Atypical Disease after *Bordetella pertussis* Respiratory Infection of Mice with Targeted Disruptions of Interferon-γ Receptor or Immunoglobulin μ Chain Genes

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Summary

Using a murine respiratory challenge model we have previously demonstrated a role for Th1 cells in natural immunity against *Bordetella pertussis*, but could not rule out a role for antibody. Here we have demonstrated that *B. pertussis* respiratory infection of mice with targeted disruptions of the genes for the IFN-γ receptor resulted in an atypical disseminated disease which was lethal in a proportion of animals, and was characterized by pyogranulomatous inflammation and postnecrotic scarring in the livers, mesenteric lymph nodes and kidneys. Viable virulent bacteria were detected in the blood and livers of diseased animals. An examination of the course of infection in the lung of IFN-γ receptor–deficient, IL-4–deficient and wild-type mice demonstrated that lack of functional IFN-γ or IL-4, cytokines that are considered to play major roles in regulating the development of Th1 and Th2 cells, respectively, did not affect the kinetics of bacterial elimination from the lung. In contrast, B cell–deficient mice developed a persistent infection and failed to clear the bacteria after aerosol inoculation. These findings demonstrate an absolute requirement for B cells or their products in the resolution of a primary infection with *B. pertussis*, but also define a critical role for IFN-γ in containing bacteria to the mucosal site of infection.

Respiratory infection with the gram-negative coccobacillus *Bordetella pertussis* results in whooping cough, a major cause of morbidity and mortality in human infants. It is well known that during colonization of the respiratory tract this bacterium can specifically adhere to ciliated epithelium, however a number of studies have suggested that *B. pertussis* may also exploit an intracellular niche during infection. Persistence of *B. pertussis* within murine and rabbit alveolar macrophages has been described (1, 2), and it has recently been reported that *B. pertussis* can invade and survive within human macrophages (3–5). Although controversial, these studies suggest that intracellular localization may be an important mechanism in the disease process.

Recovery from a primary *B. pertussis* infection provides long lasting protective immunity against subsequent disease, however it is not clear which components of the immune response confer protection or contribute to bacterial clearance. Passive and active immunization studies in mice have shown that antibody can induce varying degrees of protection against either aerosol or intracerebral challenge (6, 7). In contrast, clinical trials of an acellular pertussis vaccine consisting of chemically detoxified pertussis toxin (PT)[1] and filamentous hemagglutinin (FHA), failed to demonstrate a correlation between serum antibody responses to PT or FHA and protection (8). Furthermore, studies using a murine respiratory infection model have demonstrated a role for *B. pertussis*–specific $CD4^+$ T cells that secrete IL-2 and IFN-γ (Th1 cells) in pulmonary clearance (9–11). Adoptive transfer experiments demonstrated that $CD4^+$ T cells from convalescent mice were capable of mediating *B. pertussis* clearance from nude or sublethally irradiated mice in the absence of a detectable serum antibody response (9). However this study, as well as the results of passive immunization experiments reported by others (6, 7, 12), did not rule out a role for $CD4^+$ T cells in providing help for a protective antibody response against *B. pertussis*.

The development of mouse strains with gene-targeted disruptions in key components of the immune response has provided insights into the mechanisms of antimicrobial de-

[1] *Abbreviations used in this paper:* FHA, and filamentous hemagglutinin; IFN-γR, IFN-γ receptor; PT, pertussis toxin.

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fense and pathology (13). Studies with gene knockout mice have highlighted the central role of T cells in immunity against the intracellular pathogens *Listeria monocytogenes* and *Mycobacterium tuberculosis* (13, 14). In the present study we have employed mice with targeted disruption of the genes for IL-4 (IL-4$^{-/-}$), the IFN-γ receptor (IFN-γR$^{-/-}$), or the immunoglobulin μ-chain (Ig$^{-/-}$) to elucidate the role of cytokines and the adaptive immune response to clearance of a primary *B. pertussis* respiratory infection. We demonstrate that in addition to the known requirement for CD4$^+$ T cells, clearance of this bacterium is dependent on B cells. Studies addressing the contribution of different cytokines demonstrated that functional IFN-γ or IL-4 were not essential for bacterial clearance from the lungs, but revealed that IFN-γ was critical in preventing bacterial dissemination.

Materials and Methods

Mice. All mice used were commercially obtained (B+K Universal Ltd., Hull, UK) and bred and maintained according to the guidelines of the Irish Department of Health. The IFN-γR$^{-/-}$ mice, in which IFN-γ is nonfunctional (15), were used with the kind permission of Dr. M. Aguet (University of Zurich, Switzerland). These mice were generated from the wild-type 129Sv/Ev (H-2^b) strain (15), which were employed as the control mice in these experiments. The IL-4$^{-/-}$ mice (IL-4T strain) (16) were used with the kind permission of Dr. Werner Muller (University of Cologne, Germany), and the Ig$^{-/-}$ (μMT strain) (17) were used with the kind permission of Dr. Klaus Rajewsky (University of Cologne, Germany). The Ig$^{-/-}$ and IL-4$^{-/-}$ mice, were generated from wild-type C57BL/6 (H-2^b) mice, which were employed as control mice in these experiments. Unless otherwise stated, all mice were 8–12 wk old at the initiation of experiments.

Aerosol Infection. Respiratory infection of mice was initiated by aerosol challenge by a modification of the method described by Sato et al. (18). Phase 1 *B. pertussis* Wellcome 28 (W28; a kind gift from Keith Redhead, National Institute for Biological Standards and Control, South Mimms, Herts, UK) was grown under agitation conditions at 37°C in Stainer-Scholte liquid medium. Bacteria from a 48-h culture were resuspended at a concentration of ~2 × 10^{10} CFU/ml in physiological saline containing 1% casein. The challenge inoculum was administered to mice as an aerosol over a period of 15 min by means of a nebulizer as previously described (19). Groups of three or four mice were killed at various times after aerosol challenge to assess the number of viable *B. pertussis* in the lungs, livers, or peripheral blood.

Enumeration of Viable Bacteria. Lungs or livers were removed aseptically from infected mice and homogenized in 1 ml of sterile physiological saline with 1% casein on ice. 100 μl of undiluted homogenate or of serially diluted homogenate from individual lungs or livers were spotted in triplicate onto Bordet-Gengou agar plates and the number of CFU was estimated after 5 d of incubation at 37°C. Results are reported as the mean viable *B. pertussis* for either individual lungs or livers from at least three mice per time point per experimental group. The limit of detection was ~$\log_{10}$ 0.5 CFU per organ. In experiments on disseminating infection, blood was aseptically sampled from a peripheral vein and assessed for the presence of viable *B. pertussis*. Samples of whole blood or peripheral blood mononuclear cells, isolated by metrizamide gradient centrifugation and lysis of red blood cells as previously described (20), were plated on Bordet-Gengou agar and the mean CFU/ml determined.

Bacterial Antigens. Heat killed *B. pertussis* was prepared by incubation of cells at 80°C for 30 min. Inactivation was confirmed by demonstrating that bacteria could not be cultured from these preparations. Genetically detoxified recombinant PT (PT-9K/129G), native FHA and pertactin prepared from *B. pertussis* were kindly provided by Rino Rappuoli (Chiron, Sienna, Italy). All antigen preparations were of clinical grade, endotoxin free and produced according to good manufacturing practice.

Analysis of B. pertussis-specific Antibody Production. The levels of serum antibody to *B. pertussis* were determined by ELISA using *B. pertussis* sonicated extract (5 μg/ml) to coat the plates. Bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse IgG (Sigma Chem. Co. Poole, Dorset, UK). Mouse IgG subclasses were determined using alkaline phosphatase conjugated anti–mouse IgG1 (clone G1-65), IgG2a (clone R19-15), IgG2b (R12-3), or IgG3 (R40-8L) purchased from PharMingen (San Diego, CA). Antibody levels are expressed as the mean endpoint titers calculated by regression of the straight part of a curve of OD vs. serum dilution to a cutoff of two standard deviations above background control values.

T Cell Proliferation Assays. Spleen cells from naive and infected mice were resuspended at 2 × 10^6/ml in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 8% heat-inactivated FCS (endotoxin content 0.071 ng/ml; Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured for 4 d with heat killed *B. pertussis* (10^5, 10^6, or 10^7/ml), heat-inactivated PT (0.2–5.0 μg/ml), FHA (0.2–5.0 μg/ml), pertactin (0.2–5.0 μg/ml), or medium alone and [^{3}H]thymidine (0.5 μCi/well; specific activity 2.0 Ci/mmol) was added for the final 4 h of culture as described (9). Results are given as the mean cpm of [^{3}H]thymidine incorporation for triplicate cultures for groups of at least three mice, after subtraction of the background responses with medium alone.

Analysis of Cytokine Production. Spleen cells from infected mice were cultured with *B. pertussis* antigens as described for the proliferation assay. Supernatants were removed after 24 h to determine IL-2 and after 72 h to determine IFN-γ, IL-4, and IL-5 concentrations as previously described (9). In brief, IL-2 release was assessed by testing the ability of culture supernatants to support the proliferation of the IL-2–dependent cell line CTLL-2 and the concentrations of murine IL-4, IL-5, and IFN-γ were determined by immunoassay using commercially available antibodies (PharMingen). Concentrations were determined by comparing either the proliferation or the OD for test samples with a standard curve for recombinant cytokines of known potency and concentration.

Pathology. Killed animals from 6 experiments (60 IFN-γR$^{-/-}$, 28 Ig$^{-/-}$, 28 IL-4$^{-/-}$, 28 C57BL/6, and 54 129Sv/Ev mice) were examined for signs of pathology. Furthermore in two separate challenge experiments necropsies were specifically performed on 15 *B. pertussis* infected IFN-γR$^{-/-}$ mice, 4 wild-type 129Sv/Ev mice and 4 uninfected IFN-γR$^{-/-}$ mice after euthanasia with pentobarbitone sodium. Liver, lungs, spleens, kidneys, and brains were placed in 10% neutral buffered formalin. After fixation, the tissues were dehydrated and embedded in paraffin. Sections were stained with hematoxylin and eosin (H & E) for histopathological examination. For demonstration of *B. pertussis* antigen, paraffin sections were stained using a commercial *B. pertussis*-specific antiserum (Difco Laboratories, Detroit, MI) at a dilution of 1/200 using an avidin-biotin technique (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin.

Results

Disseminating Infection in IFN-γR$^{-/-}$ Mice after B. pertussis Respiratory Challenge. Respiratory infection with *B. pertussis* proved lethal in a number of IFN-γR$^{-/-}$ mice, particularly in mice younger than 8 wk and those that received a high challenge inoculum (Fig. 1 and data not shown). Death appeared to result from organ failure associated with disseminating disease; *B. pertussis* infected IFN-γR$^{-/-}$ mice showed abnormal pathology not observed in the wild-type 129Sv/Ev mice. Furthermore, IFN-γR$^{-/-}$ mice that survived the challenge frequently showed overt lesions, visible macroscopically, in the liver, lungs, kidneys, spleen, and mesentery. All wild-type 129Sv/Ev mice (54 mice, 6 experiments) challenged with the same inoculum of bacteria survived the infection and lesions were confined to the lungs. All Ig$^{-/-}$ and IL-4$^{-/-}$ or C57BL/6 mice survived more than 20 wk after *B. pertussis* challenge.

Examination of 60 *B. pertussis*–infected IFN-γR$^{-/-}$ mice killed between 7–100 d after challenge (6 experiments), revealed evidence of gross pathological changes outside the lungs in 80% of animals. The most common examination features were multiple pale, firm nodules up to 10 mm in diameter projecting above the capsular surface of the liver. Similar nodular lesions were visible in the kidneys, spleens, and mesentery, but these were smaller than those in the livers. In two separate experiments histopathological examination was performed on 15 *B. pertussis* infected IFN-γR$^{-/-}$ 24 d after challenge and atypical liver pathology was observed in 100% of animals. The histological appearance of the lesions was of pyogranulomatous inflammation and postnecrotic scarring. Aggregates of neutrophils and macrophages adjoined the areas of necrosis and infiltrates of lymphocytes and plasma cells (Fig. 2, *a* and *b*). Fibroblastic proliferation and collagenization were prominent particularly in the livers. Localized infiltrates of neutrophils, macrophages and lymphoid cells observed in the brains of a minority of surviving IFN-γR$^{-/-}$ mice were largely confined to the leptomeninges (Fig. 2 *c* and data not shown).

In addition to areas of pyogranulomatous inflammation, infiltrates of macrophages and neutrophils were present in the alveolar septae and lumens of the lungs. Areas of consolidation were common in the lungs of each of 15 *B. pertussis* infected IFN-γR$^{-/-}$ mice examined. Granular deposits of basophilic debris observed in the alveoli and bronchioles correlated with the distribution of *B. pertussis* antigen (Fig. 2, *d* and *e*). Deposits of *B. pertussis* antigen were also evident in macrophages in areas of pyogranulomatous inflammation in mesenteric lymph nodes (Fig. 2 *f*).

Carriage of *B. pertussis* to secondary non pulmonary foci was via the blood (Table 1). Viable bacteria could be recovered from the blood of aerosol infected IFN-γR$^{-/-}$ mice between days 3 and 11, and from the liver between days 7 and 56 after challenge, but viable bacteria could not be cultured from sites outside the lung beyond day 56. Analysis of the CFU counts from fractionated blood samples demonstrated that viable *B. pertussis* were associated with the mononuclear cell fraction (data not shown). No evidence of bacterial dissemination was observed in *B. pertussis* infected Ig$^{-/-}$ and IL-4$^{-/-}$ or C57BL/6 mice (Table I) and all animals survived the challenge. Furthermore, there was no evidence of pathological changes in uninfected IFN-γR$^{-/-}$ mice or outside the lungs of *B. pertussis* infected 129Sv/Ev, IL-4$^{-/-}$, Ig$^{-/-}$ or C57BL/6 mice. A minority of *B. pertussis* infected Ig$^{-/-}$ mice (3/28) displayed lung consolidation and granulomas, limited to a single lobe.

Clearance of B. pertussis from the Lungs of Gene Knockout Mice. To examine the role of Th1 and Th2 cytokines on the course of infection in the lungs, the kinetics of bacterial clearance was monitored by performing CFU counts on the lungs of wild-type and gene disrupted mice at intervals after challenge. Although IFN-γR$^{-/-}$ mice displayed an atypical disease which proved lethal in ~30% of infected mice, the kinetics of bacterial clearance from the lungs of surviving animals was not significantly different from the wild-type 129Sv/Ev mice (Fig. 3). There was no significant difference in the kinetics of bacterial clearance from the lungs of IL-4$^{-/-}$ and the wild-type C57BL/6 mice (Fig. 3). We have previously reported that MHC and non-MHC differences between mouse strains influence murine survival in the *B. pertussis* intracerebral challenge model (11). Here we show that unlike BALB/c (H-2^d) mice which reproducibly clear a respiratory challenge within 40 d (9), C57BL/6 and 129Sv/Ev (both H-2^b) do not completely clear a respiratory challenge until up to 100 d after challenge (Fig. 3).

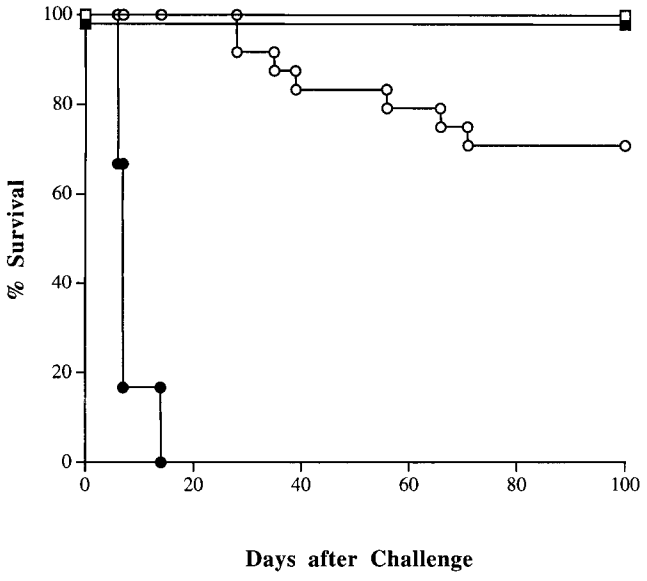

Figure 1. Survival of IFN-γR$^{-/-}$ mice after *B. pertussis* challenge. In one experiment groups of 24 IFN-γR$^{-/-}$ (○) or 24 wild-type 129Sv/Ev (□) mice were aerosol challenged with *B. pertussis* which resulted in an inoculum of 6×10^4 CFU/lung, determined from a sample group killed 2 h after challenge. In a separate experiment 6 IFN-γR$^{-/-}$ mice (●) received a higher dose challenge, which resulted in 1×10^6 CFU/lung 2 h after challenge. Six wild-type 129Sv/Ev mice (■) exposed to the higher dose of bacteria all survived the challenge (displayed offset for clarity).

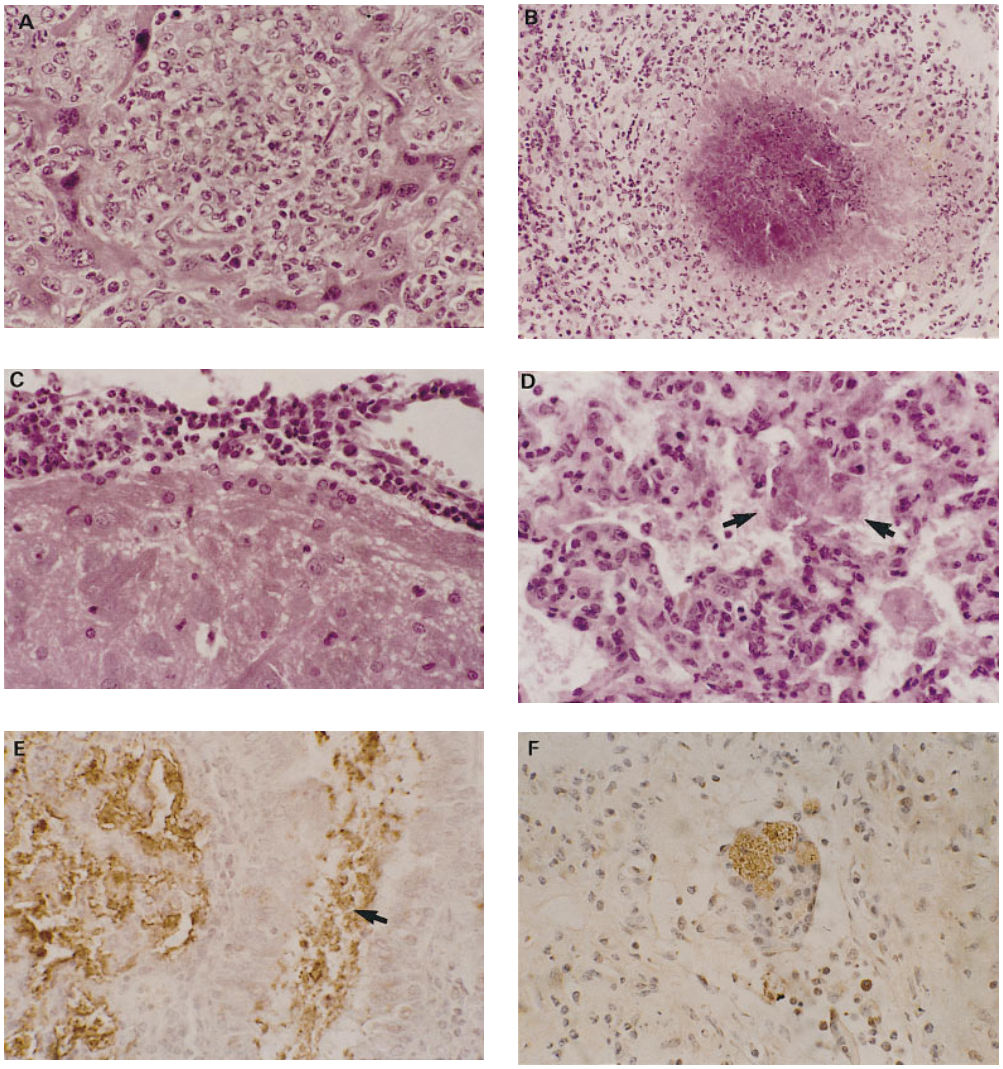

Figure 2. Pathological findings in IFN-$\gamma R^{-/-}$ mice after respiratory infection with *B. pertussis*. Mice were challenged with 2×10^{10} CFU/ml giving 6×10^4 CFU/lung 2 h later. (*A*) Liver: aggregates of neutrophils and macrophages with reactive changes in adjoining hepatocytes. (*B*) Mesentery: macrophages and neutrophils surround a central area of necrosis (necrotic pyogranuloma). (*C*) Brain: neutrophils, macrophages and fibrin deposits in the leptomeninges. (*D*) Lung: granular basophilic debris (*arrows*) in the alveolar spaces with macrophage and neutrophil infiltration primarily in alveolar walls. (*A–D*, hematoxylin and eosin [H & E] staining) (*E*) Lung: *B. pertussis* antigen in the lumen of alveoli and a bronchiole (*arrow*). Anti–*B. pertussis*, haematoxylin counterstain. (*F*) Mesentry: intracytoplasmic deposits of *B. pertussis* antigen in macrophages in an area of pyogranulomatous inflammation. Anti–*B. pertussis*, haematoxylin counterstain. Results are representative of 15 mice from two challenge experiments. Similar pathological lesions were observed in tissue from all mice examined, except for the brain, where lesions were visible in only 3 out of 7 mice examined. Original magnifications: (*A*, *C–F*) ×400; (*B*) ×200.

Aerosol infection of $Ig^{-/-}$ mice resulted in a persistent chronic lung infection and a failure to clear the bacteria from the lungs (Fig. 3 *C*). However B cell knockout mice were not overwhelmed by the infection and survived to the termination of the experiment 20 wk after challenge. The pattern of infection was similar to that seen in athymic *nu/nu* BALB/c mice (9), thus demonstrating a requirement for B cells as well as T cells in resolution of *B. pertussis* respiratory infection.

Antibody Responses in B. pertussis-infected Gene Knockout Mice. The contribution of antigen-specific B cells and specific Ig to the resolution of *B. pertussis* infection of humans and experimental animals is controversial (6–8). The development of the specific antibody response after aerosol challenge was characterized in normal mice and in mice with targeted gene disruptions. The results shown in Table 2 confirm that $Ig^{-/-}$ mice, which lack mature B cells, fail to mount an IgG antibody response. In contrast, wild-type C57BL/6 and 129Sv/Ev mice, as well as the IL-$4^{-/-}$ and IFN-$\gamma R^{-/-}$ gene knockout mice develop *B. pertussis*-specific serum antibody by day 24 after challenge, which in-

Table 1. *Disseminating B. pertussis infection in IFN-$\gamma R^{-/-}$ mice*

	B. persussis ($\log_{10}$ CFU)		
Mice	Lungs	Blood	Liver
IFN-$\gamma R^{-/-}$	6.6 (0.2)	3.0 (2.5)	2.0 (1.3)
129Sv/Ev	5.7 (0.4)	−*	−
$Ig^{-/-}$	5.8 (0.4)	−	−
C57BL/6	4.9 (0.3)	−	−

B. pertussis colony counts recovered from lungs (CFU/lung) and whole blood (CFU/ml) 10 d after challenge and from liver (CFU/liver) 24 d after aerosol challenge with 2×10^{10} CFU/ml *B. pertussis* (time points reflect peak bacterial recovery from tissue, detectable in the blood of infected IFN-$\gamma R^{-/-}$ from days 3–11 and in the liver from days 7–56 after challenge). Results are mean (SE) values for 4 mice tested individually in triplicate and are representative of two experiments. The lower detection limit was 0.5 $\log_{10}$ CFU per organ or per ml blood.
*Undetectable bacteria.

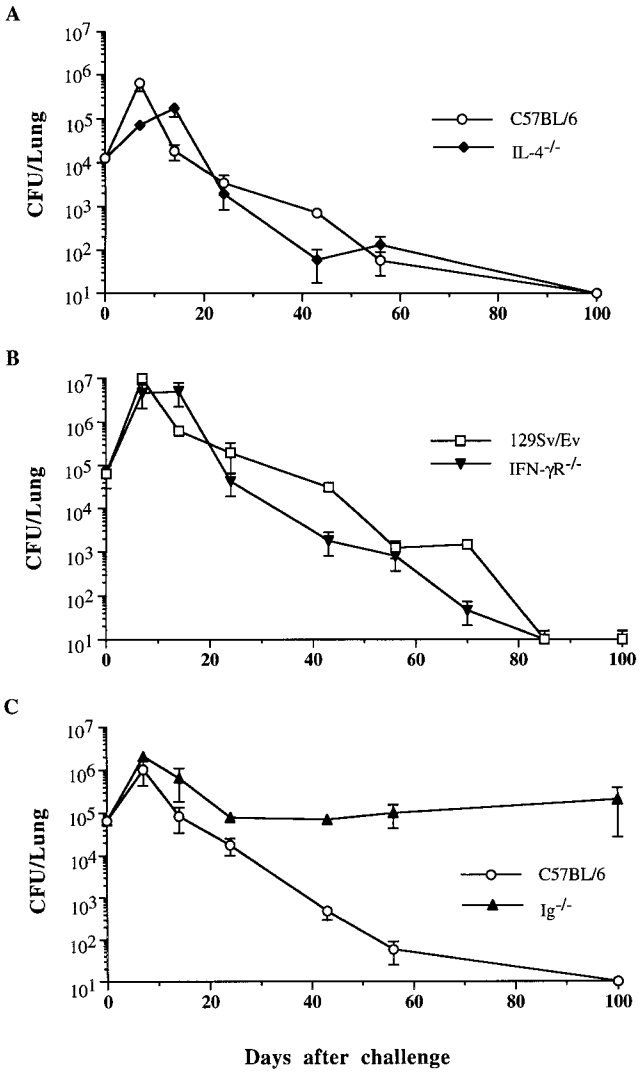

Figure 3. Course of *B. pertussis* respiratory infection in normal and gene knockout mice. IL-$4^{-/-}$ and C57BL/6 (*A*), IFN-$\gamma R^{-/-}$ and 129Sv/Ev (*B*), and $Ig^{-/-}$ and C57BL/6 (*C*) mice were infected by aerosol with 2×10^{10} CFU/ml *B. pertussis*, giving an initial colonization of $2–8 \times 10^4$ per lung. Groups of mice were killed at intervals after challenge and the number of viable bacteria estimated by performing colony counts on individual lung homogenates. Results are representative from two experiments and are presented as mean (±SE) CFU in the lungs estimated for three or four mice at each time point.

creases in titer until at least day 100. Higher antibody titers were observed early after infection in IFN-$\gamma R^{-/-}$ mice, when compared with the wild-type strain. Conversely IL-$4^{-/-}$ mice produced lower titers of specific antibody than that observed in wild-type C57BL/6 mice at early time points. An examination of the antibody isotypes revealed that the predominant IgG subclass detected was IgG2a, with little or no IgG1, except in IFN-$\gamma R^{-/-}$ mice which displayed higher titers of IgG2b and lower levels of IgG2a (Fig. 4). This pattern was consistent between analyses on serum samples recovered 24, 43, and 100 d after challenge.

Cell-mediated Immune Responses in B. pertussis-infected Gene Knockout Mice. The development of systemic cell-mediated immune responses in *B. pertussis* infected animals is thought to be central to clearance of the bacteria from the lungs (9–11). Positive spleen cell proliferative responses to heat killed *B. pertussis* could be detected in gene knockout and wild-type mice at day 14 after aerosol challenge (data not shown). Although relatively high background responses were observed with medium alone, this has previously been reported during *B. pertussis* infection of mice and children (21, 22) and may reflect activation of cells in vivo. The proliferative response to the putative protective antigens showed some variation; responses to PT were strongest in IFN-$\gamma R^{-/-}$ mice and were detectable in this group from

Table 2. *Bordetella Pertussis-specific Serum IgG Responses after Respiratory Infection of Mice with Targeted Gene Disruptions*

Days after challenge	Antibody titer*				
	129Sv/Ev	IFN-$\gamma R^{-/-}$	C57BL/6	IL-$4^{-/-}$	$Ig^{-/-}$
7	–‡	–	–	–	–
14	–	1.7 (0.1)	1.7 (0.1)	–	–
24	2.0 (0.1)	2.5 (0.1)	3.0 (0.1)	2.0 (0.1)	–
43	2.7 (0.2)	3.0 (0.1)	3.7 (0.1)	3.5 (0.4)	–
100	>4.0	>4.0	>4.0	>4.0	–

*Groups of gene knockout and wild-type mice were exposed to an aerosol challenge of virulent *B. pertussis* and the serum anti–*B. pertussis* IgG titers determined by ELISA. Results are shown for 20 mice per group from 2 experiments and are expressed as the recipocal of the mean (±SE) antibody titer ($\log_{10}$) for at least 4 mice at each time point after challenge.
‡Denotes an antibody titer <1/10.

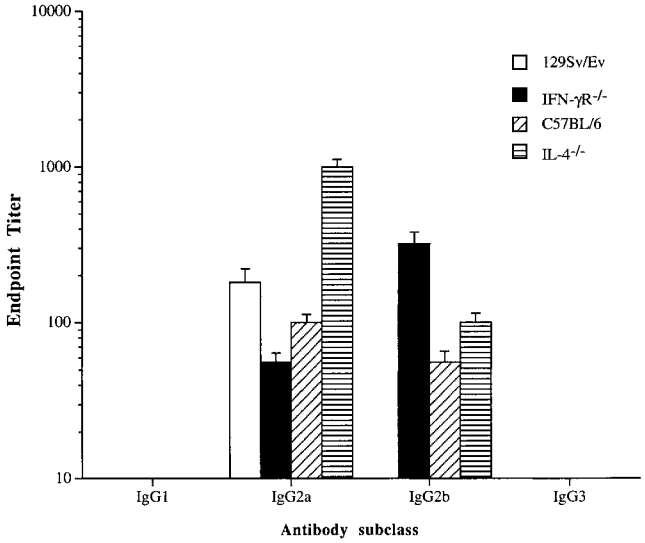

Figure 4. Subclass of *B. pertussis*–specific IgG in the serum 43 d after respiratory infection of wildtype and gene disrupted mice. Serum IgG was measured by *B. pertussis*–specific ELISA. Results are given as the geometric mean (±SE) ELISA titers from at least four mice determined in quadruplicate.

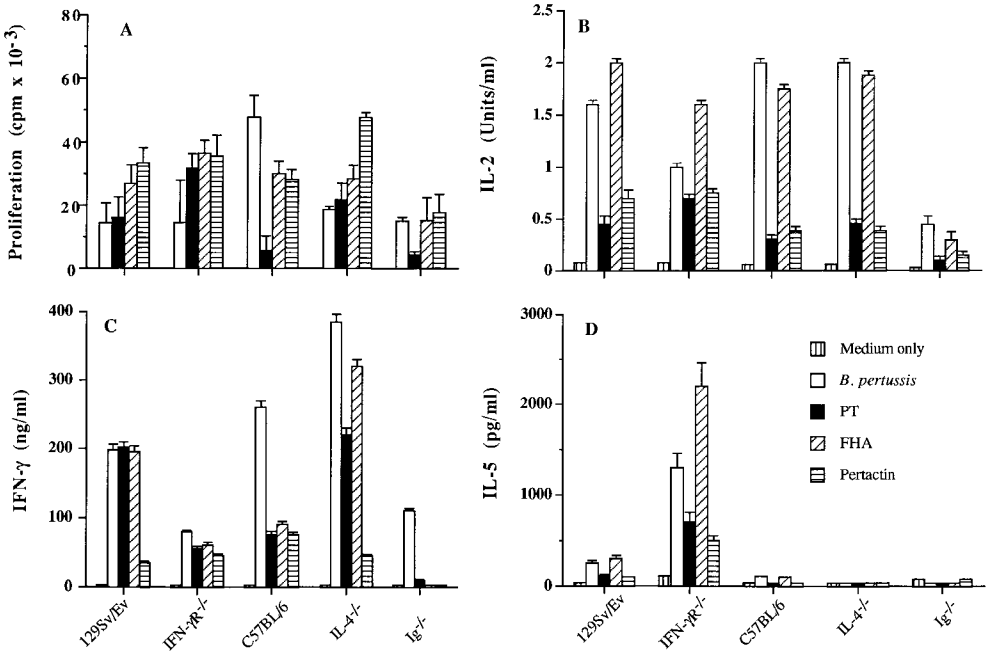

Figure 5. Cell mediated immune responses induced after *B. pertussis* infection of normal and gene disrupted mice. Proliferation (*A*), IL-2 (*B*), IFN-γ (*C*), and IL-5 (*D*) secretion by spleen cells from gene disrupted (IFN-γR$^{-/-}$, IL-4$^{-/-}$, and Ig$^{-/-}$) and wild-type (129Sv/Ev and C57BL/6) mice examined 43 d after challenge. Spleen cells (2×10^6/ml) were incubated with heat-killed whole *B. pertussis*, 1×10^6 cells/ml (*open bar*), inactivated PT 5.0 μg/ml (*solid bar*), FHA (*hatched bar*) 5.0 μg/ml, or pertactin 5.0 μg/ml (*horizontal shading*). Results are the mean responses for 4 mice per group assayed individually in triplicate. Proliferative responses are expressed as CPM (±SE) after subtraction of background responses to medium alone, which ranged from 5,000–10,000 cpm.

day 14, but were detectable in all animals by day 43 (Fig. 5 *A*). Unlike the C57BL/6 wild-type mice, responses to FHA could not be detected in IL-4$^{-/-}$ mice until 24 d after challenge. Responses to pertactin could not be detected from any mice before day 24. The *B. pertussis*-specific proliferative responses of Ig$^{-/-}$ mice were compromised compared with the wild-type C57BL/6 strain. Proliferative responses to *B. pertussis* and component antigens were detected on day 43 (Fig. 5 *A*), but this had declined by day 100 and positive proliferative responses could only be detected in Ig$^{-/-}$ mice at time points beyond 100 d against whole bacterial preparations at high concentrations. Responses to the putative protective antigens PT, FHA and pertactin could not be restored by the addition of MHC-matched APC to the cultures (data not shown).

An examination of the pattern of cytokines induced by *B. pertussis* infection of gene knockout mice and their controls revealed that spleen cell preparations from all mice secreted IL-2 in an antigen-specific manner by day 14, and this response was generally strongest at day 43 after challenge (Fig. 5 *B*). Levels of IL-2 remained high 100 d after infection in all except Ig$^{-/-}$ mice. The production of IFN-γ by spleen cells from infected animals was low at day 14 after challenge. However by day 24 and beyond antigen-specific IFN-γ production could be detected in all mice (Fig. 5 *C*). The reason for the lower level of IFN-γ production by spleen cells from IFN-γR$^{-/-}$ mice compared with the wild-type strain is not clear, but may reflect a lack of positive feedback on Th1 cells and negative regulation on the Th2 population. There was no evidence of bacterial outgrowth from spleen cell cultures derived from infected IFN-γR$^{-/-}$ mice and IFN-γ could not be detected from unstimulated control cultures of these cells (Fig. 5 *C*).

The Ig$^{-/-}$ strain showed less robust IFN-γ production with appreciable levels only reached by day 43, and only in response to whole bacteria and not the component bacterial antigens tested. The levels of the Th2 cytokine IL-4 were also tested, but as previously reported (9) no significant IL-4 was detected after a single in vitro stimulation of bulk spleen preparations (data not shown). In contrast, the Th2 cytokine IL-5 was detected in the supernatant of antigen-stimulated spleen cells from *B. pertussis* infected IFN-γR$^{-/-}$ mice, but not from the wild-type or other strains examined.

Discussion

The results of this study provide direct evidence of an obligatory role for B cells and IFN-γ in the protective immune response against *B. pertussis*. Using a murine respiratory infection model and mice with disruptions targeted to the genes for IL-4, the IFN-γ receptor, or for the immunoglobulin μ-chain (B cell knockouts) we demonstrate atypical disease resulting from disseminating infection in the absence of functional IFN-γ and a persistent infection, confined to the lungs, in the absence of antibody and B cells.

We have previously shown that effective immunization against *B. pertussis* respiratory infection in mice is dependent on the induction of cell mediated immunity (9–11). Respiratory challenge of athymic (*nu/nu*) BALB/c mice results in a persistent infection demonstrating an essential role for T cells in the clearance of a primary infection (9). Furthermore, adoptive transfer studies have shown that MHC class II–restricted CD4$^+$ cells but not CD8$^+$ T cells mediate protection against subsequent infection (9). These studies suggested a role for Th1 cells in protective immunity against *B. pertussis*, but interestingly also suggested that primed B cells and macrophages contribute to effective bacterial elimination. The present study demonstrates an essential role for B cells in bacterial clearance, with Ig$^{-/-}$

mice failing to clear a respiratory challenge, and indicates that both $CD4^+$ T cells and B cells are required for complete elimination of *B. pertussis* from the lungs. It is not clear which aspect of B cell function is central to the protective mechanism. The T cell proliferative and cytokine responses against *B. pertussis* and in particular against the soluble bacterial components are less robust than in wild-type control mice, however these responses are detectable but do not correspond with a decline in bacterial load. These findings suggest that while defective antigen presentation to T cells may contribute to the lack of clearance in $Ig^{-/-}$ mice, failure to mount an antibody response may also contribute to bacterial persistence. Virulent *B. pertussis* is also known to exhibit varying degrees of resistance to the classical complement pathway of lysis by virtue of the recently described products of the *brk* locus (23). Taken together these data suggest that the lack of specific opsonizing antibody may render $Ig^{-/-}$ mice unable to clear the bacterium. It should also be noted that bacterial numbers in the lungs of B cell knockout mice increase and then plateau at day 21. The observation that these mice are not overwhelmed by infection but survive beyond 100 d after challenge strongly supports a role for other nonhumoral immune mechanisms in limiting pulmonary infection.

The demonstration of *B. pertussis* in the liver and the early bacteremia, together with the pathological changes in the liver and other organs of aerosol infected IFN-$\gamma R^{-/-}$ mice indicate an important role for IFN-γ in the strict localization of *B. pertussis* infection to the lungs of immunocompetent animals. Although the full significance of the brain pathology observed in certain IFN-$\gamma R^{-/-}$ mice is not clear, cases of encephalitis have been associated with whooping cough in children (24). The probable hematogenic dissemination of *B. pertussis* in the absence of functional IFN-γ may be of significance to the reported isolation of *B. pertussis* from blood culture of a patient with Wegener's granulomatosis (25), and the ability of *B. holmesii*, *B. hinzii*, and *B. bronchiseptica* to cause bacteremia or sepsis (26–28). The reduced ability of the immature immune system to secrete IFN-γ and other cytokines may explain the greater susceptibility of human infants and neonatal mice to *B. pertussis* infection. Although neonatal mice were not examined in the present study, the results of preliminary experiments did demonstrate that in comparison with adult mice, younger IFN-$\gamma R^{-/-}$ mice were more susceptible to the lethal effects of bacterial dissemination.

Interestingly viable virulent bacteria were isolated from the livers of IFN-$\gamma R^{-/-}$ mice early after infection and immunohistochemical analysis of mesenteric lymph nodes demonstrated intracytoplasmic bacterial aggregates within macrophages. We found evidence of disseminating infection in IFN-$\gamma R^{-/-}$ mice before the development of *B. pertussis*-specific antibody or T cell responses in wild-type or knockout mice. This suggests that IFN-γ produced by cells of the nonadaptive immune response such as NK cells or $\gamma\delta$T cells plays an important role in containing *B. pertussis* to the lung during the initial period of infection, perhaps through the killing of bacteria exploiting an intracellular niche within macrophages (29). Atypical disease has previously been reported in disease models of obligate and facultative intracellular bacteria using gene knockout mice. Experimental *L. monocytogenes* infection of TCR $\delta^{-/-}$ mice results in large abscess-like liver lesions not seen in normal control mice (30), and disseminated tuberculosis is observed in IFN-γ gene disrupted mice infected either intravenously or aerogenically (31), highlighting the key role of IFN-γ in another important respiratory disease.

Whole *B. pertussis* and its components are known to elicit NO production by murine macrophages under a variety of conditions (32, 33). It has been reported that IFN-γ can augment bacterial killing and NO production by *B. pertussis* infected macrophages in vitro (28, 32). Furthermore, IFN-$\gamma R^{-/-}$ mice have an impaired ability to produce reactive nitrogen intermediates (34, 35 and unpublished observations). Taken together with the findings of the present study, these observations suggest that NO or reactive nitrogen intermediates may play a role in the destruction of an intracellular reservoir of *B. pertussis* and the prevention of disseminated disease. However IFN-$\gamma R^{-/-}$ mice show unimpaired bacterial clearance from the lungs suggesting that this is not an essential mechanism of pulmonary elimination during *B. pertussis* infection and that TNF-α, TNF-β, or Th2 derived cytokines may compensate for certain of the functions of IFN-γ in immunity to *B. pertussis* in the knockout animals.

It has been suggested that both Th1 and Th2 cells must interact for optimal mucosal protection against *B. pertussis* (36). Our demonstration of similar kinetics of bacterial clearance from the lungs of IFN-$\gamma R^{-/-}$ or IL-$4^{-/-}$ and wild-type mice demonstrates that there is a degree of redundancy in the cytokines or the Th subpopulations that mediate bacterial elimination from the lungs. However, IFN-γ, whether T cell derived or from other cell types, appears necessary to contain infection within the lungs. It is likely that the early production of IFN-γ by cells of the innate immune system in response to *B. pertussis* infection favors the subsequent induction of a Th1 type response. This may explain the high level of protection observed in convalescent mice or in animals immunized with pertussis vaccines formulated with IL-12 or with vaccines which include components that induce endogenous IL-12 production (19). Thus IFN-γ may function to activate the antimicrobial activity of macrophages against an intracellular reservoir of bacteria. However this does not exclude the obvious role for $CD4^+$ T cells in providing help for antibody responses of a functionally relevant isotype (37). Our demonstration of reduced levels of *B. pertussis*–specific IgG2a in infected IFN-$\gamma R^{-/-}$ mice compared with the wild-type strain is consistent with the latter possibility.

Our findings may also provide an explanation for the failure to demonstrate a serological correlate of protection induced with acellular pertussis vaccines, that have recently been shown to confer a high level of protection against disease in children. (38, 39). These vaccines induce potent, but short lived antibody responses (38, 39) and *B. pertussis*-specific T cells with a Th0 or mixed Th1/Th2 cytokine

profile (22). We have previously shown that infection preferentially induces Th1 cells and that these cells appear to play a critical role in protection against *B. pertussis* (9, 21). In the present study, the production of Th2 cytokines and the lack of functional IFN-γ did not affect the clearance of bacteria from the lung during a primary *B. pertussis* infection, but did result in dissemination of this bacterium. In conclusion, our findings support a model of bacterial clearance from the respiratory tract which requires both B cells and $CD4^+$ T cells to resolve infection and indicate that functional IFN-γ plays an essential role in confining *B. pertussis* to the mucosal site of the lung.

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