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Acellular Pertussis Vaccine Protects against Exacerbation of Allergic Asthma Due to *Bordetella pertussis* in a Murine Model

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The prevalence of asthma and allergic disease has increased in many countries, and there has been speculation that immunization promotes allergic sensitization. *Bordetella pertussis* infection exacerbates allergic asthmatic responses. We investigated whether acellular pertussis vaccine (Pa) enhanced or prevented *B. pertussis*-induced exacerbation of allergic asthma. Groups of mice were immunized with Pa, infected with *B. pertussis*, and/or sensitized to ovalbumin. Immunological, pathological, and physiological changes were measured to assess the impact of immunization on immune deviation and airway function. We demonstrate that immunization did not enhance ovalbumin-specific serum immunoglobulin E production. Histopathological examination revealed that immunization reduced the severity of airway pathology associated with sensitization in the context of infection and decreased bronchial hyperreactivity upon methacholine exposure of infected and sensitized mice. These data demonstrate unequivocally the benefit of Pa immunization to health and justify selection of Pa in mass vaccination protocols. In the absence of infection, the Pa used in this study enhanced the interleukin-10 (IL-10) and IL-13 responses and influenced airway hyperresponsiveness to sensitizing antigen; however, these data do not suggest that Pa contributes to childhood asthma overall. On the contrary, wild-type virulent *B. pertussis* is still circulating in most countries, and our data suggest that the major influence of Pa is to protect against the powerful exacerbation of asthma-like pathology induced by *B. pertussis*.

The prevalence of asthma and allergic disease has increased in many countries (5, 14), and there has been speculation as to the possible causes (4, 46, 47), including a possible role of immunization in promoting allergic sensitization (12, 55). Asthma is a chronic disease of the respiratory tract and is tightly associated with airway hyperresponsiveness, increased mucus production, and infiltration of the bronchial mucosa by $CD4^+$ T cells (59). In allergic asthma there is evidence of an altered local T-cell response in favor of Th2 cytokines (interleukin-4 [IL-4], IL-5, and IL-13), which results in B-cell isotype switching to immunoglobulin E (IgE); mast cell, eosinophil, and basophil recruitment; and the production of a wide range of inflammatory mediators (61).

Epidemiological and clinical studies have provided intriguing data that suggest that a link exists between the relative lack of infectious diseases and the increase in allergic disorders (9, 56, 57); this is referred to as the "hygiene hypothesis." According to this hypothesis, viral and bacterial infections prevent the induction of allergen-specific Th2 cells because they establish Th1-biased immunity. In particular, the prenatal period and early childhood are considered critical intervals for the establishment of a putative Th1-Th2 balance. On the other hand, several studies have suggested that viral and bacterial infections exacerbate asthma. For example, respiratory syncytial virus, commonly associated with pulmonary infections in infancy, is known to exacerbate asthma (24, 30), and recently, we have shown that *Bordetella pertussis* infection exacerbates allergic asthma in a murine ovalbumin (OVA) model (8).

B. pertussis is a gram-negative bacterium and the causative agent of pertussis, or whooping cough, a respiratory disease that remains a significant cause of morbidity and mortality among infants worldwide. It is a highly contagious disease and can occur at any age, although severe illness is more common in young unimmunized children. *B. pertussis* infection induces Th1 responses in humans and can be modeled by respiratory challenge of mice, in which the response correlates well to the responses in humans (36).

There has been speculation about the possible promotion of allergy by common childhood vaccines (21, 43). A number of studies have analyzed the prevalence of allergic sensitization and atopic disease in relation to immunization (12, 21). Gruber et al. (12) found that children with higher levels of immunization coverage seemed to acquire transient protection against the development of atopy in the first years of life. In contrast, Hurwitz and Morgenstern (21) suggested that immunization against diphtheria-pertussis-tetanus appeared to be associated with an increased risk of subsequent asthma or other allergies.

Two different types of pertussis vaccine have been used in infant immunization programs. The whole-cell pertussis vaccine (Pw) consists of heat- and formalin-inactivated virulent whole bacteria, whereas the acellular pertussis vaccine (Pa) is composed of purified components of the bacteria, typically including inactivated pertussis toxin (PT). Immunization with Pw has a high degree of efficacy and is associated with the induction of antigen-specific Th1 cells (27, 28, 53), but it has been associated with reactogenicity (3). In contrast, immunization with Pa induces Th2 responses in children and in murine models (2), but it has reduced reactogenicity (6, 34). It has been suggested that the promotion of allergy may occur directly by administering potentially proallergic vaccines or indi-

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TABLE 1. Experimental design

Description (group name)[a]	Procedure on the following day (route of administration):					
	−42	−14	0	10	24	35, 36, 37
Pa	Pa	Pa	Saline aerosol	Saline (i.p.)	Saline (i.p. and i.n.)	Saline (i.n.)
Pa and *B. pertussis* infection (PaBp)	Pa	Pa	*B. pertussis* aerosol infection	Saline (i.p.)	Saline (i.p. and i.n.)	Saline (i.n.)
Pa and OVA sensitization (PaOVA)	Pa	Pa	Saline aerosol	OVA (i.p.)	OVA (i.p. and i.n.)	OVA (i.n.)
Pa, *B. pertussis* infection, and OVA sensitization (PaBpOVA)	Pa	Pa	*B. pertussis* aerosol infection	OVA (i.p.)	OVA (i.p. and i.n.)	OVA (i.n.)
Control			Saline aerosol	Saline (i.p.)	Saline (i.p. and i.n.)	Saline (i.n.)
B. pertussis infection (Bp)			*B. pertussis* aerosol infection	Saline (i.p.)	Saline (i.p. and i.n.)	Saline (i.n.)
OVA sensitization (OVA)			Saline aerosol	OVA (i.p.)	OVA (i.p. and i.n.)	OVA (i.n.)
B. pertussis infection and OVA sensitization (BpOVA)			*B. pertussis* aerosol infection	OVA (i.p.)	OVA (i.p. and i.n.)	OVA (i.n.)

[a] Groups of female BALB/c mice (age, 6 to 8 weeks) were immunized (i.p.) and boosted with Pa on days −42 and −14. Mice were either sham infected or infected with *B. pertussis* (Bp) by aerosol exposure. On day 10 and subsequently, selected groups were sensitized to OVA by the i.p. and i.n. routes. Bacterial burdens were measured between days 0 and 37. All other readouts were on day 37. For comparison, further groups of mice were treated as follows: control mice were sham infected on day 0 and sham sensitized. A second group (group Bp) was infected with *B. pertussis* on day 0 and sham sensitized. The third group (group OVA) was sham infected but sensitized with OVA (100 μg i.p.) on days 10 and 24 and then again (50 μg i.n.) on days 24, 35, 36, and 37. A separate group (group BpOVA) was infected with *B. pertussis* on day 0 and sensitized as described above. Each experiment was repeated at least twice, with *n* > 35 mice per group on each occasion.

rectly by hindering the Th1-promoting effects of infectious agents. Pertussis vaccine acts as an adjuvant for antigen-specific responses in laboratory animals (45); active PT, known to enhance immunoglobulin E (IgE) formation in animal models (26), is widely used as an adjuvant in animal experiments to enhance the immune response to coadministered antigens (51) and has been linked with a shift toward Th2-like cytokine production in humans (39, 48).

We have previously shown that infection with *B. pertussis* modulates allergen priming and the severity of airway pathology in a murine model of allergic asthma (8). Furthermore, we have demonstrated that Th1-inducing Pw protects against exacerbation of allergic asthma due to *B. pertussis* (7). In order to test whether immunization with Pa exacerbated asthma, we used a well-characterized murine model of acellular pertussis vaccination and *B. pertussis* infection in combination with the murine OVA model of airway hyperresponsiveness. Our findings demonstrate that a Th2-inducing Pa does not enhance but protects against exacerbation of allergic asthma due to *B. pertussis*. However, we note that in the absence of *B. pertussis* infection, a Pa can enhance IL-13 expression under certain conditions.

MATERIALS AND METHODS

Animals. Female BALB/c (age, 6 to 8 weeks; Harlan, Bicester, Oxon, United Kingdom) mice were used under the guidelines of the Irish Department of Health and the research ethics committee of the National University of Ireland, Maynooth. The BALB/c strain was selected because the performance of pertussis vaccines in this strain has been shown to correlate well with the performance of the vaccines in clinical trials with humans (37) and allergic asthma in this strain has been shown to be consistent with previous models of allergic asthma (8).

Immunization and airway delivery of OVA. Four groups of at least 35 6- to 8-week-old female BALB/c mice were immunized intraperitoneally (i.p.) with 0.16 IU of Pa [The Candidate International Reference Material for Purified (Acellular) Pertussis Vaccine, 1997, National Institute for Biological Standards and Control, Potters Bar, United Kingdom], equivalent to 1/25 of the human dose, according to the schedule outlined in Table 1. The four groups consisted of mice immunized with Pa (group Pa); mice challenged with virulent *B. pertussis* via aerosol and immunized with Pa (group PaBp); mice immunized with Pa and sensitized with OVA (group PaOVA); and mice immunized with Pa, challenged with virulent *B. pertussis* via aerosol, and sensitized with OVA (group PaBpOVA). At day 0 the mice were infected with *B. pertussis*; selected groups were then sensitized with OVA. Sensitization involved 100 μg of OVA (grade V; Sigma, Dorchester, United Kingdom) emulsified in 2% Alhydrogel adjuvant (Superfos

Biosector, Kvistgaard, Denmark) administered i.p. on days 10 and 24. The control group received saline alone (i.p.). On days 35, 36, and 37, PaOVA- and PaBpOVA-sensitized mice received 50 μg of OVA intranasally (i.n.), whereas the remaining groups received saline only (Table 1). All experiments were repeated twice.

Aerosol infection. Respiratory infection was initiated by aerosol challenge with *B. pertussis* strain W28, following growth under agitation conditions at 37°C in Stainer-Scholte liquid medium. Bacteria from a log-phase culture were resuspended at a concentration of 2×10^{10} CFU/ml in 1% (wt/vol) casein in 0.9% (wt/vol) saline. The challenge inoculum was administered to groups of mice on day 0 (the group challenged with virulent *B. pertussis* via aerosol [group Bp], PaBp, and PaBpOVA). Administration was by aerosol exposure over a period of 15 min with a nebulizer. Groups of four or more mice were killed at various time points after aerosol challenge to assess the number of viable *B. pertussis* organisms in the lungs. The remaining mice received a similar aerosol of sterile saline alone.

Enumeration of viable bacteria in the lungs. Lungs were removed aseptically and placed into 1 ml of sterile physiological saline with 1% casein. One hundred microliters of serially diluted homogenate from individual lungs was placed onto triplicate Bordet-Gengou agar plates, and the number of CFU was determined after incubation at 37°C for 4 days. The results are reported as the mean number of *B. pertussis* CFU for individual lungs determined in triplicate for lungs from four or more mice per time point. All experiments were repeated twice.

Measurement of OVA- and *B. pertussis*-specific antibody concentrations. Total and OVA-specific IgE concentrations were measured by using a rat anti-mouse IgE monoclonal antibody (BD, Pharmingen, San Diego, Calif.). The IgE concentration was expressed in micrograms per milliliter after comparison to the concentrations of murine IgE standards.

Cytokine measurement from BALF specimen and spleen cell cultures. Bronchoalveolar lavage fluid (BALF) specimens were obtained by repeat administration and aspiration of 0.5-ml volumes (total, 5 ml) of phosphate-buffered saline via cannulation of the trachea of mice from three experiments (*n* = 5 mice per treatment group on each occasion). Spleen cells (2×10^6/ml) from infected, sensitized, and control mice (*n* = 4 or more mice per group on each occasion) were cultured with heat-inactivated *B. pertussis* (1×10^4 CFU/ml), OVA (20 μg/ml), concanavalin A (5 μg/ml; positive control), or medium alone (negative control). At 72 h, culture supernatants were sampled for cytokine analysis; although the kinetics of cytokine production varies at this time point, this time point has previously proved acceptable for the detection of most cytokines (19). Concentrations of IL-5, IL-10, IL-13, and gamma interferon (IFN-γ) from spleen tissue and BALF samples were assessed by an enzyme-linked immunosorbent assay (BD, Pharmingen). Cytokine concentrations were calculated by comparison with cytokine standards of known concentration, as described previously (29).

Whole-body plethysmography. Airway responsiveness was assessed by methacholine (MCh)-induced airflow obstruction in conscious mice by the use of whole-body plethysmography (Buxco Electronics, Sharon, Conn.), as described previously (16). Pulmonary airflow obstruction was measured by enhanced pause, a value that is determined from the ratio of the expiratory time and

relaxation time to the peak expiratory flow and peak inspiratory flow and that is thought to correlate with airway responsiveness. Measurements were obtained after exposure of mice to phosphate-buffered saline (baseline) for 3 min, followed by the delivery of incremental doses (3.3 to 50 mg/ml) of MCh by aerosol (16).

Respiratory tract histology. Animals ($n = 5$ mice per group per experiment) were killed on day 37. Lungs were removed; fixed in a paraformaldehyde-lysine-periodate fixative; embedded in paraffin; sectioned; and stained by the hematoxylin-eosin, Discombes (for identification of eosinophils), Alcian blue (for identification of mucus), periodic acid-Schiff (for assessment of basement membrane thickness), azure A (for identification of mast cells), and Van Gieson (for identification of fibrosis) methods. The histopathological changes that were evident were graded according to a semiquantitative scoring system as mild, moderate, or severe by two researchers without prior knowledge of the treatment group. All experiments were performed at least twice ($n = 5$) on each occasion.

Statistical methods. Results are expressed as the means ± standard errors of the means (SEMs) for the indicated number of animals. Student's t test was used to determine significance among the groups in cytokine assays, whereas two-way analysis of variance was used for plethysmography. A P value of <0.05 was considered significant. Analyses were performed with Prism software (Graph-Pad, San Diego, Calif.).

RESULTS

OVA sensitization does not impair Pa-mediated clearance of *B. pertussis*. Both Pa immunization and OVA sensitization induce powerful Th2 responses in mice (15, 27, 50), whereas *B. pertussis* infection induces strong Th1 responses in mice and children (37, 52). The goal of this study was to examine the possibility that Pa immunization enhances allergic sensitization; however, it was also necessary to examine the confounding influence of sensitizing antigen upon clearance. Therefore, the effect of OVA sensitization upon the development of a protective response to infection in Pa-immunized and nonimmunized mice was examined. Mice received combinations of OVA sensitization, Pa immunization, and aerosol challenge with virulent *B. pertussis* (Table 1). Groups of mice infected with *B. pertussis* (groups Bp and BpOVA) showed similar kinetics of bacterial clearance (Fig. 1), indicating that OVA sensitization did not measurably influence the kinetics of bacterial clearance. Likewise, OVA-sensitized and nonsensitized mice that had been immunized prior to bacterial challenge (groups PaBpOVA and PaBp, respectively) showed identical rates of clearance. No bacteria were recovered from the uninfected OVA-sensitized group or the control group (Fig. 1). The bacterial burden in the infected groups (groups Bp and BpOVA) peaked at day 10 and declined thereafter. Pa-immunized mice cleared subsequent infection by *B. pertussis* by day 7. In contrast, only unimmunized mice (groups Bp and BpOVA) showed complete bacterial clearance by day 35 (Fig. 1). Therefore, sensitization with OVA did not impair the vaccine-mediated clearance of *B. pertussis* in this model.

Pa immunization does not enhance OVA-specific IgE production. The goal of this study was to examine the influence of Pa immunization on responses associated with allergic sensitization. Although OVA-induced sensitization does not impair vaccine-mediated clearance of *B. pertussis*, it was possible that Pa influenced allergic sensitization. An increase in the concentrations of IgE antibodies specific for PT, a component of Pa, has been reported in other studies (40, 50). Therefore, the serum IgE responses of mice sensitized to OVA following Pa or sham immunization were examined. Differences were observed in the induction of IgE antibodies to the different antigens, with the concentration of OVA-specific IgE typically being greater than the concentration of IgE induced to *B.*
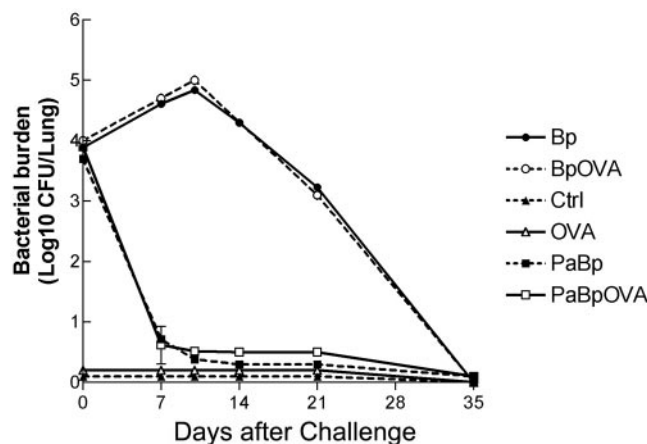

FIG. 1. Course of *B. pertussis* infection in experimental and control mice. Groups of mice were killed at various intervals after challenge, and the number of viable bacteria was estimated by performing colony counts on individual lung homogenates. Results are representative of those from at least two experiments and are presented as the mean number of CFU in the lungs, determined individually for the lungs from four or more mice per group at each time point. Data for the control and OVA groups have been offset from zero for clarity. Ctrl, control group.

pertussis (Fig. 2A and B). Pa induced little *B. pertussis*-specific IgE, and this was not altered by OVA sensitization (Fig. 2A). The only increase in *B. pertussis*-specific IgE was observed when a combination of immunization and infection prior to OVA sensitization was used. This resulted in a small but significant increase in the *B. pertussis*-specific IgE concentration detected (group PaBpOVA compared to groups PaOVA, BpOVA, and PaBp, $P = 0.0026$, 0.001, and 0.004, respectively) (Fig. 2A).

OVA sensitization induced high levels of OVA-specific IgE, as reported previously (8). Notably, Pa did not enhance the OVA-specific IgE response (Fig. 2B). It has been shown that Pw immunization of mice down-modulates the OVA-specific IgE response (7); however, Pa did not have this effect in this model: OVA-specific IgE was not significantly reduced by prior Pa immunization (groups OVA and Pa OVA) (Fig. 2B). In contrast, a combination of immunization and infection prior to OVA sensitization (group PaBpOVA) resulted in a significant reduction in the OVA-specific IgE titer compared to that in mice sensitized to OVA in the absence or the presence of Pa immunization (groups OVA and PaOVA, respectively, for which $P = 0.0447$ and 0.0413, respectively), similar to the effect previously reported (8) for infection in combination with OVA sensitization (group BpOVA) (Fig. 2B).

Pa immunization enhances OVA-induced IL-10 and IL-13. It has been shown previously (8) that *B. pertussis* infection enhances the OVA-induced IL-10 and IL-13 detectable in BALF specimens and from spleen cell cultures stimulated with specific antigen. Pa immunization is known to induce Th2 cytokines (1, 52), although its effect on IL-13 production is unknown. In order to dissect the influence of immunization on airway hyperresponsiveness, cell-mediated immune responses were examined in spleen cell preparations from the various study groups. Pa immunization alone or in combination with *B. pertussis* infection (group Pa or PaBp) induced very little IL-10,

A

Bp - specific IgE
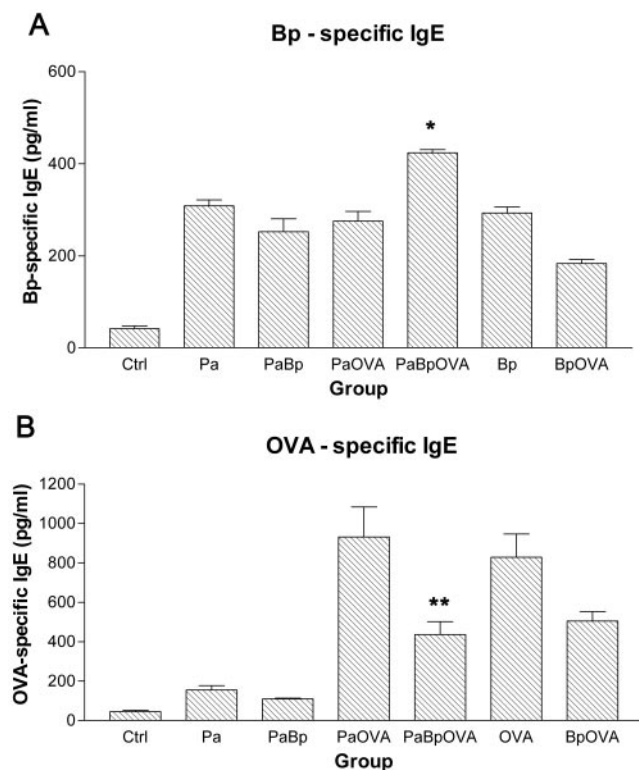

B

OVA - specific IgE

FIG. 2. Serum IgE elicited by Pa vaccination, bacterial infection, and allergic sensitization. The *B. pertussis*-specific (A) or OVA-specific (B) serum IgE responses elicited from each experimental group are shown. The experimental groups included mice receiving combinations of immunization with Pa (groups Pa, PaBp, PaOVA, and PaBpOVA), infection with *B. pertussis* (groups Bp, PaBp, BpOVA, and PaBpOVA), or sensitization to OVA (groups OVA, PaOVA, and PaBpOVA). Controls (Ctrl) were sham immunized, sham infected, or sham sensitized with saline, as appropriate. Results are expressed as the mean antibody concentrations ± SEMs. Mice that were infected only (group Bp) made no OVA-specific IgE, and mice that were sensitized only (group OVA) made no pertussis-specific IgE. The data presented are representative of those from two experiments; in each case, at least four animals were assessed, and each individual assessment was performed independently in triplicate. *, significantly greater IgE induction than that by mice in groups PaOVA, BpOVA, and PaBp ($P = 0.0026$, 0.001, and 0.004, respectively); **, significantly less IgE induction by mice in group PaBpOVA than by mice in groups OVA and PaOVA ($P = 0.0447$ and 0.0413, respectively). Data for group BpOVA are included here for comparison.

IL-13, or IFN-γ (Fig. 3). This was consistent with the protection observed earlier (Fig. 1) and previous data (28). Pa immunization in combination with OVA (group PaOVA) induced significantly higher levels of IL-10 ($P = 0.001$), IL-13 ($P = 0.007$), and IFN-γ ($P = 0.006$) (Fig. 3A to D) than OVA sensitization alone, but no differences in IL-5 levels were detected. In contrast, a combination of immunization and infection prior to OVA sensitization (group PaBpOVA) resulted in a significant reduction of IL-5, IL-10, and IL-13 levels ($P = 0.0014$, 0.0012, and 0.0158, respectively) compared to those in group PaOVA (Fig. 3A to C).

To extend these findings, we examined the levels of cytokines present in BALF specimens from each group of mice. Pa immunization alone induced no detectable IFN-γ in BALF specimens, but low levels of IL-5, IL-10, and IL-13 were re-

producibly detected (Fig. 4A to D). As expected, OVA sensitization induced IL-10 and IL-13 and very high levels of IL-5 (Fig. 4A-C); OVA sensitization also induced airway IFN-γ, as has been reported previously (8). Pa immunization influenced the levels of cytokines detected in sensitized mice (group PaOVA), reducing the levels of IL-5 and IFN-γ ($P = 0.008$ and $P = 0.0015$, respectively, compared to those in mice sensitized with OVA alone; Fig. 4A and D, respectively) but significantly increasing the levels of IL-10 ($P = 0.001$) and IL-13 ($P = 0.002$) (Fig. 4B and C, respectively). PaBpOVA mice also showed reduced IL-5 and IFN-γ levels but increased IL-10 and IL-13 levels compared to those in mice sensitized with OVA alone, although this effect was less pronounced than that seen in mice in group PaOVA (Fig. 4A to D).

Pa immunization protects against *B. pertussis* exacerbation of bronchial hyperresponsiveness and pathology but influences sensitization to OVA. It has been proposed that prior Th1 responses to bacterial infections protect against allergic disease; however, Th1-inducing *B. pertussis* infection exacerbates airway hyperresponsiveness in OVA-sensitized mice (8). We have shown previously that prior immunization with a whole-cell pertussis vaccine, which induces a very similar immune response to *B. pertussis*, did not enhance but protected against the airway hyperresponsiveness exacerbated by *B. pertussis* (7). In the present study we examined the influence of Pa immunization on physiological lung function using whole-body plethysmography to measure enhanced pause, a surrogate marker associated with airway hyperreactivity. Statistical analysis by two-way analysis of variance showed that Pa-immunized mice and/or mice challenged with *B. pertussis* (groups Pa, Bp, and PaBp) showed airway hyperreactivity comparable to that of the controls (Fig. 5A and B). As expected, mice sensitized to OVA showed significantly increased airway hyperreactivity compared to that of the controls ($P < 0.0003$) (Fig. 5A and C). As demonstrated above, *B. pertussis* infection (group BpOVA) exacerbated the OVA-induced response ($P = 0.0008$ for group BpOVA compared to mice sensitized to OVA) (Fig. 5D). The most notable influence of Pa immunization was that it significantly reduced this exacerbation (for group PaBpOVA versus group BpOVA, $P = 0.048$; Fig. 5D). Thus, Pa protects against the enhanced airway reactivity induced by the combination of infection and sensitization seen in mice in group BpOVA. Given that Pa influences airway IL-10 and IL-13 levels, we were also interested in observing the influence of Pa on OVA-sensitized mice in the absence of infection. Immunization did influence bronchial hyperreactivity upon OVA sensitization, but this increase was only evident at high concentrations of MCh exposure (Fig. 5C).

B. pertussis infection is known to modulate the quality of the OVA-induced inflammatory influx to the respiratory tract, with a marked reduction in eosinophil numbers accompanied by various degrees of epithelial hyperplasia, mucus metaplasia, and airway pathology (8). Lung tissue from experimental mice was assessed histologically (Table 2). Minimal pathology was observed in mice immunized with Pa or those immunized and infected with *B. pertussis* (group PaBp) (Table 2 and Fig. 6A and B). Pa-immunized and OVA-sensitized mice (group PaOVA) illustrated moderate mural and periairway inflammation with accompanying mild epithelial hyperplasia and smooth-muscle hypertrophy (Fig. 6C). The combination of Pa
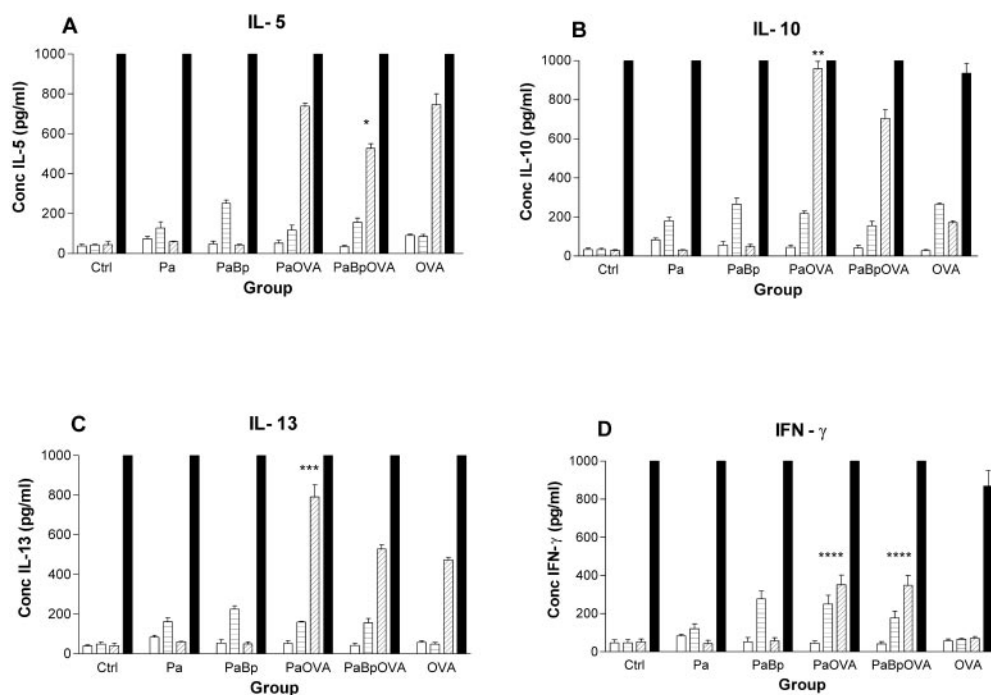
FIG. 3. Cell-mediated immune responses from the spleen elicited by Pa immunization, bacterial infection, and allergic sensitization. The IL-5 (A), IL-10 (B), IL-13 (C), and IFN-γ (D) responses from spleen cell cultures stimulated with medium alone (negative control [Ctrl]; open bars), heat-inactivated *B. pertussis* equivalent to 10^4 CFU/ml (bars with horizontal shading), OVA (bars with hatched shading), or concanavalin A (positive control; closed bars) are shown. Responses are representative of duplicate experiments, each of which was performed in triplicate with individual samples from at least four mice per group and are expressed as means ± standard errors. ∗, decreased IL-5 recall response to OVA compared to that of the PaOVA group ($P = 0.0014$); ∗∗, significantly increased IL-10 response to OVA compared to that of the PaBpOVA group ($P = 0.0012$) or the OVA group ($P = 0.001$); ∗∗∗, significantly increased IL-13 response to OVA compared to that of the PaBpOVA group ($P = 0.0158$) or the OVA group ($P = 0.007$); ∗∗∗∗, significantly increased IFN-γ response to OVA compared to that of OVA-sensitized mice ($P = 0.006$ for mice in group PaOVA; $P = 0.0056$ for mice in group PaBpOVA).

immunization, *B. pertussis* infection, and OVA sensitization induced a similar degree of airway inflammation, but with accompanying mild mucous metaplasia, moderate epithelial hyperplasia, and smooth-muscle hypertrophy (Fig. 6D). In summary, the pathology associated with immunized and sensitized mice (group PaOVA) was not greater than that seen in nonimmunized mice (group OVA), and Pa immunization (group PaBpOVA) protected against the exacerbated pathology seen in infected and sensitized mice (group BpOVA).

DISCUSSION

The present study demonstrates that Pa immunization protects against *B. pertussis* exacerbation of OVA-induced airway hyperresponsiveness in a murine model of allergic asthma. Pa immunization did not enhance OVA-specific IgE production but did enhance OVA-induced IL-10 and IL-13 in the absence of infection. Nevertheless, Pa immunization did not enhance the observed airway pathology over that seen in nonimmunized and sensitized mice. Furthermore, Pa immunization protected against the exacerbated pathology seen in infected, sensitized mice. Taken together, these results suggest that the major influence of Pas is not to promote allergic asthma but, rather, to protect against the exacerbating influence of virulent *B. pertussis* infection.

Pertussis vaccination in infancy has been discussed as a putative risk factor for bronchial asthma (18, 41, 49), presumably

through Th2-mediated mechanisms. Th2 responses are thought to be protective against parasite-mediated disease (60) but are associated with allergic disorders (59, 61). It has been suggested that induction of Th2 cells or failure to mount a Th1 or regulatory T-cell response to foreign antigens in childhood may enhance atopic disease (10, 49). It is therefore conceivable that changing patterns of microbial exposure through infection and vaccination in childhood may contribute to the observed increase in atopic disease and asthma. Active PT enhances IgE responses to foreign antigens and is widely used as an adjuvant in animal experiments to enhance the immune responses to coadministered antigens (26, 51). Inactivated PT is a component of most Pas, and it is known that booster immunization of children with an acellular pertussis vaccine enhanced Th2 cytokine production and serum IgE responses against PT (50). Circulating IgE antibodies against PT have also been found after primary immunization with Pa with or without a subsequent Pa or Pw booster (11, 17, 42). Furthermore, *B. pertussis* infection is known to modulate allergen priming and exacerbates the severity of allergen-driven pathology in a murine model (8); however, Th1-inducing vaccines (Pw) protect against this (7). In the present study we show that Pa immunization did not enhance the OVA-specific IgE response (Fig. 2B); this suggests that Pa-like vaccines are unlikely to promote atopy and is consistent with the findings of studies of Pa-immunized children, which found no significant enhancement
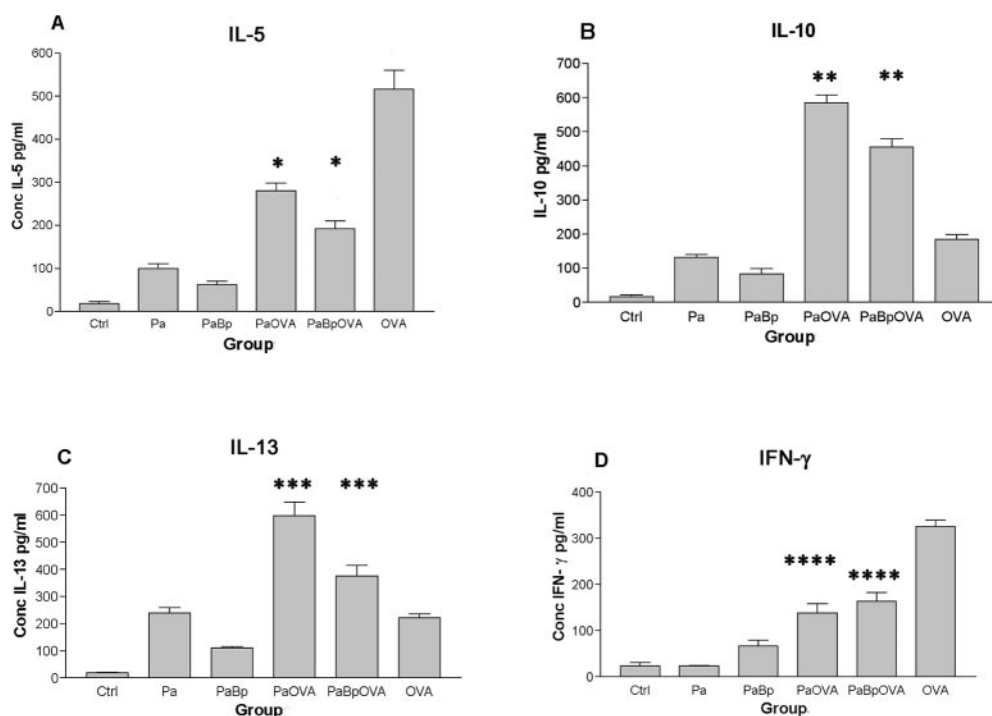
FIG. 4. Pa immunization modulates the local cytokine response to *B. pertussis* infection and OVA sensitization. Diluted BALF specimens from five mice per group were pooled, and the concentrations of IL-5 (A), IL-10 (B), IL-13 (C), and IFN-γ (D) were determined by enzyme immunoassay. The results are representative of those from duplicate experiments, each measurement was performed in triplicate, and the values are expressed as means ± SEMs. ∗, reduced IL-5 concentration compared to that in OVA-sensitized mice ($P = 0.008$ for group PaOVA; $P = 0.0025$ for group PaBpOVA); ∗∗, increased IL-10 concentration compared to that in OVA-sensitized mice ($P = 0.001$ for group PaOVA; $P = 0.001$ for group PaBpOVA); ∗∗∗, increased IL-13 concentration compared to that in OVA-sensitized mice ($P = 0.002$ for group PaOVA; $P = 0.022$ for group PaBpOVA); ∗∗∗∗, decreased IFN-γ concentration compared to that in OVA-sensitized mice ($P = 0.0015$ for group PaOVA; $P = 0.0026$ for group PaBpOVA). Ctrl, controls.

of IgE specific to third-party antigens (50). Significantly decreased levels of OVA-specific IgE were observed in mice in group PaBpOVA compared to those in either the PaOVA or the OVA group (Fig. 2B). These data are consistent with our previous findings (8) that suggest that the mechanism by which virulent *B. pertussis* infection exacerbates asthma is independent of IgE. Furthermore, this indicates that the major action of Th2-inducing Pa vaccines is likely protective rather than a contributor to the exacerbation of asthma.

Airway reactivity can be mediated by IgE-independent mechanisms that include a direct influence of IL-4 and IL-13 on airway cells (22, 23, 38, 58). It has previously been proposed (62) that IL-10 plays an essential role in countering such responses by inducing regulatory T-cell responses, evidence that suggests that allergic asthma is a disease of defective T-cell regulation. Interestingly, McGuirk and colleagues (31–33) have shown that filamentous hemagglutinin, a component of the acellular vaccine used in this study, has the net effect of inhibiting the induction, activation, and recruitment of Th1 cells and also interacts with dendritic cells to promote the production of IL-10. Our findings of elevated IL-10 levels in BALF specimens support this observation. However, it has been demonstrated recently (25) that IL-10 can induce IL-13 expression in vivo and that this is responsible for the mucus, but not the inflammatory or fibrotic effects, observed in the airways of allergen-sensitized animals. In the present study, we

show that Pa immunization prior to OVA sensitization significantly increases both IL-10 and IL-13 levels at the systemic and local levels (Fig. 3A to D and Fig. 4A to D) and that this may influence airway hyperreactivity (Fig. 5). This is also consistent with the findings of McGuirk and Mills (32) concerning filamentous hemagglutinin. Although IL-10 can act as an immune regulator, our findings suggest that IL-10 has broader functions that may contribute to inflammatory disease (8, 13). This may be through direct effects on the smooth muscle in damaged airways (13) or indirectly via induction of IL-13 (25). Although the Pa used in this study enhanced the levels of IL-10 and IL-13 production in response to the sensitizing antigen, the influences of these cytokines appear to be the greatest in the context of the widespread airway epithelial damage that occurs during breakdown of the epithelial-mesenchymal unit (20). Thus, although Pa induces these cytokines, because its major influence is to reduce bacterial carriage and, hence, pathology, the dramatic exacerbating influence on airway hyperreactivity associated with IL-13 and IL-10 induction by *B. pertussis* components during infection (8) was not observed here. Nevertheless, components that do not induce either IL-10 or IL-13 should be considered in enhanced future formulations of Pas.

A study by Gruber et al. (12) revealed no evidence for an allergy-promoting effect of common childhood vaccines in a prospectively monitored atopy risk-enhanced birth cohort. Moreover, children with better vaccination coverage seemed to
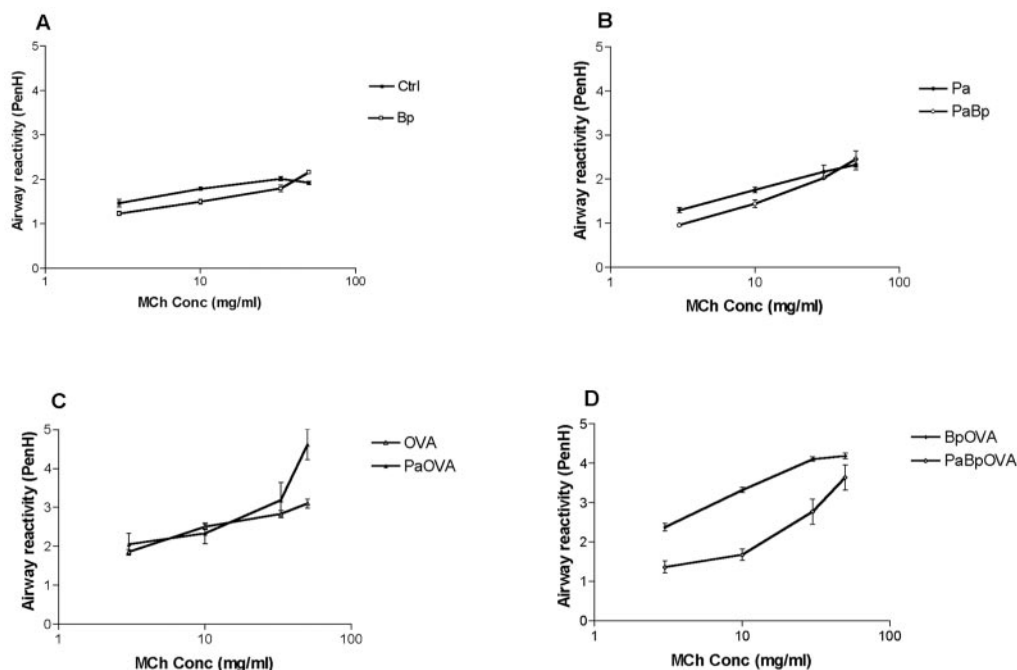
FIG. 5. Influence of Pa immunization on bronchial hyperresponsiveness. Airway hyperreactivity in response to increasing concentrations of inhaled MCh was measured by whole-body plethysmography in control (Ctrl; sham infected) or Bp-infected mice (A), mice in group Pa or PaBp (B), mice in group OVA or PaOVA (C), and mice in group BpOVA or PaBpOVA (D). Results are representative of those from two experiments ($n = 4$ or more), and values are expressed as the mean enhanced pause ± SEM.

be better protected against the development of atopy in their second and third years of life. In particular, immunizations against measles and mumps, pertussis, and diphtheria and against tetanus were associated with a transient reduction of atopy, whereas immunization against polio and *Haemophilus influenzae* had no effect (12). A conflicting study of the effects of vaccination on allergies among children in the United States has reported that diphtheria-tetanus-pertussis vaccination appeared to increase the risk of allergies and related respiratory symptoms (21). One contentious interpretation of that study is that vaccine components may be responsible for a portion of the increased prevalence of asthma and allergies in U.S. children. However, a large number of previous studies have demonstrated that Pa protects against the devastating and some-

times fatal childhood disease whooping cough (27, 44, 50, 54) and that current commercial formulations of Pa show greatly reduced reactogenicities compared to that of Pw (6). The latter data unequivocally demonstrate the benefit of Pa immunization to health and justify the selection of Pa for mass vaccination protocols. Our data do not suggest that Pa contributes to childhood asthma overall. On the contrary, wild-type virulent *B. pertussis* is still circulating in many countries, and our data suggest that the major influence of Pa is to protect against the powerful exacerbation of asthma-like pathology induced by *B. pertussis*.

We have established models that allow examination of the mechanisms of interaction between protective immunization and allergic sensitization. These are based on BALB/c mice,

TABLE 2. Histological assessment of airway pathology[a]

Treatment group	Mucous metaplasia of airway epithelium	Hyperplasia of airway epithelium	Smooth-muscle hypertrophy of airway wall	Periairway or vascular inflammation[b]					
				Overall degree	E	N	L	M	F
Control	−	−	−	−	−	−	−	−	−
Bp	−	+	+	+	−	+	+ +	−	−
OVA	+ +	+ +	+ +	+ +[I]	+	+ +	+ +[M]	−	−
BpOVA	+ + +	+ + +	+ +	+ + +[I]	+ +	+ + +	+ + +[M]	−	−
Pa	−	−	−	−	−	−	−	−	−
PaBp	−	+	+	−	−	−	−	−	−
PaOVA	−	+	+	+ +[I]	+ +	+ +	+ +[M]	−	−
PaBpOVA	+	+ +	+ +	+ +[I]	+ +	+ +	+ +[M]	−	−

[a] A semiquantitative score (−, absent; +, mild; ++, moderate; +++, severe) was assigned to the features of the airway pathology observed.
[b] Periairway or vascular inflammation was assessed in terms of overall degree; numbers of infiltrating eosinophils (E), neutrophils (N), lymphocytes, plasma cells, and macrophages (L), and mast cells (M); and circumscribing fibrosis (F). Superscript I, inflammation extending into surrounding pulmonary interstitium and alveolar spaces; superscript M, macrophage giant cells form part of the inflammatory exudates within surrounding alveolar spaces.
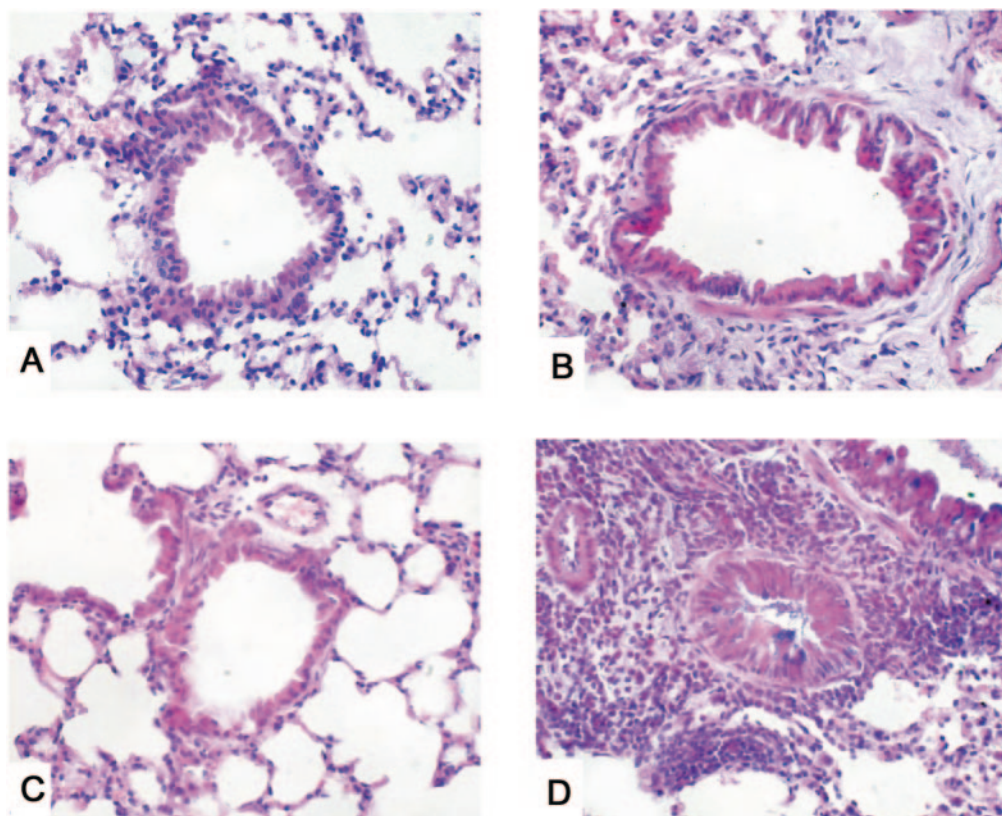
FIG. 6. Photomicrographs illustrate representative morphological changes in transverse sections of bronchioles of four treatment groups ($n = 5$ mice per group). (A) Group Pa, illustrating a normal airway morphology; (B) group PaBp, illustrating mild epithelial hyperplasia and smooth-muscle hypertrophy; (C) group PaOVA, illustrating mild airway hyperplasia and smooth-muscle hypertrophy; (D) group PaBpOVA, showing moderate mural and periairway inflammation, mild mucous metaplasia, moderate epithelial hyperplasia, and smooth-muscle hypertrophy. All sections were stained with a combination of Discombes and Alcian blue stains. Magnifications, ×400.

but qualitatively similar results are seen with alternative strains (2, 35). While we find evidence that Pa contributes to the induction of airway IL-13, we demonstrate that the main influence of acellular pertussis vaccination on asthma is to protect against the exacerbating effects of virulent *B. pertussis* infection.

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