Cytokine mRNA Expression and Proliferative Responses Induced by Pertussis Toxin, Filamentous Hemagglutinin, and Pertactin of *Bordetella pertussis* in the Peripheral Blood Mononuclear Cells of Infected and Immunized Schoolchildren and Adults

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Pertussis infection is increasingly recognized in older children and adults, indicating the need of booster immunizations in these age groups. We investigated the induction of pertussis-specific immunity in schoolchildren and adults after booster immunization and natural infection. The expression of mRNA of gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, and IL-5 in the peripheral blood mononuclear cells (PBMCs) was assayed by reverse transcription-PCR. The PBMCs of 17 children immunized with one dose of an acellular vaccine containing pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN) significantly proliferated in vitro after stimulation with the vaccine antigens. The PBMCs of seven infected individuals markedly proliferated in the presence of PT and FHA, but the cells of only two of these subjects responded to PRN. At least one of the antigens induced mRNA for IL-4 and/or IL-5 in the cells of 93% of tested vaccinees and patients, and FHA induced IFN-γ mRNA in the cells of two-thirds of them. Expression of mRNA for IFN-γ correlated with the production of the cytokine protein. Anti-FHA immunoglobulin G antibodies significantly correlated with FHA-induced proliferative responses both before and after immunization. These results show that booster immunization with acellular pertussis vaccine induces both antibody- and cell-mediated immune responses in schoolchildren. Further, booster immunization and natural infection seem to induce the expression of mRNA of T-helper 1 (Th1) and Th2 type cytokines in similar manners. This observation supports the use of acellular pertussis vaccines for booster immunizations of older children, adolescents, and adults.

Pertussis is a highly contagious respiratory disease caused by *Bordetella pertussis*, which particularly threatens nonimmunized infants. The disease has remained endemic and epidemic in immunized populations (6–8, 18, 40). Pertussis infection is increasingly recognized in older children and adults (5, 8, 19, 25, 26, 31). This indicates that the immunity imparted by childhood immunizations wanes below the protective level in these age groups and stresses the need of booster immunizations. Modern acellular vaccines, being less reactogenic than conventional whole-cell vaccines, seem to be suitable not only for primary immunization but also for boosting (11, 12, 16, 17). At present, four components of *B. pertussis*, pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (PRN), and fimbrial antigens, are regarded as candidate antigens to be included in acellular vaccines (11, 12). However, the significance of immune responses to each of these components in preventing infection and disease is not fully known.

Antibodies have been traditionally thought to play an important role in protection against pertussis, because *B. pertussis* was considered to be an extracellular pathogen. PT, FHA, and PRN, used singly or in combination, have induced good antibody responses and protective immunity in experimental animals (21, 35, 37). However, in clinical efficacy trials of acellular vaccines, no clear correlation has been found between serum antibody levels and protection (1).

Increasing evidence suggests that cell-mediated immunity is involved in immune protection against pertussis. Several reports have shown that *B. pertussis* can survive in mammalian cells, including macrophages, in vitro and in vivo (3, 13, 14, 36). Further, T lymphocytes specific for *B. pertussis* or its components have been demonstrated in humans and mice after infection (9, 15, 24, 27–30, 39). In a recent study (34), Ryan et al. demonstrated a preferential induction of T-helper 1 (Th1) cells in preschool children with *B. pertussis* infection. Zepp et al. reported that primary immunization with a tricomponent acellular pertussis vaccine induced predominantly Th1 cells in infants (41). In contrast, Ausiello et al., also by vaccinating infants, found that an acellular vaccine induced cytokines of both types, whereas a whole-cell vaccine induced cytokines of Th1 type (2). However, there are practically no studies comparing the effects of booster immunization and natural infection on cell-mediated immunity in schoolchildren and adults.

We investigated pertussis-specific cell-mediated immune responses by proliferation assay of the peripheral blood mononuclear cells (PBMCs) in schoolchildren and adults after either natural infection or booster immunization. The mRNAs of Th1 and Th2 type cytokines were assayed by reverse transcription-PCR (RT-PCR) in the PBMCs of the subjects. Gamma interferon (IFN-γ) and interleukin-5 (IL-5) were measured by an enzyme-linked immunosorbent assay (ELISA) in the culture media of the PBMCs of the adult vaccinees.
37°C in air with 5% CO₂ and 95% humidity in a CO₂ incubator. Sinki, Finland) and 1% (wt/vol) glutamine (29.2 mg/ml; Biological Industries, heat-inactivated (30 min at 56°C) human AB serum (Finnish Blood Bank, Hilden). Cells were cultured in RPMI 1640 medium containing 10% (vol/vol) of a whole-cell pertussis vaccine combined with diphtheria and tetanus toxoids and had received a dose of the bivalent acellular vaccine 6 years before this study. Of them, only V7 had received the primary three doses of the whole-cell vaccine in childhood. The eight culture-confirmed pertussis patients (three males and five females) included two adults (P1 and P2; 60 and 26 years) and six 13-year-old children (P3 to P8). P3 to P6 had been immunized with the whole-cell pertussis vaccine in childhood. P3 to P8 were all from a school class where a pertussis epidemic had occurred. At the time of sampling, P6 and P7 were asymptomatic, and the others had had cough for 1 to 17 weeks.

Twenty-five healthy subjects (10 males and 15 females) served as controls. Nine of them were randomly selected among 117 10- to 12-year-old children who had received a booster dose of the DT vaccine 1 month before testing; eight (C1 to C8) were adults (aged 20 to 40 years) recruited from the NPHI, Department in Turku. They had not received a dose of the bivalent acellular vaccine 6 years before this study. Of them, only V7 had received the primary three doses of the whole-cell vaccine in childhood. The culture-confirmed pertussis patients (three males and five females) included two adults (P1 and P2; 60 and 26 years) and six 13-year-old children (P3 to P6). P2 to P6 had been immunized with the whole-cell pertussis vaccine in childhood. P3 to P6 were all from a school class where a pertussis outbreak occurred. At the time of sampling, P6 and P7 were asymptomatic, and the others had had cough for 1 to 17 weeks.

Proliferation assays. For proliferation assay, the preliminary experiments indicated the following optimal doses of antigens: PT, 1 \( \mu \)g/ml; FHA, 8 \( \mu \)g/ml, and PRN, 2.5 \( \mu \)g/ml. The samples in which reverse transcription was omitted but all reagents were used for the proliferation assay were considered as background. The results were expressed as mean counts per minute for triplicate cultures. An antigen-negative control (cDNA) was also included in each assay. The PCR mixture of 50 \( \mu \)l contained 10 \( \mu \)M of each primer, 200 \( \mu \)M of each deoxynucleoside triphosphate (dNTP), 1 \( \mu \)l of diluted cDNA, 1 \( \mu \)l of diethyl pyrocarbonate-treated distilled water, and 2.5 units of Taq polymerase. The PCR program was started with denaturation at 94°C for 5 min and ended with final extension at 72°C for 5 min. Subsequent conditions were as follows: for β-actin, 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min; for IL-2 and IFN-γ, 35 and 40 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 2 min; for IL-4, 35 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 2 min; for IL-6, 56°C for 5 min followed by 40 cycles of 72°C for 1 min, 59°C for 1 min, and 72°C for 2 min; for IL-5, 38 cycles of 94°C for 1 min, 63°C for 30 s, and 72°C for 2 min. The resulting PCR products (16 \( \mu \)l) were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

Samples in which reverse transcription was omitted or all reagents were included were used as negative controls in the PCR. To verify the performance of the first-strand cDNA synthesis and amplification reaction, 50 ng of control RNA was used in the cDNA synthesis kit and also reverse-transcribed, and 1/20 of the cDNA obtained was amplified in the PCR. All samples were tested twice. Patients P5 and P6 were not tested for cytokine mRNA expression because not enough cells were available.

Cytokine assays. PBMCs at a concentration of 2 × 10⁶ cells were cultured in 96-well plates with the antigens or PHA as described for the proliferation assay, and supernatants were removed after 48 h. IFN-γ and IL-5 were chosen to represent type 1 and type 2 cytokines, respectively. Their concentrations were measured by a commercial ELISA kit (Quantikine, R&D Systems, Minneapolis, Minn.) with threshold detection values of 15.6 pg/ml for IFN-γ and 7.8 pg/ml for IL-5. IL-5 was selected instead of IL-4 because of well-known low sensitivity of commercial kits for IL-4 and because the level of transcription of the IL-4 gene is lower than the level of transcription of the IL-5 gene (2, 41).

Measurement of IgG antibodies. Immunoglobulin G (IgG) antibodies against PT, FHA, and PRN were measured by ELISA in the laboratory of SmithKline Beecham Biologicals as described previously (20, 33). The results were expressed as ELISA units/ml. The threshold detection level of the test was 5 ELISA units/ml.
Statistical analyses. Fisher’s exact test and the Mann-Whitney U test were used for the analysis of statistical significance, and the Spearman rank correlation coefficient was used for the analysis of correlations. A P value of <0.05 was considered statistically significant.

RESULTS

Proliferative responses to B. pertussis antigens. The PBMCs of the 17 children immunized with the DTaP vaccine showed significantly higher proliferative responses to PT, FHA, and PRN than the cells of the 9 children immunized with the DT vaccine (for PT, \( P = 0.0015 \); for FHA, \( P < 0.0001 \); for PRN, \( P = 0.0053 \) [Table 2]). The two groups of vaccinees did not differ in their PHA and pokeweed mitogen responses (data not shown). After immunization with DTaP, the proliferative responses of 14 (82%) individuals increased significantly, and all individuals showed a positive proliferative response (SI \( \geq 4 \)) to at least one of the three antigens. No corresponding conversions were seen in the proliferative responses of the control subjects immunized with DT. Three adult individuals (V6 to V8) had been immunized with the bivalent vaccine containing PT and FHA 6 years before testing. Proliferation assays of the PBMCs of these three subjects were repeated 1 year after the first testing, and very similar results were obtained (Fig. 1A). The PBMCs of two of these vaccinees (V6 and V7) responded with a significant proliferation to PT and FHA, whereas the cells of the third were totally unresponsive to these antigens. None of these three adult vaccinees had significant SI of PRN (SI \( \geq 4 \)).

Of the 26 child vaccinees, 17 immunized with DTaP and 9 immunized with DT, 16 (62%) showed proliferative responses to at least one of the three antigens before immunization: 13 (50%) responded to FHA, nine (35%) responded to PT, and seven (27%) responded to PRN.

The PBMCs of all seven pertussis patients (P1 and P3 to P8) showed positive proliferative responses to FHA and/or PT, but only two of them (P7 and P8) showed positive responses to PRN (SI \( \geq 4 \) [Fig. 1A]). Subjects immunized with DTaP showed a strong proliferative response to PRN more frequently (14 of 17) than the infected individuals (2 of 7) (\( P = 0.021 \)).

Cytokine mRNA expression induced by B. pertussis antigens. A pilot study on the kinetics of cytokine mRNA expression induced by the antigens was first carried out on the PBMCs of patient P1. The cells were cultured for 5 h, 20 h, 2 days, or 5 days and then subjected to RT-PCR (Fig. 2). PT and FHA induced expression of the IFN-\( \gamma \), IL-2, IL-4, and IL-5 mRNAs by 2 days, but PRN induced only low expression of the IL-2 mRNA (weakly positive). Spontaneous production of the IL-2 and IL-4 mRNAs reached in 5 days the levels induced by the antigens. The best discrimination between antigen-induced

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Median cpm (range) Before immunization</th>
<th>Median cpm (range) After immunization</th>
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<tbody>
<tr>
<td></td>
<td>DT</td>
<td>DTaP</td>
</tr>
<tr>
<td>Medium</td>
<td>183 (40–554)</td>
<td>95 (42–451)</td>
</tr>
<tr>
<td>PT</td>
<td>950 (183–3,622)</td>
<td>272 (66–4,642)</td>
</tr>
<tr>
<td>FHA</td>
<td>1,500 (248–2,996)</td>
<td>332 (52–2,654)</td>
</tr>
<tr>
<td>PRN</td>
<td>470 (77–3,581)</td>
<td>189 (44–8,077)</td>
</tr>
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</table>

* Seventeen immunized with a dose of DTaP vaccine; nine immunized with DT vaccine.

\( ^{b} \) No statistically significant difference was found between children who were immunized with DTaP and DT vaccines.

\( ^{c} \) Statistically significant differences were found between children who were immunized with DTaP and DT vaccines: for PT, \( P = 0.0015 \); for FHA, \( P < 0.0001 \); and for PRN, \( P = 0.0053 \).
and spontaneous production of cytokine mRNAs was obtained at 2 days of culture, and this incubation time was selected for further studies.

Expression of the cytokine mRNAs in the PBMCs of 20 subjects (8 vaccinees, 6 infected persons, and 6 controls) was measured (Table 3). PHA stimulated the production of the mRNAs of all four cytokines in the PBMCs of all tested subjects (data not shown). Various levels of IL-2 mRNA were also detected in the antigen-stimulated PBMCs of all tested subjects except the two newborns (C15 and C16). In general, the proliferative responses of the PBMCs induced were stronger the higher the IL-2 mRNA expression. Expression of the mRNA for IL-4 and/or IL-5 was induced by at least one antigen in the PBMCs of all tested vaccinees and patients except

![Table 3](image-url)  

<table>
<thead>
<tr>
<th>Subjects (no.)</th>
<th>Antigen</th>
<th>IFN-γ mRNA expression</th>
<th>IL-2 mRNA expression</th>
<th>IL-4 mRNA expression</th>
<th>IL-5 mRNA expression</th>
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</thead>
<tbody>
<tr>
<td>Vaccinees (8)</td>
<td>PT</td>
<td>38/100/38/88</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FHA</td>
<td>63/100/38/50</td>
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<td></td>
<td>PRN</td>
<td>20/100/60/80</td>
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<td></td>
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<tr>
<td>Patients (6)</td>
<td>PT</td>
<td>50/100/67/60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FHA</td>
<td>83/100/50/100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PRN</td>
<td>17/100/33/20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (6)</td>
<td>PT</td>
<td>0/67/0/0</td>
<td></td>
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<tr>
<td></td>
<td>FHA</td>
<td>0/67/0/0</td>
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<td></td>
<td>PRN</td>
<td>0/67/0/0</td>
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</table>

a Vaccinees (V1 to V5) were immunized with the DTaP vaccine containing PT, FHA, and PRN 1 month earlier; V6 to V8 had been immunized with the bivalent PT-FHA vaccine 7 years earlier and were not included in the results for responses to PRN.

b All patients were culture positive for B. pertussis. At the time of sampling, five patients (P1 to P4 and P8) had been coughing for 1 to 17 weeks, and one patient (P7) was asymptomatic. IL-2 mRNA expression was weakly positive in the PBMCs stimulated with PRN; IL-5 mRNA expression was not tested in patient P2.

c Four controls (C2 and C6 to C8) were healthy adults; two (C15 and C16) were newborns.

V8. The PBMCs of the six controls did not produce the mRNAs for IFN-γ, IL-4, and IL-5 after stimulation with any antigen. Expression of IFN-γ mRNA was less frequent after stimulation with PRN (2 of 11 tested) than after stimulation with FHA (10 of 14 tested) (\(P = 0.015\)). Expression of the cytokine mRNAs in the PBMCs of the immunized and infected subjects did not differ significantly from each other.

**Production of IFN-γ and IL-5 protein.** Antigen-induced proliferation, cytokine mRNA expression, and cytokine production of the PBMCs of four subjects, V6, V7, V8, and C2, were tested in parallel (Fig. 1 and Table 4). Significant proliferation and production of IFN-γ mRNA and IFN-γ were detected in the PBMCs of V6 and V7 after stimulation with PT and FHA but not after stimulation with PRN. The cells of V8 and C2 did not produce IFN-γ after stimulation with any antigen. IL-5 mRNA was detected in the PBMCs of V6 and V7 after stimulation with PT, but no IL-5 was detected.

**Relationship between humoral and cellular immune responses.** Immunization of the 17 schoolchildren with the DTaP vaccine induced significant IgG antibody responses to all three pertussis antigens (Table 5). Significant correlation was found between FHA-induced proliferative responses before immunization and anti-FHA IgG antibody levels after immunization (\(\gamma = 0.625, P = 0.007\)), as well as between FHA-induced proliferative responses and anti-FHA IgG antibodies after immunization (\(\gamma = 0.625, P = 0.008\)). Significant correlation was also found between anti-PRN IgG antibodies and PRN-induced proliferative responses after immunization (\(\gamma = 0.488, P = 0.047\)), whereas no correlation was found between proliferative and IgG antibody responses to PT.

**DISCUSSION**

Our results show that in schoolchildren and adults, immunization with acellular pertussis vaccine and natural infection cause arousal of cellular immune functions as assessed by antigen-induced proliferation and cytokine mRNA expression of PBMCs. The mRNA transcripts for both Th1 type cytokines,
IFN-γ and IL-2, and for Th2 type cytokines, IL-4 and IL-5, were detected in the PBMCs of the vaccinees and infected individuals after in vitro stimulation with B. pertussis antigens. These findings are in agreement with the results of earlier studies on the immune responses of infants. Ausiello et al. (2) found that the induction of Th1 or Th2 cytokines is a vaccine- and antigen-dependent phenomenon after primary vaccination with either whole-cell or acellular vaccines. The acellular vaccine induced a basically type 1 cytokine profile, accompanied by some production of type 2 cytokines. Our acellular vaccine was the same as that used by Ausiello et al. Although the concentration of the antigens included in our vaccine had been reduced to one-third, the significant immune responses after the booster immunization were induced. Ryan et al. (34) studied preschool children with B. pertussis infection and demonstrated a preferential induction of Th1 cells. In our study, the PBMCs of five of the six patients with B. pertussis infection expressed mRNA for IFN-γ, a characteristic Th1 cytokine, after stimulation by at least one of the three antigens. The responses were not, however, restricted to the Th1 type, since transcripts of the IL-4 and/or IL-5 mRNA were also detected in the antigen-stimulated cells of these patients.

Our results show that IFN-γ mRNA was expressed at the protein level in the PBMCs stimulated with B. pertussis antigens, although the production of cytokine proteins was studied in a limited number of subjects. This finding further supports the concept that the expression of IFN-γ mRNA can be used as a parameter in assessing the type of immune response. The production of IL-5 mRNA was detected in the cells stimulated with B. pertussis antigens. However, the levels of IL-5 protein remained below the detection threshold of our assay. It is possible that the number of PBMCs which could be used for technological reasons still was too low for production of measurable concentrations of IL-5. Another reason might be that the 2-day culture was not optimal for the production of this particular cytokine.

In our study, the PBMCs of all tested subjects except the two infants expressed IL-2 mRNA after stimulation with pertussis antigens. Thus, some individuals expressed IL-2 but not IFN-γ. The IL-2 in these subjects could be derived from Th2 cells rather than Th1 cells. It has been shown that Th2 cells can produce small quantities of IL-2 (32). Because our cytokine RT-PCR is not a quantitative assay, IL-2 expression could not be used to differentiate the activities of Th1 and Th2 cells.

Whole-cell pertussis vaccines have been used extensively in most industrial countries where, despite high immunization rates, pertussis remains endemic and epidemic (6–8, 18, 40). Moreover, B. pertussis infection is increasingly recognized in older children and adults (5, 8, 19, 25, 26, 31), indicating the need of repeated booster immunizations in these age groups. Our results show, for the first time, that in schoolchildren the booster immunization with the trivalent acellular vaccine induced good responses in both arms of the immune system. The expression of cytokine mRNAs induced after the booster immunization was comparable to that induced after natural infection, suggesting that the acellular vaccine with reduced antigen concentration is suitable for the boostering in this population.

It is not fully known how long the protective immunity provided by pertussis vaccines persists. We have previously shown that Finnish children become susceptible to clinical pertussis after school entry (19), suggesting that the protection persists for about 5 years after the last immunization at 2 years of age. In this study, specific cellular responses to PT and FHA were not observed in one (V8) of three adults who had received a dose of bivalent vaccine (PT and FHA) 6 years before this testing. The significant responses of his cells to tetanus toxoid and mitogens excluded the possibility of a general unresponsiveness in this subject. This result suggests that cell-mediated immunity induced by pertussis immunization starts to decrease and may even become undetectable over 6 years.

Proliferative responses to PRN were more often found after immunization than after natural infection. Since the same antigens were used for immunization and for in vitro stimulation, good responses after immunization were to be expected. The lower responses after natural infection may be due to antigenic differences between the PRN of bacteria causing infections and the PRN used for in vitro stimulation. The selection pressure caused by the immunization program of more than 40 years may have changed the PRN of B. pertussis bacteria existing in the Finnish population. The preliminary results obtained by sequencing and restriction fragment length polymorphism analysis indicate that most clinical isolates have differences in the gene encoding PRN compared to the vaccine strains (data not shown). On the other hand, the PRN used for immunizations had been treated with formaldehyde. This treatment may have destroyed some antigenic epitopes which are recognized by T cells induced during natural infection (10). However, we could not exclude the possibility that in some infected individuals, the blood samples were taken too early for any response to PRN to develop.

Our data clearly show that strong and specific cell-mediated immune responses are induced in schoolchildren and adults after B. pertussis infection or after booster immunization with an acellular vaccine containing reduced concentrations of PT, FHA, and PRN. Moreover, the expression of cytokine mRNAs induced after the booster immunization is comparable to that induced after natural infection. These results suggest that the acellular vaccine tested is suitable for the booster immunizations in these age groups.

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