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Booster immunization of children with an acellular pertussis vaccine enhances Th2 cytokine production and serum IgE responses against pertussis toxin but not against common allergens

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SUMMARY

Acellular pertussis vaccines (Pa) protect against severe pertussis in children. However, serum antibody responses decline quickly after immunization. Studies in animal models suggest that cell-mediated immunity also contributes to protection against *Bordetella pertussis*, and it has already been demonstrated that Pa induce T cells that secrete type-1 and type-2 cytokines in children. In this study we examined the persistence of the T cell response and the effect of booster immunization in 4–6-year-old children. Cell-mediated immunity to *B. pertussis* antigens was detected in a high proportion of children more than 42 months after their last immunization. Peripheral blood mononuclear cells (PBMC) from the majority of children secreted interferon-gamma (IFN-γ) and a smaller proportion IL-5, in response to specific antigen stimulation *in vitro*. However, following booster immunization, significantly higher concentrations of IL-5, but not IFN-γ, were produced by PBMC in response to *B. pertussis* antigens. Furthermore, plasma IL-4 and IL-5 concentrations were increased, whereas IFN-γ concentrations were reduced following booster immunization. It has been suggested that childhood immunization with Th2-inducing vaccines may predispose some children to atopic disease. Although we found that pertussis toxin (PT)-specific IgE was significantly increased after booster immunization in both atopic and non-atopic children, the levels of IgE to common allergens and the prevalence of positive skin prick test were unaffected by the booster vaccination. Thus, despite the enhancement of type-2 responses to *B. pertussis* antigens, booster vaccination with Pa does not appear to be a risk factor for allergy.

Keywords pertussis vaccine Th1/Th2 cell IgE allergens pertussis toxin

INTRODUCTION

In many developed countries the traditional whole cell pertussis vaccines (Pw) have now been replaced by acellular pertussis vaccines (Pa) composed of purified bacterial components. Pa protect against severe whooping cough and when compared with Pw have significantly enhanced safety profiles [1–4]. Recovery from whooping cough and immunization of children with Pw have been shown to selectively induce Th1 cells [5,6], whereas Pa induce T cells that secrete both Th1 and Th2 cytokines [6,7]. Although Pa appear to provide relatively long lasting immunity, early waning of serum antibody responses has been reported in vaccinated children [8–10]. Evidence from an animal model of *Bordetella pertussis* infection shows that the cell-mediated immune responses can provide protection from infection in the absence of antibody [11,12]. The induction of Th1 cells is associated with optimal immunity induced by infection or immunization with Pw [12,13]. Although Pa can also confer protection in mice, albeit not to as significant a level as that conferred with potent Pw, the protective mechanism appears to involve antibody and Th2 cells [13].

Th2 responses are associated with allergic disorders and it has been suggested that induction of Th2 cells or failure to mount a Th1 response to foreign antigens in childhood may enhance atopic diseases [14–18]. Although heredity factors and environmental tobacco smoke are evident risk factors and indoor humidity, poor ventilation, indoor pets and use of antibiotics are possible risk factors, they cannot explain the increase in allergy during the last decades, especially in countries with a 'western life-style'. Alternatively, changing patterns of microbial exposure through infection and vaccination in childhood may contribute to the increase in atopic diseases. Pertussis vaccination in infancy has

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been discussed as an important risk factor for bronchial asthma [18–20]. Whooping cough is considered a moderate risk factor for bronchial asthma, allergic rhinoconjunctivitis, and sensitization to inhalant allergens [20,21]. Pertussis toxin (PT) enhances IgE responses to foreign antigens and is widely used in animal experiments as an adjuvant to enhance the immune responses to co-administered antigens [22–24]. Circulating IgE antibodies to PT have been found after primary immunization with Pa and more frequently in infants given primary immunization with Pa and boosted with either Pa or Pw [2,25–27].

In this study we evaluated the persistence of cell-mediated immune responses in 4–6-year-old children who had received three doses of Pa as infants and the effect of booster immunization. Antigen-specific cytokine production was assessed in peripheral blood mononuclear cells (PBMC) pre- and post-booster immunization, administered 42–66 months after the primary course of vaccination. We also evaluated the changes in IgE antibody responses to PT and age-relevant allergens following booster vaccination with Pa in atopic and healthy children. We wished to address the hypothesis that pertussis vaccination may enhance Th2-like immunity, particularly in atopic children.

SUBJECTS AND METHODS

Subjects, vaccination and disease diagnosis

Blood samples were received from 29 healthy children from clinic 1 (Umeå, Sweden), and 55 children from clinic 2 (Linköping, Sweden) between December 1996 and January 1999. These children had received a primary course of vaccination with a five-component Pa from Connaught Laboratories, Toronto, Canada in a clinical trial at 2, 4, and 6 months of age [3]. The five-component Pa contained 10 μg glutaraldehyde-inactivated PT, 5 μg filamentous haemagglutinin (FHA), 5 μg fimbriae 2 and 3, 3 μg pertactin (PRN) and 0·316 mg aluminium phosphate per dose. All children were boosted with fresh batches of the same five-component Pa in December 1996, 1997, or 1998. T cell responses to *B. pertussis* antigens were assessed pre- and post-booster vaccination at 4–6 years of age.

To evaluate the effect of boosting with Pa on atopic disease, 55 children from clinic 2 were evaluated for symptoms of atopic disease in a prospective study from 2 months of age. Skin prick tests were performed after primary vaccination at 7 months, at 2·5 years and before and after booster vaccination. The diagnosis of atopic disease was based on questionnaires, clinical findings and information in medical records [20]. In this cohort a subgroup of 15 children (five atopic children and 10 healthy controls) was evaluated for IgE levels to PT and to common allergens and for atopic status at 2 (preimmunization), 7 (4 weeks after the third vaccination) and 30 months of age, and pre- and post-booster vaccination at 4 years of age.

The diagnosis of *B. pertussis* infection was established according to WHO criteria [28]. Telephone interviews were made with the parents every 6 weeks, and cultures and samples for serology were taken after 7 days of coughing. None of the children in the study had had *B. pertussis* infection.

Skin prick tests

Skin prick tests (SPTs) were performed with relevant food (cow's milk, egg white) and inhalant allergens (cat, timothy, birch, *Dermatophagoides pteronyssinus* and *farinae*) at 7 and 30 months of age, in accordance with guidelines [29]. The SPTs were done in duplicate on the volar aspect of the forearm with pasteurized undiluted cow's milk and hen's egg and with Soluprick SQ extracts (10 HEP; Allergologist Laboratorium A/S, Horsholm, Denmark) for the other allergens. Tests were regarded as positive if skin wheals had a mean diameter of ≥ 3 mm after 15 min. Histamine dihydrochloride 10 mg/ml and blank lancets were included as positive and negative controls, respectively. No antihistamines were used during 3 days preceding the SPT.

Bacterial antigens

Heat-killed *B. pertussis* W28 was prepared as described [5]. Purified native PT, FHA, PRN and agglutinnogens 2 + 3 (AGG) for use in T cell assays were provided by Connaught Laboratories (Toronto). PT was inactivated by heating at 80°C for 20 min to abolish mitogenic activity.

Cytokine production by PBMC

Analysis of Th1- and Th2-type T cell responses was performed by assessment of cytokine levels in the supernatant of PBMC stimulated with *B. pertussis* antigens. Fresh venous blood samples were drawn into preservative-free heparinized tubes and sent by courier to Ireland, where they were processed within 24 h. PBMC, prepared on Ficoll gradients as described [5], were cultured in RPMI 1640 medium supplemented with 8% fetal calf serum (FCS) in triplicate in 96-well microtitre plates (200 μl/well) at $1{\cdot}5 \times 10^6$ cells/ml with heat-killed bacteria (10^7 cells/ml), FHA (1 and 5 μg/ml), AGG (1 and 5 μg/ml), heat-inactivated PT (1 and 5 μg/ml), PRN (1 and 5 μg/ml), for 3 days at 37°C in a humidified CO_2 incubator. Control stimuli included medium alone or anti-CD3 (OKT3, 2·0 μg/ml) and phorbol myristate acetate (PMA, 25 ng/ml; Sigma, St Louis, MO). Supernatants were removed after 72 h and the levels of interferon-gamma (IFN-γ) and IL-5 measured by immunoassays as described [6]. The limits of sensitivity of the assays were 0·25 U/ml for IFN-γ and 15 pg/ml for IL-5. Specific cytokine production in response to antigen was calculated by subtracting concentrations measured in unstimulated cultures (i.e. background).

Analysis of circulating cytokine levels

The plasma obtained following the isolation of PBMC by density gradient centrifugation was frozen at −80°C until analysis. Plasma IL-5 and IFN-γ concentrations were determined by specific immunoassay as described above. IL-4 was also measured by specific immunoassay using matched MoAb pairs obtained from PharMingen (San Diego, CA); the limit of sensitivity of the assay was 15 pg/ml.

PT and allergen-specific IgE

PT-specific IgE was analysed by the Magic Lite System (ALK, Horsholm, Denmark), an immunochemiluminometric assay that uses PT-coupled paramagnetic particles as a solid phase. PT solution was dialysed against ammonium bicarbonate and freeze-dried. Protein was reconstituted with PBS pH 7·4 immediately before coupling to a concentration of 238 μg/ml. Resolubilized PT was coupled to paramagnetic particles with the Magic Lite SQ Allergy System Coupling Kit according to the instructions of the manufacturer. Coupling efficiency was high; no protein could be recovered from the coupling buffer. There was no non-specific binding, as assessed by running diluent against the PT solid phase. For higher sensitivity, PT-coupled particles and serum were incubated overnight at room temperature. With this method, the

detection limit was 0·5 Standardized Units (SU)/ml. IgE antibodies from egg white, birch and cat allergens were also analysed with Magic Lite system with the high sensitivity protocol as recommended by the manufacturer. The detection limit was 1 SU/mL.

Statistical analysis

Statistical differences between paired samples was determined by Wilcoxon signed rank test. Differences between the atopic and non-atopic groups were determined by Mann–Whitney *U*-test.

Ethics

The study was approved by the Human Research Ethics Committees of the Medical Faculty at the University of Linköping and at Umeå University.

RESULTS

Bordetella pertussis-*specific cytokine production by PBMC from immunized children*

Children who had received a primary course of immunization with the five-component Pa at 2, 4, and 6 months of age were examined for T cell responses immediately before and 35 days after booster vaccination with Pa at 4–6 years of age. Two cohorts of children were examined, 28 from clinic 1 (Umeå) and 55 from clinic 2 (Linköping). Analysis of cytokine production by PBMC against *B. pertussis* antigens revealed that cell-mediated immune responses were still detectable in the majority of children more than 42 months after a primary course of vaccination. PBMC from 61 (73%) of the children secreted IFN-γ and 42 (50%) IL-5 in response to one or more of the antigens PT, AGG, FHA, PRN or killed *B. pertussis* before boosting. The proportion of children (82%) secreting IL-5 in response to *B. pertussis* antigens and the mean concentrations of IL-5 produced by PBMC in response to each of the pertussis antigens were significantly increased following booster immunization. The pattern of enhanced IL-5 production was remarkably consistent between the two cohorts of children from the different clinics. In contrast, the number of children producing IFN-γ in response to *B. pertussis* antigens and the mean concentration of IFN-γ were not significantly altered following vaccination (Fig. 1). However, there was a non-significant increase in IFN-γ levels produced by antigen-stimulated PBMC from clinic 1. After booster immunization, PBMC from 92%, 90% and 81% of children secreted IFN-γ and/or IL-5 in response to at least one, two or three *B. pertussis* antigens, respectively. There was reciprocity in IFN-γ and IL-5 production in a proportion of individuals; PBMC from 63% of children produced IFN-γ and IL-5, 19% produced IL-5 only and 8% produced IFN-γ only in response to two or more of the antigens tested. Furthermore, when compared with the pre-immunization samples the concentrations of IL-5 relative to IFN-γ were enhanced after booster immunization, suggesting

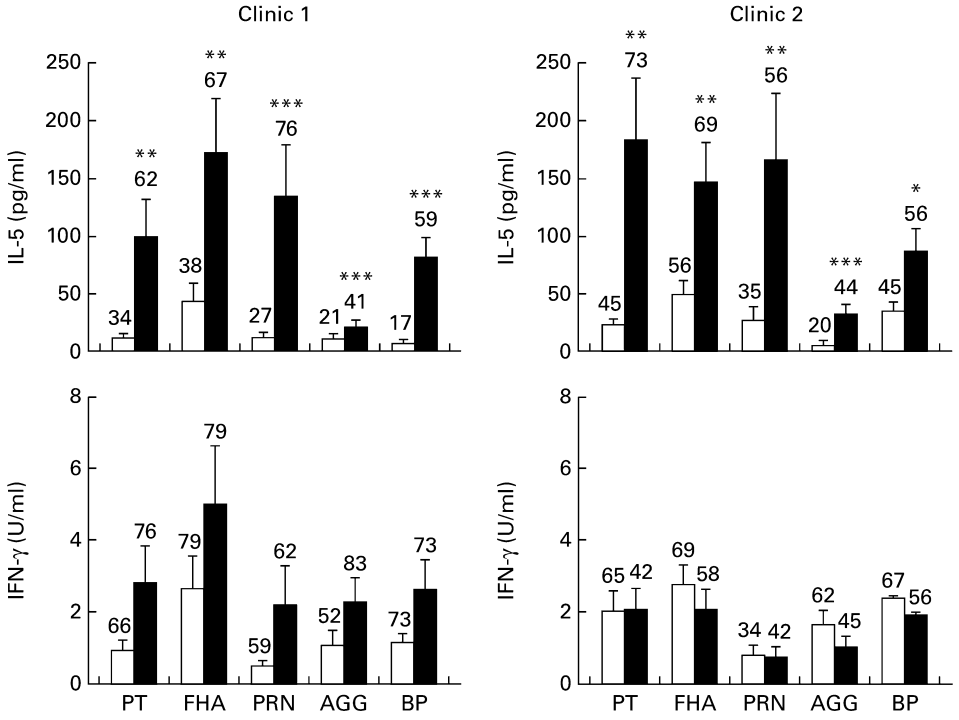

Fig. 1. Cytokine production by *Bordetella pertussis*-specific T cells in 4–6-year-old children before and after booster immunization with acellular pertussis vaccines (Pa). Two cohorts of children, 28 from clinic 1 in Umeå and 55 from clinic 2 in Linköping, received a primary course of vaccination at 2, 4, and 6 months with the Connaught five-component Pa and were boosted at 4–6 years of age with the same vaccine. Peripheral blood mononuclear cells (PBMC), prepared from the same children immediately before (□) and 5 weeks after (■) booster vaccination, were stimulated with 1–5 μg/ml of heat-inactivated pertussis toxin (PT), filamentous haemagglutinin (FHA), pertactin (PRN) or agglutinogens 2 and 3 (AGG) or 10^6-10^7/ml killed *B. pertussis* (BP). Results are mean (s.d.) IL-5 and IFN-γ concentrations in supernatants of PBMC from 28 children in clinic 1 and 55 children in clinic 2 in response to the optimum concentration of each of the antigens. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$ *versus* prebooster values. The numbers above each bar represent the percentage of responding children to that antigen.

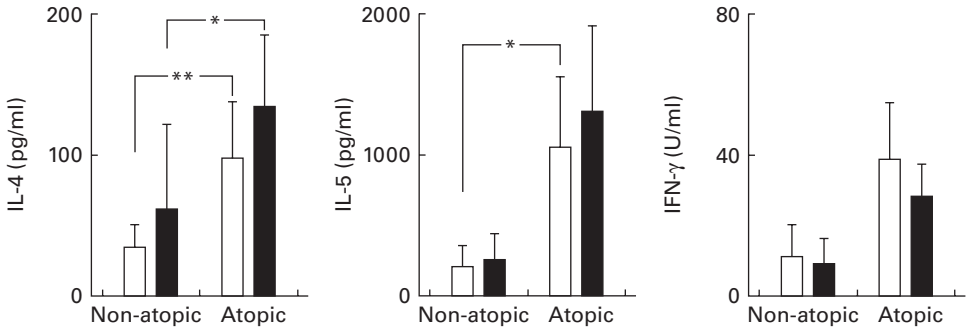

Fig. 2. Cytokine concentrations in plasma from children before (□) and after (■) booster vaccination with acellular pertussis vaccines (Pa). Children were immunized as described in Fig. 1 and plasma was prepared from blood samples taken immediately before and 35 days after booster vaccination. Results are given as mean (s.d.) concentrations of IL-4, IL-5 and IFN-γ for five atopic and 38 non-atopic children. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

selective stimulation of Th2 cells *in vivo*. PBMC from all of the children, including those that did not respond to specific antigen stimulation, secreted high levels of IFN-γ and IL-5 in response the polyclonal activators PMA and anti-CD3 (data not shown). A comparison of T cell responses in children with symptoms of atopic diseases with non-atopic children did not reveal any significant difference in IL-5 and IFN-γ production by PBMC in response to *B. pertussis* antigens (data not shown).

Plasma cytokine concentrations

We investigated the effect of boosting with Pa on circulating cytokine concentrations in both atopic and healthy children. We found significantly higher levels of Th2 cytokines in the plasma of atopic compared with healthy controls. Although circulating IFN-γ was also higher in atopic compared with non-atopic children, only the differences in IL-4 and IL-5 reached a level of statistical significance ($P < 0{\cdot}05$–$0{\cdot}01$). Following booster immunization with Pa the levels of circulating IL-4 and IL-5 did increase, though not significantly, in both atopic and non-atopic children (Fig. 2). In contrast, boosting with Pa resulted in a small reduction in the concentrations of circulating IFN-γ in both healthy and atopic children. The 43 children from the cohort of 55 for which plasma samples were available for cytokine analysis included nine children that tested positive on SPT (five of these had atopic disease), and these were also compared for pre- and post-immunization cytokine levels. After booster immunization, the levels of IL-4 were enhanced from 95 pg/ml to 130 pg/ml ($P = 0{\cdot}01$) and IL-5 from 1031 to 1298 ($P = 0{\cdot}01$), and IFN-γ reduced from 38 to 28 U/ml ($P > 0{\cdot}05$) in the nine children with positive SPTs. By comparison, IL-4 levels increased from 52 to 98 pg/ml ($P > 0{\cdot}05$) and IL-5 from 300 to 384 ($P > 0{\cdot}05$), and IFN-γ reduced from 16 to 12·9 U/ml ($P > 0{\cdot}05$) in the 34

Table 1. Classification of the children in the atopic/healthy children IgE study

Study no., female (f), male (m)	Age at booster vaccination (years; months)	Atopic disease	Skin prick test, 7 months	Skin prick test, 2·5 years	Family history of allergy
A1, f	4; 8	AD, BA?	Egg	Egg	Yes
A2, f	4; 1	AD, BA?	Egg	Egg	Yes
A3, m	4; 0	ARC	Egg, milk	Egg, birch	Yes
A4, m	4; 9	AD	Egg	No	Yes
A5, m	4; 0	AD	Egg, milk	Egg, birch	Yes
N1, m	4; 6	No	No	No	No
N2, f	4; 8	No	No	No	No
N3, m	4; 2	No	No	No	No
N4, f	4; 3	No	No	No	No
N5, f	4; 9	No	No	No	No
N6, m	4; 3	No	No	No	No
N7, f	4; 5	No	No	No	No
N8, 9	4; 5	No	No	No	No
N9, f	4; 10	No	No	No	No
N10, f	4; 10	No	No	No	No

A1–A5 are children with atopic disease and sensitized at 7 months and/or 2·5 years of age to common allergens, N1–N10 are children with no atopic disease.

AD, Atopic dermatitis; ARC, allergic rhinoconjunctivitis; BA, bronchial asthma; BA?, nocturnal dry cough (A1), or wheezing after 2 years of age (A2).

All children were vaccinated with the same five-component acellular pertussis vaccine (Pa) at 2, 4, and 6 months of age.

SPT-negative children. These findings are consistent with the cytokine production by antigen-stimulated PBMC, which suggests preferential activation of Th2 responses following booster immunization with Pa.

Influence of Pa immunization on IgE responses and allergy

The 55 children from clinic 2 were assessed for symptoms of allergy and SPT before and after vaccination. In addition, 15 were studied over the 4-year period after primary vaccination and before and after booster immunization for IgE responses to PT and to common allergens. Five of these children had atopic disease and were sensitized to common allergens and 10 were healthy controls. Four of the five children with atopic diseases had atopic dermatitis, one had allergic rhinoconjunctivitis and two symptoms of possible asthma. All were SPT-positive for egg white at 7 months of age and two were also sensitized to milk (Table 1). At 2·5 years of age four of the children were still sensitive to egg white, two of them also to birch and one did not have any positive skin tests at this age. All five children had allergic heredity, four from their mothers. No anamnestic data of atopic disease, no positive SPTs and no family history of allergy were shown among the 10 controls.

No evidence of enhanced symptoms of atopic diseases or SPT reactivity were observed in atopic or healthy children after booster vaccination with Pa, and this is consistent with a study of a larger number of children from the same clinic [20].

PT-specific IgE antibodies were detected in both atopic and non-atopic children. PT-specific IgE antibodies were present in three children at 7 months, two children at 12 months, two children at 30 months and one child prebooster (Table 2). After booster immunization with Pa, the PT-specific IgE levels had increased significantly. Fourteen out of 15 (93%) had positive PT-specific IgE, 11 out of 15 had concentrations >20 SU/ml. The highest levels were observed in two children with a history of atopic disease (Table 2).

Table 2. Pertussis toxin (PT)-specific serum IgE after primary immunization and before and after booster immunization with acellular pertussis vaccines (Pa)

Study no.	7 months	12 months	30 months	Pre-booster	Post-booster
A1	<1.43	<1.43	<1.43	<1.43	450
A2	<1.43	<1.43	<1.43	<1.43	3.08
A3	<1.43	2.44	<1.43	<1.43	116
A4	3.07	2.07	<1.43	<1.43	37
A5	6.27	–	<1.43	<1.43	495
N1	<1.43	<1.43	<1.43	<1.43	22.9
N2	<1.43	<1.43	<1.43	<1.43	42.8
N3	<1.43	<1.43	5.54	<1.43	118
N4	51.7	12.1	7.31	2.57	71.5
N5	<1.43	<1.43	<1.43	<1.43	10.4
N6	<1.43	<1.43	<1.43	<1.43	1.71
N7	<1.43	<1.43	<1.43	<1.43	43
N8	<1.43	<1.43	<1.43	<1.43	31.7
N9	<1.43	<1.43	<1.43	<1.43	<1.43
N10	<1.43	<1.43	–	<1.43	106

PT-specific IgE was assessed in serum at 7 months (after three vaccinations with the five-component Pa), at 12 and 30 months of age and pre- and post-booster vaccination with Pa in five atopic (A1–A5) and 10 normal healthy children (N1–N5) at 4 years of age.

Table 3. Allergen-specific serum IgE before and after booster immunization with acellular pertussis vaccines (Pa)

	Egg-white		Birch		Cat	
Study no.	Pre	Post	Pre	Post	Pre	Post
A1	1.1	1.2	<1	<1	<1	<1
A2	<1	<1	22.9	20.5	<1	<1
A3	1.7	1.6	91.6	87.3	7.0	7.6
A4	<1	<1	<1	<1	<1	<1
A5	3.6	3.5	84.2	64.4	<1	<1
N1	<1	<1	<1	<1	<1	<1
N2	<1	<1	<1	<1	<1	<1
N3	<1	<1	<1	<1	<1	<1
N4	3.4	4.0	<1	<1	<1	<1
N5	<1	<1	<1	<1	<1	<1
N6	<1	<1	<1	<1	<1	<1
N7	<1	<1	<1	<1	<1	<1
N8	<1	<1	<1	<1	<1	<1
N9	<1	<1	1.8	1.8	<1	<1
N10	<1	<1	<1	<1	<1	<1

IgE specific for egg-white, birch and cat allergens was assessed in serum pre- and post-booster vaccination with Pa in five atopic (A1–A5) and 10 normal healthy children (N1–N5) at 4 years of age.

No increases in IgE levels to egg white, birch or cat antigens were seen in either atopic or non-atopic children after booster vaccination with Pa (Table 3). However, we found one child with IgE antibodies to egg white and another child with IgE antibodies to birch antigen in the non-atopic group at booster vaccination. The parents of these children did not report symptoms at prebooster physician examination and the children had negative skin prick tests at previous assessments. The child with increased IgE antibodies to hen's egg with no allergic disease was one of three children with increased PT-IgE at 7 months of age (Tables 2 and 3).

DISCUSSION

Immunization with pertussis vaccines prevents severe whooping cough. However, the mechanism of protective immunity against *B. pertussis* in children has not been fully elucidated. Evidence from murine models suggests that protection involves cell-mediated as well as humoral immunity [13]. Although Pa induce potent antibody responses in children, the specific serum IgG concentrations decline rapidly after immunization and are often undetectable 2–3 years after a full course of primary vaccination [8,9]. Nevertheless, vaccinated children still appear to be protected against severe disease [10]. The results of the present study demonstrate that T cell responses persist in a high proportion of children for >4 years after vaccination and suggest that memory T cells may play a role in long-term immunity induced with Pa.

In the mouse model natural infection or immunization with Pw induces Th1 cells and confers a high level of protection against infection with *B. pertussis* [12]. Although Pa also confer protection against infection in the murine respiratory challenge model, immunity induced by these vaccines appears to be

primarily mediated by antibodies and Th2 cells [13,30]. Attempts to define a role for T cells in protective immunity against *B. pertussis* in children have focused on an examination of T cell proliferation and cytokine production by PBMC from immune donors. Consistent with the responses observed in mice, recovery from whooping cough in infants is associated with selective induction of Th1 cells. Immunization with Pw also induces Th1 cells, but Pa generate T cells with a mixed Th1/Th2 cytokine profile in children [6,7]. The findings of the present study demonstrate that this pattern of T cell cytokine production persists in a proportion of children for at least 4 years. However we observed modest levels of IFN-γ production in response to PT and/or FHA by PBMC from 70% to 80% of 4-year-old children prior to boosting with Pa. These findings are supported by a recent report [8] that the cell-mediated response to *B. pertussis* antigens in 4-year-old Italian children who had received Pa at 2, 4, and 6 months of age was predominantly Th1. The authors speculated that the persistence of the *B. pertussis*-specific Th1 responses might be due to natural boosting with subclinical infections of *B. pertussis* or *B. parapertussis* [8]. This is consistent with our earlier report of *B. pertussis*-specific Th1 responses in a proportion of naive Swedish children [5]. However, these children were studied prior to the resumption of routine pertussis vaccination in Sweden, when the incidence of pertussis was relatively high. Although it is possible that periodic boosting with subclinical infection may account for some or all of the *B. pertussis*-specific IFN-γ production detected in the present study, it is also possible that memory Th1 cells persist for at least 4 years after immunization with Pa. Indeed, this may explain the persistence of protection in Pa-immunized children despite the waning antigen-specific serum IgG responses [9,10]. This conclusion is supported by a recent study in the murine model where it has been demonstrated that protection in immunized mice after waning of antibody responses is maintained through the induction of memory B and T cells [11]. Although persistent protection was observed in mice immunized with Th1- and Th2-inducing pertussis vaccine, the rate of bacterial clearance was more rapid in the Th1-primed mice.

Despite the apparent persistence of Th1 responses for at least 4 years, our study demonstrated that booster immunization in 4–6-year-old children preferentially enhanced Th2 responses. When compared with the prebooster samples, PBMC obtained 5 weeks after booster secreted significantly higher amounts of IL-5 following *in vitro* stimulation with *B. pertussis* antigens, PT, FHA, PRN, AGG and heat-killed bacteria. Furthermore, the concentrations of serum PT-specific IgE were significantly enhanced and circulating IL-4 and IL-5 were non-significantly increased after booster immunization. In contrast, the numbers of responding children and the levels of IFN-γ produced by *B. pertussis* antigen-stimulated PBMC and in the circulation were either not significantly enhanced or reduced after booster immunization with Pa. In a previous study we had reported that PBMC from children primed with Pw and boosted with Pa secreted comparatively higher levels of IFN-γ and lower levels of IL-5 in response to *B. pertussis* antigen stimulation *in vitro* [6]. These observations suggest that the booster immunization with Pa in children primed with Pa may result in selective stimulation of Th2 cells, which may down-regulate the type-1 arm of the immune response, whereas immunization with Pw primes Th1 cells and results in less Th2-biased response after boosting with Pa. This is consistent with the dichotomy in cytokine production by T cells induced with Pw and Pa in immunized mice [30], which has been explained by the induction of endogenous IL-12 secretion by lipopolysaccharide (LPS) present in Pw [31]. IL-12 has a positive regulatory effect on the induction and activation of Th1 cells. In contrast, immunization with purified bacterial antigens adsorbed to alum and devoid of endotoxin and other residually active toxins that stimulate IL-12, favours the induction of Th2 cells [31].

It is well recognized that patients with atopic diseases mount Th2 and IgE responses to common allergens, and the results of the present study, as well as that reported by others [32,33], demonstrated that atopic patients also have elevated serum concentrations of IL-4 and IL-5. It has been suggested that Th2-mediated atopic disease may be suppressed by exposure to Th1-inducing microbes and exacerbated by exposure to Th2-inducing vaccines or parasites [14–18]. Despite the fact that primary vaccination with Pa or Pw does not result in polarized Th2 response in children, immunization with pertussis vaccines has been proposed as a possible risk factor for allergic disease, including asthma [18–20]. Furthermore, it has been demonstrated that PT has adjuvant properties for T cell and antibody responses, in particular IgE and the murine IgG1 subclass [22–24]. Consequently it has been proposed that vaccines that include PT may augment IgE and other immune responses to unrelated antigens. In this study we had the opportunity to make a thorough temporal analysis of IgE responses in a small subgroup of atopic and healthy children that formed part of the larger cohort used for the SPT and cytokine analysis. The selective enhancement of *B. pertussis*-specific Th2 after booster immunization was also reflected in significantly enhanced antigen-specific serum IgE. Anti-PT IgE antibodies are detected in about 24% of children following primary immunization with Pa compared with about 3% for Pw [27]. However, 14/15 (93%) children developed PT-specific IgE after booster immunization. PT-specific IgE were similar in the healthy and the atopic groups, but the highest levels of PT-IgE were found in two atopic children. In contrast, no increases in IgE to birch, egg white and cat allergens were seen after booster vaccination. Thus, despite the evidence of bystander effects of Th1-inducing immunogens on allergy [14] and of Th2-inducing parasites on IFN-γ production in response to bacteria [34], we found little evidence of a bystander effect on allergen-specific IgE or atopic disease in the present study. We demonstrated a small increase in circulating IL-4 and IL-5 and a reduction in IFN-γ following booster immunization with Pa. Although this was more pronounced in children with positive SPTs, it was also observed in SPT-negative children. Furthermore, we found no significant difference in the production of IL-5 and IFN-γ in PBMC stimulated by *B. pertussis* antigens from atopic and healthy children. The lack of enhancement of IgE responses to common allergens in sensitized children is consistent with the hypothesis that chemically detoxified PT, present in most Pa, does not retain adjuvant activity [24]. Although certain adjuvant activity is retained in a non-toxic PT mutant (PT-9K/129G) that lack ADP-ribosyl-transferase activity [24], the ability to enhance IgE responses appears to be dependent on an active S-1 component [35].

It has also been suggested that the local adverse reactions after booster immunization with Pa may be more common in atopic children than non-atopic children [27]. In this and a previously reported study [20], there was no evidence of increased local side-effects among the atopics or exacerbation of the symptoms of clinical allergies in children boosted with Pa. Despite the

increase in IgE to PT ([25–27] and present study) and to tetanus toxoid (TT) [36] with alum-adsorbed vaccines, local and systemic side-effects are low and hypersensitivity reactions are extremely rare [37]. Although it may be possible to enhance the efficacy of Pa by substituting alum with other adjuvants or vaccine delivery systems that induce Th1 cells [31], modifications of vaccine formulation aimed at preventing IgE production do not seem warranted.

In conclusion, our findings have demonstrated that 4–6-year-old children who were primed with Pa vaccination at 2, 4, and 6 months still have cell-mediated immunity to *B. pertussis*. Boosting with Pa selectively enhanced type-2 immune responses, as demonstrated by *B. pertussis*-specific cytokine production and IgE response and serum cytokine levels. However, no enhancement of atopic disease or IgE responses to common allergens was observed after booster vaccination. Few local and systemic side-effects were seen, with no preponderance for the atopic children. The observations here and by others [20,38] suggest that booster immunizations with Pa may not be a risk factor in atopic disease.

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REFERENCES

1 Englund JA, Decker MD, Edwards KM, Pichichero ME, Steinhoff MC, Anderson EL. Acellular and whole cell pertussis vaccines as booster doses: a multicenter study. Pediatrics 1994; **93**:37–43.

2 Greco DD, Salmaso S, Mastrantonio P *et al.* A controlled trial of two acellular vaccines and one whole cell vaccine against pertussis. New Eng J Med 1996; **334**:341–8.

3 Gustafsson L, Hallander HO, Olin P, Reizenstein O, Storsaeter J. A controlled trial of a two-component acellular, a five component acellular, and a whole cell pertussis vaccine. New Eng J Med 1996; **334**:349–55.

4 Olin P, Rasmussen F, Gustafsson L, Hallander HO, Heijbel H. Randomised controlled trial of two-component, three component and five-component acellular vaccines compared with whole cell pertussis vaccine. Lancet 1997; **450**:1569–77.

5 Ryan M, Storsaeter J, Nilsson L, Mills KHG. *Bordetella pertussis* respiratory infection in children is associated with preferential activation of type 1 Th cells. J Infect Dis 1997; **175**:1246–50.

6 Ryan M, Murphy G, Ryan E *et al.* Distinct Th cell subtypes induced with whole cell and acellular pertussis vaccines in children. Immunol 1998; **93**:1–10.

7 Ausiello CM, Urbani F, La Sala A, Lande R, Cassone A. Vaccine- and antigen-dependant type 1 and type 2 cytokine induction after primary vaccination of infants with whole-cell or acellular pertussis vaccines. Infect Immun 1997; **65**:2168–74.

8 Ausiello CM, Lande R, Urbani F, la Sala A, Stefanelli P, Salmaso P, Mastrantonio P, Cassone A. Cell-mediated immune responses in four-year-old children after primary immunization with acellular pertussis vaccines. Infect Immun 1999; **67**:4064–71.

9 Giuliano M, Mastrantonio P, Giammanco A, Piscitelli A, Salmaso S, Wassilak SG. Antibody responses and persistence in the two years after immunization with two acellular vaccines and one whole-cell vaccine against pertussis. J Pediatr 1998; **132**:983–8.

10 Salmaso S, Mastrantonio P, Wassilak SG *et al.* Persistence of protection through 33 months of age provided by immunization in infancy with two three-component acellular pertussis vaccines. Vaccine 1998; **16**:1270–5.

11 Mahon BP, Brady MT, Mills KHG. Protection against *Bordetella pertussis* in mice in the absence of circulating antibody: implications for long term immunity in children. J Infect Dis 2000; **181**: 2087–91.

12 Mills KHG, Barnard A, Watkins J, Redhead K. Cell mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect Immun 1993; **61**:399–410.

13 Mills KHG, Ryan M, Ryan E, Mahon BP. A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. Infect Immun 1998; **66**:594–602.

14 Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorder. Science 1997; **275**:77–79.

15 Burney PGJ, Chinn S, Rona RJ. Has the prevalence of asthma increased in children? Evidence from the national study of health and growth 1973–1986. Br Med J 1990; **300**:1306–10.

16 Ring J. Allergy and modern society. Does 'Western Life Style' promote the development of allergies? Int Arch Allergy Immunol 1997; **113**:7–10.

17 Farooqi IS, Hopkin JM. Early childhood infection and atopic disorder. Thorax 1998; **53**:927–32.

18 Rook GAW, Stanford JL. Give us this day our daily germs. Immunol Today 1998; **19**:113–6.

19 Henderson J, North K, Griffiths M, Harvey I, Golding J. Pertussis vaccination and wheezing illnesses in young children: prospective cohort study. Br Med J 1999; **318**:1173–6.

20 Nilsson L, Kjellman N-IM, Bjorksten B. A randomized controlled trial of the effect of pertussis vaccines on atopic disease. Arch Ped Adolesc Med 1998; **152**:734–8.

21 Schuster A, Hofmann A, Reinhardt D. Does pertussis infection induce manifestation of allergy? J Clin Invest 1993; **71**:208–13.

22 Munoz JJ, Arai H, Bergman RK, Sadowski PL. Biological activities of chrystalline pertussigen from *Bordetella pertussis*. Infect Immun 1981; **33**:820–6.

23 Lindsay DSJ, Parton R, Wardlaw AC. Adjuvant effect of pertussis toxin on the production of anti-ovalbumin IgE in mice and lack of direct correlation between PCA and ELISA. Int Arch Allergy Immunol 1994; **105**:281–8.

24 Ryan M, McCarthy L, Mahon B, Rappuoli R, Mills KHG. Pertussis toxin potentiates Th1 and Th2 responses to co-injected antigen: adjuvant action is associated with enhanced regulatory cytokine production and expression of the costimulatory molecules B7-1, B7-2 and CD28. Int Immunol 1998; **10**:651–62.

25 Hedenskog S, Bjorksten B, Blennow B, Granstrom G, Granstrom M. Immunoglobulin E response to pertussis toxin in whooping cough and after immunisation with a whole cell and an acellular pertussis vaccine. Int Arch Allergy Appl Immunol 1989; **89**:156–61.

26 Odelram H, Granstrom M, Hedenskog S, Duchen K, Bjorksten B. Immunoglobulin E and G responses to pertussis toxin after booster immunization in relation to atopy, local reactions and aluminium content of the vaccines. Pediatr Allergy Immunol 1994; **5**:118–23.

27 Nilsson L, Gruber C, Granstrom M, Bjorksten B, Kjelmman N-IM. Pertussis IgE and atopic disease. Allergy 1998; **53**:1195–201.

28 WHO meeting on case definition of pertussis: Geneva, 10–11 January, 1991. (Issue no. MIN/EPI/PERT/91.1). Geneva: World Health Organisation, 1991:4–5.

29 Dreborg S, Frew A. Allergen standardization and skin tests. EAACI Position paper. Allergy 1993; **48** (Suppl. 14):48–82.

30 Redhead K, Watkins J, Barnard A, Mills KHG. Effective immunization

against *Bordetella pertussis* respiratory infection in mice is dependent on induction of cell-mediated immunity. Infect Immun 1993; **61**: 3190–8.

31 Mahon BP, Ryan MS, Griffin F, Mills KHG. Interleukin-12 is produced by macrophages in response to live or killed *Bordetella pertussis* and enhances the efficacy of an acellular pertussis vaccine by promoting induction of Th1 cells. Infect Immun 1996; **64**:5295–301.

32 Hoekstra MO, Hoekstra Y, De Reus D, Rutgers B, Gerritsen J, Kauffman HF. Interleukin-4, interferon-gamma and interleukin-5 in peripheral blood of children with moderate atopic asthma. Clin Exp Allergy 1997; **27**:1254–60.

33 Leonard C, Tormey V, Burke C, Poulter LW. Allergen-induced cytokine production in atopic disease and its relation to disease severity. Am J Respir Cell Mol Biol 1997; **17**:368–75.

34 Brady MT, O'Neill SM, Dalton JP, Mills KHG. *Fasciola hepatica* suppresses a protective Th1 response against *Bordetella pertussis*. Infect Immun 1999; **67**:5372–8.

35 Van der Pouw-Kraan CTM, Rensink HJAM, Rappuoli R, Aarden LA. Co-stimulation of T cells via CD28 inhibits human IgE production; reversal by pertussis toxin. Clin Exp Immunol 1995; **99**:473–8.

36 Cogne M, Ballet JJ, Schmitt C, Bizzini B. Total and IgE antibody levels following booster immunization with aluminum absorbed and non-absorbed tetanus toxoid in humans. Ann Allergy 1985; **54**:148–51.

37 Jacobs RL, Lowe RS, Lanier BR. Adverse reactions to tetanus toxoid. JAMA 1982; **247**:40–42.

38 Gifford CG, Gonsior EC, Villacorte GV, Bewtra A, Townley RG. Pertussis booster vaccination and immediate hypersensitivity. Ann Allergy 1985; **54**:483–8.