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Identification of Human T-Cell Epitopes on the S4 Subunit of Pertussis Toxin

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Ten adult humans were vaccinated with the Japanese acellular pertussis vaccine JNIH-3, containing detoxified pertussis toxin (PT), formaldehyde, and filamentous hemagglutinin. The vaccination induced a specific antibody response to PT and filamentous hemagglutinin, and a Western blot (immunoblot) analysis of the antibody response to PT revealed antibodies to PT subunits S1, S2, S3, S4, and S5. The response of peripheral lymphocytes to PT was assessed in an in vitro proliferation assay. A proliferative response to detoxified PT and PT dimers S2-S4 and S3-S4 was found, and it was further demonstrated that the proliferative response to detoxified PT and dimer S2-S4 was mediated by T cells of the $CD4^+$ phenotype. The specificity of the proliferative response to subunit S4 was analyzed with a range of synthetic peptides synthesized on the basis of the primary sequence of subunit S4. The proliferative response to the peptides revealed two major and one minor T-cell epitope located in the NH_2-terminal end of subunit S4.

Pertussis toxin (PT) is considered one of the main virulence factors produced by *Bordetella pertussis* (52). Active or passive immunization with PT is protective in humans against disease and in animal models against lethal infection with virulent *B. pertussis* (1, 33, 44). Toxoided forms of PT are the principal components of acellular pertussis vaccines currently in use or under development (41). PT, which is composed of five subunits, termed S1 through S5, exhibits the A-B structure characteristic of many bacterial toxins (50). Subunit S1, the A protomer, is the enzymatically active moiety of PT (50). It is responsible for most of the biological activities induced by the toxin, e.g., lymphocytosis, neurotoxicity, hypoglycemia, adjuvanticity, and histamine sensitization (38). Subunits S2 through S5 constitute the B oligomer, with subunit S4 being present in a molar ratio of 2:1 with respect to the other subunits (50). The B oligomer mediates the binding of the toxin to the surface of the eukaryotic cell (50) and the introduction of the A protomer into the cytosol (32). Both the A protomer and the B oligomer are able to induce neutralizing antibodies in mice and to protect against challenge with PT (2, 15, 36) or infection with virulent *B. pertussis* (3, 36, 49). Pertussis infection or vaccination of humans gives rise to antibody responses primarily against subunit S1 (40, 51). However, monoclonal antibodies (MAbs) against subunits S1, S2, S3, and S4 are able to neutralize the biological effects of PT (14, 24, 43) and to protect against lethal infection of mice with virulent *B. pertussis* (14, 42, 43).

Identification of B- and T-cell epitopes on PT able to induce suitable protective immune responses may in the future stimulate the development of a completely or a partially synthetic pertussis vaccine. Potent B- and T-cell epitopes have been identified on PT subunits S1, S2, and S3 (4–6, 8, 10, 11, 17, 21, 22, 34, 35, 39, 45–48). Subunit S4 has until recently gained little attention. In only a single published study have synthetic peptides been used to identify B-cell epitopes on S4 (48), and only in one instance has specific T-cell reactivity to subunit S4 been demonstrated (11).

The aim of this study was to identify human T-cell epitopes on PT subunit S4. With the purpose of inducing an immune response to PT, 10 adults were vaccinated with an acellular pertussis vaccine containing detoxified PT (dPT) in addition to filamentous hemagglutinin (FHA). The antibody response to PT was directed against subunits S1, S2, S3, and S4. A proliferative response of peripheral lymphocytes to dPT, dimer S2-S4, and dimer S3-S4 was demonstrated, and the proliferative response to dPT and dimer S2-S4 was shown to be mediated by T cells of the $CD4^+$ phenotype. The specificity of the proliferative response to subunit S4 was analyzed with a range of synthetic peptides synthesized on the basis of the primary sequence of subunit S4. The proliferative response to the peptides revealed two major and one minor T-cell epitope located in the NH_2-terminal end of subunit S4.

MATERIALS AND METHODS

Donors. Six female and five male healthy adult volunteers, aged 27 to 51 years, participated in this study. The donors were assigned the letters A through K. Cord blood from six newborn infants was used.

Vaccination. Donors A through K (except E) received one dose (0.5 ml) of acellular pertussis vaccine JNIH-3 from the Japanese Institute of Health; this vaccine contains dPT (7.5 μg of protein nitrogen, FHA (7.5 μg of protein nitrogen), aluminium (0.2 mg), formaldehyde (15 μg), thiomersal (0.1 mg), and Hemacell (20 mg). The vaccine was given as a deep subcutaneous injection in the upper arm. Donor F had received one dose of the same vaccine in an earlier study. Donor E had received a whole-cell pertussis vaccine at about age 7 and was known to have peripheral lymphocytes with a high level of specific reactivity to pertussis antigens.

Antigens. (i) Purified antigens. PT and FHA were obtained from *B. pertussis* Tohama grown in modified Stainer Scholte

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medium supplemented with heptakis(2,6-*O*-dimethyl)β-cyclodextrin (18, 19). The two antigens were extracted from the fermentation culture and purified by adsorption, hydrophobic, and gel filtration chromatographies (9). PT subunits S1 and S5 and dimeric subunits S2-S4 and S3-S4 were purified by haptoglobin-Sepharose 4B and DEAE–Sepharose CL-6B chromatographies after exposure of PT to 5 M urea (9). PT subunit S4 was purified by haptoglobin-Sepharose 4B and DEAE–Sepharose CL-6B chromatographies after exposure of PT to 8 M urea (9). PT detoxified by hydrogen peroxide was a generous gift from The North American Vaccine Corp., Washington, D.C. The purity of all antigen preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. Tuberculin purified protein derivative (PPD) and tetanus toxoid (TT) were prepared at the Tuberculin Department and the Bacterial Vaccine Department, respectively, of Statens Seruminstitut, Copenhagen, Denmark.

(ii) Peptide synthesis. Solid-phase peptide synthesis was performed with the fluorenylmethyloxycarbonyl (Fmoc) strategy. The 11 peptides that were 25 or 26 residues in length, that covered the S4 subunit, and that overlapped 15 or 16 amino acids of the adjacent peptide and the 9 peptides that covered the sequences from 59 to 72, 72 to 88, 93 to 106, 18 to 28, 40 to 46, 53 to 65, 73 to 80, 84 to 92, and 101 to 110 were synthesized by the continuous-flow version of the polyamide solid-phase method (13) on a fully automated peptide synthesizer developed in our laboratory as previously described (30). The 50 12-residue peptides were synthesized on a parallel multiple-column peptide synthesizer developed in our laboratory (16, 27). Fmoc amino acid pentafluorophenyl esters and Pepsyn KA were purchased from Milligen. Dimethylformamide was distilled at reduced pressure and analyzed for free amines by the addition of 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-OH prior to use. High-pressure liquid chromatography (HPLC) was performed on a Waters HPLC system with a C_{18} reversed-phase column (Hamilton PRP-3; flow rate, 1.5 ml/min, for analytical separations), buffer A (0.1% trifluoroacetic acid), and buffer B (0.1% trifluoroacetic acid and 10% water in acetonitrile), and amino acid analyses were performed with a Waters PICOTAG system. A detailed analysis will be published elsewhere (16a). The peptide sequences that were possible B-cell epitopes were predicted by use of the Surfaceplot computer program (Synthetic Peptides Inc., Edmonton, Alberta, Canada).

(iii) Whole *B. pertussis* (WB). The Danish whole-cell pertussis vaccine, consisting of killed, phase-1 *B. pertussis* (16×10^9 cells per ml) from vaccine strains 3803, 3825, 3843, and 3860 and 0.1 mg of thiomersal per ml, was washed twice in saline before use as an antigen for in vitro stimulation.

ELISA. An enzyme-linked immunosorbent assay (ELISA) was performed with polystyrene Maxisorb microtiter plates (Nunc, Roskilde, Denmark). For PT, subunit S1, dimers S2-S4 and S3-S4, and FHA, the wells were coated with antigen in carbonate buffer (pH 9.6) (PT, 2.2 μg/ml; FHA, 2.0 μg/ml; S1, 0.9 μg/ml; S2-S4, 1 μg/ml; S3-S4, 1.7 μg/ml). The plates were incubated overnight at 4°C. The human plasma samples were prediluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 1% Triton X-100, diluted twofold in eight wells, and incubated for 2 h at room temperature. U.S. reference human pertussis antiserum (lot no. 3; Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Md.) containing 200 U of anti-PT immunoglobulin G (IgG) per ml and 200 U of anti-FHA IgG per ml was run as a standard on each plate. Horseradish peroxidase-conjugated rabbit anti-human IgG (P214; DAKO, Glostrup, Denmark) was diluted 1/1,000 in the same buffer as the plasma, and the plates were incubated for 1 h at room temperature. Following this step, substrate buffer (0.7% citric acid, 1.2% Na_2HPO_4 [wt/vol] [pH 5]) was applied to the wells and allowed to stand for 5 min. Finally, a dye-substrate solution (0.4 mg of *O*-phenylenediamine per ml, 0.04% H_2O_2) was added and allowed to react for 30 min. Color development was stopped with 1 N H_2SO_4, and the A_{490} was read. Total IgG antibodies to PT, PT components, and FHA in the test samples were calculated by a computerized parallel-line assay and expressed as units per milliliter. For TT, the wells were coated with TT in carbonate buffer (pH 9.6) (0.2 μg/ml). The plates were incubated overnight at 4°C. The human plasma samples were prediluted in PBS containing 1% BSA and 1% Triton X-100, diluted twofold in eight wells, and incubated overnight at 4°C. Human reference serum containing 8.82 IU of human anti-TT immunoglobulin (Ig) per ml was run as a standard on each plate. Horseradish peroxidase-conjugated rabbit anti-human Ig (P212; DAKO) was diluted 1/5,000 in the same buffer as the plasma, and the plates were incubated for 90 min at room temperature. Following this step, the plates were treated as for the determinations of antibodies to PT, PT components, and FHA. Total Ig antibodies to TT in the test samples were calculated by a computerized parallel-line assay and expressed as international units per milliliter.

Electrophoresis and Western blotting (immunoblotting). PT and molecular weight standards (SDS-PAGE standards, high range [161-0303] and low range [161-0304]; Bio-Rad, Richmond, Calif.) were heated to 100°C for 2 min in 0.02 M Tris buffer containing 2% (wt/vol) SDS, 20% glycerol, 0.006 M EDTA, 0.05% bromophenol blue and, for the molecular weight standards, 20 mM dithiothreitol. Electrophoresis was performed on a 15% polyacrylamide gel as described by Laemmli (23). PT was applied at 21.5 μg per gel. PT was transferred from polyacrylamide gels to nitrocellulose sheets (0.45-μm-pore size; BA 85; Schleicher & Schuell, Dassell, Germany) at a constant voltage of 50 V for 3 h in a Tris-HCl–glycine–ethanol buffer (25 mM Tris-HCl, 192 mM glycine, 20% ethanol [vol/vol/vol] [pH 9.0]). Following this step, the nitrocellulose sheets were blocked for 30 min in TBS-skim milk buffer (TBS-SKM) (20 mM Tris–500 mM NaCl [pH 7.5] with 5% skim milk). The antigen lanes were incubated overnight with either MAbs as hybridoma supernatants or plasma samples diluted in TBS-SKM at room temperature. The MAbs were designated and diluted as follows: anti-PT S1, 51.1.D3, 1/2; anti-S2, 47.3.C4, 1/2; anti-S3, 47.1, 1/10; anti-S4, 56.2.E4, 1/10; and anti-S5, 50.1.C2, 1/2. The plasma samples were diluted 1/50. After being washed, the antigen lanes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse Ig (P260; DAKO) or rabbit anti-human Ig (P212; DAKO), each diluted 1/1,000 in TBS-SKM, for 2 h at room temperature. Finally, the antigen lanes were developed with dioctyl sodium sulfosuccinate and 3,5,3′,5′-tetramethylbenzidine.

Lymphocyte stimulation test (LS test). Peripheral blood lymphocytes were obtained from heparinized (0.3% heparin per ml of blood; Noparin, 5,000 IU/ml; NOVO Industri A/S, Copenhagen, Denmark) venous blood from adult human donors or from cord blood from newborn infants, diluted 1:1 in saline, and separated by Lymphoprep (Nycomed AS, Oslo, Norway) density gradient (density = 1.077 mg/ml) centrifugation. The cells were collected at the interphase layer, washed twice in complete medium (Dulbecco's modified Eagle's medium supplemented with 1% penicillin-strep-

TABLE 1. Pre- and postvaccination levels of antibodies to PT, PT components, FHA, and TT

Antigen	Vaccination[a]	Antibody level in donor:										
		A	B	C	D	E[b]	F[c]	G	H	I	J	K
PT[d]	–	9	10	43	2	0		3	14	3	3	13
	+	1,061	1,796	1,678	153		244	799	1,142	330	96	1,402
S1[e]	–	1	5	20	3	4		2	43	3	5	3
	+	259	1,849	892	65		196	143	1,047	247	90	458
S2-S4[e]	–	11	9	33	1	2		2	4	2	4	13
	+	1,262	666	955	108		170	562	384	245	95	994
S3-S4[e]	–	14	11	37	2	5		3	8	4	4	16
	+	1,617	660	741	65		153	519	255	255	54	842
FHA[d]	–	18	23	68	39	83		7	10	17	49	13
	+	5,776	1,221	2,080	2,084		470	2,357	1,190	1,884	611	6,671
TT[f]	–	0.0	1.3	2.9	3.3	2.9		2.0	0.6	4.0	3.2	8.3
	+	0.0	0.7	3.4	3.4		5.1	2.5	0.7	3.9	3.0	8.4

[a] –, prevaccination serum sample; +, postvaccination serum sample.
[b] Donor E was vaccinated with a whole-cell pertussis vaccine at age 7.
[c] Donor F had earlier received one dose of an acellular pertussis vaccine. No prevaccination serum sample was available from this donor.
[d] IgG, determined in units per milliliter, relative to U.S. reference human pertussis antiserum (lot no. 3).
[e] IgG, determined in units per milliliters, relative to U.S. reference human pertussis antiserum (lot no. 3). Because the IgG content of U.S. reference human pertussis antiserum is defined against PT and FHA only, the content of IgG against S1, S2-S4, and S3-S4 was arbitrarily determined in units per milliliter.
[f] Ig, determined in international units per milliliter, relative to human anti-TT reference serum.

tomycin [10,000 U/ml; Seromed]), and counted. The cells were cultured in flat-bottomed microtiter plates (Nunc), at 10^5 cells per well, in a volume of 200 μl of complete medium containing 10% human AB serum without antigen (control), with 1 μg of phytohaemagglutinin (PHA) (HA 17; Wellcome, Beckenham, United Kingdom) per ml as a positive control for cell reactivity and viability, or with antigen. All tests were carried out in triplicate. Cultures were incubated for 7 days at 37°C in an atmosphere of 5% CO_2 in humidified air. At 22 h prior to harvest, 0.25 μCi of [^{3}H]thymidine (TRA 120; Radiochemical Centre, Amersham, United Kingdom) was added to each well. The cells were harvested on fiberglass paper, and the incorporated radioactivity was measured in a liquid scintillation counter. The proliferative responses were expressed in counts per minute.

Depletion of CD4$^+$ and CD8$^+$ T cells. Suspensions of peripheral lymphocytes were depleted of CD4$^+$ and CD8$^+$ T cells by treatment with Dynabeads M-450 CD4 (111.06; Dynal, Oslo, Norway) and Dynabeads M-450 CD8 (111.08; Dynal), respectively. In brief, the cells were isolated as described for the LS test, and washed in PBS-HS (PBS [pH 7.4] containing 2% human AB serum [vol/vol]). The beads were washed in PBS-HS before use. Cells and beads were mixed in a cell/bead ratio of 1:10 and incubated for 30 min on ice with gentle agitation. Cell depletions were performed on a magnetic particle concentrator (120.02; Dynal) for 2 min. The remaining cells were washed in complete medium and used for antigen stimulation in the LS test.

Tissue typing. HLA class II typing was performed essentially as described by Bidwell et al. (7) with the DNA typing system restriction fragment length polymorphism (RFLP).

Sequence analysis of peptides. The primary sequence of subunit S4 as well as those of peptides 1 and 4 were analyzed by use of Sequence Analysis Software Package Version 7.0 from the University of Wisconsin Genetics Computer Group (12).

Statistical methods. To evaluate the results of LS tests with the 25- or 26-residue peptides, we performed an analysis of variance with peptides (m = 11), experimental subjects (m = 11 donors), and dilution levels (m = 3) of peptides as factors. On these bases, least-square estimates and confidence intervals for peptide stimulation levels were calculated, allowing a comparison of peptide stimulation levels. The proliferative responses to the S2-S4 and S3-S4 dimers were compared by an analysis of variance. Index values (counts per minute in antigen-stimulated cultures/counts per minute in nonstimulated cultures) were used as the bases for all calculations.

RESULTS

Antibody responses. To induce an immune response to PT, we immunized 10 human adult volunteers with Japanese acellular pertussis vaccine JNIH-3, containing dPT, formaldehyde, and FHA. Samples of peripheral blood were drawn immediately before and 2 weeks after the vaccination. In addition, a donor known to have peripheral lymphocytes with a high level of specific reactivity to pertussis antigens but not to have been recently vaccinated against pertussis (donor E) participated in this study. Table 1 shows the quantitative antibody response to PT, PT subunit S1, PT dimers S2-S4 and S3-S4, FHA, and TT, as measured by an ELISA and calculated relative to U.S. reference human pertussis antiserum (PT components and FHA) or human anti-TT reference serum. It should be noted that the specific IgG content of the U.S. reference human pertussis antiserum is defined against PT and FHA only. For convenience, the content of specific IgG against S1, S2-S4, and S3-S4 is defined as 200 U/ml in this study. The vaccination induced a marked antibody response to PT, S1, and the dimers for donors A, B, C, H, and K and to FHA for all vaccinated donors, except for donor J. Donors D, E, F, G, I, and J reacted with an intermediate to low or no antibody response to PT and its components. The levels of antibody to TT were not affected by the vaccination, demonstrating the specificity

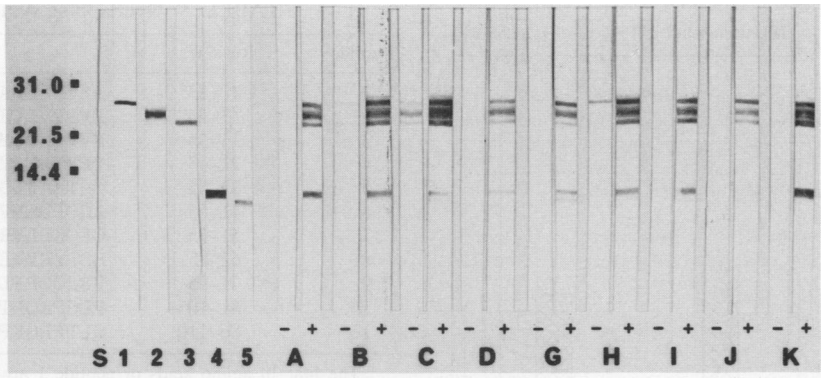

FIG. 1. Specificity of the antibody response to PT in sera from nine donors vaccinated with an acellular pertussis vaccine. Lanes: 1 to 5, MAbs to PT subunits S1 to S5; −, prevaccination sera; +, postvaccination sera. Numbers at left indicate molecular mass.

of the immune response. The antibody response to purified S4 and S5 was likewise tested in an ELISA with pre- and postvaccination sera, but only a negligible or no response to these subunits could be detected in either group.

Western blotting of pre- and postvaccination sera. To test the possibility that the weak responses to the S4 and S5 subunits in the ELISA were due to conformational changes in the two subunits caused by immobilization on the ELISA plates, we performed Western blotting with pre- and postvaccination sera from nine of the vaccinated donors with native PT as the antigen (Fig. 1). MAbs against the PT subunits were applied to ensure the presence and positions of the subunits. These MAbs also recognized the PT subunits in the ELISA reported above. In all donors, an antibody response to subunits S1, S2, S3, and S4 was generated by the vaccination, except for donor J, in whom no reactivity to subunit S4 was detected. In donors C and G, a weak response to subunit S5 was also detected.

Proliferative response to PT, PT subunits, and FHA. Peripheral lymphocytes isolated from all of the participating donors were stimulated in vitro with PHA, PT detoxified by hydrogen peroxide, the PT dimers, FHA, WB, TT, and PPD (Table 2). The peripheral lymphocytes from all of the donors showed a proliferative response to PHA, TT, and PPD, indicating that the lymphocytes were viable and able to show an antigen-specific proliferative response under the culture conditions chosen. Lymphocytes from donors A, B, C, E, and H showed a strong proliferative response to dPT, whereas the proliferative response of the remaining donors was weak. All of the donors showed a vigorous reaction to the S2-S4 dimer. The response to the S3-S4 dimer was significantly weaker ($P < 0.02$) than the response to the S2-S4 dimer. The response to FHA was strong for all of the donors, except for donors I and K.

Depletion of $CD4^+$ and $CD8^+$ T cells. To identify the subpopulation of T cells that proliferated in response to dPT and dimer S2-S4, we depleted the peripheral lymphocytes from donors A, B, and C of $CD4^+$ or $CD8^+$ T cells or left them untreated (controls) and stimulated them in the LS test with dPT or dimer S2-S4 (Fig. 2). The specific antigen response was abrogated by depletion of the $CD4^+$ population. The depletion had no effect on the response to PHA. Depletion of the $CD8^+$ population had no effect on the response to dPT, dimer S2-S4, or PHA. On the basis of these results, it was concluded that the cells proliferating in response to dPT and dimer S2-S4 were T cells of the $CD4^+$ phenotype.

Synthetic peptides. With the purpose of studying the proliferative response to subunit S4 in detail, we synthesized a range of synthetic peptides on the basis of the primary sequence of subunit S4 (25, 31): (i) 50 peptides 12 amino acid residues in length, covering the entire S4 subunit sequence, and overlapping 10 amino acids of the adjacent peptide; (ii) 11 peptides 25 or 26 amino acid residues in length, covering the entire S4 subunit sequence, and overlapping 15 or 16 amino acids of the adjacent peptide (Table 3); (iii) 3 peptides predicted to be putative helper T-cell epitopes by the computer algorithm developed by Margalit et al. (26) (these

TABLE 2. In vitro proliferative response of peripheral lymphocytes to purified antigens

Antigen (μg/ml)	Proliferative response (mean cpm)[a] in donor:											Common SE[b]
	A	B	C	D	E	F	G	H	I	J	K	
Control	354	508	167	119	221	100	114	371	142	182	322	135
PHA (1)	125,972	51,222	80,018	99,293	71,739	103,191	50,892	89,706	97,273	91,431	67,907	8,020
dPT (10)	5,466	3,786	6,252	390	5,573	629	972	7,062	163	318	1,223	855
S2-S4 (5)	31,928	65,576	64,529	50,096	54,578	22,670	21,344	12,391	12,950	26,195	32,326	11,387
S3-S4 (5)	13,378	8,363	12,657	5,566	14,556	2,391	4,743	9,928	172	932	1,231	3,165
FHA (10)	14,658	15,656	10,958	2,782	16,365	8,707	3,855	12,585	445	4,247	4,457	4,136
WB[c]	12,628	23,027	14,836	2,927	33,556	15,904	4,943	8,233	881	4,715	4,740	2,794
TT (20)	21,899	21,259	6,411	4,718	44,741	3,961	2,782	2,756	1,848	20,068	22,265	2,301
PPD (1)	6,145	20,576	21,533	31,167	42,954	11,146	40,948	27,112	622	13,696	18,144	4,345

[a] From three independent experiments.
[b] For each antigen, calculated by an analysis of variance.
[c] 12.5×10^7 bacteria per ml.

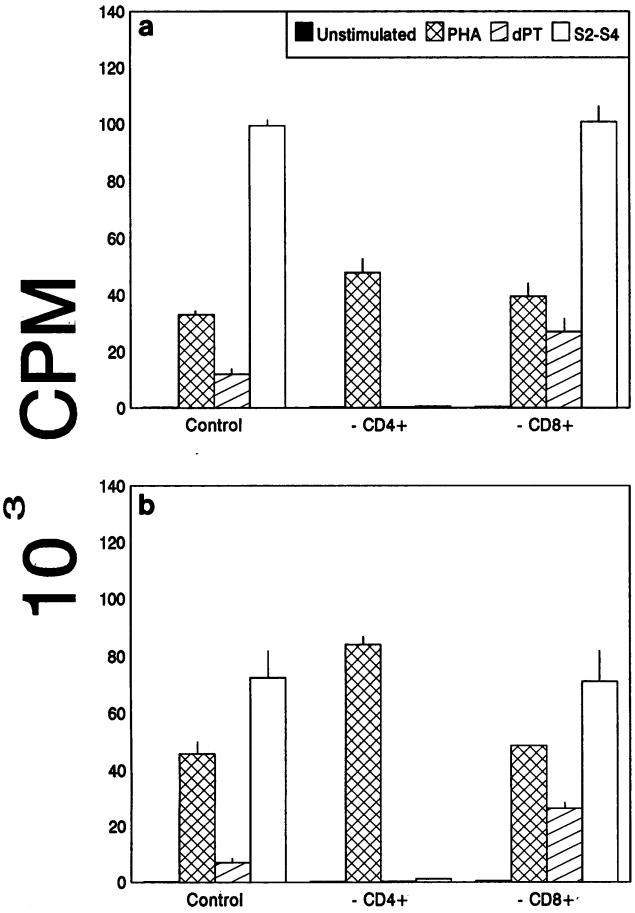

FIG. 2. Proliferative response of untreated (control) or $CD4^+$ (− CD4+)- or $CD8^+$ (− CD8+)-depleted cultures to stimulation with PHA, dPT, or PT dimer S2-S4. (a) Donor B. (b) Donor C. The experiments were performed two or three times for donors A, B, and C and revealed essentially similar results. Thin bars show standard errors for triplicate cultures.

peptides covered the sequences from 59 to 72, 72 to 88, and 93 to 106); and (iv) 6 peptides predicted to be putative B-cell epitopes by the Surfaceplot computer program (these peptides covered the sequences from 18 to 28, 40 to 46, 53 to 65, 73 to 80, 84 to 92, and 101–110).

Proliferative response to synthetic peptides. Peripheral lymphocytes from the 11 donors were stimulated in vitro with the synthetic peptides at concentrations of 50, 10, and 1 μg/ml. None of the 50 different 12-residue peptides, the 3 computer-predicted T-cell epitopes, or the 6 computer-predicted B-cell epitopes showed any reproducible proliferative response when used for in vitro stimulation in the LS test (results not shown). However, a proliferative response was found following stimulation with the 25- or 26-residue peptides. Since these peptides were found to differ in dose optima, we chose to depict the results obtained with all three in vitro concentrations (Fig. 3).

Lymphocytes from all of the donors showed a proliferative response to peptide 4, and in 10 of the 11 donors a proliferative response to peptide 1 was also observed. In donors A, E, F, I, and K, the strongest response was to peptide 4, and in donors C, D, G, and H, the strongest response was to peptide 1. The remaining donors showed equivalent reactions to the two peptides. Donors A, B, C, D, H, and J showed a stronger response to peptide 6 than to peptide 5. A statistical analysis including the 11 donors, the 11 peptides, the three dilution levels for the peptides, and the three individual experiments performed for each donor showed that the responses to peptides 1 and 4 were significantly higher ($P < 0.05$) than those to the other peptides. The response to peptide 6 was likewise significantly ($P < 0.05$) higher than the responses to the other peptides, except for peptides 1 and 4. These results indicate the existence of two dominating T-cell epitopes on PT subunit S4, located near peptides 1 and 4, and a minor T-cell epitope, located near peptide 6 and separate from the major epitope located near peptide 4.

TABLE 3. Overlapping synthetic peptides

Peptide	Residues	Sequence
1	−10–15[a]	AMTHLSPALADVPYVLVKTNMVVTS
2	1–26	DVPYVLVKTNMVVTSVAMKPYEVTPT
3	11–35	MVVTSVAMKPYEVTPTRMLVCGIAA
4	21–45	YEVTPTRMLVCGIAAKLGAAASSPD
5	31–55	CGIAAKLGAAASSPDAHVPFCFGKD
6	41–65	ASSPDAHVPFCFGKDLKRPGSSPME
7	51–75	CFGKDLKRPGSSPMEVMLRAVFMQQ
8	61–85	SSPMEVMLRAVFMQQRPLRMFLGPK
9	71–95	VFMQQRPLRMFLGPKQLTFEGKPAL
10	81–105	FLGPKQLTFEGKPALELIRMVECSG
11	86–110	QLTFEGKPALELIRMVECSGKQDCPA

[a] The first 10 amino acids of peptide 1 are leader peptide residues, not expected to be present in the native S4 molecule.

The proliferative responses to the purified antigens and the synthetic peptides are antigen specific. Since purified *B. pertussis* antigens may contain trace amounts of PT, which is mitogenic for T and B cells, and synthetic peptides theoretically may be mitogenic or act as superantigens, it was necessary to investigate whether the proliferative responses were antigen specific. Therefore, cord blood lymphocytes isolated from newborn infants were stimulated in vitro with the panel of purified antigens and synthetic peptides (Fig. 4). Except for WB and TT, none of the purified antigens or the synthetic peptides gave rise to significant proliferative responses of cord blood lymphocytes, thereby excluding mitogenicity.

Tissue typing of the donors. The 11 participating donors were tissue typed with respect to HLA-DR-B, HLA-DQ-A, and HLA-DQ-B (Table 4). Most donors were, as expected, found to differ with respect to tissue type, except for donors H and J, who appeared to possess identical tissue types. These two donors, however, differed in their responses to the synthetic peptides. The stimulation results obtained with this panel of donors indicated that peptides 1 and 4 were able to act as antigens in the context of a relatively broad range of HLA types.

Sequence similarity of subunit S4, peptide 1, and peptide 4 with other proteins. If synthetic peptides are to be used in complete or partially synthetic vaccines, it is of importance to know whether they will cross-react with other antigens, especially human tissue antigens. The sequences of subunit S4, peptide 1, and peptide 4 were therefore tested by a computerized search of an international data base for similarity with other published protein sequences (results not shown). No similarities between subunit S4 and other proteins were found. The sequences of peptide 1 and peptide 4 showed up to 70 and 67% similarities, respectively, with the sequences of other proteins. However, the positions of the

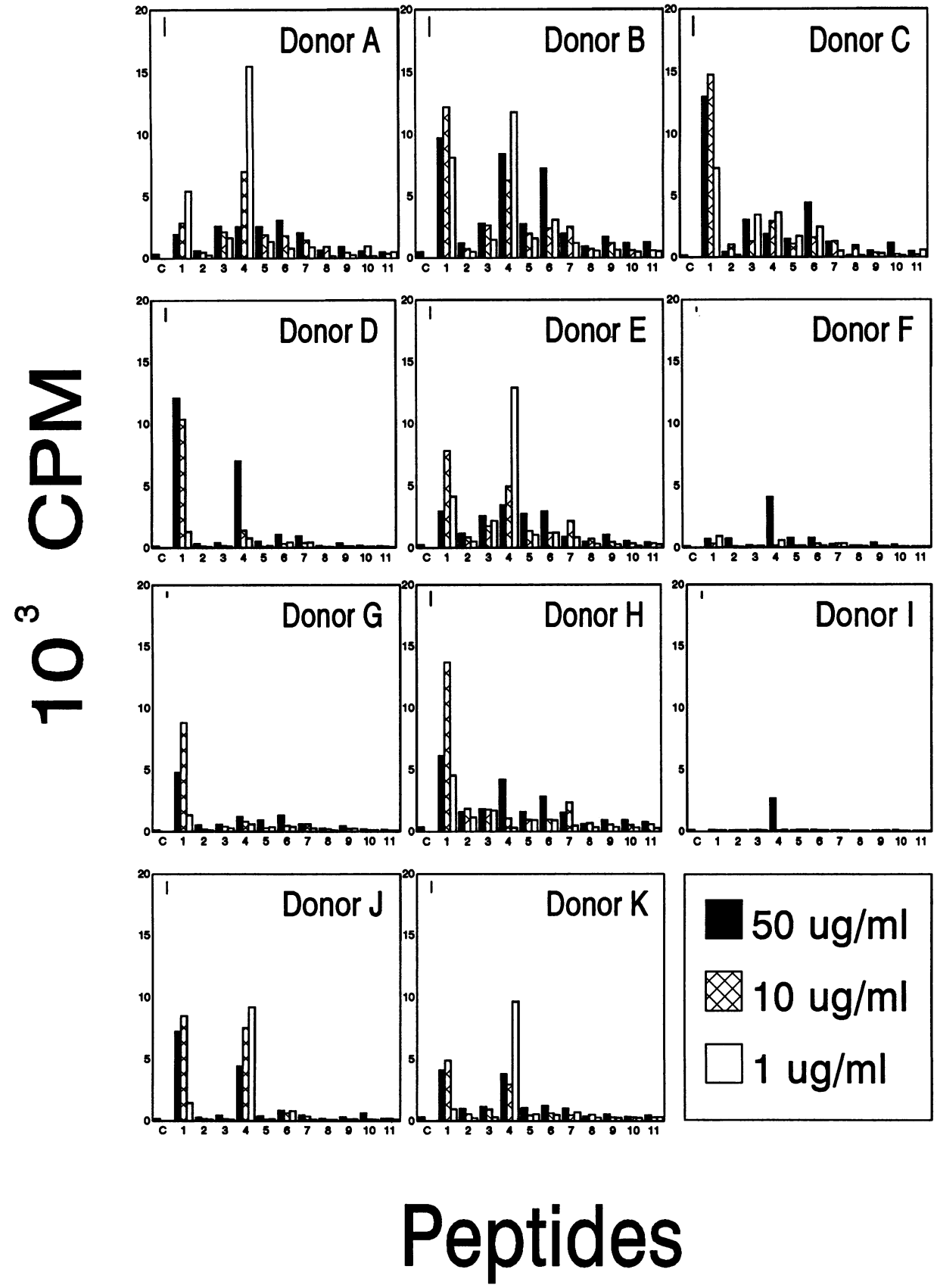

FIG. 3. Proliferative response to synthetic peptides of peripheral blood lymphocytes isolated from donors A to K. For vaccination details, see Materials and Methods. C, unstimulated cultures. The results shown are the mean counts per minute for three independent experiments. Thin bars in the upper left corner of each panel show the common standard error, calculated by an analysis of variance.

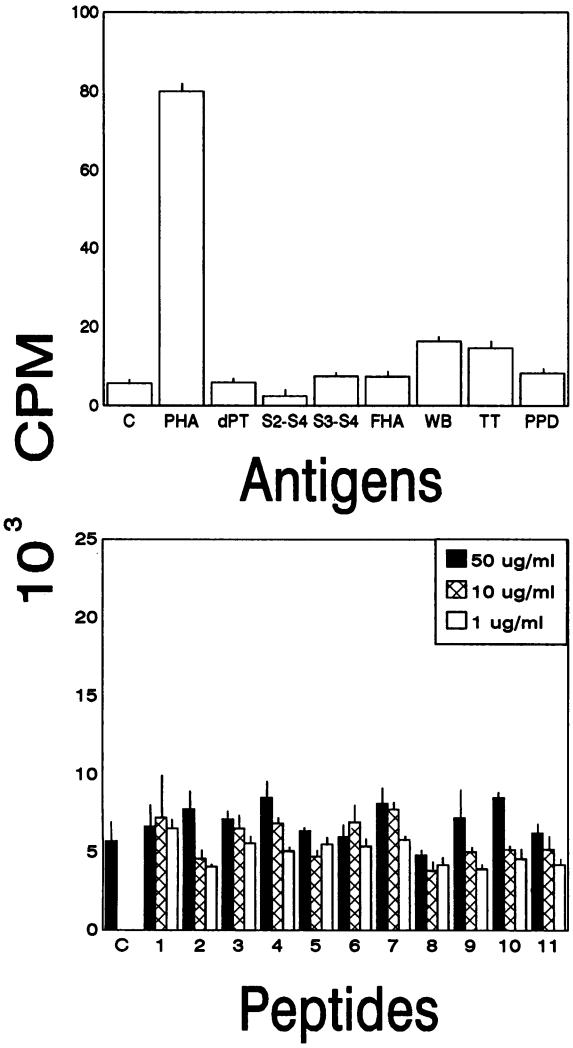

FIG. 4. Proliferative responses of cord blood lymphocytes to purified antigens and synthetic peptides. For concentrations of purified antigens, see Table 2. The results shown are representative of three (antigens) or six (peptides) independent experiments. C, unstimulated cultures. Thin bars show standard errors for triplicate cultures.

homologous amino acid residues were scattered over the 25-residue lengths of the peptides, and in no instances were the similarities found in a stretch of more than 4 amino acid residues.

DISCUSSION

The development of a synthetic vaccine against pertussis requires the identification of B-cell epitopes for antibody synthesis and T-cell epitopes that might serve as carriers for B-cell epitopes or inducers of cell-mediated immunity. Recently, several studies focused on the identification of T- and B-cell epitopes on the subunits of PT. Potent B-cell epitopes (4–6, 8, 10, 17, 21, 35, 39) as well as T-cell epitopes (10, 11, 22, 34, 35) have been identified on subunit S1. A few studies have identified B-cell epitopes on subunits S2 and S3 (45–47), and T-cell reactivity to subunits S2 and S3 also has been shown (11). Until now, subunit S4 has gained little attention. To our knowledge, only one study has used synthetic peptides based on the primary sequence of subunit S4 for the identification of B-cell epitopes (48). Another study has shown T-cell reactivity to subunit S4 (11).

TABLE 4. Tissue typing of donors

Donor	HLA tissue type[a]		
	DR-B	DQ-A	DQ-B
A	11; 15	1B; 3B	1B; 3B
B	1; 11	1A; 3B	1A; 3B
C	7.2; 14A	1A; 3	1A; 2B
D	4; 7.2	3	2B; 3B
E	8; 11	3B; 4	3A; 3B
F	4; 9	3	3A
G	15; 17.2	1B; 2	1B; 2B
H	13A3; 15	1B	1A; 1B
I	4; 7.2	3	2B; 3B
J	13A3; 15	1B	1A; 1B
K	4; 7.2	3	3A

[a] The donors were tissue typed with the DNA typing system RFLP (7).

The aim of the present study was to identify T-cell epitopes on the S4 subunit, with the long-range purpose of using this knowledge in the future formation of synthetic vaccines against pertussis.

In an earlier study (37), specific antibodies and proliferative responses of peripheral lymphocytes to PT were induced in adult humans by vaccination with Japanese acellular pertussis vaccine JNIH-3, containing dPT, formaldehyde, and FHA. In the present study, we used a similar vaccination schedule with the purpose of inducing an immune response to PT. The vaccination was found to induce humoral immunity to PT and FHA. The antibody response to PT was directed against subunits S1, S2, S3, and S4. Anti-subunit S4 antibodies were not reported by Thomas et al. (51) and Redhead (40), a result that might have been due to differences in the immunogens used (whole-cell vaccine or infection versus acellular vaccine).

Stimulation of peripheral lymphocytes from the donors with purified dPT and dimers S2-S4 and S3-S4 resulted in a proliferative response for most of the donors, and the responses to dPT and dimer S2-S4 were shown to be mediated by T cells of the $CD4^+$ phenotype. Although the PT in the vaccine was detoxified by formaldehyde, PT detoxified by hydrogen peroxide was chosen as an antigen for in vitro stimulation, since it has been shown that PT detoxified by formaldehyde is less suitable as an in vitro antigen (37). The fact that all of the donors showed a reaction to dimer S2-S4 but not to dPT can be explained by the partial destruction of T-cell epitopes on dPT during the detoxification process. As a whole, these results therefore indicate that the proliferative responses of the peripheral lymphocytes from the participating donors to PT components were mediated by T cells of the $CD4^+$ phenotype.

To identify T-cell epitopes on subunit S4, we synthesized a series of overlapping synthetic peptides on the basis of the primary sequence of subunit S4 (25, 31). The results of the in vitro stimulation with the synthetic 25- or 26-residue peptides indicate the existence of two major human T-cell epitopes, located near the sequences corresponding to peptide 1 and peptide 4, and a minor T-cell epitope, located near the sequence corresponding to peptide 6. The T-cell epitope located near peptide 1 must be contained in the last 15 amino acids of this peptide, since the first 10 amino acids were deduced to be the leader peptide. Surprisingly, peptide 2,

which also contains these 15 amino acids, resulted in only a minor proliferative response. This result indicates that a synthetic peptide not only may be too short but also may be too long to function as an antigen for T cells.

The response to dPT and dimer S2-S4 was solely mediated by $CD4^+$ T cells. This result suggests that the response to the synthetic peptides may also be mediated by T cells of this phenotype. However, differences in the processing of the peptides, dPT, and S2-S4 may activate different T-lymphocyte populations.

The fact that we found no proliferative response to the 12-residue peptides, several of which covered parts of the designated T-cell epitopes, is very likely explained by the length of the peptides, 12 amino acids in some cases being too few to make up a T-cell epitope (20). The three peptides predicted to be T-cell epitopes by the computer algorithm were all located in the COOH-terminal end of the subunit S4 sequence. This end of the sequence seems to be devoid of T-cell epitopes when the overlapping synthetic peptide approach is used. The six peptides predicted to be B-cell epitopes probably were too short to function as T-cell epitopes. This suggestion explains the lack of response to the computer algorithm-predicted peptides.

To rule out the possibility that the proliferative response to the purified *B. pertussis* antigens was due to trace amounts of PT, which is mitogenic for human T cells (29), or that the response to the synthetic peptides was due to mitogenicity or the peptides acting as superantigens (28), we used cord blood lymphocytes as responder cells in vitro. Cord blood lymphocytes are the only human lymphocytes that, with certainty, can be considered not to be immune to *B. pertussis* antigens. In an earlier study, we showed that cord blood lymphocytes are fully reactive to the mitogenic effects of PT (37). Stimulation of cord blood lymphocytes with the purified *B. pertussis* antigens clearly showed that none of the purified antigens or synthetic peptides used in this study induced any nonspecific proliferative response. The specificity of the response to the PT components is further supported by the fact that depletion of $CD4^+$ cells abrogated the antigen-specific response but had no effect on the mitogenic response to PHA.

If short peptides are to be considered suitable as candidates for a synthetic vaccine, they must be recognized by a broad range of human major histocompatibility complex class II types. Typing of the participating donors with respect to HLA-DR and HLA-DQ revealed, as expected, that the donors represented a range of different tissue types. Donors H and J had identical tissue types but differed in their reactivity to the synthetic peptides. This result may be explained by the influence of genes outside the major histocompatibility complex, e.g., the T-cell receptor gene complex. The fact that peptide 1 and peptide 4 in combination were able to stimulate a proliferative response in all the participating donors implies that these two peptides are candidates in further studies for suitability as components of a synthetic pertussis vaccine. In this context, it is important that cross-reactivity with human tissue proteins can be ruled out. No homology between the sequence of subunit S4 and the published sequences of other proteins was found.

These results raise the possibility that peptide 1 and/or peptide 4 coupled to relevant B-cell epitopes can function as carriers in a future completely or partially synthetic pertussis vaccine. The use of pertussis antigens as carriers would be of great advantage, since pertussis infections would probably boost the memory cells specific for the carrier peptides. Choosing T-cell epitopes from subunit S4 would further provide the advantage that each PT molecule presents two identical epitopes to the immune system (50), whereas the other subunits only present one epitope. In ongoing studies, we are investigating the possibility of using peptides 1 and 4 as carriers for B-cell epitopes in the murine system.

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