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Pertussis-Specific Cell-Mediated Immunity in Infants after Vaccination with a Tricomponent Acellular Pertussis Vaccine

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The aim of this study was to investigate pertussis-specific cell-mediated immunity in infants vaccinated with a tricomponent acellular vaccine. Infants were investigated during a primary vaccination schedule from the third month of life to the sixth month as well as before and after a booster at 15 to 24 months. This is the first report of specific cell-mediated immune responses to pertussis-related antigens in infants below the age of 12 months. Our data show that the vaccine induces T-cell responses specific for the vaccine components, detoxified pertussis toxin, filamentous hemagglutinin, and pertactin, that increase progressively over the course of the vaccination schedule. In contrast to declining antibody titers, cell-mediated immune responses are stable over the postprimary to prebooster period. Vaccination results in a progressive increase in the number of T cells that express activation marker CD45RO preferentially on CD4-positive T cells after stimulation with pertussis antigens. Measurements of cytokine secretion profiles demonstrated a preferential induction of interleukin 2- and gamma interferon-producing T-helper 1 cells and only low production of interleukin 10. The observed persistence of the specific cell-mediated immunity may have a bearing on the protective mechanisms induced by pertussis vaccination. Cell-mediated immunity requires further study, particularly to improve our understanding of the persistence of protection afforded by vaccination up to the administration of booster doses.

Recovery from infection with *Bordetella pertussis* leads to immunity which provides temporary, but not lifelong, protection against subsequent disease. High levels of immunity can also be conferred by immunization with whole-cell vaccines (20, 27). Concerns over the reactogenicity of whole-cell vaccines (5, 41, 43) led to the development of acellular pertussis vaccines, which are composed of defined purified antigens, designed to achieve protection while minimizing adverse side effects (15).

The nature of immunity against infection and disease is still poorly understood. This especially concerns the lack of available information on the specific immunity of infants vaccinated for pertussis within the first year of life. Research on protection has traditionally been focused on the role of humoral pertussis-specific antibodies, which are induced in humans and rodents following natural infection or immunization with whole-cell vaccines or defined pertussis antigens (3, 33, 46, 53). In animal models, passive transfer of antibodies against several pertussis antigens (25, 48, 52) as well as antibodies elicited after active immunization with whole-cell or acellular vaccines (4, 10, 43) can confer different degrees of protection against subsequent intracerebral or respiratory challenge. However, such models are not truly representative of human pertussis.

Clinical trials in Sweden (2, 21), Italy (19), and Germany (50) as well as active immunization experiments with rodents (52) were unable to demonstrate a clear correlation between serum antibody levels to *B. pertussis* antigens and protective efficacy. While circulating antibodies may play a role in toxin neutralization and prevention of bacterial attachment to respiratory epithelial cells, animal experiments are providing increasing evidence that other immunological mechanisms such as cell-mediated immunity (CMI) may also be necessary for complete, long-term protection against *B. pertussis* (30).

In mice, pertussis-specific T-cell-mediated immune responses were demonstrated after infection (29) or following immunization (36, 37). Mills et al. (30) recently demonstrated that the adoptive transfer of splenic T cells or purified CD4-positive T cells from immune mice can confer protection against *B. pertussis* to immunodeficient mice in the absence of a detectable antibody response. The presence of pertussis antigen-specific T cells after natural infection in humans has been reported (12, 14, 17). In adults, CMI against pertussis toxoid (PT), PT subunits, and filamentous hemagglutinin (FHA) following vaccination with whole-cell or acellular vaccines has been described (38, 40). However, the data from these subjects are not helpful with regard to immunity induced after primary vaccination, and data from infants during the first year of life are still lacking.

The relevance of CMI in protection against *B. pertussis* is further supported by observations of pertussis in a previously immunized patient with AIDS (1) as well as by the observation that the bacterium is a facultative intracellular organism (7). This suggests that T cells are induced by immunization and infection and that these T cells may play a role in the termination of primary infection and in protection against subsequent challenge by facilitating clearance of intracellular pathogens.

Since there is no information currently available on the pertussis-specific CMI response of the primary target group for acellular pertussis vaccination, i.e., infants in their first year of life, we performed the present study to investigate the pertus-

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sis-specific CMI in infants vaccinated with a candidate tricomponent acellular pertussis vaccine and to compare it with humoral immune responses. The infants were vaccinated in a primary schedule at 3, 4, and 5 months of age with a booster at 15 to 24 months as part of a larger vaccine efficacy trial, results of which have been reported previously (50).

MATERIALS AND METHODS

Vaccine. The diphtheria-tetanus-acellular pertussis vaccine (Infanrix) was manufactured by SmithKline Beecham Biologicals (Rixensart, Belgium) and consisted of 25 μg of PT, 25 μg of FHA, and 8 μg of pertactin (PRN) absorbed to 0.5 mg of aluminum (as hydroxide), together with ≥30 IU of diphtheria toxoid and ≥40 IU of tetanus toxoid.

Study population. Participants were enrolled in an acellular pertussis vaccine efficacy study conducted in six areas of Germany from 1992 and 1995 involving 22,205 healthy children who were vaccinated at 3, 4, and 5 months of age (primary vaccination) and boostered at age 15 to 19 months of age. All participants in our study were recruited from one center (Children's Hospital, University of Mainz) consecutively in order of their presentation at the center. Only infants shown to be seronegative for immunoglobulin G (IgG) antibodies specific for PT, FHA, and PRN before primary vaccination were included in the final evaluation to eliminate the possibility of interference by maternally derived pertussis-specific antibodies. In fact, fewer than 3% of the infants were positive for maternally derived antibodies before primary immunization.

Proceeding. For preparation of peripheral blood lymphocytes (PBL), heparinized blood (2.5 ml with 20 IU of heparin per ml) was drawn immediately before and 4 weeks after the primary and booster immunizations. These time points are referred to as preprimary, postprimary, prebooster, and postbooster, respectively. For the evaluation of the vaccine-induced immune responses, the preprimary study group served as a negative control, since these individuals were naive with regard to contact to pertussis-related antigens. Anti-pertussis antigen-specific IgG antibodies were measured in sera prepared from an additional 2.0 ml of blood obtained without additives at each time point.

The study was approved by the appropriate local ethical review board and performed according to the requirements of the German Drug Act, the European Community Good Clinical Practice guidelines, and the WHO Declaration from Hongkong-Helsinki. Written informed consent was provided by the parents of all children prior to enrollment in the study.

Pertussis antigens. Purified detoxified PT (248 μg/ml), FHA (283 μg/ml), and PRN (285 μg/ml), identical to those present in the vaccine, were kindly provided by SmithKline Beecham. PT was detoxified by treatment with glutaraldehyde followed by formalin (45). FHA and PRN were treated with formalin to inactivate traces of residual PT if present.

Proliferation assay. Blood samples were processed within 1 hour, and PBL were prepared by gradient sedimentation on Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden). PBL were prepared in cold (4°C) RPMI 1640 medium without glutamine (Gibco) but supplemented with 10% heat-inactivated (30 min at 56°C) AB serum, 1 mM sodium pyruvate (Gibco), 1× minimal essential medium amino acids (Gibco), 2 mM L-glutamine (Gibco), 100 IU of penicillin per ml, and 100 μg of streptomycin (Gibco) per ml. The cells were washed twice in complete medium and used at a final concentration of 2×10^6 cells per ml.

The capacity of PBL to respond to the pertussis antigens (PT, FHA, and PRN) was investigated by measurement of antigen-specific proliferation. Lymphocytes (2×10^5) were cultured in round-bottom microtiter plates (Nunc, Roskilde, Denmark) in a volume of 250 μl per well in the presence of antigen. Antigen concentrations yielding the highest proliferative responses after a culture period of 6 days were determined in dose-response studies. The following antigen concentrations were used: PT, 10.0 μg/ml; FHA, 10.0 μg/ml; PRN, 15.0 μg/ml.

Cultures with 4 μg of purified phytohemagglutinin (Wellcome) per ml served as positive controls for cell reactivity; cultures without antigens served as negative controls. All cell cultures were performed in triplicate in a humidified atmosphere at 37°C and 5% CO_2. After a culture period of 6 days for antigen-specific responses and of 2.5 days for purified phytohemagglutinin, 1 μCi of [^{3}H]thymidine (21.5 Ci/mmol; NEN) was added per well for an additional 16 h. Thereafter, [^{3}H]thymidine incorporation was evaluated in a scintillation counter (Canberra Packard), and the results were expressed as mean counts per minute.

An individual who did not develop a proliferative response at least threefold higher than background proliferation or whose response was below 1,500 cpm was considered to be nonimmune or a nonresponder. The data on these individuals are reported separately. Because of the limited numbers of PBL available from small infants, not all of the individuals could be tested for each antigen. As a basic quality criterion for the proliferation assays, the proliferative response to purified phytohemagglutinin at 72 h had to exceed 50,000 cpm.

Lymphokine assays. To determine antigen-specific cytokine production, a 100-μl aliquot of supernatant from cultures established for the measurement of lymphocyte proliferation (see above) was harvested at 48 h, quick-frozen, and stored at −70°C. In pretrials, the culture period of 48 h had been determined as the optimal time point for cytokine measurement. Cultures were refilled with complete RPMI 1640 medium and kept until day 6 as described above.

Cytokine determination was performed for interleukin 2 (IL-2), IL-10, and gamma interferon (IFN-γ) with commercially available enzyme-linked immunosorbent assay (ELISA) kits (Dianova, Hamburg, Germany) as described in the manufacturer's instructions.

Immunofluorescence and flow cytometric analysis. Immunophenotyping of PBL and investigation of the expression of activation markers after antigen stimulation was achieved by flow cytometric analysis. Monoclonal antibodies (MAb), labeled directly with either fluorescein isothiocyanate or phycoerythrin, to the surface structures CD3, CD4, and CD8 (all from Dianova) and to CD45RO and CD14/CD45 (Becton Dickinson, Heidelberg, Germany) were used. Nonspecific isotype mouse MAb were used as negative controls.

Cultures for flow cytometric evaluation of lymphocyte phenotype and activation marker were established under conditions similar to those described for the lymphocyte proliferation assay. Cells were analyzed before and after stimulation with pertussis antigens over 36 h, which was identified as the optimal time point in pretrials. When the cultures were terminated, the cells were recovered from the wells and washed twice in complete medium. The cells were then stained with the appropriate MAb for flow cytometric evaluation by standard procedures.

To saturate nonspecific binding sites, 50 μl of cell suspension was incubated with 50 μl of human immunoglobulin (Polyglobin N; Troponwerke, Cologne, Germany) diluted 1:10. Thereafter, the samples were washed and incubated with the appropriate MAb. Between the staining procedures, the cells were washed twice with cold phosphate-buffered saline (PBS). Finally, the samples were fixed with 1% formaldehyde in PBS and stored at 4°C until the flow cytometric analyses were performed (analyses were completed within 24 h).

Samples were analyzed on a FACScan flow cytometer (Becton Dickinson) with data stored in list mode files. Ten thousand viable cells, as defined by forward-scatter and right-angle intensity as well as propidium iodide exclusion, were analyzed for each sample. A forward-side scatter gate was used to ensure that measurements were not biased by polymorphonuclear leukocytes. Lymphocyte gates were established by CD14/CD45 staining. Isotype- and fluorochrome-matched control MAb were used in each experiment to determine nonspecific background MAb binding.

Serum antibody determination. IgG and IgA antibodies to PT, FHA, and PRN were determined by a standardized ELISA procedure (8) at SmithKline Beecham in a blinded manner with respect to the vaccination status of the participants (pre- and postprimary and pre- and postbooster). Results were calculated with a reference line method and four-parameter logistics with a pooled human serum as a reference and serum numbers 3 and 4 of the Laboratory of Pertussis, Food and Drug Administration, Bethesda, Md., as controls. Antibody concentrations were expressed in ELISA units (EL.U) per milliliter the limit of detection being arbitrarily set at 5 EL.U/ml for all antibodies.

Data analysis and statistical methods. CMI data and flow cytometric results are presented as box plots.

Data were analyzed by the Kruskal-Wallis one-way analysis of variance on ranks. In the case of statistical significance, an all-pair-wise multiple-comparison procedure was performed by Dunn's method. Results from cytokine measurements were analyzed by a paired *t* test or, in cases of nonnormal distribution, with a Wilcoxon signed rank test. Significant differences are indicated in the graphs by brackets or given in the text and legends.

RESULTS

Lymphocyte proliferation. To investigate the induction of a pertussis-specific CMI after vaccination with the acellular pertussis vaccine, antigen specific T-cell responses were measured in a proliferation assay. The results are given in Fig. 1.

None of the three pertussis antigens induced a significant proliferative response in 3-month-old infants before vaccination (Fig. 1). However, 4 weeks after the primary vaccination course, a strong T-cell proliferation was detectable for all three antigens (Fig. 1). The pertussis antigen-specific T-cell response was stable for more than 12 months, with proliferative responses equal to or even higher than those determined in the postprimary vaccination group obtained from children before booster vaccination at the age of 15 to 24 months. Booster vaccination led to a further increase in the level of T-cell proliferation in response to all antigens tested (Fig. 1).

On average, 13% (3 of 37 for PT, 12 of 80 for FHA, 5 of 36 for PRN) of the children at the postprimary sampling failed to demonstrate a cell-mediated response on the basis of our definition. This proportion had decreased to 9% (3 of 43 for PT, 4 of 41 for FHA, 3 of 33 for PRN) in the prebooster group. The observation of a decrease in the numbers of nonresponders at the prebooster time point indicates that some of the subjects who were scored initially as negative 4 weeks after the primary vaccination may have developed a delayed cell-mediated re-

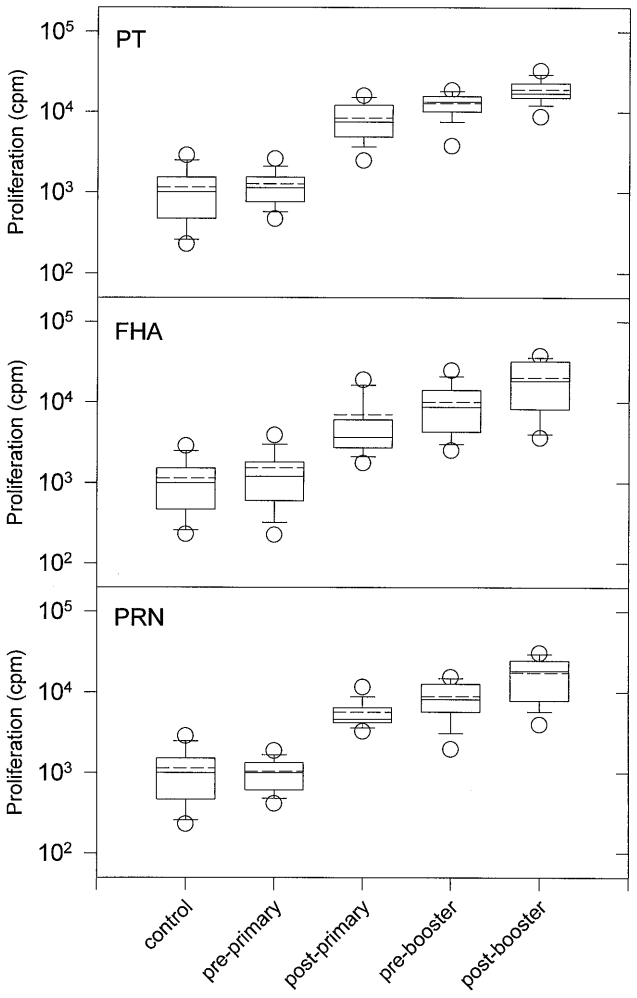

FIG. 1. Proliferation of PBL from infants before and after primary and booster vaccination with a tricomponent acellular pertussis vaccine. As described in Materials and Methods, cells were harvested from peripheral blood, stimulated with PT, FHA, and PRN over 6 days, and then pulsed with [^{3}H]thymidine for 16 h. The results are presented as Tukey box blots. The limits of the boxes represent the 25th and 75th percentiles of the results. Broken lines indicate the mean values, and closed lines indicate the medians. Additional symbols indicate the 5th and 95th percentiles (circles) and the 10th and 90th percentiles (bars). The first box plot depicts results from negative controls, i.e., cultures that were not stimulated with antigen ($n = 94$). The second plot (preprimary; $n = 80$) depicts the results from nonvaccinated infants. The third to fifth box plots give the results after antigen stimulation of cells from the postprimary ($n = 80$), prebooster ($n = 43$), and postbooster ($n = 33$) study groups, respectively. Vaccination with the acellular pertussis vaccine induces an antigen-specific CMI response against PT, FHA, and PRN that increased continuously during the vaccination schedule. Significant increases in proliferation occurred between the control as well as the preprimary groups in comparison with the postprimary, prebooster, and postbooster groups for PT, FHA, and PRN ($P < 0.001$). In addition, there was a significant difference between the results from the postprimary and postbooster groups for all three antigens used ($P < 0.001$).

sponse. However, the number of subjects investigated to date is too low to substantiate this hypothesis. Indeed, natural exposure to pertussis infection could not be excluded. In the postbooster group, no nonresponders were identified, all infants having a demonstrable CMI response to the pertussis antigens.

Antibody response following vaccination. The serum concentrations of antibodies against the three individual antigens of the acellular pertussis vaccine before and after primary vaccination as well as before and after booster vaccination are documented in Table 1. Four weeks after the third primary dose of the diphtheria-tetanus-acellular pertussis vaccine, a significant rise in the levels of circulating IgG antibodies specific for PT, FHA, and PRN was observed. Although anti-PT, anti-FHA, and anti-PRN were decreased 1 year later at the prebooster sampling, booster vaccination increased the levels of circulating antibodies to concentrations higher than those observed after primary vaccination, thus demonstrating efficient priming. Correlation of individual antibody titers with individual CMI results did not reveal any correlation between the quality of humoral and CMI responses.

TABLE 1. Seropositive status of the vaccinees and the geometric mean titers with respect to the three specified antigens before and after the primary and booster vaccinations[a]

Sampling point	PT		FHA		PRN	
	%	GMT	%	GMT	%	GMT
Postprimary	98.4	49	96.6	89	98.1	124
Prebooster	64.5	7	95.9	27	92.4	18
Postbooster	99.5	109	98.5	627	99.0	864

[a] Seropositivity was defined as antibody titers above 5 EL.U/ml. Since only initially seronegative infants were included in this study, the preprimary group, by definition, had a titer of <5 EL.U/ml.

Phenotype and expression of activation markers on T cells. To further define the nature of T-cell responses and T-cell subpopulations activated by the acellular vaccine, we examined lymphocyte phenotypes and the expression of cell surface activation markers after in vitro antigen-specific stimulation of lymphocytes obtained from vaccinated infants. Expression of CD45RO on CD4- and CD8-positive T cells was determined separately by two-color flow cytometry (Fig. 2). For PT, FHA, and PRN, a preferential up-regulation of CD45RO on CD4-positive T-helper cells was observed. Again, the proportion of cells specifically activated by the antigens increased with the progression of the vaccination schedule. These results suggest that the T cells activated by the acellular pertussis vaccine are preferentially confined to CD4-positive T cells.

Cytokine production after vaccination. Distinct T-helper subpopulations can be characterized by their cytokine production profiles (32). T-helper 1 (Th1) cells preferentially produce IFN-γ and IL-2 and are thought to regulate cell-mediated effector responses. T-helper 2 (Th2) cells produce IL-4, IL-5, and IL-10 and mainly control antibody production. To identify the type of immune response induced by the acellular vaccine, secretion profiles of IL-2, IFN-γ, and IL-10 by antigen-stimulated T lymphocytes were evaluated, and the results are illustrated in Fig. 3.

It is evident that the level of cytokine production exhibits a large variation between different individuals (data are depicted in a log scale). A threefold increase above background production was defined as a positive cytokine response. Only IL-2 and IFN-γ production were statistically significantly increased after stimulation with the pertussis antigens. Cells from vaccinated individuals secreted moderate to large amounts of IFN-γ in response to PT, FHA, and PRN. Ten of 12 individuals (83%) scored positive for IFN-γ production after stimulation with the three antigens. Production of IL-2 was proven in 15 of 19 subjects (79%) for PT, in 16 of 19 (84%) for PRN, and in all individuals (100%) for FHA. For IL-10, the majority of vaccinees investigated produced only low levels of response to the antigenic stimuli: IL-10 scored positive in 6 of 19 subjects (32%) for PT, 5 of 19 subjects (26%) for FHA, and 7 of 19 subjects (37%) for PRN.

In summary, these results indicate a preferential, but not

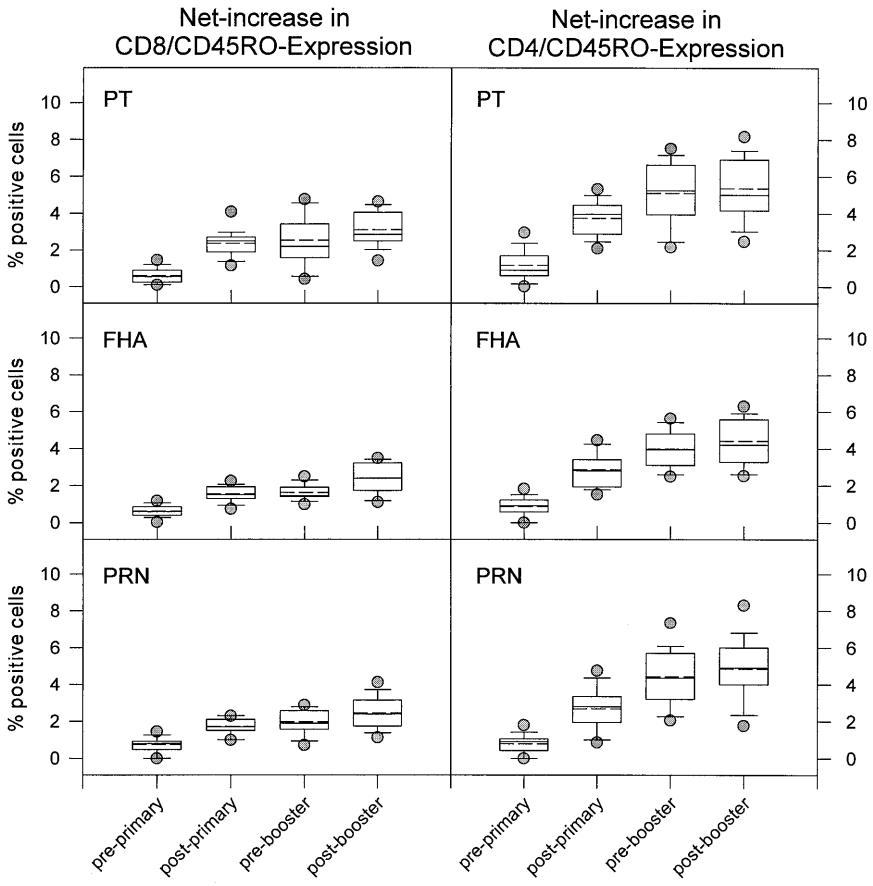

FIG. 2. Pertussis antigen-induced up-regulation of CD45RO on CD8-positive cytotoxic T cells or CD4-positive T-helper cells from infants before and after primary and booster vaccinations with a tricomponent acellular pertussis vaccine. Cells were prepared and cultured with pertussis antigens as described in the legend to Fig. 1. After a culture period of 36 h, the cells were harvested, stained with fluorescein isothiocyanate- and phycoerythrin-labeled MAb (CD4, CD8, and CD45RO), and evaluated by two-color flow cytometry. The results are presented as Tukey box plots (for legend to Fig. 1). The box plots illustrate the net increase of activation marker expression between cells from antigen-stimulated cultures and unstimulated controls ($N = 15$). Vaccination with the acellular pertussis vaccine preferentially induced an up-regulation of CD45RO on CD4-positive T-helper cells in response to PT, FHA, and PRN, while CD8-positive T cells remained mainly unaffected. Significant increases were observed for CD45RO expression on $CD4^+$ T cells between the preprimary and postprimary, prebooster, and postbooster groups ($P < 0.001$) for all antigens investigated. In addition, there was a significant difference between the postprimary and postbooster results ($P < 0.001$).

exclusive, induction of IL-2- and IFN-γ-producing Th1 cells by the acellular pertussis vaccine.

DISCUSSION

Despite increasing evidence that T-cell-mediated immune responses are important for complete termination of *B. pertussis* infections, no information is currently available on the primary CMI response to pertussis antigens in small infants. A large, ongoing efficacy trial for acellular pertussis vaccines in Germany between 1992 and 1995 provided a unique opportunity to analyze specific CMI responses in a population treated with an exactly defined quantity of pertussis-related antigens. Emerging immune responses could be clearly correlated to the immunization process and the progression of the vaccination schedule. Our study provides the first evidence for the induction of pertussis antigen-specific CMI responses in infants below the age of 12 months immunized with a tricomponent acellular pertussis vaccine.

A strong T-cell-proliferative response against PT, FHA, and PRN was already induced after the primary vaccination course at the age of 6 to 7 months. Specific CMI responses increased continuously with the ongoing vaccination schedule and remained stable for more than 12 months. In contrast to declining humoral antibody levels before the booster dose, the level of cell-mediated responses was the same or higher than after the primary vaccination and could be further stimulated by the booster.

The only available study on pertussis vaccine-related CMI responses in infants has been conducted by a Japanese group with children above the age of 18 months (54). In this study, a specific cellular immune response was observed only for FHA. For native PT, high levels of proliferation were found in both immunized and nonimmunized children, and the authors failed to demonstrate specific responses against native PT. Two factors may have contributed to this observation. First, the failure to detect a PT-specific response may be related to the low concentrations of the antigens in the vaccine (2 μg of PT and 8 μg of FHA). Second, only native PT, which is known to display mitogenic activities on lymphocytes (31, 36), was used for the in vitro testing of T-cell reactivity. Consequently, detection of specific responses may have been hampered by nonspecific T-cell proliferation due to the toxin.

In contrast to these observations, we could clearly demonstrate a PT-specific T-cell response following vaccination when nonmitogenic, detoxified PT was used. This is further supported by published studies which demonstrated specific CMI responses to PT and PT subunits S1, S2, S3, and S4 in adults

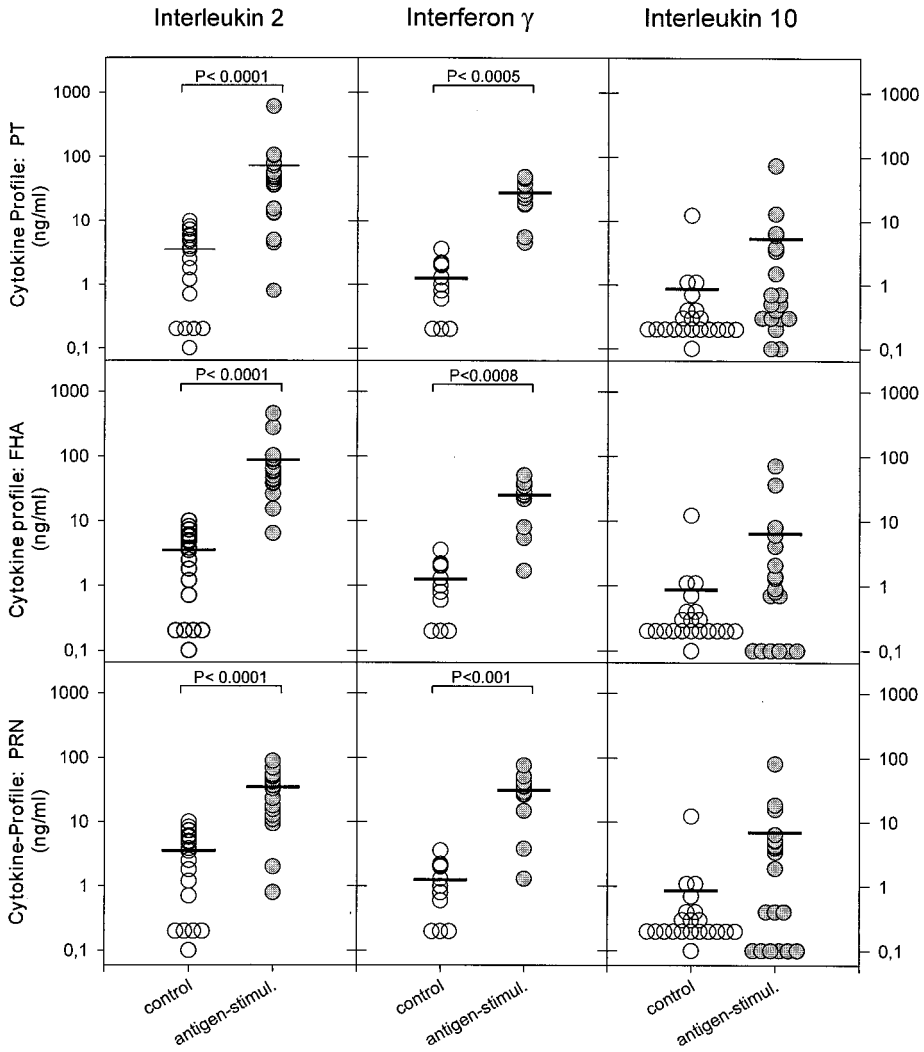

FIG. 3. Detection of pertussis antigen-induced cytokine production (IL-2, IFN-γ, and IL-10) by T cells from infants before and after primary and booster vaccinations with a tricomponent acellular pertussis vaccine. Cells were prepared and cultured in the presence of pertussis antigens PT, FHA, and PRN as described in the legend to Fig. 1. Culture supernatants were harvested after 48 h, and cytokine contents were determined by ELISA. Data are depicted as Tukey box plots in a log scale. The graph shows the results of unstimulated cultures (control) (○) and antigen-stimulated cultures (PT, FHA, and PRN) (◍) for vaccinated infants ($n = 19$ for IL-2, $n = 12$ for IFN-γ, $n = 19$ for IL-10). In vaccinated infants, a significant increase in IL-2 and IFN-γ production in response to PT, FHA, and PRN was observed. In contrast, IL-10 production occurred only in a minority (3 of 19) of the individuals. The percentages of positive cytokine production for each cytokine and antigen are given in Results. Significant differences are indicated by brackets in the graphs.

after infection (11–14, 17) or following vaccination (38, 40). Similarly, CMI responses have been demonstrated in mice following parenteral (36, 37) or mucosal (29) immunization or after infection (39). The reported variability of CMI responses to detoxified PT, especially in animal models (29, 36, 37), may be related to a different alteration of the toxoid by the detoxification process that in consequence may have influenced its quality as a specific antigen. Furthermore, experimental pertussis in mice (34, 47) located in the upper respiratory tract and lungs differs from infection in humans, which is almost exclusively located in the ciliated epithelium of the upper respiratory tract (25). These factors may significantly influence the development of PT-specific T-cell responses. Our data clearly document that the formaldehyde- and glutaraldehyde-detoxified PT present in the acellular vaccine used in this study induced a strong and specific response against PT in infants.

The finding of specific T-cell responses for FHA and PRN is supported by several studies conducted with adult humans (14, 17, 35, 54) and rodents (9, 23, 29). In extension of these observations, we were able to demonstrate for the first time a specific T-cell response for these two antigens in children within the first year of life and as a response to immunization with an acellular vaccine.

Most remarkably, our study showed that CMI against pertussis antigens persisted until the booster date, an observation not previously reported. Indeed, the level of the responses tended to increase between the primary course and the booster dose. Our data are currently insufficient to conclude whether this phenomenon was related to the increased age of the children before the booster or if the results documented after the primary vaccination did not represent the peak of the T-cell responses. Nevertheless, the results clearly indicate that the acellular vaccine induced a strong, long-lasting, and stable cellular immune response. This finding is particularly interesting in light of declining antibody titers and the recent observation in several trials (2, 19, 21, 50) that there is no apparent correlation between serological antibody titers and protection. There was no correlation between the quality of humoral and

CMI responses in our study population. Since the capacity of pertussis vaccines to confer protection (2, 19, 21, 50) is well documented, CMI may prove to be a more reliable measure of protection than humoral responses.

To further characterize the nature of CMI elicited by the acellular pertussis vaccine, we investigated lymphocyte phenotypes and expression of activation marker CD45RO on CD4- or CD8-positive T cells. CD45RO is up-regulated on activated T cells but is also considered to be an indicator for the generation of memory T cells (18, 22).

T cells from infants who were seropositive after vaccination responded with an up-regulation of the activation marker CD45RO when stimulated with pertussis antigens. Analogous to the increasing T-cell proliferation, the number of cells that could be activated by PT, FHA, and PRN increased during the vaccination protocol. This trend was maintained even during the period between primary and booster doses, further supporting our observation of a long-lasting and stable T-cell response. PT, FHA, and PRN preferentially stimulated CD4-positive T cells, while CD8-positive T cells remained almost unaffected. This indicates that CMI initiated by the acellular pertussis vaccine used in our study is primarily confined to the CD4-positive T-helper population. The results corroborate data obtained in mice, which showed that only immune CD4-positive T cells were able to confer protection to immunodeficient nude mice (30). In addition, Petersen et al. (36, 39), using anti-CD4 or anti-CD8 MAb, demonstrated that the relevant T-cell response to pertussis was restricted to the CD4-positive subpopulation.

Recently, the existence of distinct subsets of T-helper cells, Th1 and Th2, which can be distinguished by their cytokine secretion profiles (6, 32, 44), has been proven. Peppoloni et al. (35) reported that T-cell clones isolated from pertussis convalescents with specificity for PT and other pertussis antigens secreted IL-2 and IFN-γ but low or undetectable levels of IL-4. Studies with T cells from mice recovering from infection or following immunization with whole-cell vaccines revealed cytokine profiles characteristic for Th1-type responses (30, 36, 39), i.e., cells producing high amounts of IFN-γ and IL-2 but no IL-4 or IL-5.

The evaluation of cytokine production in our study revealed a more heterogeneous pattern. Although a preferential production of IFN-γ and IL-2 in response to pertussis antigens was observed in T cells obtained from immune infants, lower levels of IL-10 were found in several of these individuals. In contrast to models using inbred mice or cloned human T cells, a greater variability of cytokine responses must be expected in bulk cultures of human peripheral blood T lymphocytes. Indeed, a strict delineation to either Th1 or Th2 responses would be an extremely unlikely event. The majority of infants displayed not only cell-mediated responses but also pertussis-specific antibodies. Therefore, Th1 and Th2 cells had to be activated to at least some extent. Our data support a preferential induction of Th1 cells following immunization with the acellular vaccine but by no means rule out the concomitant activation of Th2 cells. These observations paralleled results observed in individuals after natural infection (35; and unpublished observations).

Animal studies on cytokine production after vaccination are still controversial. While some indicate a Th1 response after vaccination with whole-cell or acellular vaccines (9), others report a Th2 profile in mice after vaccination with acellular vaccines (42). In contrast to our results, Redhead et al. (42) observed a Th1-type response in inbred mice only after natural infection or vaccination with whole-cell vaccines, while acellular pertussis vaccines consisting of PT and FHA tended to induce a Th2-type cytokine profile. As already mentioned, experimental pertussis in mice differs in several aspects from pertussis in human infants. Moreover, since the mice were vaccinated intraperitoneally, an entirely different route of antigen processing could have been engaged. Thus, it is quite likely that humans immunized by the intramuscular route will utilize different antigen-presenting cells than those engaged in processing vaccine antigens administered intraperitoneally. Furthermore, the antigen load per gram of body weight in mice was enormously higher (half the human dose of vaccine) than that given to human infants. Our data show that in the human situation, vaccination with the acellular pertussis vaccine, like natural infection, will result in a broad activation of Th1- and Th2-type cells, with a preference for the Th1 population. Further definition of the relevant type of response will require a formal frequency analysis at the clonal level.

The observation that CMI with predominance of Th1-type CD4-positive cells is the preferred type of immune response induced by the acellular pertussis vaccine poses the question of how these cells contribute to protection from *B. pertussis*. Recent reports suggest that although *B. pertussis* was formerly considered to be noninvasive, it is not exclusively an extracellular pathogen and can be taken up and survive within mammalian cells (16, 26, 49). Moreover, *B. pertussis* has been detected within alveolar macrophages from the lungs of human immunodeficiency virus-infected individuals (7). In light of this evidence, it is conceivable that Th1-type lymphokines are needed to recruit and activate phagocytes such as macrophages or neutrophils (51) to facilitate clearance of intracellular pathogens (24, 28).

In conclusion, there is increasing evidence supporting a role for a specific CMI response in the complete elimination of *B. pertussis* and subsequent protection against the disease. Our study documents for the first time that vaccination of infants with an acellular pertussis vaccine induces a strong and persisting T-cell response to all vaccine antigens. This response is preferentially mediated by CD4-positive T cells that predominantly display a Th1-type cytokine profile. Thus, the vaccine creates an immune response pattern that may be relevant for protection against the disease. Measurement of CMI may prove to be a reliable tool to assess protective potency in future investigations.

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