeks postinfection, with complete resolution of infection 6 to 8 weeks postinfection. In contrast, neither adult SCID mice (which lack mature B and T lymphocytes) nor adult nude mice (which lack mature T lymphocytes) controlled *B. pertussis* infection, and both strains died within 3 to 5 weeks postinfection. Short-term T-cell lines generated from the draining lymph nodes of the lungs of infected BALB/c mice were found to be $CD4^+$ and produced IFN-γ but no detectable interleukin-4. Analyses of IFN-γ mRNA induction in the lungs of mice following *B. pertussis* infection showed that in both BALB/c and C57BL/6 mice, IFN-γ mRNA levels increased sharply by 1 week postinfection and then subsequently declined. Further exploration of a potential role for IFN-γ demonstrated that infection of adult BALB/c mice depleted of IFN-γ in vivo with anti-IFN-γ monoclonal antibodies resulted in greater numbers of bacteria recovered from the lungs than in infected, control BALB/c mice, although IFN-γ-depleted mice could subsequently clear the infection. Infection of mice which have a disrupted IFN-γ gene resulted in bacterial clearance with a time course similar to those seen with IFN-γ-depleted mice. These results indicate that IFN-γ plays a role in controlling *B. pertussis* infection.

Bordetella pertussis is a gram-negative bacterium that was first isolated by Bordet and Gengou in 1906. *B. pertussis* infects humans via inhalation of aerosol droplets and preferentially associates with the cilia of the respiratory epithelia lining the nasopharynx, trachea, and bronchial tree. During the course of disease, *B. pertussis* infection remains localized to the respiratory tract and does not progress to bacteremia or meningitis (4).

The nature of protective immunity against *B. pertussis* infection and disease is poorly understood. In animal models of infection, antibody-mediated protection, to multiple antigen specificities, has been observed. Passive transfer of monoclonal antibodies to pertussis toxin, pertactin, and lipooligosaccharide, as well as polyclonal antibodies to filamentous hemagglutinin, has been shown to protect against *B. pertussis* infection (7, 10, 15, 17, 19). However, recent clinical trials of acellular pertussis vaccines have failed to show any correlation between protection and antibody level in postvaccination sera (1).

Recently, it has been observed that pertussis-specific T-cell immunity can be detected following pertussis infection and following vaccination with whole-cell pertussis vaccine (9, 13). *B. pertussis*-specific T cells in humans have been demonstrated after immunization or infection (6). Transfer to mice of a murine $CD4^+$ T-cell clone specific for *B. pertussis* filamentous hemagglutinin, decreases peak *B. pertussis* infection by 2 $\log_{10}$ CFU (9). Further, T cells derived from mice infected with *B. pertussis* or immunized with whole killed bacteria produced gamma interferon (IFN-γ) and tumor necrosis factor alpha (11, 12).

We have been using a mouse model of aerosol *B. pertussis* infection to study the mechanism of immunity responsible for controlling *B. pertussis* infection. Following respiratory infection of adult mice with 5×10^4 CFU of *B. pertussis*, the bacteria multiply to 10^7 CFU at 7 to 10 days postinfection and then are subsequently cleared. In contrast, infection is not controlled in mice younger than 19 days of age and these mice die within 21 days postinfection (18). The purpose of this study was to investigate the role of IFN-γ in controlling infection.

MATERIALS AND METHODS

Mice. Specific-pathogen-free BALB/cAnNcR, C57BL/6, BALB/c.*nu*/*nu* (nude), and BALB/c.*scid* (SCID) mice were obtained at 5 weeks of age from either the Animal Production Program, Division of Cancer Research Treatment, National Cancer Institute, Frederick, Md., or Charles River, Portage, Mich. Mice (C57BL/6J background) deficient in the gene encoding IFN-γ were a generous gift from Ronald Schwartz, National Institute of Allergy and Infectious Diseases, Bethesda, Md. All mice were maintained in microisolators under specific-pathogen-free conditions.

Antigens. *B. pertussis* 18323 was grown on Bordet-Gengou agar plates and then transferred to liquid Stainer-Scholte media. After reaching stationary phase the bacteria were fixed with 0.2% formalin with gentle shaking at 24°C overnight, washed once in phosphate-buffered saline (PBS), and resuspended to 440 μg of protein per ml in PBS with 0.04% sodium azide as a preservative. Before use in T-cell stimulation assays, preparations of formalin-fixed *B. pertussis* (FFBP) were washed by centrifugation several times in PBS to remove residual sodium azide.

Aerosol challenge. A 21-h of culture *B. pertussis* 18323 was suspended in a solution of sterile PBS at a concentration of approximately 10^9 CFU/ml. This inoculum was administrated to mice as an aerosol by the use of a standard nebulizer as previously described (18). Mice were removed from the chamber 1 h after termination of the aerosol challenge. For each aerosol challenge performed, two mice were sacrificed upon removal from the chamber to determine the number of viable *B. pertussis* cells in the lungs; all animals tested had approximately 5×10^4 CFU in their lungs 1 h after aerosol challenge. The lungs of infected animals were aseptically removed, homogenized in sterile PBS, serially diluted, and then plated on Bordet-Gengou agar plates to determine bacterial recoveries at different time points during the course of infection.

Establishment of short-term *B. pertussis*-specific T-cell lines. Short-term T-cell lines were prepared according to a procedure previously described (2). T cells

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were prepared from the draining lymph nodes of the lungs of mice 8 weeks postinfection. Twenty four hours prior to sacrifice, the mice were injected intraperitoneally (i.p.) with carbon soot encapsulated in lecithin (Thies Technologies, St. Louis, Mo.) in order to visualize the draining lymph nodes of the lung. Following anesthetization of the same mice with 0.4 ml of a 2% solution of 2,2,2-tribromoethanol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) given i.p., the tracheobronchial and hilar lymph nodes were removed and single-cell suspensions were prepared. Cells were passed through a nylon wool (2 g) column equilibrated with 10% fetal calf serum in RPMI 1640, and the eluted T cells were collected. The T cells were incubated at 4×10^6 cells per well in 24-well plates with 2×10^5 irradiated syngeneic spleen cells and FFBP at 50 μg/ml in complete medium, consisting of RPMI 1640 (GIBCO laboratories, Grand Island, N.Y.), 10% fetal calf serum (HyClone Laboratories, Logan, Utah), 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of gentamicin per ml. Four days later, T-cell blasts were harvested from the wells and purified from dead cells by Ficoll density-gradient centrifugation. The cells were cultured at 2×10^5 cells per well with 4×10^6 irradiated syngenic spleen cells for 10 days. Then 5×10^6 irradiated splenocytes with FFBP were added to begin another round of stimulation and rest. After two or 3 rounds of stimulation all of the responding cells were $CD4^+$ T cells, as determined by flow cytometry conducted using a FACScan. Cells were stained using fluorescein isothiocyanate-labeled anti-CD4 and phycoerythrin-labeled anti-CD8 (each purchased from PharMingen, San Diego, Calif.).

Detection of cytokines secreted by short-term T-cell lines. After 10 days of culture, T cells were harvested from 24-well plates and put in culture at 2×10^5 cells per well with 4×10^5 irradiated splenocytes and 50 μg of FFBP per ml. The supernatants were removed 24 and 48 h after stimulation for cytokine detection by enzyme-linked immunosorbent assay. IFN-γ, interleukin-4 (IL-4), and IL-2 levels in harvested supernatants were assayed using pairs of cytokine-specific monoclonal antibodies in a sandwich enzyme-linked immunosorbent assay according to the manufacturer's recommendations (PharMingen).

In vivo neutralization of IFN-γ. IFN-γ was neutralized by using an anti-IFN-γ monoclonal antibody (MAb R46A2; American Type Culture Collection, Rockville, Md.). The antibody was purified from ascites by ammonium sulfate precipitation as described previously (14). Mice were injected i.p. with 1 mg (in a total volume of 0.5 ml) of MAb R46A2 or an isotype-matched control, MAb 11B11 (American Type Culture Collection) which is specific for IL-4, at both 3 and 1 days before infection and then at days 3, 10, and 17 postinfection.

RNA isolation. A small amount of tissue (50 mg) was taken from the right medium lobe of the lungs of mice at various time points during the course of *B. pertussis* infection. Each portion of lung tissue was homogenized in 1.5 ml of RNAzol B (Tel-Test, Inc. Friendswood, Tex.) according to the manufacturer's instructions. RNA was extracted by isopropanol precipitation. The individual RNA pellets were each dissolved in 10 μl of diethyl pyrocarbonate-treated H_2O. RNA concentration was determined by measuring absorbance at 260 nm, and the concentration of RNA in each sample was adjusted to 0.6 μg/μl. Contaminating proteins in the RNA preparations were detected by measuring the ratio of absorbances at 260 and 280 nm. If the 260/280 ratio was less than 1.8, the sample was extracted again by isopropanol precipitation.

Detection of mRNA. A reverse transcriptase-PCR (RT-PCR) procedure was performed to determine the levels of mRNA for IFN-γ and hypoxanthine-guanine phosphoribosyltransferase (HPRT) during *B. pertussis* infection. Reverse transcription of 3.6 μg of RNA was performed as previously described (20). The resulting cDNA was used for PCR. Primers and probes were prepared on a DNA synthesizer (Applied Biosystems) according to published sequences. IFN-γ and HPRT sequences for primers and probes were from Svetic et al. (20). Primers were chosen such that the product amplified from cDNA could be distinguished from amplified genomic DNA because of a difference in size due to introns found only in genomic DNA. For each mRNA, the optimal number of cycles used for RT-PCR was determined empirically and was defined as the number of cycles that would achieve an optimal signal that exhibited a linear relationship to the initial concentration of RNA. The number of cycles for specific mRNAs were 25 for IFN-γ and 16 for HPRT. PCR conditions were identical for each mRNA. After an initial incubation at 95°C for 5 min, temperature cycling was initiated with each cycle as follows: 95°C for 1 min (denaturation), 55°C for 1 min (annealing of primers), and 72°C for 1 min (primer extension). The total reaction volume was 50 μl. In experiments that utilized C57BL/6 or GKO mice, RNAs purified from lung tissue of three or four individual mice were pooled, and the pooled sample was examined in triplicate using RT-PCR in order to determine mRNA levels. In experiments that utilized BALB/c, nude, or SCID mice, RNAs from different mice were not pooled; instead, RNAs from three to five individual mice were examined separately and in duplicate using RT-PCR in order to determine mRNA levels. To verify that equal amounts of RNA were used for each PCR reaction, primers for HPRT were used to amplify cDNA reverse transcribed from the total RNA.

Detection and quantitation of PCR products. The PCR product (40 μl) was subjected to electrophoresis on a 2% agarose gel. The cDNA was then transferred to a Hybond membrane (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). Specific probes were end labeled with fluorescein-11-dUTP, using the ECL detection system according to manufacturer's instructions (Amersham). Autoradiographs were exposed at room temperature from 2 to 20 min using Hyperfilm-ECL (Amersham). Quantitation of mRNA was performed by densitometry using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). For each mRNA, the maximal level measured over the time course of the experiment was arbitrarily given a value of 100%. Levels of the same mRNA at other time points were then expressed relative to the maximal level.

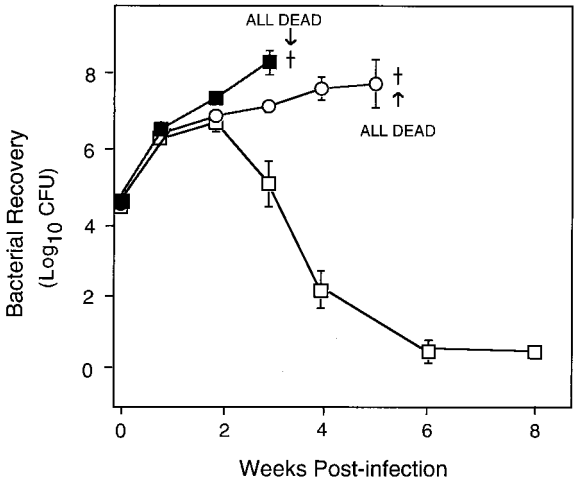

FIG. 1. Recovery of *B. pertussis* from the lungs of mice after aerosol challenge. BALB/c (□), nude (○), and SCID (■) mice were infected with an aerosol of *B. pertussis* as described in Materials and Methods. At each time point, the geometric mean and standard deviation of bacterial recoveries from the lungs of five mice were individually determined. No deaths due to infection occurred in the group of BALB/c mice, whereas nude mice died due to infection with a mean time to death of 32.8 ± 1.9 days postchallenge and SCID mice died due to infection with a mean time to death of 26.3 days ± 1.5 days postchallenge.

Statistics. Differences between mean values were analyzed by Student's *t* test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Nude and SCID BALB/c mice do not clear *B. pertussis* infection. Aerosol challenge of euthymic BALB/c mice with *B. pertussis* 18323 for 30 min resulted in a highly reproducible infection (18). Bacterial recovery from the lungs of BALB/c mice increased from an initial level of 4×10^4 CFU/lung determined 1 h after aerosol challenge to 7×10^6 CFU/lung determined 2 weeks after challenge (Fig. 1). Thereafter, bacterial recovery slowly declined until 8 weeks, when viable bacteria were no longer detected in lungs. In contrast to the euthymic mice, athymic T-cell-deficient nude and lymphocyte-deficient SCID mice do not clear the infection. In these mice, bacterial recovery in the lung continues to increase at a time when the euthymic mice have begun to clear the infection. Nude and SCID mice die 3 to 5 weeks postinfection.

Lymphokine secretion by *B. pertussis*-specific $CD4^+$ $CD8^-$ T cells. In order to investigate the role of specific immunity to *B. pertussis*, we generated short-term T-cell lines specific for *B. pertussis* antigens. These lines were generated using draining lymph nodes of the lungs of convalescent mice, 8 weeks after aerosol infection with *B. pertussis*. Cells from lymph nodes of convalescent mice responded to FFBP in vitro. After several rounds of stimulation, all of the proliferating cells were $CD4^+$ $CD8^-$ T cells. Forty-eight hours after stimulation of the *B. pertussis*-specific T cells in vitro with FFBP, IFN-γ was readily detected in the cell supernatant at a concentration of 20 ng/ml. In contrast, neither IL-4 nor IL-2 was detected in the supernatant (data not shown).

IFN-γ mRNA levels during the course of *B. pertussis* infection. In the mouse aerosol challenge model of pertussis, the

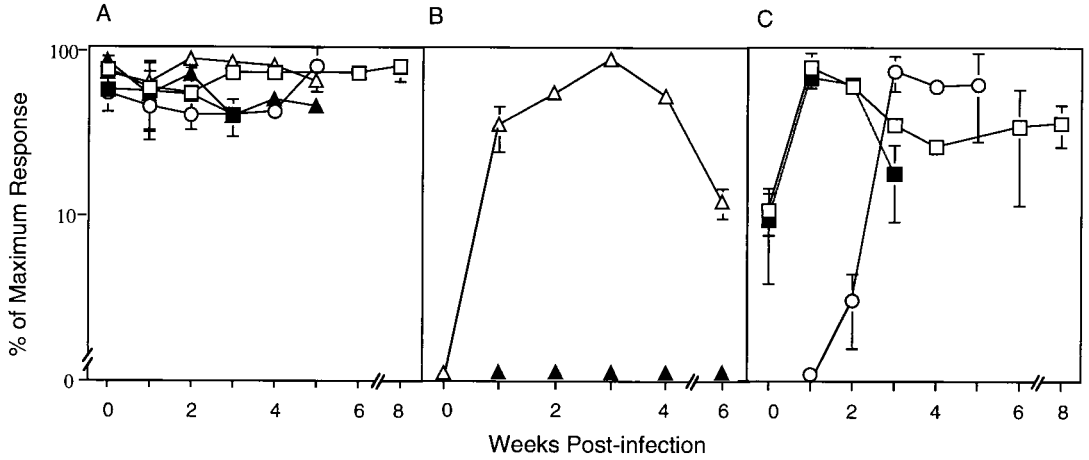

FIG. 2. Time course of IFN-γ mRNA expression in the lungs of mice after aerosol infection with *B. pertussis*. The mean and standard deviation of mRNA levels derived from lungs of C57BL/6 (△), GKO (▲) BALB/c (□), nude (○), and SCID (■) mice are shown for each time point. Determinations of mRNA levels were made as described in Materials and Methods. (A) HPRT mRNA levels; (B and C) IFN-γ mRNA levels.

lungs are the major site of *B. pertussis* infection. This infection is characterized by pulmonary infiltrates, composed mostly of mononuclear cells with lymphocyte morphology and polymorphonuclear leukocytes (8). After aerosol challenge of mice with *B. pertussis*, their lungs were excised and levels of IFN-γ mRNA were determined each week after infection until death (for nude and SCID mice) or for the duration of the experiment. *B. pertussis* infection induced IFN-γ mRNA in all mouse strains examined (Fig. 2) except for the IFN-γ-knockout or GKO mice, as expected since these mice have a defective IFN-γ gene (5). As a control, levels of HPRT mRNA, which is transcribed from a housekeeping gene that should not be affected by infection with *B. pertussis*, were measured, and these mRNA levels were found to remain constant throughout the course of the experiment. Induction of IFN-γ mRNA is apparent within 1 week after infection for most mouse strains, with the exception of nude mice, which exhibited induced levels of mRNA only 2 weeks after infection. IFN-γ mRNA levels for C57BL/6 mice peaked at 3 weeks postinfection; thereafter, levels slowly declined. IFN-γ mRNA levels peaked one week postinfection in the lungs of BALB/c and SCID mice and declined thereafter. Levels remained high in the lungs of nude mice after initial induction until death of the animal approximately 5 weeks postinfection.

Course of *B. pertussis* infection in mice depleted of IFN-γ or lacking a functional IFN-γ gene. The high level of IFN-γ production by *B. pertussis*-specific $CD4^+$ T-cell lines and the observed increases in IFN-γ mRNA levels in the lungs of mice during the course of infection indicate a possible role for IFN-γ in controlling *B. pertussis* infection. Our first approach to examining the role of IFN-γ in *B. pertussis* infection was to deplete IFN-γ levels by treating BALB/c mice with R46A2, a monoclonal antibody specific for IFN-γ which has previously been shown to efficiently neutralize murine IFN-γ in vivo (14).

BALB/c animals received 1 mg of MAb R46A2 at both 3 days and 1 day before infection and at days 3, 10, and 17 following infection. Controls animals were either left untreated or received an isotype-matched monoclonal antibody, MAb 11B11, which is specific for IL-4, at the same time points. The subclass of both monoclonal antibodies used in this study was rat immunoglobulin G1 (14).

The BALB/c animals that received weekly injections of the anti-IFN-γ antibody exhibited a 2-$\log_{10}$ increase in bacteria recovered from the lungs as compared with matched, untreated animals during the time course of 3 weeks. After 2 weeks, some of the mice began to clear the infection, but not as effectively as control animals (Fig. 3). Animals treated with the anti-IL-4 antibody cleared pertussis infection as well as control, untreated animals (data not shown). Thus, neutralization of IFN-γ impaired the ability of BALB/c mice to control *B. pertussis* infection.

To further examine the role of IFN-γ in bacterial clearance during *B. pertussis* infection, we determined the course of infection in GKO mice in which the IFN-γ gene had been disrupted (5). These mice, which have normal T and B cells, were exposed to an aerosol infection with *B. pertussis*. The course of *B. pertussis* infection in the GKO and normal control mice was monitored by examining bacterial recovery from the lungs of infected mice for 6 weeks after aerosol challenge with *B. pertussis*. As shown in Fig. 4, the GKO mice exhibited increased bacterial recovery from their lungs at 1 week postinfection compared to normal control animals. Although both strains of

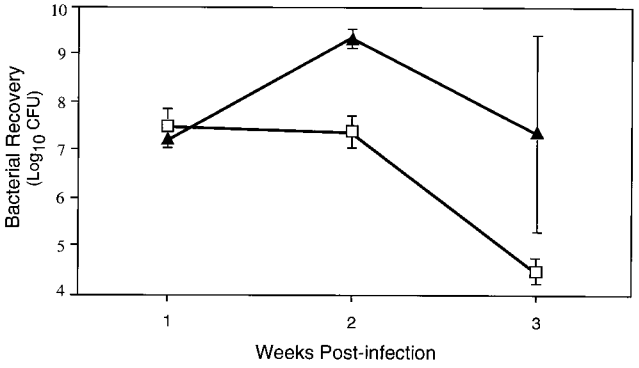

FIG. 3. Recovery of *B. pertussis* from the lungs of BALB/c mice depleted of IFN-γ, determined after aerosol infection. At each time point, the geometric mean and standard deviation of bacterial recoveries from the lungs of control, untreated mice (□) or mice depleted of IFN-γ (▲), using a monoclonal specific for IFN-γ as described in Materials and Methods, were determined. Each point represents the average bacterial recovery from the lungs of five mice. $P < 0.05$ when values for the 2- and 3-week time points for IFN-γ-depleted mice are compared to the corresponding values for control mice.

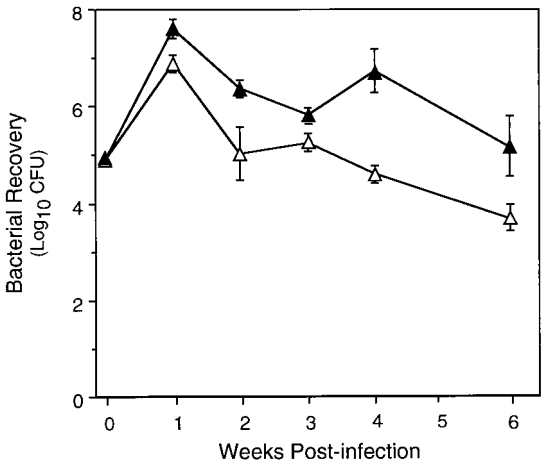

FIG. 4. Recovery of *B. pertussis* from the lungs of mice with a defective IFN-γ gene after aerosol infection. At each time point, the geometric mean and standard deviation of bacterial recoveries from the lungs of C57BL/6 mice (△) and GKO mice (▲) after aerosol challenge with *B. pertussis* were determined. Each point represents the average recovery from the lungs of three mice. $P < 0.05$ when values for the 1-, 2-, 4-, and 6-week time points for the GKO mice are compared to the corresponding values for the control mice.

mice began to clear *B. pertussis* infection after 1 week, the difference in bacterial recovery from the lungs of GKO mice compared to that from control animals remained constant over the 6-week course of the experiment.

DISCUSSION

In order to further elucidate the immune mechanisms responsible for natural clearance of infection, we examined the role of IFN-γ in the response of mice to an aerosol challenge of *B. pertussis*. While aerosol infection of adult mice is not a model of whooping cough per se, the model does reflect the initial stages of infection (i.e., colonization and outgrowth) and provides insights into the host-pathogen interactions that mediate colonization, disruption of host defense by bacterial toxins, and the specific immune mechanisms that lead to clearance of infection. Adult BALB/c mice are able to clear a *B. pertussis* infection within 6 weeks after aerosol challenge; however, nude mice (which lack mature T lymphocytes) and SCID mice (which lack mature B and T lymphocytes) have higher bacterial burdens in the lungs, cannot clear the infection, and die within 3 to 5 weeks after challenge.

Our work, as well as previous work (9), demonstrates that IFN-γ is produced upon stimulation of T cells obtained from mice recovering from *B. pertussis* infection. Moreover, we observed that IFN-γ mRNA levels increased in the lungs of BALB/c, nude, SCID, and C57BL/6 mice after aerosol infection with *B. pertussis*. For most mouse strains studied, IFN-γ mRNA levels increase rapidly after challenge and remain elevated for at least 4 weeks postinfection. Nude mice, however, exhibit a lag in induction of IFN-γ mRNA levels in that maximal levels are not achieved until 3 weeks postinfection. While this observation is reproducible, the basis for the delayed response is not known.

The production of IFN-γ by T cells obtained from mice recovering from *B. pertussis* infection as well as the observation that IFN-γ mRNA increased during the course of infection prompted us to pose the question of whether IFN-γ plays a role in controlling the infection. We found that mice depleted of IFN-γ exhibit a significant increase in bacteria recovered from the lungs after aerosol challenge compared to normal controls. Decreasing IFN-γ levels by two independent methods, antibody neutralization of IFN-γ or knockout of the IFN-γ gene, yielded similar results. While IFN-γ can affect clearance of *B. pertussis* from the lungs of infected mice, an IFN-γ response must represent only part of the protective immune response since mice with depleted levels of IFN-γ will begin to clear the infection by 2 to 3 weeks after exposure of the mice to the bacteria. The observation that nude and SCID mice, which die 3 to 5 weeks after infection, also exhibit an induction of IFN-γ mRNA in their lungs after infection suggests the possibility that while IFN-γ may help control the infection, it is not sufficient, although care should be taken when interpreting these results since mRNA levels are not necessarily reflective of protein levels. Thus, while IFN-γ clearly plays a role in controlling *B. pertussis* infection, it may be neither necessary nor sufficient to control infection. Such a result is not surprising since redundant mechanisms have likely evolved to offer optimal protection against microorganisms.

The mechanism by which IFN-γ may control infection remains unknown. IFN-γ is known to activate macrophages and stimulate nonspecific defense mechanisms such as phagocytosis (3, 16), which may operate here to control *B. pertussis* infection. While much is left to learn about the immune response to *B. pertussis* infection, elucidation of a role for IFN-γ in protection represents an initial step in dissecting the protective immune response to *B. pertussis.*

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