Cell-Mediated Immune Response of Healthy Adults to *Bordetella pertussis* Vaccine Antigens

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Cell-mediated immunity (CMI) to *Bordetella pertussis* and acellular pertussis vaccine constituents (pertussis toxin, pertactin, and filamentous hemagglutinin) were studied in peripheral blood mononuclear cells (PBMC) and T cell cultures from healthy adults with no record of vaccination against, or history of, pertussis. Similarly to stimulation with common recall antigens, PBMC proliferation was induced in 80%–100% of the cultures, depending on the specific *B. pertussis* stimulant. Proliferation did not occur when antigen-presenting cells were ablated by chemical or physical methods or with naive cord blood lymphocytes. *B. pertussis* antigen stimulation resulted in a preferential induction of type 1 cytokine profile, as shown by interferon-γ and interleukin-2 (but no interleukin-4 or interleukin-5) gene transcripts and actual cytokine production by T cells. The data suggest that most healthy adults are repeatedly exposed to *B. pertussis*, with natural acquisition of antigen-specific CMI and a putatively protective type 1 cytokine pattern.

The mechanisms of protective immunity against *Bordetella pertussis* infection following natural exposure or vaccination are still unclear. Immunogenicity studies during efficacy trials of pertussis vaccines in infants suggested that antibodies are not the sole determinants of resistance to this pathogen (reviewed in [1–3]). Consequently, cell-mediated immunity (CMI) has been addressed, both in murine models of *B. pertussis* respiratory infection and in children recovering from, or vaccinated against, pertussis [4–8]. These studies suggest that CMI is probably an important host determinant of anti-pertussis resistance.

CMI responses against *B. pertussis* antigens in adults have been little explored. Recent reports demonstrate that when pertussis vaccination of infants is largely implemented, adolescents and adults remain susceptible to infection by *B. pertussis* and actually constitute the reservoir for children’s infection [9, 10]. This makes it plausible to offer a booster dose of vaccine for adolescents and adults, also with the goal of preventing pertussis transmission from adults to infants. Knowledge of the CMI response in adults may give some critical insights into the acquisition and degree of natural or artificial sensitization to *B. pertussis*, hence into the rationale of adult vaccination. In this pilot study, we have addressed peripheral blood mononuclear cell (PBMC) proliferation and cytokine production by T cells of blood donors with no history of pertussis or pertussis vaccination.

**Methods**

**Antigens.** *B. pertussis* whole cells (strain 18323 [ATCC 9797, agglutinogen 1,3]; in-house preparation), pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN) (all *B. pertussis* soluble antigens were provided by Chiron-Biocine, Siena, Italy) were used as antigens. All preparations were heat-inactivated at 86°C for 1 h.

**Subjects.** Peripheral blood from 16 volunteers (mean age, 40 years; range, 24–57) with no record of pertussis vaccination or pertussis was used.

**PBMC isolation, T cell enrichment, proliferation, and cytokine assay.** PBMC were isolated by centrifugation on density gradients and suspended in complete RPMI medium (GIBCO, Grand Island, NY) supplemented with 5% pooled AB serum and antibiotics. In other experiments, mononuclear cells from cord blood (CBMC) were used [11]. T cell–enriched cultures (hereafter referred to as T cells or T cell cultures) were obtained by immunomagnetic negative selection with Dynabead particles (Dynol, Oslo), as previously described [12]. The enrichment was considered to be effective when T cells were >90% CD3 and <1% CD14 and CD19, as determined by immunofluorescence assays on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and the culture did not proliferate (see below for definition) on stimulation by anti-CD3 monoclonal antibody [12]. In some experiments, PBMC were depleted of antigen-presenting cells (APC) by treatment with 1-leucine methyl ester (Sigma, St. Louis) [13].

Cell proliferation was assayed by using 10^6 cells/mL in 0.2 mL of complete medium in the presence of a predetermined optimal dose of each stimulant. The plates were incubated at 37°C in 5% CO_2_. DNA synthesis was evaluated by counting [3H]thymidine incorporation. Unless otherwise indicated, the data were expressed as mean counts per minute (cpm) × 10^3 of triplicate values ± SD. Lymphoproliferation was defined as positive when the mean cpm was >3 × 10^3 after subtraction of the cpm of the unstimulated control cultures [5, 8].

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Cytokines in culture supernatants were measured by ELISA (Quantikine; R&D Systems, Minneapolis).

Polymerase chain reaction (PCR)–assisted mRNA amplification. Total cellular RNA was extracted by the guanidinium isothiocyanate method, reverse-transcribed, and subjected to PCR reactions, as described elsewhere [11]. The reaction product was visualized by electrophoresis. The relative density of the ethidium bromide–stained PCR reaction products was determined by using a Scanning Densitometer CS-9301PC (Shimadzu, Columbia, MD). The actual densitometric values for each cytokine mRNA were normalized against $\beta$-actin band density taken as the 100% reference, and all other values were expressed as a percentage of this figure [12].

Results

In preliminary experiments, the optimal concentration of each B. pertussis stimulant for both PBMC proliferation and cytokine production was established ($10^6$ cfu/mL for B. pertussis and 10, 20, and 20 $\mu$g/mL for PT, PRN, and FHA, respectively). These experiments also showed that kinetics of PBMC proliferative response to all preparations reached the maximum values after 7 days, as typical of memory recall antigen stimulation. Thus, all subsequent experiments were done under the above experimental conditions.

PBMC proliferation. The PBMC of all 16 subjects proliferated in response to B. pertussis and PRN, while those of 12 and 14 of 15 subjects examined responded to PT and FHA, respectively. The degree of proliferation induced by PT or FHA (mean $\pm$ SD, 9.5 $\pm$ 1.5 and 18.5 $\pm$ 3.5 x $10^3$ cpm, respectively) was significantly lower than that induced by PRN (30.3 $\pm$ 4.7) or B. pertussis (34.6 $\pm$ 3.6). (All values are given after subtraction of the cpm of the unstimulated control).

To rule out a possible mitogenic or superantigenic activity of B. pertussis antigens (despite their use as heat-inactivated preparations), naive lymphocytes from cord blood (CBMC) were isolated and their proliferation tested in comparison with PBMC from a subject responsive to all B. pertussis antigens. Figure 1A shows that these antigens induced very low, if any, CBMC proliferation, compared with the high proliferative responses of PBMC. In contrast, CBMC were, as were PBMC, fully responsive to the mitogenic stimulation exerted by interleukin (IL-2) (100 U/mL).

Treatment of PBMC with the lysosomotropic agent, l-leucine methyl ester, which ablates APC [13], drastically reduced PBMC proliferation induced by B. pertussis antigens. APC reintegration of l-leucine methyl ester–treated PBMC cultures by the addition of irradiated PBMC resulted in a full recovery of proliferative PBMC potential (figure 1B [left]). On the other hand, the mitogenic proliferation induced by phytohemagglutinin stimulation was not inhibited but, rather, appreciably increased by l-leucine methyl ester treatment (figure 1B [right]).

Figure 1C shows that T cells prepared from PBMC of B. pertussis antigen–responsive donors and physically ablated of...
conventional APC (see Methods) did not proliferate in response to *B. pertussis* antigen stimulation. However, when irradiated APC were added to these cultures, cell proliferation occurred (data not shown). Altogether, these results indicate that *B. pertussis* preparations acted as antigens rather than mitogens.

**Cytokine profile in *B. pertussis* antigen–activated PBMC and T cells.** The cytokine profile in *B. pertussis* antigen–stimulated cultures was studied as mRNA expression and protein secretion.

Figure 2 shows the cytokine mRNA signals in PBMC and in T cell cultures from a representative donor. It shows that most cytokines tested were expressed in *B. pertussis* antigen–activated PBMC, and the intensity of the signals depended on the single stimulating antigen. However, IL-4 and IL-5 signals had low or no intensity, whichever the *B. pertussis* antigen used. Figure 2 also shows that granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, and interferon (IFN)-γ, but not IL-6, tumor necrosis factor-α, and IL-10, genes were expressed by T cells. In particular, the IL-2 signal was more intense in T cells than in PBMC, and, in the former, both the IL-2 and the IFN-γ signals were definitely less intense on stimulation by PT than by any other *B. pertussis* antigen. This qualitative analysis was confirmed by densitometric quantitation of the cytokine mRNA signals against β-actin mRNA with representative data obtained from PBMC of 5 donors (figure 3, upper left) and the corresponding T cell cultures (figure 3, lower left).

To confirm that gene expression was actually translated into the cytokine products and reflected the differential cytokine gene expression patterns shown by proliferating PBMC and nonproliferating T cell cultures, we assayed the supernatants of both PBMC and T cell cultures of the same 5 subjects for production of representative Th1 and Th2 cytokines. Despite large subject-to-subject variations, figure 3 (right) confirms that both IFN-γ and IL-10 were produced by *B. pertussis* antigen–stimulated PBMC, whereas only the former was present in the supernatant of T cell cultures. No or minimal IL-5 was produced by either PBMC or T cells. Together, these data confirm the cytokine gene expression patterns and demonstrate that IL-10, IL-6, and tumor necrosis factor-α are not produced by T cells under our stimulation conditions.

Addition of irradiated APC to *B. pertussis*–stimulated T cells did not affect the amount of IFN-γ production, nor did it stimulate appreciable IL-5 production (data not shown).

**Discussion**

Recent investigations in immunized children demonstrate that CMI responses to *B. pertussis* antigens constitute a relevant feature of pertussis vaccine immunogenicity and, as suggested by animal studies [14], may contribute to the vaccine efficacy [5, 6, 8]. Similarly, determinations of *B. pertussis*–specific CMI responses in adults may provide valuable information for any potential adult immunization.

Very little is indeed known about CMI responses to *B. pertussis* antigens in adults. One study has been done with T cell lines and clones from a single adult who had pertussis in his childhood, and other data by the same group of investigators demonstrated the persistence of antibody and CMI levels to *B. pertussis* antigens in adult recipients of acellular pertussis vaccines during a phase I clinical trial [15].

Although limited to a small sample population, the observation that all healthy adults investigated here were primed against *B. pertussis* antigens suggests a situation substantially comparable to that seen with common recall antigens of human-indigenous microorganisms or that following repeated exposures to, or vaccination with, microbial antigens. This priming was antigen-specific, as lymphocyte proliferation required APC, and was not mediated by a mitogenic effect, as shown.
Figure 3. Cytokine production in peripheral blood mononuclear cells (PBMC) and T cells stimulated by *B. pertussis* antigens. Cultures of PBMC or T cells obtained from 5 independent donors were unstimulated or stimulated by pertussis toxin (PT), pertactin (PRN), filamentous hemagglutinin (FHA), or *B. pertussis* (BP). After 48 h, cells were harvested and RNA was extracted and reverse-transcribed. cDNA obtained was assayed for presence of specific cytokine mRNA by polymerase chain reaction. Reaction products were run on 1% agarose gel. For each cytokine signal, data are % absorbance (mean ± SE) of β-actin signal as control of in-house gene expression (left). Cytokines: IL, interleukin; TNF, tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon. Cytokine production by *B. pertussis* antigen–stimulated PBMC and T cells was quantified as described for cytokine mRNA quantitation. Amount of IFN-γ, IL-10, and IL-5 in culture supernatants was assayed by ELISA. Data are expressed as pg/mL (mean ± SE) (right).

by the lack of lymphoproliferation of mitogen-responsive, naive cord blood cells; resulted in a typical Th1 cytokine profile (abundant IFN-γ and presence of IL-2 transcripts, with no or very low production of IL-4 or IL-5); and was reflected in the ability of T cells to produce type 1 cytokines on stimulation by *B. pertussis* antigens even without cell proliferation.

The extensive priming of Italian adults to *B. pertussis* antigens is unlikely to depend on vaccination or pertussis in infancy, given the random selection of the blood donors in this study, the absence of a record of vaccination, and the low anti-pertussis vaccination coverage in our country, as well as the negative history of pertussis. Notably, in a previous study of humoral and cellular markers of prevalence of exposure to *B. pertussis* in 62 military recruits in Italy [16], we found that the PBMC of the large majority of them proliferated in response to the *B. pertussis* antigens, and the degree of proliferation was comparable in those with or without history of pertussis. It is also unlikely that *B. pertussis*–specific CMI in adults depends exclusively or predominantly on exposure to cross-reacting antigens, as was also seen with PT and PRN, which are specific *B. pertussis* antigens. It more likely reflects the acquisition of exposure-induced immunity,
which is presumably high in a country with low vaccine coverage of infants.

In a recent study by Ryan et al. [7], PBMC from unvaccinated, uninfected naive children >4 years old showed very low or no proliferation to *B. pertussis* cells. In keeping with the high incidence of *B. pertussis* in adolescence [9], this suggests that natural immunization and CMI priming in adults might occur during adolescence and be of long duration, probably because of repeated, natural boosters. The data of our study, while awaiting confirmation by further investigations with a higher number of subjects, would nonetheless argue against the need for primary immunization in adults while suggesting that a booster dose of pertussis vaccine in adolescents might be beneficial against pertussis in adults.

Ryan et al. [7] also demonstrated a type 1 cytokine profile in acutely infected and pertussis-convalescent children. The similarity with our data on healthy adults suggests that natural exposure to *B. pertussis* substantially equals, as far as CMI priming is concerned, the infection itself. This scenario is somewhat different from that observed in children receiving acellular pertussis vaccines. Particularly in the recipients of a vaccine with higher antigen content, appreciable IL-5 levels and IL-4 mRNA transcripts, mostly in response to PRN, were detected [8]. Together with the data from experimental infections [14], this suggests that acellular pertussis vaccines, though being protective against pertussis, do not exactly mimic the cytokine pattern stimulated by pertussis or exposure to *B. pertussis* without clinically overt disease.

A rather surprising finding of our investigation was that T cell-enriched cultures from *B. pertussis*-sensitized donors express type 1 cytokines and GM-CSF gene transcripts as well as actual IFN-γ production in the absence of a detectable proliferative response. Interestingly, proliferation was absent despite the transcription of the IL-2 gene and the likely production of this cell proliferation–essential cytokine. Although monocytes and B cells were efficiently eliminated from our populations of T cells, other cells, such as NK lymphocytes or even some T cells, could be acting as APC for cytokine production but not for proliferation. A full interpretation of these data would require the exact identification of the cytokine-producing cell among the various T cell populations of our cultures. It remains, however, remarkable that *B. pertussis*-stimulated T cells of unvaccinated but naturally primed adults do not apparently require clonal expansion to produce cytokines such as IFN-γ, and probably also IL-2 and GM-CSF, which are all highly effective in stimulating the antimicrobial activity of professional or nonprofessional phagocytic cells, upon which eradication of *B. pertussis* ultimately relies.

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References