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Vaccine- and Antigen-Dependent Type 1 and Type 2 Cytokine Induction after Primary Vaccination of Infants with Whole-Cell or Acellular Pertussis Vaccines

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Cytokine profiles were examined 1 month after primary vaccination of infants with a whole-cell pertussis vaccine (wP) (Connaught) or either of two acellular pertussis vaccines, aP-Chiron Biocine (aP-CB) or aP-SmithKline Beecham (aP-SB), each combined with diphtheria-tetanus toxoids (DT), in *Bordetella pertussis* antigen-stimulated or unstimulated peripheral blood mononuclear cells (PBMC). Pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN) were used as antigens, and the children were defined as responsive when their PBMC proliferated in response to these antigens. The controls were either children who received only DT or children who received pertussis vaccine but whose PBMC did not proliferate upon stimulation with *B. pertussis* antigens (unresponsive children). Antigen-stimulated PBMC of responsive wP recipients were characterized by an elevated production of T-helper-cell type 1 cytokines gamma interferon (IFN-γ) and interleukin 2 (IL-2), low to minimal production of IL-5, and no production of IL-4. The PBMC of aP vaccine-responsive recipients showed, in addition to the elevated IFN-γ production, a consistent, antigen-dependent production of type 2 cytokines (IL-4 and IL-5), with PRN being the most and PT being the least effective antigen. Type 2 cytokine induction was more pronounced in aP-SB than in aP-CB recipients, as shown by the presence of IL-4 mRNA transcripts and higher IL-5 production in the former (161.6 ± 36 and 47.9 ± 44 pg/ml [mean ± standard error for five subjects each], respectively, after PRN stimulation). Appreciable, antigen-unstimulated (constitutive) IFN-γ production was also detected in PBMC cultures of all vaccinees. However, this spontaneous IFN-γ production was, in most vaccinees, significantly lower than the antigen-driven cytokine production. In contrast, no constitutive type 2 cytokine production was ever observed in any vaccine group. PBMC from the two control groups (either DT or pertussis vaccine recipients) did not show any type 2 cytokine production, while IFN-γ production was comparable in both antigen-stimulated and unstimulated conditions. Absence of type 2 cytokines and low levels of constitutive IFN-γ production were also seen in prevaccination children. Thus, pertussis vaccines induce in infants a basically type 1 cytokine profile, which is, however, accompanied by some production of type 2 cytokines. The latter are more expressed by aP-SB than by aP-CB recipients, and with PRN than with other antigens, and they are minimally expressed in wP recipients and with PT as antigen. Our data also highlight a constitutive IFN-γ production in infancy, which might reflect natural immunization and/or the burden of concomitant vaccinations and which may have an impact on T-helper-cell cytokine pattern polarization consequent to pertussis vaccination.

The mechanism by which immunization with antipertussis vaccines induces protection against pertussis in children is still unknown. Large clinical trials of pertussis vaccines did not provide evidence for a direct correlation between serum antibody titers and protection from disease (1, 13, 15). Thus, other factors might be involved in addition to, or even in place of, the importance of antibodies in vaccine-induced antipertussis immunity. One such factor may be cell-mediated immunity (CMI), which appears to persist much longer than antibody levels in most children receiving a primary vaccination cycle with the highly efficacious acellular pertussis (aP) vaccines (10, 13, 34). CMI could be particularly important for the eradication of intracellular *Bordetella pertussis* or to sustain an adequate production of opsonic and complement-fixing immunoglobulin isotypes (8, 16, 31).

A role for CMI in controlling *B. pertussis* infection is rather clear from various studies with murine models (20, 28, 30). In particular, it has been suggested that $CD4^+$ cells, T helper cells of type 1 subset which secrete interleukin 2 (IL-2) and gamma interferon (IFN-γ), may be responsible for protection against respiratory *B. pertussis* infection in mice (20, 22). Type 1 cytokines are strong activators of natural antimicrobial effector cells such as macrophages and polymorphonuclear cells which are ultimately critical for *B. pertussis* elimination (9, 28). However, the significance of these data in relation to the immunity following pertussis vaccination remains uncertain as there are marked differences in the cytokine profiles of spleen cells of mice immunized with different pertussis vaccines, which vaccines are, however, of equal efficacy in children (20, 22).

The Italian efficacy trial of antipertussis vaccines (10, 13) provided an opportunity to study prospectively and under controlled conditions the cytokine profile in children undergoing primary vaccination with two acellular pertussis (aP) and one whole-cell pertussis (wP) vaccine. In a previous study (10) substantial, vaccine-dependent, differences in *B. pertussis* antigen-stimulated lymphoproliferative responses (10) were observed, a fact that could imply vaccine-related but also antigen-related differential cytokine production. Determination of cytokine profiles could therefore contribute to knowledge of

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the immunological basis of the immunogenicity differences of the various pertussis vaccines used in infants and provide important clues as to the mechanisms of vaccine-induced protection and overall vaccine quality.

MATERIALS AND METHODS

Subjects, vaccines, and vaccination. The subjects entering this study were part of a group of participants in a previous assessment of the CMI response in a randomized, double-blind, placebo-controlled clinical trial of antipertussis vaccines in Italy (10, 13). In the previous study, the CMI response was assessed only for the proliferation of peripheral blood mononuclear cells (PBMC) and not for cytokine production (10). In principle, children who donated a sufficient amount of blood for the assay of both lymphoproliferation and cytokine response (mRNA and/or actual cytokine production) were considered for evaluation in the present study. However, the logistics of this study, in particular the need to use frozen PBMC collected from the various clinical centers involved in the previous trial (13), limited the number of suitable PBMC samples. Within these limitations, all children with sufficient cell recovery from each blood donation were studied, without any a posteriori selection, and all tests were made under blinded conditions. The children were given either aP or wP vaccines, each of which was combined with diphtheria-tetanus toxoids (DT). The aP vaccines were manufactured by Chiron Biocine (Siena, Italy) and SmithKline Beecham (Rixensart, Belgium) and are hereafter referred to as aP-CB and aP-SB, respectively. Each vaccine contained inactive pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN). Each dose of aP-CB contained 2.5 μg of FHA, 2.5 μg of PRN, and 5 μg of genetically inactivated PT, while each dose of aP-SB contained 25 μg of FHA, 8 μg of PRN, and 25 μg of formalin-glutaraldehyde inactivated PT. The wP vaccine was manufactured by Connaught Laboratories, Inc. (Swiftwater, Pa.) and contained heat-inactivated cells of *B. pertussis*. A control group consisted of children who received only DT vaccine (manufactured by Chiron Biocine). The vaccination schedule consisted of three doses of vaccine administered intramuscularly at 2, 4, and 6 months of age.

Three heparinized venous blood samples were taken (one just before vaccine administration and one at 1 month and one at 12 months after completion of the vaccination schedule) to study the lymphoproliferative response (10). However, the cytokine profile was mainly studied using the first postvaccination sample, which was taken from 7-month-old children (see also below). Each blood sample (5 ml) was sent, within 5 h of collection, to one of the Regional Laboratory Centers participating in the Italian pertussis vaccine efficacy trial (10, 13). PBMC were separated on a Ficoll-Hypaque density gradient and counted and frozen in dimethyl sulfoxide according to routine procedures. The frozen PBMC samples were sent to the Laboratory of Bacteriology and Medical Mycology, Istituto Superiore di Sanità, Rome, Italy (Central Laboratory) where lymphoproliferation and cytokine assays were performed.

Informed, written consent was obtained from the parents or guardians of children enrolled in this study. This study was approved by the bioethical committee of the Italian trial of pertussis vaccine efficacy.

Antigens. The *B. pertussis* soluble antigens were supplied by Chiron Biocine. Because PT possesses a high mitogenic potential and FHA and PRN may have a low PT contamination (25), the antigens were heat-inactivated for 1 h at 96°C. All antigen batches were also quality tested in independent proliferation assays before use. Preliminary experiments indicated the following optimal doses of stimulant: PT, 10 μg/ml; PRN, 30 μg/ml; and FHA, 50 μg/ml. PBMC from a healthy, adult donor responsive to all three *B. pertussis* antigens were included in each test as a positive control for all experiments performed with PBMC from the study children. Accordingly, a sufficient number of PBMC from this donor were separated and frozen exactly as described for the PBMC from children, and an aliquot were thawed and used in each assay.

Culture conditions to assess cytokine profile. Cytokine mRNA expression and secretion in cultured PBMC were measured in the presence of *B. pertussis* antigens. Pilot kinetics experiments served to establish an optimum timing of the culture for both mRNA and actual cytokine measurement (see below). Briefly, PBMC were quickly thawed and then washed in 15-ml conic tubes with 12 ml of RPMI 1640 medium. Cells were resuspended in 1 ml of RPMI complete medium containing RPMI 1640 medium (ICN-FLOW) supplemented with 1% (vol/vol) antibiotics (penicillin; 500 IU/ml; streptomycin, 0.5 mg/ml [FLOW]), 2% HEPES (FLOW), 1% nonessential amino acids (GIBCO BRL), and 5% human AB serum. The cells were diluted 1:10 in trypan blue, immediately counted, and brought to 10^6 cells/ml in complete medium.

For cytokine determination cells were cultured in 5-ml tubes (Falcon; Becton Dickinson, Lincoln Park, N.J.) at 10^6 cells/ml in 1 ml of complete medium for 48 h at 37°C in a 5% CO_2 atmosphere. Culture supernatants were collected and used to measure cytokine secretion, while the cells were used to extract RNA (see below). IFN-γ and IL-5 secretion were measured by enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D Systems, Minneapolis, Minn.), with threshold detection values of 1 and 3 pg/ml, respectively.

PCR-assisted mRNA amplification. Total cellular RNA was extracted, starting from 10^6 cells/assay, by the guanidium-isothiocyanate method (11) with tRNA (5 μg) (Yeast tRNA; GIBCO BRL) as carrier. RNA was retrotranscribed by using Moloney murine leukemia virus reverse transcriptase, as previously reported (2), and following the manufacturer's instructions (GIBCO BRL). PCR was carried out with a 20-μl volume with 2 μl of cDNA (GeneAmpkit; Perkin-Elmer Cetus, Norwalk, Conn.) (19). Cytokine-specific primer pairs were synthesized according to published sequences (DNA Synthesizer; Applied Biosystems, Inc., Foster City, Calif.) (12, 29). PCR was performed in a 9600 Perkin-Elmer thermal cycler, and each cycle comprised 40 s of denaturation at 94°C, 40 s of annealing at 62°C, and 1 min of extension at 72°C. The numbers of PCR cycles for each primer pair, optimized to avoid the PCR plateau effects (2, 19), were 28 for IL-2, IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) and 30 for IL-4 and IL-5. β-Actin was amplified (25 cycles) for each sample as an internal cDNA control and for adjustment of the amount of cellular RNA. The reaction product was visualized by electrophoresis using 10 μl of the reaction mixture. The specificity of amplified cDNA sequences was validated by correspondence to predicted gene size and by restriction mapping (19). Omission of reverse transcriptase (RT) from the RT mixture or of cDNA (replaced by water) from the PCR mixture served as controls for contaminants. After electrophoresis, the relative densities of the ethidium bromide-stained PCR products were determined by using a Scanning Densitometer CS-9301PC (Shimadzu, Columbia, Md.). The actual densitometric values for each cytokine mRNA were normalized by using β-actin densitometry as the 100% reference, and all other values are expressed as percentages of this figure (3, 4). Two experiments with exactly the same experimental conditions were performed for each PCR determination to ensure reproducibility of the assay.

Lymphoproliferation assay. Culture conditions for the cell proliferation assay were as reported elsewhere (10). Briefly, cells were incubated in a volume of 0.2 ml/well, in triplicate, in the absence or presence of the relevant antigen stimulant for 7 days at 37°C in a 5% CO_2 atmosphere. Methyl-[^{3}H]thymidine (Amersham TRK120; specific activity, 2.5 Ci/mmol) was added to the culture at a final concentration of 0.5 μCi/well 18 h before cell harvesting with a semiautomatic harvester (Pharmacia, Uppsala, Sweden). DNA synthesis was evaluated by assessing the [^{3}H]thymidine incorporation with a Beta plate counter (Pharmacia). The lymphoproliferation values are reported as mean (± standard errors [SE]) counts per minute × 10^3 after subtraction of the counts per minute of antigen-unstimulated cultures.

Definitions, data presentation, and statistical analyses. Children whose PBMC proliferated in response to *B. pertussis* antigen were defined as CMI^+ or responsive children. Lack of PBMC proliferation with the same antigenic stimulation defined the CMI^- or unresponsive children. The cutoff value for positive lymphoproliferation was set at 3×10^3 cpm after subtraction of the cpm of the antigen-unstimulated culture (10). Since the cpm of the latter rarely exceeded 10^3, the lowest stimulation index of a responsive PBMC culture was 4. The PBMC of both CMI^+ and CMI^- children responded equally well to mitogenic stimulation with PHA (PBMC proliferation $\geq 40 \times 10^3$ cpm). Data from all cytokine determination experiments (ELISA and RT-PCR) were recorded in a computerized database, and descriptive statistical analyses were carried out using the SPSS statistical package. The Mann-Whitney U test was used to compare different levels of cytokine mRNA and actual cytokine production. Each difference at a P value of <0.05 (two tails) was taken as significant. When not otherwise indicated, all average values are expressed as means ± SE and the differences were not statistically significant.

RESULTS

Because of the limited availability of PBMC samples, of the 142 children enrolled and previously evaluated at both postvaccination timepoints for PBMC proliferation to *B. pertussis* antigens it was possible to study 46 children at the first postvaccination assay and only 18 at the second postvaccination assay (10). The PBMC from six children before vaccination were also examined. Unless otherwise stated, the data refer to the first postvaccination assay. No PBMC from PT-CMI^+ children given the wP vaccine could be examined because of the very low number of these PT-responsive children (10).

Table 1 shows the distribution of PBMC responsiveness per vaccine, the antigen to which the PBMC were responsive, and the average lymphoproliferation values at the first postvaccination assay. The degree of PBMC proliferation of responsive cultures was generally the lowest in wP and the highest in aP-SB recipients, although statistical significance was reached only in a few cases (Table 1).

Cytokine profile as determined by cytokine mRNA expression in PT-, PRN-, or FHA-stimulated PBMC. Type 1 (IFN-γ and IL-2) and type 2 (IL-4 and IL-5) cytokine mRNA expression was studied by a semiquantitative RT-PCR technique and expressed as a percentage of the β-actin band intensity. Figure 1 shows a representative profile of three children vaccinated

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TABLE 1. PBMC proliferation response to *B. pertussis* antigens

Vaccine	No. of children tested	No. of unresponsive children	No. of responsive children (PBMC proliferation value)[a] to:		
			PRN	FHA	PT
wP	10	5	5 (8 ± 1)	4 (5 ± 0.4)	0 (—)[b]
aP-CB	15	4	8 (23 ± 6)	11 (11 ± 3)	7 (24 ± 4)
aP-SB	17	10	5 (32 ± 11)	4 (31 ± 11)	5 (13 ± 4)
DT[c]	4	4	0 (—)	0 (—)	0 (—)
None[d]	6	6	0 (—)	0 (—)	0 (—)

[a] Data expressed as mean (± SE) × 10^3 cpm. Statistically significant differences in PBMC proliferation values between wP and aP-CB recipients (PRN stimulation) and between wP and aP-SB recipients (FHA stimulation).
[b] —, no lymphoproliferation of the PBMC culture.
[c] None of the DT recipients was CMI^+ to any *B. pertussis* antigen.
[d] Prevaccination children.

with wP, aP-CB, or aP-SB, upon stimulation with *B. pertussis* antigens. Table 2 summarizes the results of IFN-γ, IL-2, IL-4, and IL-5 mRNA expression in PBMC from all CMI^+ children at the first postvaccination assay.

The data revealed similarities but also vaccine- and antigen-related differences in cytokine messages. When stimulated by PRN, the PBMC of all children transcribed a marked IFN-γ and a less marked yet consistent IL-2 message, with no significant differences among the three vaccine groups, provided

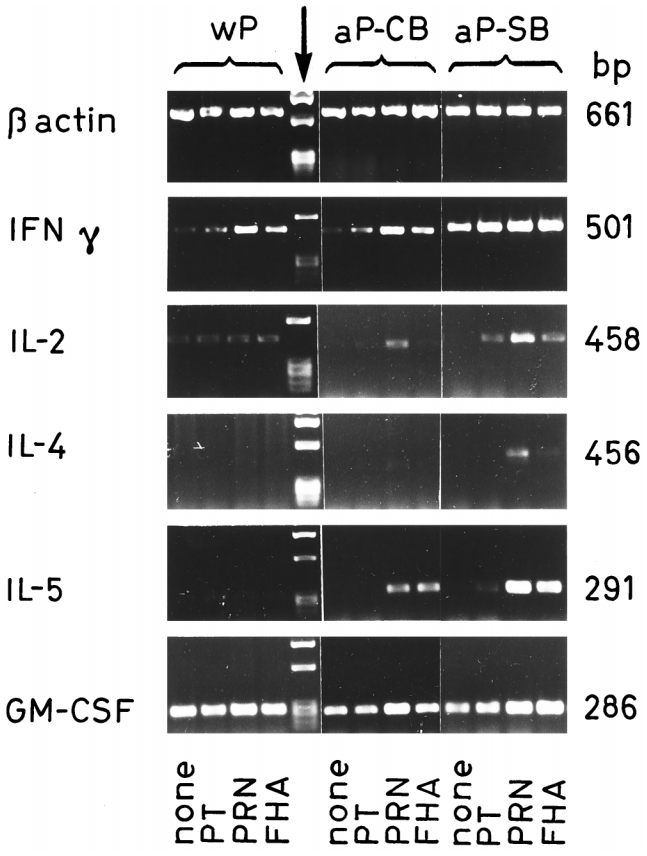

FIG. 1. Representative profile of cytokine mRNA expression in PBMC from three children vaccinated with wP, aP-CB, or aP-SB. PBMC were either unstimulated (none) or stimulated with PT, PRN, or FHA and cultured for 48 h. Cytokine mRNA expression was assayed by RT-PCR, as described in Materials and Methods. β-Actin was the positive control. The arrow indicates molecular size markers.

that the spread of individual values and the constitutive cytokine mRNA expression were considered (see also below). In contrast, a difference among the three vaccine groups was found in the expression of type 2 cytokine mRNAs. In fact, the IL-4 mRNA band, averaging slightly less than 20% of the β-actin signal, was present only in PBMC from aP-SB recipients. The IL-5 mRNA signal was measurable in all vaccinees, but its intensity was higher in aP-SB than in aP-CB or wP recipients (70% of the β-actin band versus 31 and 24%, respectively).

This trend of cytokine mRNA production was essentially similar when the PBMC were stimulated with FHA instead of PRN, i.e., rather comparable type 1 message intensities and relatively more abundant type 2 cytokine messages in aP-SB recipients. In this case, however, expression of type 2 cytokine mRNA was essentially limited to IL-5. Moreover, the PBMC cultures from the three recipients of wP examined did not express either IL-4 or IL-5 mRNA at all (while expressing a strong IFN-γ mRNA signal).

For the reasons stated above, only the aP vaccine recipients were studied for their cytokine profile in response to PT. The stimulation with this antigen substantially induced only the expression of IFN-γ mRNA signal, regardless of the vaccine received.

In all vaccinees, GM-CSF message was efficiently expressed (see Fig. 1 for representative subjects).

The expression of constitutive IFN-γ mRNA was a rather constant feature of all vaccine recipients. Such expression was particularly abundant (averaging from 37 to about 70% of the β-actin signal intensity, depending on the antigen) in aP-SB recipients, and although constitutive IFN-γ mRNA was less expressed in the PBMC from the other vaccinees, it was always translated into the actual cytokine gene product (see below). In contrast, no IL-2, IL-4, or IL-5 was constitutively produced under any conditions (Table 2).

Definitely fewer CMI^+ children, and only those who received an aP vaccine, could be studied to determine cytokine mRNA profile (in response to PT or PRN) at the second postvaccination assay. These cultures transcribed significantly less IFN-γ mRNA than those of the first postvaccination assay, even though their proliferation levels did not differ significantly. Under the strongest induction conditions, i.e., with PRN as antigen, the levels of IFN-γ mRNA synthesized by PBMC of aP-CB and aP-SB vaccinees were 16 and 13%, respectively, of the β-actin mRNA signal compared to 35 and 36% at the first postvaccination assay (all values after subtraction of the level of constitutive IFN-γ mRNA, which was also significantly lower at the second postvaccination assay). PBMC from the second postvaccination assay did not express IL-4 mRNA, whatever the antigenic stimulus, while IL-5 mRNA was detected at low levels and only in PRN-stimulated cells of aP-SB recipients (data not shown).

Actual cytokine production. Actual production of IFN-γ and IL-5, chosen as representative type 1 and type 2 cytokines, respectively, was also assessed by ELISA. IL-5 was selected instead of IL-4 because of both the well-known low sensitivity of commercial kits to the latter cytokine and the lower levels of transcription of this cytokine gene compared to the IL-5 gene, even after polyclonal stimulation (3, 4, 33). The data in Fig. 2 confirmed the propensity of PRN-stimulated PBMC cultures to produce elevated amounts of IFN-γ in all vaccine recipients (the average approached 1 ng of IFN-γ/ml of PBMC culture after subtraction of the unstimulated IFN-γ production). In contrast, only the PBMC from the aP vaccine recipients consistently produced IL-5, which, despite the expected spread of the individual values, was significantly higher in the cultures

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TABLE 2. Densitometric assessment of cytokine gene expression in CMI^+ children receiving pertussis vaccines

Vaccine	Antigen	No. of children tested	Cytokine mRNA signal intensity[a] in unstimulated (Ag^-) or antigen-stimulated (Ag^+) PBMC cultures							
			IFN-γ		IL-2		IL-4		IL-5	
			Ag^-	Ag^+	Ag^-	Ag^+	Ag^-	Ag^+	Ag^-	Ag^+
wP	PRN	4	8 ± 0.3	68 ± 25	<5	18 ± 6	<5	<5	<5	24 ± 18
	FHA	3	8 ± 0.3	55 ± 28	<5	13 ± 3	<5	<5	<5	<5
aP-CB	PRN	4	9 ± 3	44 ± 14	<5	18 ± 4	<5	<5	<5	31 ± 9
	FHA	3	11 ± 3	33 ± 4	<5	<5	<5	<5	<5	23 ± 12
	PT	6	6 ± 0.4	35 ± 9	<5	7 ± 3	<5	<5	<5	6 ± 3
aP-SB	PRN	4	39 ± 16	75 ± 16	<5	22 ± 12	<5	17 ± 9	<5	70 ± 28
	FHA	3	70 ± 3	122 ± 30	<5	24 ± 12	<5	7 ± 4	<5	63 ± 37
	PT	4	37 ± 19	68 ± 40	<5	7 ± 4	<5	<5	<5	6 ± 4

[a] Data expressed as percentage of the β-actin band intensity.

from aP-SB than from aP-CB recipients (161.6 ± 36 and 47.9 ± 44 pg/ml, respectively; five subjects each). The highest level of IL-5 production was detected in those PBMC cultures which also showed the most elevated lymphoproliferation (e.g., in aP-SB recipients upon stimulation with PRN or FHA; see also Table 1). Marked levels of IFN-γ production were also observed in FHA-stimulated cultures, and this was again accompanied by some IL-5 production (32.4 ± 16.8 pg/ml in aP-SB recipients).

Finally, appreciable levels of IFN-γ were produced in PT-stimulated cells from aP recipients. In this case, however, IL-5 production was minimal (close to the threshold of detection).

Confirming the observations made with the RT-PCR technique (see above), there was a nonneglectable constitutive IFN-γ production in PBMC cultures from all vaccinees. Here again, the highest level of constitutive IFN-γ production was seen in children who received the aP-SB vaccine (Fig. 2).

Overall, considering the results in terms of the IL-5/IFN-γ ratio as an expression of the type 2-type 1 cytokine balance, it was clear that PRN was the most effective and PT was the least effective type 2 cytokine inducer. For instance, the ratio was 1.92 (RT-PCR) or 0.21 (ELISA) for PRN-stimulated PBMC versus 0.13 or 0.01, respectively, for PT-stimulated cells of aP-SB recipients. Moreover, PRN-stimulated cultures had two (RT-PCR) or more than four (ELISA) times higher an IL-5/IFN-γ ratio in aP-SB than in aP-CB recipients.

As stated in the section on cytokine mRNA expression, few CMI^+ children, and only those who received an aP vaccine, could be assessed for cytokine production at the second postvaccination timepoint. In keeping with lower IFN-γ mRNA levels (both antigen induced and constitutive) compared to the first postvaccination timepoint, actual IFN-γ production was also lower. Under the strongest induction conditions, i.e., with PRN as antigen, the IFN-γ produced by PBMC of aP-CB and aP-SB vaccinees was 428 ± 206 and 339 ± 182 pg/ml, respectively (four subjects each). No IL-4 was detected, and IL-5 was produced only in cells of aP-SB recipients after PRN stimulation (25.7 ± 13 pg/ml; three subjects each).

Cytokine production in CMI^- children. Production of IFN-γ and IL-5 was also assessed in control CMI^- children, which group included subjects who received only a DT vaccine as well as some recipients of wP or aP vaccines whose PBMC did not respond to the stimulation with *B. pertussis* antigens. As shown in Fig. 3, the aP vaccine recipients showed comparable levels of constitutive IFN-γ secretion (268 ± 105 and 298 ± 190 pg/ml in 10 and 4 aP-SB and aP-CB vaccinees, respectively). Antigen stimulation led to cytokine levels which did not significantly differ from those produced constitutively. In wP

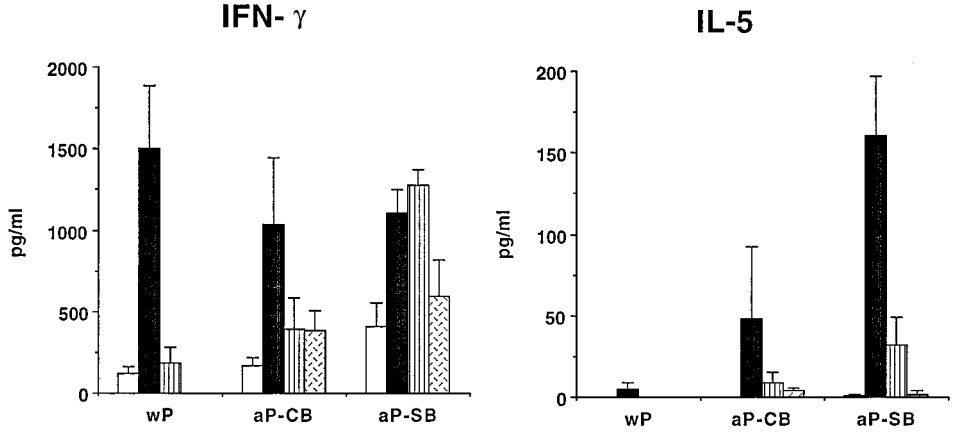

FIG. 2. IFN-γ and IL-5 production in PBMC cultures from CMI^+ children receiving pertussis vaccines. (For the number of children for each vaccine, see Table 1.) PBMC were unstimulated (□) or stimulated with PRN (■), FHA (▥), or PT (▨) and cultured for 48 h. The cytokines were determined by ELISA and expressed as picograms per milliliter of culture supernatant. Differences in IL-5 production (in response to PRN) between wP and either aP-CB or aP-SB were significant, as was the difference between aP-CB and aP-SB recipients. The difference in IFN-γ production (in response to FHA) between aP-CB and aP-SB recipients was significant. Differences in the levels of constitutive and antigen-stimulated IFN-γ production were significant for the following antigen-vaccine combinations: PRN-wP; PRN–, FHA–, and PT–aP-CB; and PRN– and FHA–aP-SB.

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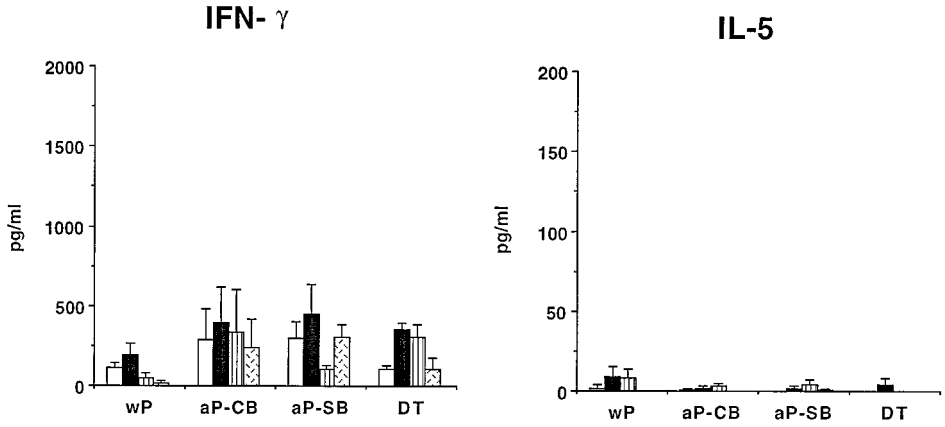

FIG. 3. IFN-γ and IL-5 production in PBMC cultures from CMI$^-$ children receiving either a control vaccine (DT) or a pertussis vaccine as indicated. (For the number of children receiving each vaccine, see Table 1.) PBMC were unstimulated (□) or were stimulated with PRN (■), FHA (▥), or PT (▨) and cultured for 48 h. The cytokines were determined by ELISA and expressed as picograms per milliliter of culture supernatant. The difference in IFN-γ production between unstimulated and PRN-stimulated PBMC from DT recipients was significant.

recipients, the IFN-γ levels were somewhat lower than in aP recipients, but they were still comparable in unstimulated and antigen-stimulated PBMC. In DT subjects, PRN induced a level of IFN-γ production which was significantly higher ($P < 0.05$) than that of the constitutive one but which was similar to the constitutive cytokine levels in aP vaccinees. No consistent production of IL-5 was ever detected in these cultures, either with or without *B. pertussis* antigen stimulation (Fig. 3).

Assessment of IFN-γ production in PBMC from six children before vaccination showed values which were substantially comparable to those of the CMI$^-$ children described above, and no IL-4 or IL-5 was produced by antigen-stimulated or unstimulated cells of these children. In particular, three of these children (two wP recipients and one aP-CB recipient) were assessed for IFN-γ and IL-5 production (in response to PRN) both before and after vaccination. The two wP recipients produced no IL-5 and 246.5 ± 71 and 2,221 ± 49 pg of IFN-γ/ml of PBMC culture before and after vaccination, respectively. The aP-CB recipient produced 10 and 1,991 pg of IFN-γ/ml of PBMC culture and <5 and 80.4 pg of IL-5/ml of PBMC culture before and after vaccination, respectively.

DISCUSSION

To contribute to the understanding of immunogenicity determinants and correlates of protection conferred by pertussis vaccines in infancy, we have recently studied CMI responses to the pertussis whole-cell or acellular vaccines used in the Italian efficacy trial (13). These responses are an essential but rather neglected component of the immunity conferred by bacterial vaccines in current use. The CMI response to *B. pertussis* antigens, as assessed by PBMC proliferation in vitro, was compared to the serum antibody levels against the same antigens. It was shown that CMI is efficiently induced by the aP vaccines and persists much longer than the rapidly waning serum antibody titers. We therefore suggested that CMI responses could be a better correlate of long-term immunity to *B. pertussis* than antibodies (10).

Since CMI is induced, and regulated, by cytokines, we have now studied the profiles of the major cytokines constitutively expressed or elicited by in vitro PBMC restimulation with *B. pertussis* antigens. We were particularly interested in assessing the balance between representative type 1 (IFN-γ and IL-2) and type 2 (IL-4 and IL-5) cytokines because of the current awareness of the critical importance of this balance for protection against infections (18, 24, 32). Evidence from murine models suggests that control of *B. pertussis* respiratory infection can be better achieved by immunization schedules favoring type 1 cytokine production (20, 28, 30).

There is now a rather wide consensus that T-helper-cell type 1 cytokines, in particular IFN-γ, are strong macrophage activators, facilitating the destruction of intracellular microorganisms, but which also promote the secretion of immunoglobulin G2a antibodies which are optimal for opsonization of extracellular bacteria. On the other hand, type 2 cytokines favor B-cell maturation and production of antibody isotypes appropriate for toxin-adhesin neutralization (18, 24, 32). On merely theoretical grounds, therefore, a well-balanced combination of type 1 and type 2 responses could be ideal for preventing or controlling pertussis. Although *B. pertussis* is indeed predominantly extracellular, it may also reside, at least in some categories of patients, intracellulary (8, 15, 22). Therefore, not only toxin-neutralizing but also opsonizing and possibly complement-fixing cytolytic antibodies may be required for optimal protection (32).

Although the restrictions in blood supply imposed by the nature of the trial (10, 13) were such that a relatively low number of PBMC cultures could be examined for cytokines produced in response to each antigen, we believe the design of our study has the following positive aspects. First, the children were randomly selected from among those of a double-blind, randomized, placebo-controlled clinical efficacy trial which demonstrated a high (84%), equal efficacy of the two aP vaccines and a low efficacy of the wP vaccine (13). Thus, we could assess the cytokines produced upon immunization with vaccines of different efficacy in well-controlled and definitely comparable groups of subjects. Moreover, a very active surveillance to identify the pertussis cases was implemented throughout the trial so that vaccine-independent or vaccine-confounding immune responses following postimmunization disease could be identified and excluded. Second, a previous large study of CMI induction (as lymphoproliferation) in infants receiving various pertussis vaccines (10) demonstrated that the recipients of the two aP vaccines differed significantly in their CMI responses to the three *B. pertussis* antigens (PT, PRN, and FHA). It was of obvious interest to try to relate these differences to possibly different cytokine patterns following antigen stimulation of specific T cells. Last, but not least, this study has permitted the

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assessment of cytokines produced by PBMC of infants who had been vaccinated at 2, 4, and 6 months of age, when cytokine production has rarely been studied (22, 31).

Our findings suggest the existence of potentially important differences in the cytokine profiles of different groups of *B. pertussis* antigen-primed children following primary antipertussis vaccination at an average age of 7 months. The most important of these differences might be the one between recipients of the wP and aP vaccines. In the former, the cytokine profile was of type 1 exclusively, as inferred from the elevated IFN-γ production and very low, if any, IL-4 and IL-5 production. In contrast, the aP vaccines appear to induce a mixed cytokine profile, with quantitatively predominant IFN-γ production (associated with IL-2), low but appreciable IL-4 mRNA transcripts, and more abundant IL-5 production. This difference between the wP and aP vaccines was consistent with both the antigens (PRN and FHA) against which the comparison could be made. The very low (with PRN as antigen) or undetectable (with FHA) type 2 cytokine level in PBMC from wP recipients was not due to accelerated cytokine destruction, binding, or disposal in these cultures but to low or no cytokine gene expression, as demonstrated by the specific mRNA signals.

Type 2 cytokine production in aP vaccine recipients was clearly *B. pertussis* antigen dependent, as no constitutive production of IL-4 or IL-5 mRNA was ever observed. Importantly, in none of the control cultures (either from prevaccination or DT vaccine recipients or even from children who, although receiving a pertussis vaccine, were CMI^-) was there any consistent production of these cytokines. In addition, no IL-2 production was detected in antigen-stimulated PBMC cultures from all these control subjects, and the measured levels of IFN-γ were comparable to those of *B. pertussis* antigen-unstimulated cells (constitutive IFN-γ production; see below). All this demonstrates the specificity of *B. pertussis* antigen-induced type 1 and type 2 cytokine production and its acquisition by pertussis vaccination, a conclusion strengthened by the comparison of pre- and postvaccination levels of both IFN-γ and IL-5 in PRN-stimulated PBMC of the few children that could be examined longitudinally. In a very recent study (34), Zepp et al. report the preferential induction of a type 1 cytokine pattern in aP-SB recipients, with no evidence of any consistent, vaccination-acquired, specific type 2 cytokine induction. However, the choice of IL-10 as a representative type 2 cytokine in their study may be questionable because IL-10 is also produced by monocytes and no specific type 2 IL-4 or IL-5 gene expression was addressed.

Another potentially relevant difference in the type 1-type 2 cytokine balance in our study children was observed between the two groups of aP vaccine recipients. Although both groups could induce IL-5 production (mostly in response to PRN), a trend for a somewhat increased production of type 2 cytokines was noticed in aP-SB compared to aP-CB vaccinees. As a matter of fact, the only cultures in which appreciable IL-4 mRNA transcripts were detected were the PRN-stimulated PBMC from the aP-SB vaccinees. Moreover, the actual production ratio between IL-5 and IFN-γ was, with PRN as antigen, higher in PBMC cultures from aP-SB than aP-CB recipients. Some limited data on cytokine production at the second postvaccination assay, i.e., 1 year after vaccination, suggest that the difference between the two aP vaccines could be more marked later on. At that time, no IL-5 was produced by antigen-stimulated cells from any aP-CB recipient, while some IL-5 was still produced by PRN-stimulated cells of some aP-SB recipients.

The reasons for these differences between the two aP vaccines are not known. One possible explanation is the higher antigen dosage in the vaccine formulation of the aP-SB than the aP-CB vaccine (13). In murine models, it has been shown that by increasing the antigen dose, a preferential development of T helper cells producing type 2 cytokines occurs even at equal levels of T-cell proliferation (5, 17, 21). In addition, the aP-SB vaccine also contains, as adjuvant, more ionic aluminum, which has long been known to facilitate type 2 responses (7). Other even subtler differences between the two vaccines, in composition and antigen inactivation methods, could also play a role (13, 29) (see below).

Our study also demonstrates some antigen-related differences in quantitative cytokine profile. No or very low IL-4 or IL-5 was ever produced by PT-stimulated cells, even those from aP-SB vaccine recipients. This was not due to a general hyporesponsiveness either in proliferation or cytokine production. In fact, PT-stimulated cultures from children who received either aP vaccine proliferated rather extensively, and the amounts of both IFN-γ and GM-CSF produced in these cultures were comparable to those produced in response to FHA or PRN. The PT used in the formulation of aP-CB and aP-SB vaccines differs substantially both in the amount and method of inactivation (genetic in aP-CB and chemical in the aP-SB vaccine [13]). It has been suggested that genetic inactivation is more suitable for PT to activate a type 1 cytokine pattern (29). Our results seem to suggest that PT, whatever the inactivation method, is per se an antigen which almost exclusively stimulates a type 1 cytokine response. Interestingly, this seems to contrast with PRN, which is the most effective type 2 cytokine-fostering antigen. In general, of the three *B. pertussis* antigens, PRN proved to be the most effective cytokine inducer. Only PRN induced the same high IFN-γ levels in all subjects, regardless of the vaccine received. In some control subjects (e.g., those vaccinated with DT) who had unresponsive levels of lymphoproliferation with this antigen, PRN was the sole antigen to induce significantly more IFN-γ production than that produced under unstimulated conditions. Clearly, these remarkable properties of PRN warrant further investigation.

The observation that antigen-unstimulated or unresponsive (in terms of cell proliferation) PBMC constitutively produce appreciable levels of IFN-γ is intriguing. This result could not be attributed to the culture conditions themselves because the level of IFN-γ production was high in the PBMC from aP-SB recipients and low or absent in equally prepared cultures from other children. Moreover, in control experiments conducted with *B. pertussis*-responsive PBMC from adults, no or low (average, <50 pg/ml) spontaneous IFN-γ production was observed, under identical culture conditions, inclusive of the freezing stage in PBMC preparations (3, 4, 19). Finally, naive T lymphocytes from cord blood mononuclear cells did not show any constitutive IFN-γ production (data not shown) (12). Interestingly, some constitutive IFN-γ production (between 50 and 150 pg/ml) could be detected in prevaccination children (2 months of age).

It is noteworthy that the children under study received, concomitantly with pertussis vaccines, other vaccines, including viral ones (polio and hepatitis) which are mandatory in Italy. In addition, spontaneous IFN-γ production was present also in *B. pertussis*-CMI^- children, and at levels that were, at the first postvaccination assay, substantially comparable to those constitutively expressed by CMI^+ children. Theoretically, this constitutive IFN-γ production might have a remarkable influence on the cytokine patterns pursuant to pertussis vaccination, considering the importance of the preconstituted cytokine environment in polarizing the response toward either cytokine type pattern following neo-antigen stimulation (18, 24, 26).

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Before this study, very little was known about the cytokine profiles of *B. pertussis* antigen-activated T cells from infants following either pertussis or immunization with pertussis vaccines (34). Pioneering studies by Mills and coworkers (6, 20, 22, 30) suggest important differences between immune responses following infection and those after vaccination. Some preliminary data of theirs show the elicitation of a mixed type 1-type 2 cytokine profile in aP-SB recipients (23). Cytokine patterns in *B. pertussis* antigen-specific T-cell clones from subjects after natural infection have also been investigated (27). Our study, which is the first that compares cytokine profiles after primary vaccination with three different pertussis vaccines, demonstrates that upon a basic type 1 cytokine profile, there are vaccine-, antigen-, and possibly dose-related differences in the relative amount of type 2 cytokine component. This latter seems to be minimal or nil in wP vaccine recipients and the highest in aP-SB vaccinees.

The quantitative differences in cytokines produced by aP-CB and aP-SB recipients may impact on the long-term protection in infants, which protection appears to differ somewhat between children receiving the two aP vaccines (14) and should also be considered in the final assessment of the overall quality of each vaccine. However, future studies are certainly needed for a full appreciation of these different cytokine profiles and for the determination of whether they are instrumental to long-term protection of children from pertussis.

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REFERENCES

1. **Ad hoc Study Group for the Study of Pertussis Vaccines.** 1988. Placebo-controlled trial of two acellular pertussis vaccines in Sweden—protective efficacy and adverse events. Lancet **31:**955–960.
2. **Ausiello, C. M., F. Urbani, S. Gessani, G. C. Spagnoli, M. J. Gomez, and A. Cassone.** 1993. Cytokine gene expression in human peripheral blood mononuclear cells stimulated by mannoprotein constituents from *Candida albicans*. Infect. Immun. **61:**4105–4111.
3. **Ausiello, C. M., F. Urbani, A. la Sala, A. Funaro, and F. Malavasi.** 1995. CD38 ligation induces discrete cytokine mRNA expression in human cultured lymphocytes. Eur. J. Immunol. **25:**1477–1480.
4. **Ausiello, C. M., A. la Sala, C. Ramoni, F. Urbani, A. Funaro, and F. Malavasi.** 1996. Secretion of IFN-γ, IL-6, granulocyte-macrophage colony-stimulating factor and IL-10 cytokines upon CD38 ligation. Cell. Immunol. **173:**192–197.
5. **Bancroft, A. J., K. J. Else, and R. K. Grencis.** 1994. Low-level infection with *Trichuris muris* significantly affects the polarization of the CD4 response. Eur. J. Immunol. **24:**3113–3118.
6. **Barnard, A., B. P. Mahon, J. Watkins, K. Redhead, and H. G. Mills.** 1996. Th1/Th2 cell dichotomy in acquired immunity to *Bordetella pertussis*: variables in the *in vivo* priming and *in vitro* cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 or Th0. Immunology **87:**372–380.
7. **Bomford, R.** 1980. The comparative selectivity of adjuvants for humoral and cell-mediated immunity. Clin. Exp. Immunol. **39:**435–441.
8. **Bromberg, K., G. Tannis, and P. Stainer.** 1991. Detection of *Bordetella pertussis* associated with the alveolar macrophages of children with human immunodeficiency virus infection. Infect. Immun. **59:**4715–4719.
9. **Cassatella, M. A.** 1995. The production of cytokines by polymorphonuclear neutrophil. Immunol. Today **16:**21–26.
10. **Cassone, A., C. M. Ausiello, F. Urbani, A. la Sala, R. Lande, A. Piscitelli, M. Giuliano, S. Salmaso, and Progetto Pertosse-CMI Working Group.** 1997. Cell mediated and antibody responses to *Bordetella pertussis* antigens in children vaccinated with two acellular or one whole-cell pertussis vaccines within the Italian trial. Arch. Pediatr. Adolesc. Med. **151:**283–289.
11. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
12. **Ehlers, S., and K. A. Smith.** 1991. Differentiation of T cell lymphokine gene expression: the in vitro acquisition of T cell memory. J. Exp. Med. **173:**25–36.
13. **Greco, D., S. Salmaso, P. Mastrantonio, M. Giuliano, A. E. Tozzi, A. Anemona, M. L. Ciofi degli Atti, A. Giammanco, P. Panei, W. C. Blackwelder, D. L. Klein, S. G. F. Wassilak and Progetto Pertosse Working Group.** 1996. A controlled trial of two acellular and one whole cell vaccine against pertussis. N. Engl. J. Med. **334:**341–348.
14. **Greco, D., S. Salmaso, P. Mastrantonio, M. Giuliano, A. Giammanco, P. Stefanelli, A. Anemona, A. E. Tozzi, M. L. Ciofi degli Atti, P. Panei, and S. Wassilak.** A difference in the relative efficacy of two DTaP vaccines in continued blinded observation of children following a clinical trial, p. 173A. Abstracts of the Annual Meeting of the Society for Pediatric Research, Washington, D.C., May 6–10, 1996.
15. **Gustafsson, L., H. O. Hallander, P. Olin, E. Reizenstein, and J. Storsaeter.** 1996. A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. N. Engl. J. Med. **334:**349–335.
16. **Hazenbos, W. L. W., B. M. van den Berg, J. W. van't Wout, F. R. MooI, and R. van Furth.** 1994. Virulence factors determine attachment and ingestion of nonopsonized and opsonized *Bordetella pertussis* by human monocytes. Infect. Immun. **62:**4818–4824.
17. **Hostien, N. A., K. Shibuya, A. W. Heath, K. M. Murphy, and A. O'Gatta.** 1995. The effects of antigen dose on CD4+ T cell phenotype development in a αβ TCR transgenic mouse model. J. Exp. Med. **182:**1579–1584.
18. **Kauffmann, S. H. E.** 1993. Immunity to intracellular bacteria. Annu. Rev. Immunol. **11:**129–163.
19. **la Sala, A., F. Urbani, R. Lande, and C. M. Ausiello.** 1995. A semiquantitative reverse transcriptase-polymerase chain reaction method to detect low abundance cytokine mRNA. Minerva Biotech. **7:**207–214.
20. **Mahon, B. P., M. S. Ryan, F. Griffin, and K. H. G. Mills.** 1996. 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. **64:**5295–5301.
21. **Mencacci, A., R. S. Spaccapelo, G. Del Sero, K.-H. Enssle, A. Cassone, F. Bistoni, and L. Romani.** 1996. $CD4^+$ T-helper-cell responses in mice with low-level *Candida albicans* infection. Infect. Immun. **64:**4907–4917.
22. **Mills, K. H. G., A. Barnard, J. Watkins, and K. Redhead.** 1993. Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect. Immun. **61:**399–410.
23. **Mills, K. H. G.** 1996. Cell mediated immunity studies from infants immunized in Sweden and pertussis vaccine efficacy studies in normal and gene knockout mice, p. 27–29. Abstracts of the National Institutes of Health Pertussis Conference, Washington, D.C., June 3–5, 1996.
24. **Mosmann, T. R., and R. L. Coffman.** 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. **7:**145–173.
25. **Munoz, J. J., H. Aral, R. K. Bergman, and P. L. Sadowski.** 1981. Biological activities of crystalline pertussigen from *Bordetella pertussis*. Infect. Immun. **33:**820–826.
26. **O'Gara, A., and K. Murphy.** 1994. Role of cytokines in determining T-lymphocyte function. Curr. Opin. Immunol. **6:**458–465.
27. **Peppoloni, S., L. Nencioni, A. Di Tommaso, A. Tagliabue, P. Parronchi, S. Romagnani, R. Rappuoli, and M. T. De Magistris.** 1991. Lymphokine secretion and cytotoxic activity of human $CD4^+$ T-cell clones against *Bordetella pertussis*. Infect. Immun. **59:**3768–3773.
28. **Petersen, J. W., P. H. Ibsen, K. Hasløv, and I. Heron.** 1992. Proliferative responses and gamma interferon and tumor necrosis factor production by lymphocytes isolated from tracheobroncheal lymph nodes and spleens of mice aerosol infected with *Bordetella pertussis*. Infect. Immun. **60:**4563–4570.
29. **Rappuoli, R.** 1994. Toxin inactivation and antigen stabilization: two different uses of formaldehyde. Vaccine **12:**579–581.
30. **Redhead, K., J. Watkins, A. Barnard, and K. H. G. Mills.** 1993. Effective immunization against *Bordetella pertussis* respiratory infection in mice is dependent on induction of cell-mediated immunity. Infect. Immun. **61:**3190–3198.
31. **Sieling, P. A., X. H. Wang, M. K. Gately, J. L. Oliveros, T. McHugh, P. F. Barnes, S. F. Wolf, L. Golkar, M. Yamamura, Y. Yogi, K. Uyemura, T. H. Rea, and R. L. Modlin.** 1994. IL-12 regulates T helper type 1 cytokine responses in human infectious disease. J. Immunol. **153:**3639–3647.
32. **Taylor, C.** 1995. Cytokines as adjuvants for vaccines: antigen-specific responses differ from polyclonal responses. Infect. Immun. **63:**3241–3244.
33. **Weinberg, A. D., M. English, and S. L. Swain.** 1990. Distinct regulation of lymphokine production is found in fresh versus in vitro primed helper T cells. J. Immunol. **144:**1800–1806.
34. **Zepp, F., M. Knuf, P. Habermehl, H. J. Schmitt, C. Rebsch, P. Schmidtke, R. Clements, and M. Slaoui.** 1996. Pertussis-specific cell-mediated immunity in infants after vaccination with a tricomponent acellular pertussis vaccine. Infect. Immun. **64:**4078–4084.

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