DISSECTING HUMAN T CELL RESPONSES AGAINST *BORDETELLA* SPECIES

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Pertussis (whooping cough) is an infectious disease caused by *Bordetella pertussis*, which primarily affects infants and young children. The infection results in a severe paroxysmal cough persisting for weeks and can cause death when acquired within the first year of life. The cellular vaccine introduced in the forties drastically reduced morbidity and mortality due to whooping cough. The efficacy of the vaccine, composed of inactivated whole cells of *B. pertussis*, usually administered with diphtheria and tetanus toxoids adsorbed with alum, is considered to be rather high (1, 2). As a consequence of the disappearance of pertussis in many countries after extensive vaccination campaigns, public fears about adverse reactions to vaccination have grown. Indeed, these reactions can range from frequent fever and local tenderness, to severe neurological complications and death. Thus, the use of the vaccine has decreased in several countries including Sweden, England, and Japan, with a resulting increase in infant mortality from the disease (3). It is therefore evident that there is an urgent need for a new, safer and effective vaccine. Indeed, a new acellular vaccine, which includes soluble pertussis toxin (PT)[1] and filamentous hemagglutinin (FHA), has been introduced in Japan, but the first controlled field trial performed in Sweden is providing controversial results (4). Again, this new generation of pertussis vaccines will be the result of an empirical approach. In fact, very little is as yet known about the host immune response to *B. pertussis*. This makes the rational design of effective new vaccines very difficult, even though recombinant proteins from *B. pertussis* are now available (5–8).

The aim of this study was to investigate humoral and cellular responses in adults who had suffered from pertussis in childhood. On the basis of the high degree of protection in previously infected people, these studies were expected to help to define the minimal antigenic structures that may be worthy of consideration for introduction into a third generation recombinant or synthetic vaccine.

Materials and Methods

Donors. PBMC and sera were collected from 52 healthy donors (aged 30–45 yr) with negative or positive anamnesis for whooping cough in childhood, but never vaccinated against *B. pertussis*.

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[1] *Abbreviations used in this paper:* FHA, filamentous hemagglutinin; PT, pertussis toxin.

ELISA Against PT. Sera were stored at −80°C to be used in the ELISA. The ELISA method was a modified version of that described by Engvall and Perlman (9). Flat-bottomed polystyrene microtest plates (Dynatech Laboratories, Inc., Alexandria, VA) were preincubated for 30 min at 37°C in a humidified chamber to minimize the electrostatic activity of the solid phase. The wells of each plate were then coated with 100 μl of purified goat anti-PT Igs (prepared at Sclavo Research Center, Siena, Italy) and diluted 1:2,000 in PBS. Plates were incubated for 3 h at 37°C. After incubation, the wells were washed with 150 μl of 0.15 M PBS containing 0.05% Tween and 0.02% sodium azide (PTA), and then 6 ng of PT (affinity purified on a fetuin column as described by Sekura [10]), in 100 μl of 0.05 M carbonate buffer (pH 9.6) was added to each well. Plates were incubated overnight at room temperature in a humidified chamber. The coating buffer was aspirated, and wells were washed with 150 μl of PTA. To minimize nonspecific adsorption of serum proteins to the plastic, wells were coated with 150 μl of a blocking solution consisting of 2% BSA in PBS, and then incubated for 2 h at 37°C. Plates were then washed three times in PTA and 100 μl of fourfold diluted test serum (1:20–1:1,280 dilution) and eight twofold serial dilutions of reference serum (1:20–1:1,280 dilution) in 0.15 M PBS containing 0.1% BSA and 0.2% sodium azide (PBS-BSA) were added to the wells. The U. S. Reference Pertussis Antiserum (human) containing 200 U/ml of IgG anti-PT was kindly provided by the Center for Drugs and Biologics, Bethesda, MD. After incubation for 1.5 h at 37°C, plates were washed three times with PTA and 100 μl of *p*-nitrophenyl phosphate substrate (Sigma Chemical Co., St. Louis, MO). 1 mg/ml in 1 M diethanolamine, pH 9.8, containing 1 mM $MgCl_2$, was added to each well. The enzyme-substrate reaction, which developed at room temperature, was stopped after 30 min, and the OD of the samples was measured at 405 nm against a blank (substrate in diethanolamine, pH 9.8) on a Titertek Multiskan (Flow Laboratories, Inc., McLean, VA). Controls for each plate included wells with serum samples but no antigen. Each serum sample was tested in duplicate, and absorbance values were averaged. The calculation of the ELISA antibody units in the test samples was determined in relation to the U. S. Reference Pertussis Antiserum (11).

Antigens. Virulent (phase I) and nonvirulent (phase III) *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* strains were used together with transposon-induced or deletion *B. pertussis* mutants lacking one or more virulence-associated antigens (see Table I and Fig. 1 for summary). *Bordetella* strains were grown on Bordet-Gengou agar at 35°C for 72 h and *Salmonella typhi* on LB agar at 37°C overnight. For inactivation, bacteria were harvested from plates, washed in PBS, and resuspended in a 0.5% formalin solution in PBS and left overnight on a mixer (Coulter Electronics Ltd., Luton, UK). After extensive washing, bacteria were resuspended in PBS and stored at 4°C. The OD_{525} of the suspensions was determined, and the number of cells per milliliter was calculated on previously established standard curves.

Production of hemolysis on BG agar and/or toxic effect on CHO cells (12) were adopted as parameters for the control of phase I *Bordetella* species.

Soluble PT was purified from *B. pertussis* phase I culture supernatant as described by Sekura (10), precipitated in ammonium sulfate, and kept at 4°C. Before use, it was resuspended in PBS and heat inactivated (at 100°C for 45 min).

The 69-kD protein, purified from *B. Pertussis* outer membrane as previously described (13), was kindly provided by Dr. Novotny (The Wellcome Research Laboratories, Beckenham, UK).

Media for Cell Stimulation and Growth. The culture medium was RPMI 1640 (Gibco Laboratories, Paisley, Scotland) supplemented with L-glutamine (2 mM), 1% nonessential amino acids, 1% sodium pyruvate, 50 μg/ml gentamycin, 5×10^{-5} M 2-ME, and 10% heat-inactivated pooled human AB serum (RPMI-HS) or FCS (RPMI-FCS). To support the antigen-independent growth of T cell clones, RPMI-HS was supplemented with 50 U/ml human rIL-2 (Hoffman-La Roche, Inc., Nutley, NJ).

Proliferation Assays. Fycoll-Hypaque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) -separated PBMC were incubated (10^5 cells/well in RPMI-HS) for 6 d with different antigen concentrations in 96-well flat-bottomed Cluster 96 plates (Costar, Cambridge, MA). When antigen-stimulated T lymphocyte clones were tested, they were incubated at 2×10^4 cells/well for 3 d in the presence of 2×10^4 mitomycin C–treated EBV-transformed autologous B lymphocytes (EBV-B cells) and antigens in RPMI-FCS (14). All assay cultures were pulsed for

TABLE I
Bacteria Used as Antigens in Proliferation Assays

Bacterium	Properties
B. pertussis Tohama (BP)	Virulent (phase I)
B. parapertussis 3715 (BPP)	Virulent (phase I)
B. bronchiseptica CCUG 7865 (BB)	Virulent (phase I)
B. bronchiseptica 7866	Nonvirulent (phase III) variant of 7865 obtained at Sclavo
B. bronchiseptica ATCC 4617	Nonvirulent (phase III)
B. pertussis Tn5-induced mutants (18):	
Strains:	Phenotype:
BP 353	reduced production of filamentous hemagglutinin (FHA^-)
BP 356	PT subunit 3 deficient ($S3^-$)
BP 348	adenylate cyclase and hemolysin deficient (AC^-, HLY^-)
BP 347	*vir*-regulated proteins deficient (vir^-), equivalent to phase III
B. pertussis FHA deletion mutant*	
Strains:	Genotype:
BP 536 fha 101	2.4 kb in frame deletion (Fig. 1, ⌒) in FHA structural gene (*fhaB*)
BP 536 fha 102	3.4 kb deletion (Fig. 1, ⌒) in *fhaB* with the 3′ region out of frame (Fig. 1; ---)

BP, *B. pertussis*.
* David Relman, Stanford University, Stanford, CA, personal communication.

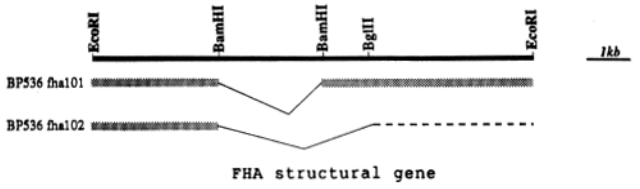

FIGURE 1. *B. pertussis* FHA deletion mutant.

the last 16–18 h with 1 μCi of [^{3}H]thymidine (sp act, 185 GBq/mmol; Amersham International, Amersham, UK). Cells were then harvested on glass-fiber filters with a cell harvester (Skatron, Lier, Norway), and incorporated radioactivity was determined by liquid scintillation counting.

Cloning of B. pertussis-specific T Lymphocytes. PBMC isolated from heparinized blood of donor R.R. by Ficoll-Hypaque were cultured in flat-bottomed Cluster 96 plates at the concentration of 10^5/well in 0.2 ml RPMI-HS in the presence of 10^6 *B. pertussis* cells. This antigen concentration was found to induce a strong proliferation response by PBMC from this donor. After 7 d, the activated T cells were expanded in medium supplemented with rIL-2, and after another 7 d, lymphoblasts were cloned by limiting dilution (14). Cells were seeded at 0.3 per well in Terasaki trays in the presence of 10^5 allogeneic mitomycin C–treated PBMC, in RPMI-HS containing PHA (1 μg/ml) and IL-2. About 50% of the clones obtained proved antigen specific when tested in proliferation assays against *B. pertussis*. These clones were maintained in culture by periodic restimulation in the presence of allogeneic mitomycin C–treated PBMC, PHA, and rIL-2. T lymphocyte clones were analyzed for cell surface phenotype by direct immunofluorescence on a FACS (FACStar, Becton Dickinson & Co., Erembodegem,

Belgium). Both phycoerythrin- and fluorescein-conjugated OKT3, OKT4, and OKT8 mAbs (Ortho Diagnostic Systems, Inc., Raritan, NJ) were used.

Immortalization of B Lymphocytes with EBV. PBMC from donor R.R. were transformed with EBV as described (15). Briefly, 10^7 PBMC were resuspended in 10 ml RPMI-FCS containing 30% supernatant of the EBV-producing marmoset cell line B95.8 and 600 ng/ml cyclosporin A (Sandoz, Basel, Switzerland) and were distributed in a flat-bottomed 96-well plate at 5×10^4/well.

Statistical Analysis. The calculation of the ELISA antibody units was based on the parallel line bioassay procedure described by Brownlee (16) using an ELISA unitage calculation program distributed by the Laboratory of Pertussis, Center for Drugs and Biologics, Bethesda, MD. An increase in ELISA units of more than mean negative controls plus 3 SD was arbitrarily considered as a significant level of anti-PT antibodies.

Results of proliferation assays are presented as the mean cpm of triplicate cultures ± SD. Statistical significance was calculated by student's *t* test.

Results

Identification of Immune Donors Against B. pertussis. Although no specific serological or cellular tests have been established for the direct measurement of host immunity to pertussis infection, donors with positive and negative anamnesis for whooping cough in childhood were screened for antibody titers against PT by ELISA, and for their proliferative response to *B. pertussis*. The aim was to identify donors suitable for the generation of T cell clones specific for *B. pertussis* antigens. As shown in Fig. 2, all donors with negative anamnesis had very low levels of anti-PT antibodies, when compared with a standard positive reference serum (see reference 11 and Materials and Methods). On the other hand, the levels of anti-PT antibodies in the group with positive anamnesis varied from 0 to 130 U/ml. This clearly indicates that anti-PT antibodies can be found only in a proportion of adults affected by pertussis in childhood (27.7%), considering as a significant increase in antibody values those higher than the mean of the group with negative anamnesis, plus 3 SD.

Mononuclear cells from peripheral blood of 9 out of 36 positive donors and of 5 out of 16 negative donors were tested for proliferation in response to inactivated whole *B. pertussis*. As shown in Fig. 3, a significant proliferation in a dose-dependent fashion was observed in the majority of donors with positive anamnesis; however, some of the negative donors also showed a low, but consistent increase in proliferation in response to *B. pertussis* antigens. The ELISA units and the maximal proliferation for all the donors tested are reported in Table II, which shows that in ~50% of the positive donors, these two parameters correlate, but this is not a general feature. Therefore, these results further confirm the difficulties in identifying immunological tests predictive of the host response against *Bordetella* infections.

Generation of T Cell Clones Against Whole Inactivated B. pertussis Bacteria. On the basis of these results, a donor with positive anamnesis and high ELISA titers as well as proliferative responses was chosen for the generation of T cell clones. Thus, PBMC from donor R.R. were stimulated in vitro for 7 d with formalin-killed *B. pertussis* phase I. The antigen-stimulated cells were then expanded in rIL-2-containing medium and cloned in the presence of PHA and nonautologous feeder cells (14). Specific clones were identified by means of proliferation in response to killed *B. pertussis* as shown in Fig. 4. A total of 12 *B. pertussis*-specific clones were obtained. Their phenotype was observed to be $CD4^+8^-$, and clones were MHC class II restricted, as

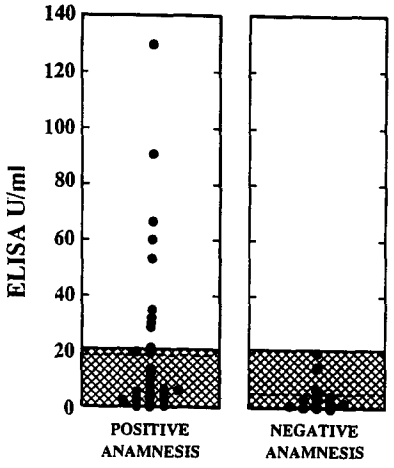

FIGURE 2. Antibody levels against PT in serum from normal adults with positive and negative anamnesis for whooping cough during childhood. Shaded area represents mean of negative controls ± 3 SD.

demonstrated by inhibition of antigen-induced proliferation by anti-HLA-DR mAbs (data not shown).

Recognition of Antigens from Whole Inactivated Bacteria by T Cell Clones. To analyze further the fine specificity of the anti-*B. pertussis* clones, a panel of *Bordetella* wild-type strains, as well as mutants, was used as antigen in proliferation tests together with a negative control such as *Salmonella typhi* (Table I). A representative experiment with four different T cell clones is shown in Fig. 5. As expected, the proliferative response of the different clones varied according to the panel of bacterial species used as antigens. A summary of 30 experiments performed is shown in Table III with a tentative prediction of the antigen recognized. On the basis of the reactivity patterns of the clones against *Bordetella* species and mutants, it can be suggested that clone 6 may recognize FHA. In fact, this clone proliferated in response to phase I *Bordetella* species, but did not recognize the FHA low producer *B. pertussis* 353 (7) and the phase III *B. bronchiseptica* and *B. pertussis* 347, both totally lacking FHA.

Clones 23 and 32 recognize *B. pertussis*-specific antigens, since they did not proliferate in response to *B. pertussis* and *B. bronchiseptica*. Furthermore, the negative results with phase III *B. bronchiseptica* and *B. pertussis* 347 suggest that the antigen(s) recognized is virulence associated. Clones 7, 8, 31, and 34 recognize virulence-associated *Bordetella* antigens, since they failed to proliferate in response to phase III *B. bronchiseptica* and *B. pertussis* 347. Finally, clones 9, 17, 26, 28, and 30 recognize nonvirulence-

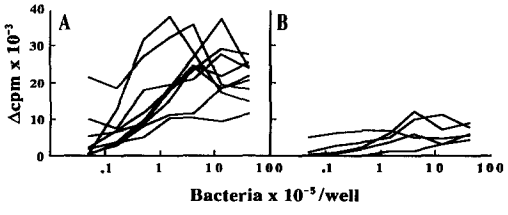

FIGURE 3. Proliferation of PBMC in response to inactivated *B. pertussis* from normal adults with positive (*A*) or negative (*B*) anamnesis for whooping cough. Background proliferation ranged from 0.3 to 0.6 cpm × 10^{-3} in all donors.

TABLE II

Humoral and Cellular Immunity to B. pertussis Antigens in Adults with Positive and Negative Anamnesis for Whooping Cough

	Positive donors			Negative donors	
	ELISA	Max cpm ($\times 10^{-3}$)*		ELISA	Max cpm ($\times 10^{-3}$)
	U/ml			*U/ml*	
M.M.‡	130	37	G.S.‡	4	6
E.B.‡	66	24	D.A.	2	11
R.R.‡	53	29	D.R.‡	1	7
F.P.‡	32	11	G.R.‡	3	4
A.T.	20	25	M.P.	7	11
P.B.	6	36			
S.N.	6	38			
M.R.	3	28			
G.G.	3	21			

* The background proliferation of cells without antigen was subtracted.
‡ Indicates correspondence between antibody levels and cellular response.

associated *Bordetella* antigens, since they proliferated in response to all the *Bordetella* species, regardless of their virulence and antigenic deficiencies. All the clones tested are specific for *Bordetella* species since they did not recognize inactivated *S. typhi*.

Since clone 6 was likely to recognize FHA, the analysis of its fine specificity was further extended using two new *Bordetella* mutants with defined deletions in the structural gene for FHA (see Table I). As shown in Table IV, clone 6 is capable of recognizing *B. pertussis* FHA 101, but not FHA 102. This would indicate that the determinant recognized by clone 6 is likely to be coded for by the sequence downstream from the second Bam HI site on the FHA structural gene, which represents approximately the COOH-terminal half of the protein. This part is missing in the mutant FHA 102.

Recognition of Soluble Bacterial Antigens by T Cell Clones. The clusters of clones against *B. pertussis*-specific antigens (cluster 2) and virulence-associated *Bordetella* antigens (cluster 3) were then tested against soluble antigens such as PT, which is present only on *B. pertussis*, and the 69-kD outer membrane protein, which is present on

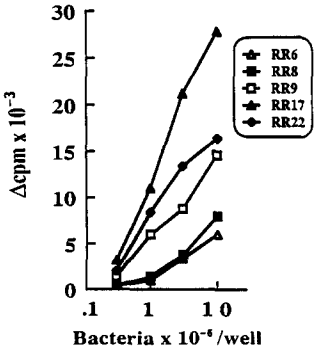

FIGURE 4. Proliferative response of T cell clones in response to inactivated *B. pertussis*.

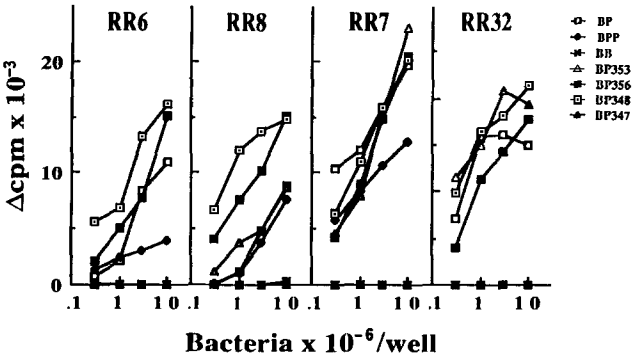

FIGURE 5. Proliferative response of T cell clones in response to inactivated *Bordetella* species and mutants.

all the three virulent *Bordetella* species, although with slightly different molecular weights (13). It was found that clones 23 and 32 from cluster 2 do not proliferate in response to inactivated PT, whereas clones 7, 31, and 34 from cluster 3 were able to recognize the 69-kD protein (Table V). However, clone 8 from cluster 3 did not proliferate in the presence of the 69-kD protein. Thus, this result would suggest that clone 8 may differ from the other clones of cluster 3. Its specificity would be directed against an additional virulence-associated, still unknown, *Bordetella* determinant.

T Cell Clones Against PT. Based on the experiments with *Bordetella* wild species and mutants and on those with soluble bacterial antigens, it can be concluded that none of the T cell clones obtained are against PT. To verify whether this was due to a low frequency or a total lack of such memory T lymphocytes or rather to a deficiency in antigen presentation when whole bacteria were used to stimulate immune PBMC, a new generation of T cell clones from the same R.R. donor was obtained by stimulating his PBMC in vitro with inactivated PT. Table VI shows that, indeed, T cell clones can be obtained against PT. Therefore, a T cell memory exists against PT, but it cannot be evoked by whole bacteria used as antigen.

Discussion

The extraordinary developments in protein chemistry and molecular biology have offered new possibilities in producing safe and effective vaccines. Important results have been obtained regarding methods for development of new vaccines for human and animal diseases, and a recombinant vaccine against hepatitis B is already in use.

Among the priority targets indicated by the World Health Organization to obtain safe and effective vaccines, one of particular interest for both developed and developing countries, is the prevention of pertussis. In fact, this disease had already been efficiently reduced by the cellular vaccine consisting of inactivated *B. pertussis* bacteria; however, due to its side effects, this vaccine was withdrawn in many countries, resulting in the return of a significant mortality from whooping cough even in highly developed countries (3). Therefore, pertussis represents on the one hand an interesting experimental system, since it is already known that it is possible to actively immunize against the disease and give protection; furthermore, it is also extremely important to obtain a safe vaccine with high compliance to continue the vaccination campaign. However, the identification of the simplest immunogenic structures capable of conferring a protective immunity is not easy, due to the complexity of the bacterial struc-

TABLE III

Recognition of Bordetella Species and Mutants by Human T Cell Clones Against B. pertussis

		Bacterial target									
		BP	BPP	BB		BP 353	BP 356	BP 348	BP 347		
Cluster	Clone No.	I*	I	I	III	(FHA^-)	(S_3^-)	(AC^-, HLY^-)	(vir^-)	*S. typhi*	Prediction of the antigen recognized
1	6	+	+	+	−	−	+	+	−	−	FHA
2	23, 32	+	−	−	−	+	+	+	−	−	*B. pertussis* specific
3	7, 8 31, 34	+	+	+	−	+	+	+	−	−	Virulence-associated *Bordetella* specific
4	9, 17, 26, 28, 30	+	+	+	+	+	+	+	+	−	Nonvirulence-associated *Bordetella* specific
Number of responding clones:‡		12	10	10	5	11	12	12	5	0	

BP, *B. pertussis*; BPP, *B. parapertussis*; BB, *B. bronchiseptica.*

* Phase.

‡ Out of 12.

TABLE IV
Proliferation of the FHA-reactive Clone RR6

Antigen	Proliferative response
None	4.5 ± 0.9*
B. pertussis	11.8 ± 1.7‡
B. parapertussis	7.4 ± 0.1‡
B. bronchiseptica (phase III)	3.5 ± 0.3
BP 353	3.7 ± 0.5
BP 347	3.6 ± 0.2
B. pertussis "FHA 101"	22.6 ± 2.5‡
B. pertussis "FHA 102"	3.9 ± 0.3

BP, *B. pertussis*.
* Mean cpm ($\times 10^{-3}$) ± SD of triplicate cultures.
‡ $p < 0.01$ vs. control.

tures. In the last few years, the genes of some among the most important proteins of *B. pertussis* have been cloned (5–8) and recombinant proteins are now becoming available. Our study was begun with the goal of providing useful tools to identify on a rational basis the immunogenic structures of *B. pertussis* proteins that might form a new synthetic and/or recombinant vaccine against pertussis.

A first problem that we tried to solve was the immunological characterization of adults who experienced whooping cough during childhood. As also observed by others, we found that the humoral response against PT seldom correlates with the anamnesis for pertussis. More interestingly, the majority of donors with positive anamnesis showed a significant in vitro proliferation against killed *B. pertussis* bacteria. This parameter was higher in donors with positive anamnesis compared with those with a negative one. However, this last group also showed some proliferation in response to bacterial antigens. This might be due to known crossreactivities between *B. pertussis* and other *Bordetella* species such as *B. bronchiseptica* and *B. parapertussis*, and/or crossreactivities with other bacteria (17). It is however of interest that a cell-mediated

TABLE V
Specificity of Anti-B. pertussis Human T Cell Clones Against PT and 69-kD Outer Membrane Protein

			Antigen			
Exp.	Cluster	Clone	None	*B. pertussis*	PT (1 μg/ml)	6.9×10^4 MW (10 μg/ml)
1	2	RR 23	4.0 ± 0.2*	17.5 ± 0.4‡	3.7 ± 0.1	ND
	2	RR 32	4.4 ± 0.6	28.7 ± 4.3§	4.2 ± 0.4	ND
2	3	RR 7	2.8 ± 0.1	10.1 ± 0.7‡	ND	17.6 ± 0.1‡
	3	RR 31	3.1 ± 0.6	16.0 ± 0.5‡	ND	20.2 ± 4.5§
	3	RR 34	2.5 ± 0.1	8.1 ± 1.2‡	ND	11.1 ± 1.8‡
3	3	RR 8	0.8 ± 0.3	6.2 ± 0.8‡	ND	0.8 ± 0.1

* Mean cpm ($\times 10^{-3}$) ± SD of triplicate cultures.
‡ $p < 0.01$ vs. control.
§ $p < 0.05$ vs. control.

TABLE VI
Response of Human T Cell Clones to Purified PT

Clone	Antigen	
	None	PT
RR-T106	2.9 ± 0.3*	5.1 ± 0.8‡
RR-T207	2.3 ± 0.4	7.7 ± 1.6§
RR-T209	2.4 ± 0.5	5.8 ± 0.7‡
RR-T215	2.3 ± 0.2	55.8 ± 2.9‡
RR-T216	2.3 ± 0.3	5.8 ± 0.9‡
RR-T218	2.1 ± 0.2	11.3 ± 0.7‡
RR-T219	2.6 ± 0.2	9.9 ± 2.1§
RR-T220	1.2 ± 0.1	14.2 ± 2.1‡
RR-T226	2.4 ± 0.2	57.3 ± 3.0‡
RR-T227	2.8 ± 0.2	12.0 ± 2.7§
RR-T229	2.5 ± 0.0	32.6 ± 2.0‡

* Mean cpm ($\times 10^{-3}$) ± SD of triplicate cultures.
‡ $p < 0.01$ vs. control.
§ $p < 0.05$ vs. control.

response such as specific lymphocyte proliferation seems to better correlate with an anamnesis for pertussis and further studies on this aspect are in progress.

These preliminary experiments on the characterization of immune responses against *B. pertussis* antigens formed the basis for the identification of an immune donor in order to increase the probability of obtaining T cell clones against pertussis antigens. Thus, donor R.R. was chosen, and his peripheral blood was used to obtain a first generation of T cell clones directed against *Bordetella* antigens. To analyze the specificity of these clones, it was decided first to stimulate them with a panel of *Bordetella* wild-type strains and mutants deficient for some antigens (18). This approach resulted in clustering the 12 clones obtained in four different groups, which recognized (1) FHA; (2) *B. pertussis*-specific antigen(s); (3) virulence-associated *Bordetella* antigen(s); and (4) nonvirulence-associated *Bordetella* antigens. The prediction that cluster 1 recognizes FHA was further confirmed by using two new *B. pertussis* mutants (D. Relman, unpublished results) that allowed the clone's specificity to be defined for the COOH-terminal portion of the FHA protein. Interestingly, the antigen recognized by three clones of cluster 3 was also positively identified as the 69-kD outer membrane protein described by Novotny et al. (13), while the specificity of the fourth clone seems to be directed to a virulence factor different from the 69-kD protein. In addition, the possibility that cluster 2 recognizes PT, suggested by the experiments with whole inactivated bacteria, was disproved by using soluble PT as antigen. Since cluster 4 recognizes nonvirulence-associated *Bordetella* antigens, one could conclude that PT is not the most immunogenic structure of inactivated whole *B. pertussis* recognized in vitro by memory T cells from a donor who had whooping cough during childhood. However, in a new series of experiments, it was possible to obtain T cell clones from donor R.R. against PT by stimulating his PBMC in vitro with soluble PT. This result would suggest that the disease may be capable of generating immunological memory against a variety of *B. pertussis* antigens, including PT. The extrapolation of our in vitro result to an in vivo vaccination with whole inactivated *B. pertussis* might suggest that the protective immunity generated by the vaccine may not be

directed against PT, but rather against other bacterial antigens. Thus, antigens such as FHA and the 69-kD protein may be sufficient to induce protection against the disease if used in an acellular vaccine. However, this conclusion does not imply that a vaccine composed of only PT would not be efficient. Indeed, this has been the case when tested in an animal model (19), which is considered rather artificial but also predictive for vaccine effectiveness.

The approach of generating T cell clones against *B. pertussis* antigens from immune donors can therefore be considered a useful tool for the development of a safer and more effective vaccine against whooping cough.

Summary

To identify the minimal structures that may be important for the creation of a synthetic and/or recombinant vaccine against whooping cough, human T cell clones were obtained against *Bordetella* antigens. Cloned peripheral blood T lymphocytes from an immune donor were grown in IL-2 and tested for proliferation in response to inactivated *Bordetella* species (*B. pertussis, B. parapertussis,* and *B. bronchiseptica*) and mutants deficient for the expression of virulence-associated antigens. All the T cell clones obtained were $CD4^+8^-$ and recognized specifically the *Bordetella* antigens when presented by autologous B cells. On the basis of the responsiveness to the whole inactivated bacteria, it was possible to cluster the 12 clones obtained into four groups with the following specificity: (1) filamentous hemagglutinin (FHA); (2) *B. pertussis*-specific antigens; (3) virulence-associated *Bordetella*-specific antigens; and (4) nonvirulence-associated *Bordetella*-specific antigens. Using two new *B. pertussis* deletion mutants, clone 6 (representative of cluster 1) was found to recognize the COOH terminus of FHA. Furthermore, three out of four clones of cluster 3 were specifically stimulated by the soluble 69-kD protein from the outer membrane of *B. pertussis*. Surprisingly, none of the twelve clones obtained by stimulation in vitro with whole inactivated bacteria recognized pertussis toxin (PT), which is believed to be the most important protein to be included in an acellular vaccine. However, when a new generation of clones was obtained using soluble PT as the in vitro stimulus, it was observed that 11 clones of this group recognized this antigen. Thus, PT does not seem to be the most representative antigen on the whole inactivated bacteria, although T cell memory against PT exists in a donor who had the disease several years ago.

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