HUMAN T CELL CLONES DEFINE S1 SUBUNIT AS THE MOST IMMUNOGENIC MOIETY OF PERTUSSIS TOXIN AND DETERMINE ITS EPITOPE MAP

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Whooping cough is an ubiquitous respiratory infection that primarily affects young children. A cellular vaccine, composed of inactivated *Bordetella pertussis* cells, was introduced in the 1940s and has been successfully used for many years in prevention of whooping cough (1). However, in recent years the side effects associated with the cellular vaccine (2, 3) have decreased its use in several countries, with the result of a sharp increase in infant mortality caused by the disease (1, 4). A more defined vaccine, composed of protective *B. pertussis* antigens, could be safer and more reliable to reintroduce mass vaccination. There are several candidate antigens for this approach: pertussis toxin (PT)[1], filamentous haemagglutinin, 69-kD outer membrane protein, and pili (5, 6). Indeed, a cell-mediated immunity against most of these antigens has been shown in human donors immunized by the disease (7). In particular, PT has been shown to be highly protective in animal models (8) and a recent field trial held in Sweden has confirmed that PT has a protective role also in humans (9).

PT is an oligomeric protein organized in five subunits that can be divided into two functionally different moieties: part A, composed of subunit S1, and part B, composed of subunits S2, S3, S4, and S5 (10). In analogy with exotoxin A from *Pseudomonas aeruginosa* and diphtheria and cholera toxins, the A moiety has ADP ribosyltransferase activity, while the B moiety binds the receptor(s) onto the surface of eukaryotic cells and allows the entry of the catalytic subunit S1 into the cell. The several biological effects exerted by PT (8) are mediated by the enzymatic activity of subunit S1, which has as target a family of GTP-binding proteins involved in the transduction of signals across the cell membrane of eukaryotic cells (11, 12).

Although the genes encoding the five subunits of PT have been cloned and sequenced (13, 14), all attempts to express the complete PT in prokaryotic vectors have so far failed (15). One way to overcome these problems and to obtain a vaccine that can be produced on a large scale may be to develop immunogenic recombinant or synthetic derivatives of PT. Subunit S1 may be the best candidate for this purpose. In fact, mAbs against S1 have been shown to neutralize the toxin in vitro and protect

This research project was supported by the contract "Programma Nazionale di Ricerca per la Chimica: Tema 3, Linfochine e Vaccini sintetici," granted to Sclavo S.p.A. by the Italian Ministry of Scientific Research and Technology. Address correspondence to M. Teresa De Magistris, Laboratory of Immunopharmacology, Sclavo Research Center, Via Fiorentina 1, 53100 Siena, Italy.

[1] *Abbreviations used in this paper:* PT, pertussis toxin; SI, stimulation indices.

mice against the intracerebral challenge with virulent *B. pertussis* (16). Furthermore, by raising polyclonal and monoclonal antibodies against the holotoxin, it was found that S1 is the most immunogenic among PT subunits (16, 17, Rappuoli et al., unpublished data). However, since recombinant S1 by itself has been shown unable to induce protective antibodies in mice because of conformational problems (15, 18), it is worthy of investigation to assess whether oligopeptides from S1 subunit may function in inducing protection towards the infection, circumventing the structural problems. Therefore, the rational development of a peptide-based vaccine requires the identification of B and T cell epitopes.

As an extension of our previous studies on B cell epitopes of the subunit S1 (18), in this study we have raised human T cell clones against PT and S1 from the blood of a donor immune to pertussis and have analyzed their fine specificity. PT-specific clones were tested for recognition of the five recombinant subunits of PT, and S1-specific clones were used to map T cell epitopes on S1 by using recombinant fragments and synthetic peptides homologous to this protein, as well as mutated S1 subunits deriving from two bacterial species related to *B. pertussis*.

Materials and Methods

Antigens. PT was purified from *B. pertussis* phase I culture supernatant as described by Sekura et al. (19), precipitated in ammonium sulfate, and kept at 4°C. Before use, PT was resuspended in PBS and heat inactivated (at 100°C for 45 min) to eliminate its mitogenic effect (20).

Subunits S1, S2, S3, S4, S5 from *B. pertussis*, BPPS1, BBS1, and nine fragments of BPS1 were expressed in *Escherichia coli*. Each protein was fused to the NH_2-terminal 98 amino acids of the MS2 polymerase (15, 21, 22). Recombinant plasmids expressing NH_2-terminal and COOH-terminal deletions of the S1 were obtained by standard DNA manipulation techniques as described (22). All the fusion proteins were partially purified by lysing the bacteria and collecting the inclusion bodies (15). The total protein concentration obtained was generally between 0.5 and 1 mg/ml with the 10–50% represented by the fusion protein (see Fig. 1 for BPS1 and its fragments). Inclusion bodies were resuspended in PBS and stored at −20°C. Recombinant proteins were used in proliferation assays without any further purification, at a final concentration of ~1 μg/ml of the fusion protein. Subunit S1 was further purified by electroelution as described (15) to stimulate the generation of specific T cell clones from PBMC.

Synthetic peptides of subunit S1 were obtained from different sources. Peptide 2–15 was purchased from Novabiochem (Läufelfingen, Switzerland). Peptides 6–17 and 27–39 were kindly donated by D. Burns (Center for Drugs and Biologics, FDA, Bethesda, MD). Peptides 8–18, 171–194, and 171–185 were prepared at the Sclavo Research Center by R. Presentini. Peptide 212–227 was purchased from G. Corradin (Université de Lausanne, Epalinges, Switzerland).

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 (Hoffmann-La Roche, Inc., Nutley, NJ).

Cloning of Antigen-specific T Lymphocytes. T cell clones were obtained from peripheral blood of donor R.R., previously used to identify antigens from *Bordetella* species (7). His HLA class II phenotype was defined as DR 1, 2, DR w16, DQ w1, DQ w5. PBMC isolated from heparinized blood of donor R.R. by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) were cultured in 96-well flat-bottomed plates (Costar, Cambridge, MA) at the concentration of 2×10^5/well in 0.2 ml RPMI-HS, in the presence of heat-inactivated PT or S1 at different concentrations (threefold dilutions from 10 to 0.1 μg/ml, in the final volume).

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After assessment of antigen-induced proliferation by [^{3}H]thymidine incorporation, rIL-2 was added on day 7 to expand the proliferating cells. After another 7 d, lymphoblasts from several wells were pooled and cloned by limiting dilution. Cells were seeded at 0.3 per well in Terasaki trays in the presence of 10^4 allogeneic mitomycin C–treated PBMC, in RPMI-HS containing PHA (1 μg/ml), and rIL-2 (23). These clones were maintained in culture by periodic restimulation in the presence of allogeneic mitomycin C–treated PBMC, PHA, and rIL-2. Clones of T lymphocytes 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 (24). 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.

Proliferation Assays. Clones of T lymphocytes were incubated at 2×10^4 cells/well for 3 d in the presence of 2×10^4 mitomycin C–treated or 10^4 irradiated EBV-transformed autologous B lymphocytes (EBV-B cells) and antigens in RPMI-FCS, in 96-well flat-bottomed plates. In the experiment determining DR restriction, two allogeneic DR homozygous EBV-B cell lines (kindly provided by E. Goulmy, Leiden, The Netherlands) were used: EDR (DR 1) and NOL (DR 2). Cells were first pulsed for 2 h with 10 μg/ml of peptide, then thoroughly washed and irradiated before being used in the proliferation assays. All assay cultures were pulsed for the last 16–18 h with 1 μCi of [^{3}H]thymidine (sp. act. 185 GBq/mmole; Amersham International, Amersham, UK). Cells were then harvested on glass fiber filters with a cell harvester (Skatron, Inc., Lier, Norway) and incorporated radioactivity was determined by liquid scintillation counting. Results of proliferation assays are presented as the mean cpm of triplicate cultures ± SD. Statistical significance was analyzed by student's t test for stimulation indices (SI) <3, where SI = cpm of cultures with antigen/cpm of cultures without antigen. A large number of experiments indicated that SI>3 is always statistically significant. Positive values in the tables are boxed.

Inhibition Assay with mAb Anti-HLA Class II Molecules. 2×10^4 cells from each clone were cultured with 10^4 autologous irradiated EBV-B cells. Appropriate dilutions of the peptides 27–39 or 171–194 were added simultaneously with 8 μg/ml final dilution of mAb anti-DR (L243), anti-DP (B7.21), or anti-DQ (Leu-10) (Becton Dickinson & Co., Müchenstein, Switzerland), in a final volume of 250 μl. After 48 h cultures were pulsed with [^{3}H]thymidine and harvested as described in the previous section.

Results

Fine Specificity of Human T Cell Clones Against PT. In a previous study (5), an adult donor (R.R.) was identified as being immune against *B. pertussis*. In fact, he had suffered from pertussis in his childhood and exhibited significant humoral and cellular responses against *B. pertussis* antigens. Thus, T cell clones against PT were obtained from his PBMC (5). Here, the fine specificity of T cell clones against PT was further analyzed using recombinant PT subunits (13). 10 of 12 clones (83%) were specific for S1 (Table I), suggesting that this subunit might be immunodominant in this individual. Of the other two clones, T106 is directed to S4 and T207 recognizes a crossreactive determinant on subunits S2 and S3, which share 67% amino acid homology. None of the clones proliferated to S5. To have a suitable number of clones neccesary for mapping the S1 epitopes, PBMC were stimulated in vitro with recombinant S1, obtaining eight additional specific clones (Table I). All the clones used have phenotype $CD3^+4^+8^-$.

Mapping T Cell Epitopes on S1. To identify the regions of S1 containing the epitopes recognized by the S1-specific T cell clones, recombinant proteins representing

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TABLE I

Proliferation of Human T Cell Clones to Purified PT and its Recombinant Subunits

Clones	Antigen						
	None	PT	S1	S2	S3	S4	S5
Raised against PT:							
RR-T106	2.9 ± 0.3*	5.1 ± 0.6	2.2 ± 0.2	2.2 ± 0.0	2.4 ± 2.7	9.4 ± 1.4	2.6 ± 0.2
RR-T207	2.3 ± 0.4	7.7 ± 1.3	1.9 ± 0.1	41.6 ± 3.5	39.4 ± 0.4	2.1 ± 0.1	2.5 ± 0.3
RR-T209	2.4 ± 0.5	5.8 ± 0.5	18.7 ± 1.7	2.2 ± 0.0	2.4 ± 0.0	2.4 ± 0.2	2.6 ± 0.3
RR-T215	2.3 ± 0.2	55.8 ± 2.4	59.4 ± 1.6	ND	2.7 ± 0.3	2.1 ± 0.1	2.2 ± 0.2
RR-T216	2.3 ± 0.3	5.8 ± 0.8	14.6 ± 0.2	2.2 ± 0.2	2.5 ± 0.3	2.5 ± 0.1	1.9 ± 0.2
RR-T217	2.0 ± 0.7	4.7 ± 0.8	16.1 ± 2.3	1.3 ± 0.1	1.9 ± 0.3	1.6 ± 0.1	1.4 ± 0.1
RR-T218	2.1 ± 0.2	11.3 ± 0.6	20.5 ± 2.6	3.2 ± 0.4	2.5 ± 0.0	2.1 ± 0.0	2.1 ± 0.3
RR-T219	2.6 ± 0.2	9.9 ± 1.7	10.4 ± 0.4	2.2 ± 0.0	2.1 ± 0.1	1.9 ± 0.0	1.8 ± 0.0
RR-T220	1.2 ± 0.1	14.2 ± 1.7	18.9 ± 0.6	1.7 ± 0.1	1.5 ± 0.0	1.3 ± 0.2	1.4 ± 0.2
RR-T226	2.4 ± 0.2	57.3 ± 2.4	56.0 ± 2.2	3.2 ± 0.8	2.3 ± 0.2	2.1 ± 0.1	2.2 ± 0.0
RR-T227	2.8 ± 0.2	12.0 ± 2.2	12.7 ± 2.1	2.0 ± 0.1	2.5 ± 0.0	2.4 ± 0.2	2.4 ± 0.1
RR-T229	2.5 ± 0.1	32.6 ± 1.6	31.7 ± 1.3	4.6 ± 0.8	2.4 ± 0.1	2.5 ± 0.2	2.3 ± 0.4
Raised against S1:							
RR-S105	2.2 ± 0.3		12.8 ± 2.3				
RR-S106	2.7 ± 0.2		63.5 ± 2.8				
RR-S201	0.7 ± 0.1		13.4 ± 1.0				
RR-S208	2.5 ± 0.2		29.3 ± 1.0				
RR-S213	0.9 ± 0.1		46.0 ± 4.8				
RR-S223	2.5 ± 0.3		15.3 ± 1.4				
RR-S229	2.9 ± 0.4		9.0 ± 0.3				
RR-S232	3.0 ± 0.5		10.6 ± 0.8				

Boxed values are statistically significant ($p \leq 0.05$).

* Mean cpm ($\times 10^{-3}$) ± SD of triplicate cultures.

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NH_2- and COOH-terminal deletions of S1 (Fig. 1, Fig. 2, and reference 18) were assayed for their ability to stimulate T cell clones. Differential recognition of the recombinant fragments by 10 representative clones of the 18 tested is reported in Table II. In addition to the truncated fragments, the recombinant full-length S1 from both *Bordetella parapertussis* (BPPS1) and *Bordetella bronchiseptica* (BBS1), were used as antigen in proliferation assays. These two proteins differ from BPS1 for some amino acid substitutions along their sequence (10 substitutions in BPPS1 and four in BBS1) (Fig. 3 and reference 21). 11 of 18 clones tested map at the NH_2 terminus of S1 (Fig. 2). In fact, they recognize the NCO, NRU, BAL, and SPH fragments, but none of the fragments with deletions of the NH_2-terminal portion of the protein. The shorter deletion at the NH_2 terminus that abolishes clone proliferation is that of fragment 34a, which is 42 residues long. The sequence 1–42 is therefore likely to contain the epitope(s) recognized by these clones, although NH_2-terminal residues of fragment 34a may also be involved. The unresponsiveness to BPPS1 and BBS1 of 5 of these 11 clones (S229, S232, T215, T219, and T226) suggests that at least two different specificities are represented in this group. The mutation Asp → Glu^{34}, present in both BPPS1 and BBS1 (Fig. 3), is therefore crucial for recognition of the protein.

On the basis of their pattern of recognition of the recombinant fragments (Table II and Fig. 2), the remaining seven clones were found to map at the COOH terminus of S1, since they recognized all the fragments truncated at the NH_2-terminal end, including fragment 18, which contains only amino acids 150–235 (Fig. 2). Of the fragments deleted in COOH terminus, five clones (S105, S201, T209, T217, and T227) recognized only NCO and 34a. This allows the exclusion of the last 24 residues of S1 as the sequence containing the epitopes recognized by these clones. Such epitopes may instead be comprised between the COOH end of NRU and the last COOH-terminal residue of NCO, in region 181–211, although some COOH-terminal residues of NRU may also be part of an incomplete epitope. Finally, clones S223 and T216 map at the very COOH-terminal end of S1. In fact, both recognize all the fragments

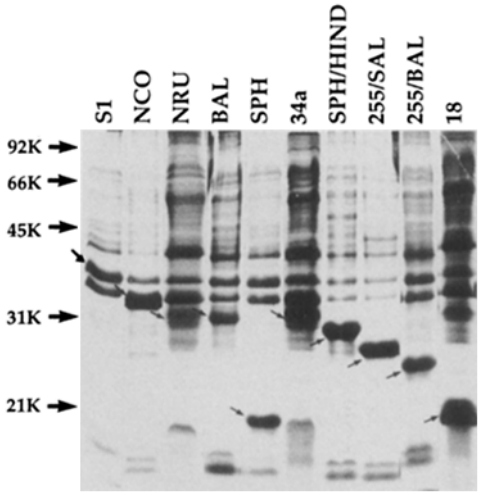

FIGURE 1. Comassie blue-stained SDS-PAGE of the *E. coli* inclusion bodies containing the recombinant BPS1 subunit and its fragments (*arrows*) used in the proliferation assays.

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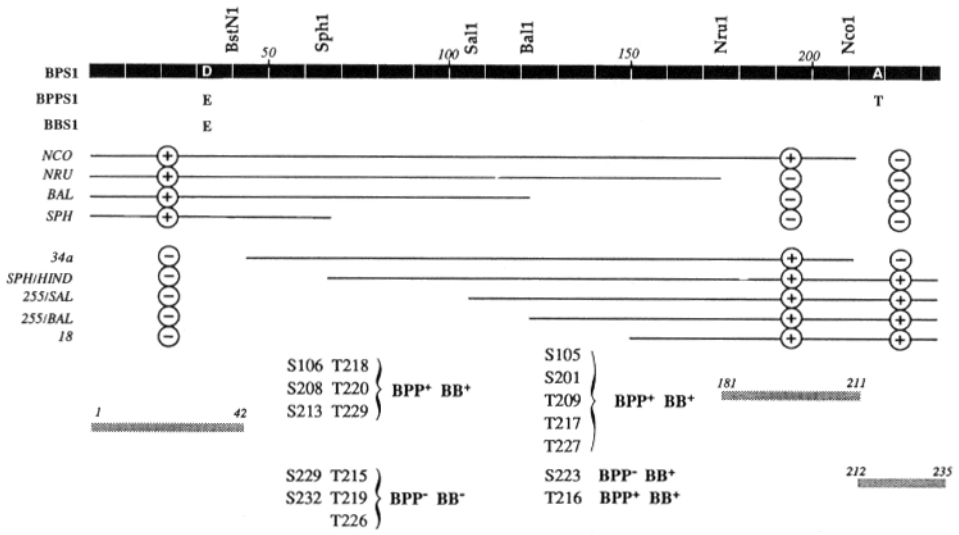

FIGURE 2. Mapping of T cell epitopic regions of S1 with recombinant fragments and mutated S1 from *B. parapertussis* and *B. bronchiseptica* (BPPS1 and BBS1). The thick black line indicates *B. pertussis* S1. Above are reported the restriction enzymes used to obtain the recombinant fragments (22). Letters below the black line indicate the two amino acid substitutions of BPPS1 and BBS1 that caused unresponsiveness to these two proteins by the clones listed in the lower part of the figure and designed with BPP^- and BB^-. The thin lines represent BPS1 recombinant fragments with COOH-terminal and NH_2-terminal truncations; their names are indicated on the left. Specificity of the clones was the following: clones S106, S208, S213, T218, T220, and T229 recognized BPPS1, BBS1, and truncated fragments indicated with ⊕ on the left part of the figure. Clones S229, S232, T215, T219, and T226 recognized the same truncated fragments of the upper reported clones, however, the mutation D → E impaired recognition of BPPS1 and BBS1. Shaded 1-42 segment represents the region of BPS1 deduced to contain T cell epitopes for these eleven clones. Clones S105, S201, T209, T217, and T227 recognized BPPS1, BBS1, and truncated peptides indicated with ⊕ in the middle-right part of the figure. Shaded 181-211 segment represents the region deduced to contain T cell epitopes for these five clones. Clones S223 and T216 both recognized BBS1 and truncated fragments indicated with ⊕ on the right-end part of the figure, however mutation A → T of BPPS1 impaired recognition of BPPS1 by clone S223. Shaded 212-235 segment represents the region deduced to contain T cell epitopes for these last two clones.

truncated at the NH_2 terminus and do not recognize the fragments truncated at the COOH terminus. This indicates that the deletion 212-235 at the COOH end of NCO and 34a is enough to abolish recognition of S1 and is therefore likely to contain the epitopes recognized by these two clones. The specificity of the two clones is different, since S223 does not recognize BPPS1. The mutation Ala → Thr^{219}, peculiar to BPPS1, is very likely the cause of this unresponsiveness.

Recognition of Synthetic Peptides. In the attempt to identify the minimal sequence capable of stimulating T cells in the regions 1-42, 181-211, and 212-235, synthetic peptides homologous to NH_2-terminal and COOH-terminal portions of BPS1 (Fig. 3) were used as stimulators in proliferation assays in the presence of EBV-B cells. Clones mapping in the NH_2-terminal region 1-42 were stimulated with the overlapping peptides 2-15, 6-17, 8-18, and with the peptide 27-39, which is homologous to the sequence that in BPPS1 and BBS1 contains the mutation Asp → Glu^{34}. As shown in Table III, three of the five clones tested (which were able to recognize BPS1,

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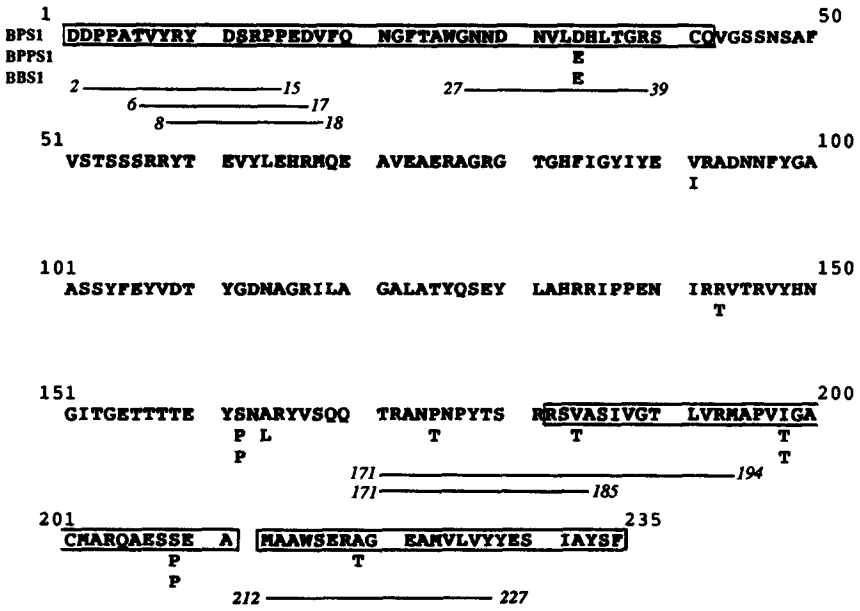

FIGURE 3. Amino acid sequence of BPS1 according to Nicosia et al. (13) and amino acid mutations in BPPS1 and BBS1 sequences. The synthetic peptides homologous to BPS1, used to stimulate T cell clones, are also shown. Boxed areas represent the regions 1-42, 181-211, and 212-235 that contain T cell epitopes.

but not BBS1 or BPPS1) specifically proliferated to peptide 27-39. Therefore, the epitope recognized by clones S232, T215, and T226 is contained within a sequence of 13aa. On the other hand, clone S229 and T219 did not respond either to 27-39 or to any other peptide. In this regard, these clones introduce a different specificity in the group of clones mapping at the NH_2 terminus. The other six clones mapping in the region 1-42, all responsive to both BPPS1 and BBS1, did not recognize any of the peptides listed in Table III (data not shown).

The five clones mapping in the COOH-terminal region 181-211 were tested for recognition of the peptides 171-185 and 171-194, which are homologous in the first 15 amino acids. Table III shows that four of the five clones proliferated to peptide 171-194, but not 171-185. This indicates that clones S105, S201, T209, and T217 map in a region of 24 residues (171-194), but suggests that the epitope recognized is shifted on the second half of the sequence 171-194. Clone T227, which did not recognize either of the previous two peptides, introduces a different fine specificity in the group of clones mapping in the region 181-211.

Clones S223 and T216, mapping in the sequence 212-235, were not stimulated by the peptide 212-227, which includes the Ala^{219}, which, when substituted with Thr, abolishes the response of S223 to BPPS1.

Determination of HLA Class II Restriction. An inhibition assay with mAbs against HLA class II molecules DR, DP, and DQ was performed to determine the restriction element used by the clones recognizing the stimulatory peptides 27-39 and 171-194. Clone T215 (specific for 27-39) and clones S105 and T217 (both specific

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TABLE II

Recognition of Mutated S1 and Truncated Fragments

	Antigens												
Clones	None	BPS1	BPPS1	BBS1	NCO	NRU	BAL	SPH	34a	SPH-HIND	255SAL	255BAL	18
RR-S106	2.7*	63.5	99.8	97.6	85.5	110.8	110.8	94.9	2.6	2.4	3.1	ND	2.9
RR-T229	1.4	9.5	8.9	11.3	9.1	16.4	8.1	11.0	1.5	1.1	1.1	2.3	1.3
RR-S229	2.9	9.0	2.3	3.2	10.0	13.0	9.0	10.7	2.2	2.1	2.5	4.4	2.4
RR-T219	1.1	17.7	0.8	3.1	20.2	27.1	20.5	38.5	0.8	0.8	0.7	1.7	1.0
RR-T226	2.2	8.6	2.5	3.2	9.4	9.0	9.3	13.2	2.1	1.6	1.9	3.3	2.4
RR-S105	2.2	12.8	4.7	33.3	33.9	2.5	2.0	2.4	16.4	52.0	58.9	55.3	46.0
RR-T209	1.2	13.6	13.4	24.0	34.9	1.4	1.2	1.2	ND	118.3	98.0	63.0	53.3
RR-T227	1.2	12.6	24.1	36.3	17.8	1.2	0.8	0.9	ND	51.8	44.1	26.2	16.4
RR-S223	2.4	15.3	2.7	24.5	2.6	1.7	2.9	2.9	2.0	56.3	55.0	39.8	32.7
RR-T216	1.7	16.5	19.9	25.8	2.4	2.5	1.6	3.4	1.6	33.1	37.8	24.3	21.9

Boxed values are statistically significant ($p \leq 0.05$).

* Mean cpm ($\times 10^{-3}$) of triplicate cultures.

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TABLE III

Proliferation of T Cell Clones Induced by Synthetic Peptides Homologous to S1

Clone*	Peptides (μg/ml) None	2-15 (10)	6-17 (10)	8-18 (10)	27-39 (10)
RR-S229	1.6 ± 0.3‡	1.4 ± 0.2	1.0 ± 0.2	1.6 ± 0.1	0.7 ± 0.1
RR-S232	0.8 ± 0.2	ND	0.9 ± 0.1	ND	9.2 ± 1.1
RR-T215	1.8 ± 0.4	1.5 ± 0.1	1.3 ± 0.1	2.3 ± 0.4	45.9 ± 10.4
RR-T219	1.4 ± 0.2	1.1 ± 0.1	1.3 ± 0.8	2.0 ± 0.9	2.4 ± 2.6
RR-T226	0.9 ± 0.2	ND	0.7 ± 0.1	ND	44.3 ± 4.2

Clone§	Peptides (μg/ml) None	171-194 (1)	171-185 (10)	212-227 (10)
RR-S105	1.7 ± 0.2‡	96.5 ± 12.3	1.4 ± 0.1	ND
RR-S201	2.6 ± 0.3	20.4 ± 2.6	2.2 ± 0.1	ND
RR-T209	1.5 ± 0.2	72.5 ± 7.8	1.0 ± 0.1	ND
RR-T217	3.5 ± 0.9	47.5 ± 5.0	2.6 ± 0.1	ND
RR-T227	2.5 ± 0.3	2.2 ± 0.1	2.4 ± 0.1	ND
RR-T216	0.5 ± 0.1	ND	ND	0.6 ± 0.1
RR-S223	0.6 ± 0.1	ND	ND	0.7 ± 0.1

Boxed values are statistically significant ($p \leq 0.05$).

* Clones mapping at the NH_2 terminus.

‡ Mean cpm ($\times 10^{-3}$) ± SD of triplicate cultures.

§ Clones mapping at the COOH terminus.

for 171-194) were cultured with autologous EBV-B cells, threefold dilutions of the appropriate peptide, and mAbs. Only anti-DR antibodies showed an inhibitory effect on antigen-induced proliferation. In fact, the response of clones T215 and S105 was strongly inhibited by these antibodies and the response of clone T217 was even abolished (Fig. 4). Therefore, these three clones recognize the respective peptides in the context of DR molecules. In a second experiment, designed to determine the DR allele involved, two allogeneic lymphoblastoid cell lines, EDR homozygous for DR1 and NOL homozygous for DR2, were used to present the peptides to clones S232, T215, T226, and T217. The cell lines were first pulsed with one or the other peptide to allow binding of the peptides to class II molecules, then irradiated and cultured with the clones. Table IV shows that only the line EDR (DR1) was capable of presenting the antigen to the clones as the autologous EBV-RR. This indicates that DR1 is the restriction element used by the clones tested.

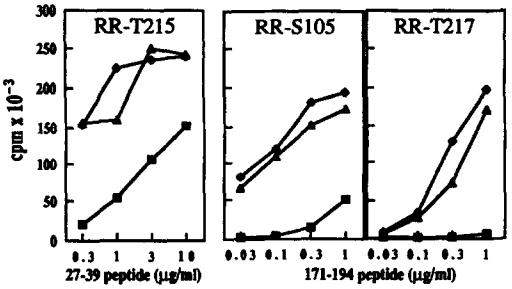

FIGURE 4. Inhibition of peptide recognition by anti-class II mAbs (■, anti-DR; ◆, anti-DP; Δ, anti-DQ). This experiment was performed with autologous irradiated EBV-B cells. cpm of controls without antigen of the three clones were, respectively, 1.6, 1.9, and 1.8 × 10^3. Anti-DP and anti-DQ antibodies were not inhibitory vs. control (not shown).

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TABLE IV

Recognition of Peptides Presented by an Allogeneic DR 1,1 EBV-B Cell Line

Clone	Peptide	APC		
		EBV-RR (DR 1,2)	EDR (DR 1,1)	NOL (DR 2,2)
RR-S232	27-39	64.5* ± 16.3	151.4 ± 20.1	0.3 ± 0.4
RR-T215	27-39	206.4 ± 17.9	176.5 ± 45.8	1.0 ± 0.2
RR-T226	27-39	166.3 ± 6.3	200.7 ± 47.0	0.0 ± 0.6
RR-T217	171-194	82.2 ± 5.3	44.8 ± 8.0	1.4 ± 0.4

Boxed values are statistically significant ($p \leq 0.05$).

* Δcpm ($\times 10^{-3}$) ± SD of duplicate cultures (background proliferation values ranged between 6.2 and 8.4 cpm $\times 10^3$).

Discussion

We have shown that the S1 subunit of PT is recognized by most (10 of 12) of the human T cell clones against PT. This result suggests immunodominance of the S1 over the other four subunits of PT in humans. The immunodominance of this enzymatically active subunit, previously shown in mice (16, 17), goats, rabbits (Rappuoli et al., unpublished data), and here, in humans, renders this toxin quite unique among the other bacterial toxins with A-B structure, as diphtheria and cholera toxins where the B oligomer has been demonstrated to be far more immunogenic than A (25-27). Based on this, subunit S1 seems to be a good candidate for a recombinant or synthetic vaccine against whooping cough. However, since recombinant S1 failed to induce protection, probably due to a different folding caused by the expression systems (15, 18), it remains to identify the short peptides of S1 recognized by humoral and cellular immunity. A major protective B cell epitope of the subunit S1, which is composed of at least two noncontiguous regions mapping at the NH_2 terminal and COOH terminal of the protein, was previously reported by our group (18). The identification of some T cell epitopes of the subunit S1 was here performed using the 10 above-mentioned S1-specific T cell clones plus eight newly generated against S1 itself, all with the helper/inducer phenotype $CD3^+CD4^+CD8^-$. Clones were first tested for recognition of nine recombinant fragments of S1, representing deletions of different lengths at the NH_2-terminal and/or COOH-terminal regions of the protein. Three regions containing antigenic sites were identified: fragment 1-42 (recognized by 11 clones) at the NH_2 terminus and fragments 181-211 and 212-235 (recognized by five and two clones, respectively), which are adjacent and situated at the COOH terminus of S1. It is noticeable that anti-S1 clones raised either against the purified PT or the recombinant S1 showed similar patterns of specificity. This would indicate that the fusion of S1 to the polymerase of phage MS2 does not alter antigen processing of the protein. Clones were also tested for recognition of two mutated S1 proteins deriving from two *B. pertussis*-related species, i.e., *B. parapertussis* and *B. bronchiseptica*. Although these species contain transcriptionally silent PT genes (21), the respective S1 proteins can be expressed in *E. coli* (21). The recombinant proteins obtained are enzymatically active and differ from BPS1 only for few amino acid substitutions along their sequence (21). Two mutations (Asp → Glu^{34} and Ala → Thr^{219}) completely abolished proliferation of some clones, indicating that those residues might be part of an epitope.

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Synthetic peptides were then assayed for their capability to stimulate the clones. Peptide 27–39 was recognized by three of the five clones affected in the antigen recognition by the substitution Asp → Glu^{34}. This suggests that an immunodominant site is located around residue 34 and that a polyclonal T cell response is directed to this antigenic site. It is not clear, however, whether the other six clones mapping in region 1–42, but which are not affected by the substitution Asp → Glu^{34}, are also directed to this immunodominant region or rather recognize a distinct NH_2-terminal sequence. At the other end of the protein, peptide 171–194 was recognized by four of the five clones mapping in the sequence 181–211, and there is evidence that the antigenic site may be localized in the COOH-terminal half of the sequence 171–194.

The primary sequence of the operationally defined T cell epitopes was analyzed a posteriori with the two most common methods for predicting immunogenic sites (28, 29). In peptide 27–39 residues Asn-Val-Leu-Asp (31–34) correspond to a consensus motif (charged or glycine-hydrophobic-hydrophobic-polar or charged), suggested by Rothbard (28) as being common to a majority of T cell determinants. Moreover, the optimized algorithm for predicting T cell antigenic sites (developed by Margalit et al.) (29) was applied to S1 sequence. Residues 26–42 can form an amphipatic helix and can be accommodated in a helical wheel such that residue 34 is on the hydrophilic side, as might be anticipated if Asp^{34} was associated with TCR interaction. Peptide 171–194 contains part of the sequence 181–198, which has a high amphipatic score (29). This supports the hypothesis that a T cell epitope is localized in the second half of the sequence 171–194. There are no consensus sequences according to Rothbard et al. (28) in peptide 171–194.

In a very recent study, Oksenberg et al. (30) reported the mapping of T cell epitopes of S1 obtained with the approach opposite to ours, that is prediction of immunogenic peptides followed by in vitro confirmation experiments. It was therefore of interest to compare effectiveness of the two approaches. While both studies find a major epitope in the NH_2-terminal region, Oksenberg et al. (30) described also antigenic peptides homologous to the central region of S1, where none of our clones map. It could be that some of those peptides are not generated during the natural processing of the S1 subunit. Furthermore, the COOH-terminal region of S1 was recognized by our clones, but it was not included in the other study (30). Thus, it can be concluded that computer prediction can be useful, but not satisfactory if taken on its own.

The development of a peptide-based vaccine requires either a universal T cell epitope, which is recognized by all the HLA types, or a mixture of peptides containing enough epitopes to cover the majority of the population (31). The peptides described here are DR1-restricted, and while further studies will establish whether they are also recognized in the context of other HLA types, it is important to include them in a vaccine made by a pool of peptides. Thus, taking together this observation, our previous findings about the B cell epitopes of S1, and the two peptides, 64–75 and 151–161, described by Oksenberg et al. (30), which are immunogenic in a panel of individuals with different HLA genotypes, a peptide-based vaccine seems ready to be tested for its capability of inducing protection from the infection in experimental systems. Meanwhile, the possibility of obtaining an improved acellular pertussis vaccine containing engineered PT lacking of the S1 enzymatic activity is being investigated (32–34). These mutated molecules should be engineered in a way that they will still contain the protective epitopes. Our study shows that some of the mutations de-

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scribed, which abolish the enzymatic activity of S1 (22, 32), fall in a region that does not contain T cell epitopes. Therefore, such vaccines are theoretically feasible.

Summary

Human T lymphocyte clones specific for pertussis toxin (PT) were used to analyze the fine specificity of the response to PT, the basic component of new acellular vaccines against whooping cough. The majority (83%) of the clones specific for PT recognized S1, the subunit that in animal models has been shown to be highly immunogenic. To map T cell epitopes on S1, 18 S1-specific clones were tested for recognition of recombinant fragments representing NH_2-terminal and COOH-terminal deletions of S1 and two recombinant S1 subunits containing amino acid substitutions. This approach led to the identification of three regions of the protein as the sequences containing T cell antigenic sites: 1–42, 181–211, and 212–235. Synthetic peptides were eventually used for a finer localization of the T cell epitopes. Two peptides, one of 13 residues (27–39) at the NH_2 terminus and one of 24 residues (171–194) at the COOH terminus, stimulated proliferation of three and four clones, respectively. Both peptides are recognized in association with HLA DR1 molecules. These results stress the role of S1 in the immune response to PT and provide data useful for the development of a recombinant or synthetic antipertussis vaccine containing T cell epitopes from S1.

The authors are indebted to Antonio Lanzavecchia (Basel Institute for Immunology, Basel, Switzerland), Jonathan Rothbard (ICRF, London, England), and Diana Boraschi (Sclavo) for critically reading the manuscript, and to Jay Berzofsky (NCI, NIH, Bethesda, MD) for providing computer prediction of T cell antigenic sites of subunit S1. Furthermore, we wish to thank Sandra Nuti for performing the FACS analysis, Giorgio Corsi for artwork, and Catherine Mallia for typing the manuscript.

Received for publication 29 December 1988 and in revised form 30 January 1989.

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