CONCISE COMMUNICATION

Protection against *Bordetella pertussis* in Mice in the Absence of Detectable Circulating Antibody: Implications for Long-Term Immunity in Children

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Most vaccines used for humans work through humoral immunity, yet many appear to be protective even after specific circulating antibody levels have waned to undetectable levels. Furthermore, it has been difficult to define a serologic correlate of protection against a number of infectious diseases, including those caused by *Bordetella pertussis*. *B. pertussis* clearance in immunized mice has been shown to correlate with pertussis vaccine efficacy in children. This murine respiratory challenge model was used to demonstrate persistent vaccine-induced protection against *B. pertussis* in the absence of circulating antibody at the time of challenge. Whole-cell and acellular pertussis vaccines induced persistent memory T and B cells and anamnestic antibody responses after challenge. The findings suggest that immunologic memory is more significant in protection than is the induction of immediate antibody responses and imply that vaccinated children still may be protected against disease following the disappearance of specific serum IgG.

Whole-cell pertussis vaccines appear to induce long-lasting immunity against severe whooping cough in children and young adults. However, concern about the safety of whole-cell vaccines has resulted in the development of acellular pertussis vaccines prepared from purified components of *Bordetella pertussis.* The new vaccines, which have a higher safety profile, have replaced whole-cell vaccines in many developed countries. Results from clinical trials have indicated that acellular pertussis vaccines confer relatively high levels of protection against severe disease [1–4], but these studies have not provided definitive information about a relationship between antibody levels against protective antigens and immune protection. Follow-up studies have indicated that antibody responses in immunized children decline to low or undetectable levels 2 years after a complete course of vaccination [5, 6]. These children do not appear to be developing overt disease [7], but, without a history of exposure and the application of sensitive indicators of infection in large postimmunization surveillance programs, it is impossible to determine whether they are immune to infection and to establish the mechanisms that mediate protection. Although these fundamental questions are difficult to answer in a clinical setting, we have been able to address them by use of a well-established animal model.

By use of a murine respiratory challenge model of *B. pertussis* infection, in which protection correlates with vaccine efficacy in human clinical trials, together with gene knockout mice, we have identified complementary roles for cellular and humoral immunity in protection [8–11]. In our study, we used this model to examine the relationship between the persistence of the immune response and the development of immunologic memory and protection against respiratory infection.

Materials and Methods

Immunization. BALB/c mice were immunized intraperitoneally (at 0 and 4 weeks) with 0.04 human dose of either Wellcome whole-cell pertussis vaccine (British Reference reagent 88/522; National Institute for Biological Standards and Control, Potters Bar, Hertsfordshire, UK) or Chiron acellular pertussis vaccine (Chiron Vaccines, Siena, Italy), equivalent to 0.2 μg of recombinant pertussis toxin (PT), 0.1 μg of filamentous hemagglutinin (FHA), and 0.1 μg of pertactin (PRN). Control mice were immunized with adjuvant (alum) only.

Challenge and course of B. pertussis *infection.* Respiratory infection of mice with *B. pertussis* was initiated by aerosol challenge as described elsewhere [8]. The kinetics of bacterial clearance were determined by counting colony-forming units (cfu) in the lungs on days 0, 3, 7, 14, and 21 after challenge [8]. Results are reported as the mean number of viable *B. pertussis* for individual lungs from at least 4 mice per time point per experimental group.

Antibody responses. The levels of serum antibody to *B. pertussis* and bacterial components were determined by ELISA as described elsewhere [9]. *B. pertussis* sonicate (5.0 μg/mL) or PT,

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PRN, or FHA (1.0 μg/mL of each) were used to coat plates. Bound antibodies were detected by use of alkaline phosphatase–conjugated anti–mouse IgG. Antibody levels are expressed as the mean end-point titer (±SE), determined by extrapolation of the straight part of the titration curve to 2 SE above the background value (OD ≤0.2) obtained with nonimmune mouse serum. Assays were standardized with reference antiserum prepared from mice immunized with the British reference preparation of whole-cell pertussis vaccines [9]. Titers ≥1/20 (1.3 $\log_{10}$) were considered positive.

T cell cytokine assays. Spleen cells from individual mice were tested at different time points after immunization and after challenge for in vitro cytokine production in response to heat-killed *B. pertussis* (10^6/mL), PT (5.0 μg/mL; heat-inactivated at 80°C for 20 min), FHA (5.0 μg/mL), PRN (5.0 μg/mL), concanavalin A (2 μg/mL), or medium alone as control. Supernatants were removed from triplicate cultures after 72 h, and interferon-γ and interleukin-4 concentrations were determined by immunoassay [7].

Assessment of memory B cells. Antibody-forming cells were quantified by an insoluble substrate *B. pertussis*–specific ELISPOT. The wells of plates (Multiscreen HA; Millipore, Bedford, MA) were coated overnight with 100 μL of sonicated *B. pertussis* or inactivated PT (5 μg/mL). Both surfaces of the membrane were washed 5 times aseptically with PBS and then were blocked for 1 h with 8% (vol/vol) fetal calf serum in RPMI medium. After blocking, spleen or peripheral blood mononuclear cell preparations were added to quadruplicate wells at concentrations from 1×10^4 to 1×10^7/mL and were incubated at 37°C with 5% CO_2 for 2 days. After being washed 6 times in PBS, plates were incubated for 2 h with horseradish peroxidase–conjugated goat anti–mouse IgG (Sigma, Dorset, UK). Plates were washed 3 times more before the addition of 200 μL/well 3-amino-9-ethylcarbazole substrate buffer. After 45 min, this was removed, and the plates were washed once with water and allowed to air dry. Spots representing individual antibody-forming cells were counted by use of a zoom stereo microscope.

Results

Acellular and whole-cell pertussis vaccines protect against B. pertussis *after the disappearance of serum IgG responses.* Immunization of mice with 2 low doses of either a whole-cell or an acellular pertussis vaccine induced strong serum IgG responses against PT, FHA, and PRN, but antibody levels declined rapidly after 3 months and were undetectable by 6–9 months (figure 1*A*). Immunized mice were challenged with *B. pertussis* either at the peak of the antibody response 6 weeks after primary immunization or once circulating antibody titers had waned, 44 weeks after primary immunization. Despite the decline in *B. pertussis*–specific serum IgG to undetectable levels, both vaccines still conferred protection against *B. pertussis* when challenged 44 weeks after immunization (figure 1*B*). The numbers of bacteria in the lungs were significantly higher in control than in either whole-cell vaccine– or acellular vaccine–immunized mice at all time points tested ($P < .01$–$.001$). In particular, mice immunized with whole-cell pertussis vaccines displayed a high level of protection that was equivalent to that observed at the peak of the antibody response 6 weeks after immunization; counts of cfu were significantly greater in whole-cell vaccine– than in acellular vaccine–immunized mice 3, 7, and 10 days after challenge at 44 weeks.

Immunization induces memory T and B cells. Recall T cell responses to *B. pertussis* antigens persisted in whole-cell vaccine– and acellular vaccine–immunized mice, and, on the day of challenge at 44 weeks after primary immunization, *B. pertussis*–specific T cell responses clearly were detectable in immunized mice (figure 2*A*). Whole-cell pertussis vaccine induced a Th1-dominated response, whereas acellular pertussis vaccine induced a Th2-type response, and these patterns of cytokine secretion persisted for at least 44 weeks. However, 14 days after challenge, the cytokine profile of *B. pertussis* antigen–stimulated spleen cells from mice immunized with acellular pertussis vaccine had switched to a mixed Th1/Th2-type response (18–33 ng/mL interferon-γ and 100–217 pg/mL interleukin-4), whereas the Th1 response was maintained in mice immunized with whole-cell pertussis vaccine (58–160 ng/mL interferon-γ and undetectable interleukin-4).

Memory B cells specific for *B. pertussis* also were induced by immunization with whole-cell and acellular pertussis vaccines and persisted for at least 44 weeks after vaccination. By use of an ELISPOT technique, specific antibody-forming cells were detected in peripheral blood mononuclear cells and spleen cells after stimulation with PT or *B. pertussis* sonicate (figure 2*B*). Significantly greater numbers of *B. pertussis*–specific B cells could be detected in the blood and spleens of immunized mice than in those of controls. Memory induction was confirmed by the observation of an anamnestic antibody response to *B. pertussis* antigens in immunized mice after challenge (figure 1*A*). IgG responses to *B. pertussis* could not be detected in unimmunized control mice for at least 21 days after challenge. In contrast, 7 days after challenge, circulating antibodies were detected against PRN and *B. pertussis* sonicate in whole-cell vaccine–immunized mice and against PRN and PT in acellular vaccine–immunized mice.

Discussion

Our study demonstrates that circulating antibody, considered to be the major effector mechanism against viruses and bacteria, need not be detectable at the time of exposure for the maintenance of vaccine-induced protective immunity against a bacterial pathogen of humans. Protective levels of circulating antibody have been identified for certain pathogens, such as poliovirus, the agents of diphtheria and tetanus, and *Haemophilus influenzae.* However, certain persons who have antibody levels below these threshold values appear to be protected. Furthermore, it has been difficult to establish protective levels of antibody induced with certain vaccines, including those against pertussis [1–4]. Recent reports from household contact studies have indicated that antibody responses to PT, PRN, and fim-

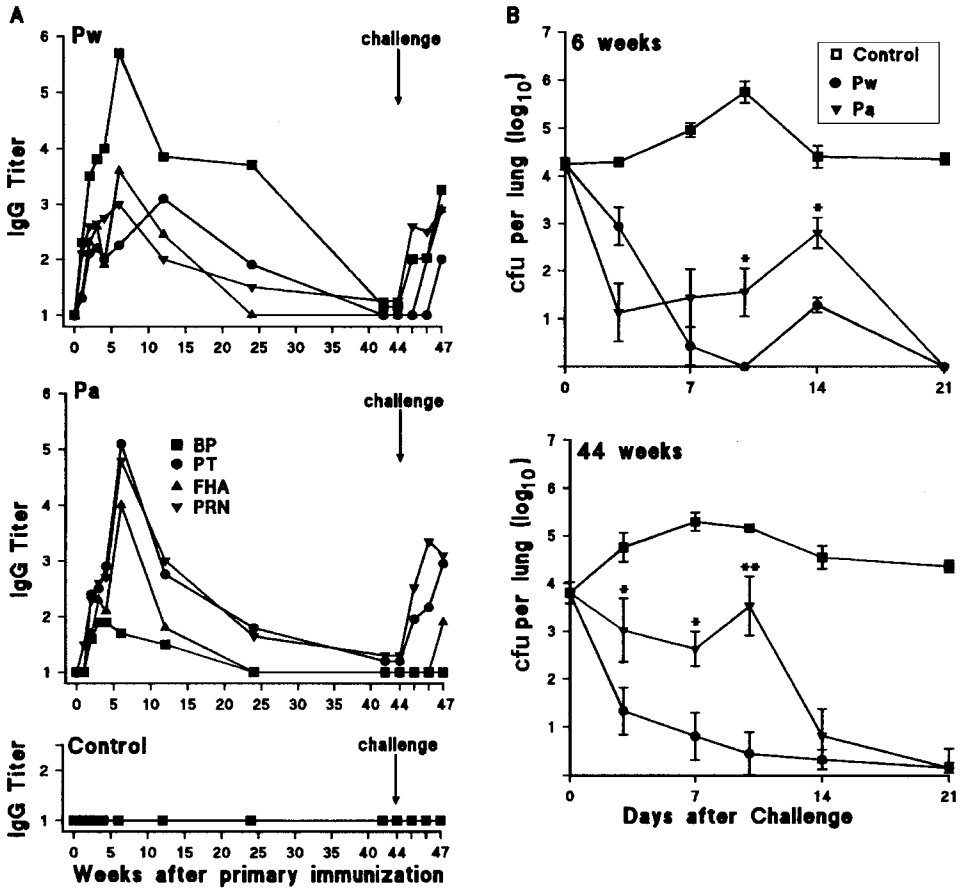

Figure 1. Persistence of protection against *Bordetella pertussis* respiratory infection after decline in specific serum IgG to undetectable levels. Mice were immunized at weeks 0 and 4 with 0.04 human dose of whole-cell pertussis vaccine (Pw) or acellular pertussis vaccine (Pa) or with adjuvant only as control. *A,* Serum antibody responses to *B. pertussis* (BP) sonicate, pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN), detected by ELISA at intervals after immunization and 1, 2, and 3 weeks after respiratory challenge. *B,* Immunized and control mice after exposure to aerosol challenge with *B. pertussis* 6 or 44 weeks after primary immunization. Results are mean (±SE) colony-forming units (cfu) in lungs, estimated for 4 mice/group at each time point (error bars are smaller than symbols at certain time points for control mice). $*P < .05$; $**P < .01$, whole-cell vs. acellular vaccine (Mann-Whitney *U* test).

briae may correlate with absence of severe disease [7, 12]. Although these studies suggested that antibodies to PRN and fimbriae are the most important, a monocomponent pertussis toxoid vaccine conferred 71% protection against severe disease in a clinical trial [4]. Furthermore, antibody levels in children decline rapidly after vaccination [5, 6, 13]. The results of the present study suggest that, if exposed to *B. pertussis,* these children still would be protected against developing whooping cough.

There are a number of explanations for the maintenance of protection after the decline in circulating anti–*B. pertussis* antibody. It is possible that antibody does not play a role in protection and that immunity is conferred exclusively by cellular mechanisms. Analysis of cell-mediated immunity in mice demonstrated that specific T cell responses were maintained up to the time of challenge, 44 weeks after immunization. Whole-cell pertussis vaccines selectively induced *B. pertussis*–specific Th1 cells, whereas acellular pertussis vaccines induced T cells more polarized to the Th2 subtype, which is consistent with reports on short-term immunization [8, 9]. We have suggested elsewhere that the Th1 subpopulation confers optimal protection against *B. pertussis* [8–11] and that these cells may function by promoting the antibacterial activity of macrophages and neutrophils and by stimulating opsonizing antibodies. However, acellular pertussis vaccines also can confer a high level of protection against severe disease, and this appears to be mediated solely by antibody with help from Th2 cells. The slightly lower levels of bacteria in the lungs of whole-cell vaccine–immunized mice, compared with acellular vaccine–immunized mice after challenge at 44 weeks, is consistent with previous studies on short-term protection with a range of whole-cell and acellular pertussis vaccines [8] and may reflect the contribution of cell-mediated immunity to protection. Nevertheless, the results suggest that the Th1/Th2 dichotomy does not impinge on memory

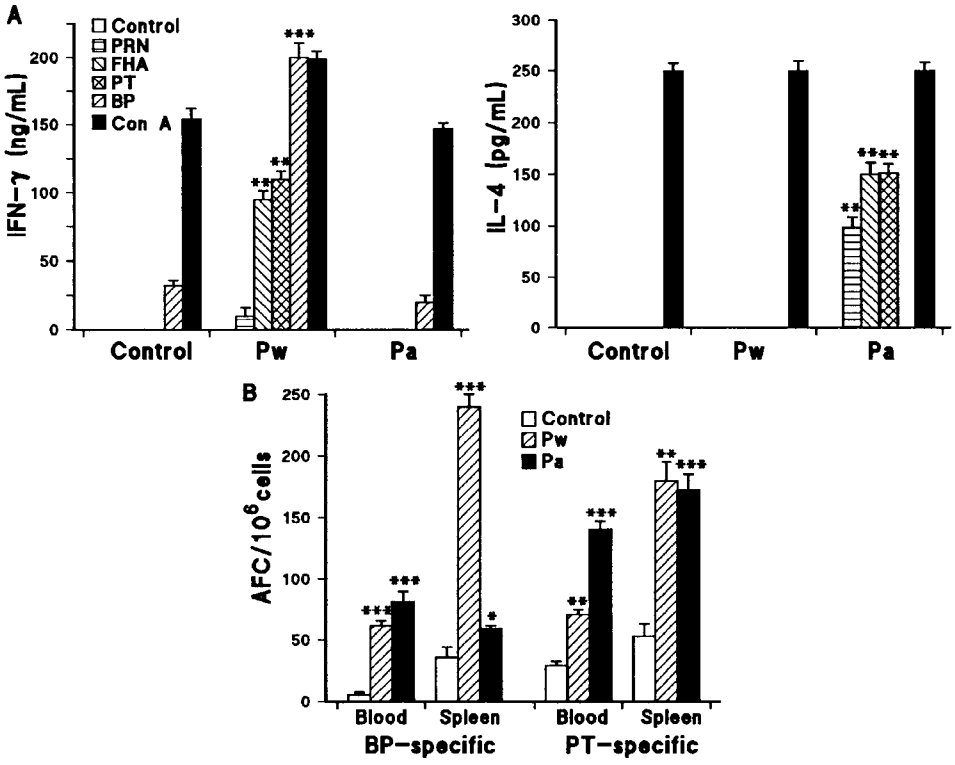

Figure 2. Recall T and B cell responses to *Bordetella pertussis* antigens after immunization with whole-cell pertussis vaccine (Pw) or acellular pertussis vaccine (Pa). *A,* 44 Weeks after immunization, spleen cells (2×10^6/mL) were incubated with heat-inactivated pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (PRN), heat-killed *B. pertussis* (BP), concanavalin A (Con A) or medium only as positive and negative controls, respectively. Supernatants were removed after 72 h and assessed for interferon-γ (IFN-γ) and interleukin-4 (IL-4). Results are mean (±SE) cytokine concentrations for 4 mice/group, assayed in triplicate. Cytokines were undetectable in supernatants from cells stimulated with medium alone. *B,* 44 Weeks after immunization, peripheral blood mononuclear cells and spleen cells from immunized or control mice were incubated in plates coated with *B. pertussis* sonicate (BP) or inactivated PT, and nos. of specific antibody-forming cells (AFC) were determined by ELISPOT. Results are mean values (±SE) for triplicate determinations from 4–6 mice/group. Cytokine and AFC data are representative of 4 different time points after immunization in 2 independent experiments, with similar levels of statistical significance at each determination. $*P < .05$; $**P < .01$; $***P < .001$ vs. control (Student's *t* test).

induction. Furthermore, although these patterns of cytokine secretion are stable, they are not immutable or "locked in." After bacterial challenge, there is a broadening of the pattern of cytokine responses observed in acellular pertussis vaccine–immunized mice, from a Th2 response 7 days after challenge to a mixed Th1/Th2 or Th0-type response thereafter. In a study of children immunized with acellular pertussis vaccines, the mixed Th1/Th2 profile observed early after vaccination [14, 15] had switched to a Th1-type response 3–4 years later [13]. It was suggested that exposure to subclinical infections, which would enhance Th1 responses, may explain the persistent protection against typical pertussis, despite a substantial waning of both antibody and T cell responses induced by the primary immunization [13].

An alternative, but not mutually exclusive, hypothesis to explain long-term protection in children after declining antibody responses is that immune effector mechanisms generated from memory B and T cells after bacterial exposure are capable of clearing the bacteria from the respiratory tract. This is consistent with our study and with recent suggestions that antibodies against multiple antigens can mediate protection against *B. pertussis* [3, 8]. Given the long incubation period of *B. pertussis* infection, anamnestic antibody responses after exposure may be sufficient to protect against severe whooping cough. However, it appears that current pertussis vaccines do not confer sterilizing immunity against *B. pertussis.* These vaccines are capable of preventing severe disease but are less efficient when judged on the criterion of preventing infection or mild disease, even in the presence of high levels of circulating antibody at the time of infection. Although the induction of a given concentration of circulating antibody early after vaccination may still provide a useful correlate of protection, in that it may predict the level of B cell priming, it is not clear that it will predict the extent of memory T and B cell induction. Although we do not yet have definitive information on the relative importance of T or B cell memory, we have demonstrated else-

where that short-term memory B and T cells both contribute to protection induced by natural infection [10].

Our findings have important implications for pertussis vaccination policy in relation to the level of protection in the face of waning antibody levels and the need for booster immunization. The data from the mouse model suggest that persistence of circulating antibody is not a prerequisite for the maintenance of protective immunity. These observations also have implications for the evaluation and development of vaccines against a range of infectious diseases. Although circulating neutralizing antibodies clearly are important to confer sterilizing immunity, especially against viral infections, it also appears that certain vaccines can protect by recall of immunologic memory at the T and B cell level.

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