MHC-RESTRICTED RECOGNITION OF IMMUNOGENIC T CELL EPITOPES OF PERTUSSIS TOXIN REVEALS DETERMINANTS IN MAN DISTINCT FROM THE ADP-RIBOSYLASE ACTIVE SITE

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Pertussis toxin (PT),[1] a protein released by *Bordetella pertussis* organisms, is a major virulence factor in whooping cough (1). A wide variety of biological effects have been attributed to the toxin, which include lymphocytosis, histamine release, insulin secretion, stimulation of IgE production, and adjuvanticity (2–4). An mAb to PT protects mice challenged intracerebrally (i.c.) with virulent B. pertussis (5). A toxoided PT was therefore selected to be one of the two main component of an acellular whooping cough vaccine, now in clinical trials (6). However, the toxin is thought to be responsible for the harmful side effects associated with the current vaccine. There is speculation that active toxin present in whole cell vaccine, and perhaps chemically toxoided PT in acellular vaccine, may cause rare but serious reactions including a state of hypotonia and hyporesponsiveness, convulsions, and encephalopathy (7, 8).

The genes coding for the five subunits of PT have been cloned and sequenced (9, 10). It was proposed that it may be possible to develop a safe but nonreactogenic vaccine by genetic manipulation of the toxin gene. We have recently demonstrated that site-specific mutants of the S1 subunit of PT were deficient in pathogenic activities associated with *B. pertussis* including leucocytosis, potentiation of a shock-like state with convulsions, and stimulation of histamine sensitivity and adjuvanticity (11). However, genetic constructs of PT with an insertion or deletion of a site from residues 140–142 in the S1 subunit, the probable active ADP-ribosylase site, were reduced in their protective capacity in the i.c. challenge test in mice (11). We have investigated whether the immune response to the S1 subunit in man is directed to sites distinct from the ADP-ribosylation locus.

T lymphocytes recognize fragments of native immunogen, termed epitopes, physically bound to elements of MHC proteins. The empirical analysis of peptide epitopes that were recognized by T cells reveals that they share several common features. Accordingly, a number of investigators proposed different models to predict

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[1] *Abbreviations used in this paper:* HLA, human leukocyte antigens; i.c., intracerebral challenge; PT, pertussis toxin.

the immunodominant epitopes of an antigen. DeLisi and Berzofsky postulated that helper T cells preferentially recognize amphipathic helices (12). Rather than consider secondary structure, Rothbard and his collegues observed that a large percentage of antigenic sites contain a linear pattern composed of a charged residue or a glycine followed by two hydrophobic residues (13). We have recently used the Rothbard algorithms to detect the encephalitogenic domains of myelin basic protein causing autoimmune encephalomyelitis in mice (14), and the immunogenic domains of acetylcholine receptor in patients with myasthenia gravis (15). Synthetic peptides corresponding to chosen epitopes are capable of eliciting a specific immune response to the intact native protein. Consequently, it is feasible to use synthetic peptide vaccines to induce protective immunity against infections by microorganisms. In the present work we use two alogrithms to predict antigenic sites in the S1 subunit of the PT molecule, and analyze the HLA restriction elements in the response patterns.

Materials and Methods

Synthetic Peptides. Peptides were synthesized by solid-phase technique (16) on a peptide synthesizer fabricated at SRI International (Menlo Park, CA), starting with commercially available *t*-Boc amino acid polystyrene resin and *t*-Boc protected amino acids with the following side-chain protecting groups: O-benzyl esters for Asp and Glu, O-benzyl ethers for Ser and Thr, tosyl for Arg and His, *p*-methoxybenzyl for Cys, ortochlorobenzyloxycarbonyl for Lys, and 2,6-dichlorobenzyl for Tyr. All couplings were performed using 2.5 molar excess of *t*-Boc amino acid and dicyclohexyldocarbodiimide (DCC) over the number of milliequivalents of amino acid on the resin. In the case of Asn and Gln, a 2.5 molar excess of *N*-hydroxybenzotriazole (HOBT) was added. If the peptides had His in their sequence, then for Asn and Gln, active ester (*p*-nitrophenyl) couplings were performed. All the couplings were monitored by the ninhydrin test. 40% TFA/dichloromethane containing 0.1% indole was used for Boc deprotection. After synthesis, the peptides were cleaved from the resin using anhydrous hydrofluoric acid (HF) in HF-Reaction Apparatus Type II (Peninsula Laboratories, Inc., Belmont, CA). The peptides were separated from the various organic side products by extraction with ether and isolated from the resin by extraction with 50% acetic acid, diluted, and lyophilized. Crude peptides were purified by gel filtration on Sephadex LH-20. Purity of the peptides was checked by analytical reverse-phase HPLC and amino acid analysis.

Subjects. PBL of healthy adult volunteers previously immunized with the whole cell *B. pertussis* vaccine were isolated by centrifugation over Histopaque gradients (Sigma Chemical Co., St. Louis, MO). HLA-DR, DQ typing was performed on nylon-wool T cell-depleted, B cell-enriched lymphocytes by the National Institutes of Health standard complement-dependent extended microcytotoxicity technique.

Proliferation Assay. PBL from HLA-DR, DQ typed donors were cultured with different concentrations of the synthetic peptides in round-bottomed microtiter plates at a concentration of 2×10^5 cells in 0.2 ml of culture medium. Culture medium was composed of RPMI 1640 (Mediatech, Washington, DC) supplemented with 20% heat-inactivated pooled human serum (Irvine Scientific, Santa Ana, CA), 2 mM L-glutamine, and antibiotics. After 72 h, cultures were pulsed with [^{3}H]thymidine (1 mCi/well, sp act 5 Ci/mM). 18 h later the cells were harvested and thymidine incorporation was measured in a liquid scintillation counter.

Preparation of APCs and T Lymphocytes. APCs were prepared from PBL as described by Freundlich and Avdalovic (17). Briefly, the method involves the incubation for 120 min and adherence of cells bearing receptors for fibronectin to plastic petri dishes coated with gelatin and autologous plasma. An enriched T lymphocyte population was obtained by passage of the fibronectin-nonadherent cells through a nylon wool column. Using a panel of mAbs we were able to show that the fibronectin adherent cells are positive for HLA class I and class II antigens and do not express T cell markers (18).

Antibody Blocking Experiments. Fibronectin adherent APCs or nonadherent T cells were incubated for 60 min with various concentrations of the different mAbs. Cells were exten-

sively washed before coculture. Blocking was determined by the percent of the response obtained in the presence of antigen plus mAb, divided by the response with antigen alone. The following mAbs were used: L243, an anti-HLA-DR (mouse IgG2a) reactive with a monomorphic HLA-DR epitope; SK10, an anti-HLA-DQ (Leu-10) (mouse IgG1), which recognizes a common polymorphic epitope on DQw 1 and DQw 3 molecules; B7/21, an anti-HLA-DP (mouse IgG1), which recognizes a monomorphic epitope present on DP molecules of cells expressing DP1, DP2, DP3, DP4, or DP5; and anti-CD4 (Leu-3a) (Becton Dickinson & Co., Mountain View, CA). Polyclonal rabbit anti-PT was obtained by consecutive injection of PT (islet-activating protein; List Biological Laboratories, Campbell, CA) in CFA. Rabbits were injected intramuscularly with 3 ml of the emulsified antigen (200 ng/ml). Immunization was repeated after 1 mo, and rabbits were bled 2 wk later. Boosting and bleeding cycle was repeated after a 3-wk resting period.

Bromodeoxyuridine and Light Treatment. Responder lymphocytes (10^7/flask) and X-irradiated stimulator lymphocytes (10^7/flask) were cocultured at 37°C, 5% CO_2 in 20 ml of culture medium. 2 mg/ml of 5-bromo-2'-deoxyuridine (Brd Urd; Sigma Chemical Co.) were added to the cultures at 48 h. At 72 h the cultures were illuminated for 180 min by a fluorescent light source to eliminate alloreactive clones (19). This procedure diminished the specific allogeneic response by ~75-85%. The surviving clones were then incubated with peptide primed APC to test genetic restriction. It should be noted that the ability of these cells to respond to PHA and nonrelevant allogeneic stimulation was not compromised by the treatment.

Results

T cells recognize linear sequences of 8-12 amino acids within native protein molecules. The peptide fragments are bound to HLA molecules, and the T cell recognizes the peptide plus HLA in a three-body interaction. Two predictive templates were used to generate potential T cell epitopes of PT. One algorithm used criteria for predicting regions that would react with antibody (20). Those linear peptides most likely to elicit B cell responses are in regions of high flexibility. T cell epitopes are frequently near the B cell sites (21, 22). These regions include the NH_2-terminal portion of the protein and regions of predicted β turns (23, 24) (residues 44-54, 64-75, 87-98, 104-116, and 206-218). Thus, use of this algorithm might predict some potential T cell epitopes that overlap B cell epitopes. The second method for predicting T cell epitopes involves sequences with a charged amino acid or glycine, followed by two or three hydrophobic amino acids, and then a polar amino acid or glycine (13) (residues 133-146, 151-161, 169-179, 180-190, 223-233).

Based on the above criteria the peptides detailed in Table I were synthesized. Healthy

TABLE I

PT (Subunit 1) Peptides Used for T Cell Stimulation

Residues	Peptide
44-54	NH_2-Arg-Tyr-Asp-Ser-Arg-Pro-Pro-Glu-Asp-Val-Phe-COOH
64-75	NH_2-Asp-Asn-Val-Leu-Asp-His-Leu-Thr-Gly-Arg-Ser-Cys-COOH
87-98	NH_2-Thr-Ser-Ser-Ser-Arg-Arg-Tyr-Thr-Glu-Val-Tyr-Leu-COOH
104-116	NH_2-Glu-Ala-Val-Glu-Ala-Glu-Arg-Ala-Gly-Arg-Gly-Thr-Gly-COOH
206-218	NH_2-Arg-Ala-Asn-Pro-Asn-Pro-Tyr-Thr-Ser-Arg-Arg-Ser-Val-COOH
133-146	NH_2-Gly-Ala-Ala-Ser-Ser-Tyr-Phe-Glu-Tyr-Val-Asp-Thr-Tyr-Gly-COOH
151-161	NH_2-Arg-Ile-Leu-Ala-Gly-Ala-Leu-Ala-Thr-Tyr-Gln-COOH
169-179	NH_2-Arg-Ile-Pro-Pro-Glu-Asn-Ile-Arg-Arg-Val-Thr-COOH
180-190	NH_2-Arg-Val-Tyr-His-Asn-Gly-Ile-Thr-Gly-Glu-Thr-COOH
223-233	NH_2-Gly-Thr-Leu-Val-Arg-Met-Ala-Pro-Val-Ile-Gly-COOH

adult volunteers previously immunized with *B. pertussis* vaccine as children were tested. It was of interest to determine the efficiency of the two different algorithms used to predict the immunogens. Fig. 1 illustrates a representative dose-response experiment in which PBL of donor JO (HLA-DR 3,8; DQw 2) responded to 3 of the 5 peptides selected by the first algorithm (Fig. 1 *A*), and to 2 of the 5 peptides that were selected by the Rothbard rules (Fig. 1 *B*). It should be noted that p64-75 (Fig. 1 *A*) includes a charged, hydrophobic, hydrophobic, glycine amino acid sequence (MLTG). The efficiency of the two methods is relatively similar and demonstrates the complex immunological properties of the PT molecule.

Individual responses to various peptides differed markedly (Fig. 2). Each individual could be stimulated by more than one peptide, but none of the peptides elicited a response in the entire panel of responders. However, it was possible to show that by simultaneous stimulation with the combination of two peptides, p64-75 and p151-161, it is possible to cover the entire responder panel.

For a peptide to be considered as a putative vaccine, homology with other antigens, including self antigens, must be considered (25). In a computerized search for similarities with other published protein sequences, homology was found between p151-161 and DNA-directed RNA polymerase and gas vesicle protein of Calothrix species:

DNA-directed RNA polymerase	602-DILS I PLV I YQ
PT	151-RILAGALATYQ
Gas vesicle protein	44-RIV I ASVETYL

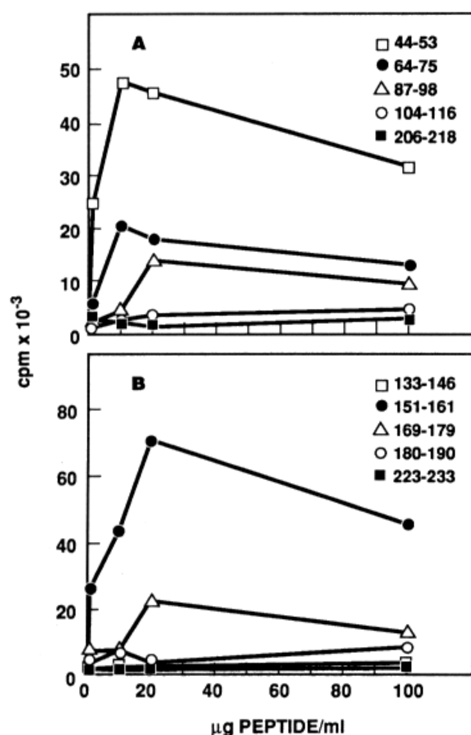

FIGURE 1. Dose-response to PT peptides chosen according to the (*A*) algorithm for high antigenic index, or (*B*) by a sequence described by Rothbard and Taylor (13) (*B*). Each point represents the mean of three different triplicate determinations, and SD values never exceeded the 15% of the mean cpm.

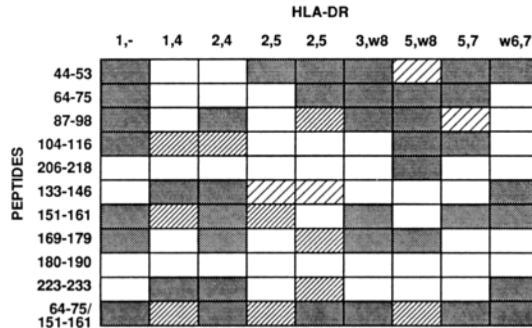

FIGURE 2. Lymphocyte proliferation indices from nine normal HLA-DR typed individuals to the PT peptides at 2, 5, 10, 20, and 100 μg/ml. Results are given for the peptide concentration that elicited the greatest proliferation, which was usually 10 or 20 μg/ml. Background cpm (no peptide) ranged from 150 to 2,500 cpm. High responders are indicated as (▤, SI > 15); intermediate responders (▨, SI > 6 and < 15); low responders (▨, SI > 3 and < 6); no responders (□, SI < 3).

Because individuals who share HLA genotypes would not be expected to share TCR genotypes since they segregate on different chromosomes, it is not surprising that even individuals sharing HLA types might differ in their response to specific peptides (26). We noted, for example, that individuals sharing the HLA-DR 2,5 genotype both responded to p44–54 and p133–146, but did not both respond to several other peptides, p64–75, p87–98, p151-161, p169–179, and p223–233 (Fig. 2). To analyze the role of HLA molecules in response to PT peptides we used two different approaches: blockade of the response to a given peptide with mAb directed to HLA-D molecules and use of HLA-D matched APC for presentation of peptides to heterologous purified T cells. As shown in Fig. 3, with three different individuals, presentation of peptides 44–54, 104–116, 206–218, 133–146, 151–161, and 223–233 by fibronectin-adherent APC was largely inhibited by the anti-HLA-DR mAb, while anti-HLA-DQ was only able to suppress certain responses by ~10–20%. Leu-10 mAb (anti-HLA-DQw1,w3) blocked the responses to peptides 64–75, 87–98, and 169–179,

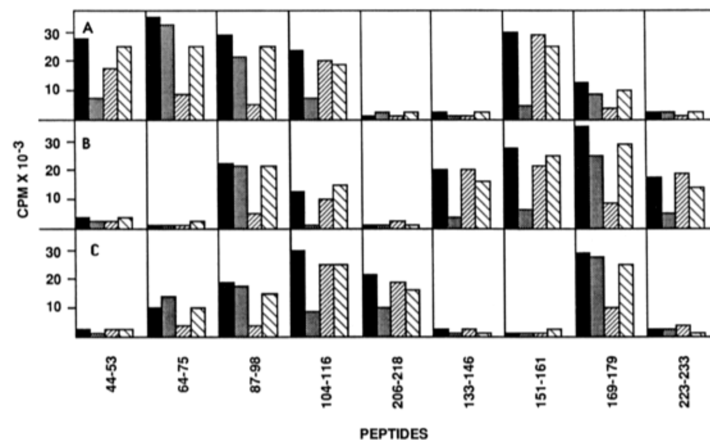

FIGURE 3. Antibody blocking of PT peptide presentation to three different individuals. The response without antibody is indicated as (■). Fibronectin-adherent APC were treated with anti-HLA-DR (▤), anti-HLA-DQ (▨), or anti-HLA-DP (▧), at a final concentration of 1:100. Bars represent mean cpm of two different triplicate determinations, and SD values never exceded the 15% of the mean cpm.

while the anti-HLA-DR mAb was associated with a much smaller reduction in the response to these peptides. Treatment of APC with anti-HLA-DP or control rabbit anti-PT antibodies caused only marginal reductions in the responses. It appears from these results that different epitopes of the PT molecule associate with either HLA-DR or DQ determinants. Our antibody blocking studies showed also that peptide stimulation could be blocked by treatment of nonadherent responder lymphocytes with anti-CD4 but not anti-HLA class II mAb (Fig. 4).

The second experimental approach involved use of heterologous APC and T cells, which differ or share HLA-DR,DQ determinants. Alloreactive T cell clones were inactivated by Brd Urd and light treatment, which eliminated irrelevant proliferative reactions. As shown in Table II, only combinations in which the APC shared HLA-class II antigens with the responder T cells resulted in significant peptide responses. Analyses of Table II and Fig. 2 reveals the possible role of the T cell repertoire in the peptide response. Combinations of a nonresponder APC and responder T cells gave positive responses, while responder APC and nonresponder T cells yielded negative responses. For example, HLA-DR 2,4; DQw1,w3 APC effectively presented p104–116, p133–146, p151–161, p169–179, and p223–233 to HLA-DR 1,4; DQw1,w3 T cells. The individual who was HLA-DR 2,4; DQw1,w3 responded to p87–98 and p169–179 as well as to p104–116, p133–146, p151–161, and p223–233 (Fig. 2), but the HLA-DR 2,4; DQw1,w3 APC could not effectively present p87–98 or p169–179 to the T cells of the HLA-DR 1,4; DQw1,w3 individual, who is a nonresponder to these peptides. A similar pattern was seen with HLA-DR 5,7 APC and HLA-DR w6,7 T cells, where responses were effectively mounted to p44–54, p133–146, p151–161, and p223–233, but not to p64–74 and p104–116, which effectively stimulated the donor of the APC, but not the donor of the T cells. To demonstrate further the role of T cell phenotype in the response to various pertussis peptides we also examined HLA-DR 2,5 T lymphocytes with HLA-DR 5,7 APC. The HLA-DR 2,5 individual did not respond to p104–116, but did respond to 44–54, 133–146, and 151–161. Thus, the failure of DR 5,7 APC to present p104–116 to DR w6,7 T cells is not due to the lack of complete class II MHC matching.

Discussion

There is serious concern about the potential reactogenicity of *B. pertussis* vaccine. The DPT vaccine is associated with convulsions in 1 of 1,750 doses, and permanent neurologic damage occurs in 1 of every 310,000 doses (7, 27). In response to pub-

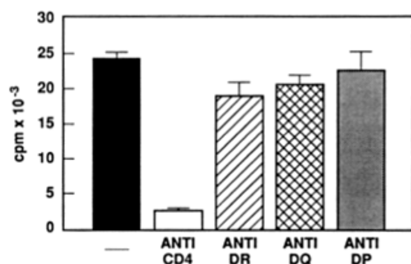

FIGURE 4. Antibody blocking of T cell proliferative responses to 10 mg/ml of PT peptide 151–161. Fibronectin nonadherent lymphocytes were treated with anti-CD4 (▧), anti-HLA-DR (▨), anti-HLA-DQ (▩), or anti-HLA-DP (▤), at a final concentration of 1:100.

TABLE II

MHC Restriction and T Cell Reactivity after Brd Urd and Light Treatment

	T cells				
	APC HLA-DR 2,4; DQ w1,w3		APC HLA DR 5,7; DQ w2,w3		
T cells[*]	HLA-DR 1,4 DQ w1,w3	HLA-DR 3,w8 DQ w2	HLA-DR w6,7 DQ w1,w3	HLA-DR 2,5 DQ w1,w3	HLA-DR1,- DQ w1
Peptides	*cpm (SI)*			*cpm (SI)*	
44-54	1,260[‡](0.95)[§]	1,670 (1.05)	12,830 (4.48)	7,830 (8.20)	2,910 (1.71)
64-75	1,230 (1.04)	2,560 (0.67)	725 (0.69)	1,230 (1.50)	3,860 (1.54)
87-98	1,920 (0.83)	1,880 (1.89)	854 (0.64)	960 (0.55)	1,910 (1.59)
104-116	16,730 (5.46)	2,960 (1.27)	710 (1.01)	2,110 (2.34)	660 (0.67)
206-218	1,870 (1.80)	2,490 (1.74)	530 (0.47)	1,530 (1.20)	960 (1.77)
133-146	31,125 (12.20)	860 (0.77)	24,860 (8.63)	3,765 (3.44)	3,820 (2.74)
151-161	9,920 (11.40)	1,780 (0.89)	25,330 (8.40)	7,810 (6.35)	4,190 (1.63)
169-179	4,010 (5.07)	2,280 (1.78)	830 (0.77)	1,960 (2.20)	750 (0.96)
180-190	1,210 (1.07)	1,975 (2.29)	1,930 (1.94)	2,210 (1.85)	920 (0.87)
223-233	22,860 (11.72)	3,320 (1.44)	48,960 (13.19)	2,810 (2.20)	1,970 (1.28)

[*] Purified T lymphocytes after Brd Urd and light treatment subsequently stimulated with peptide-primed heterologous APC.

[‡] Each value represents the mean of two different triplicate determinations. Standard deviation values never exceeded the 12% of mean cpm.

[§] Stimulation Index (SI) = (Experimental cpm + antigen)/(Control cpm − antigen).

[‖] Responses SI >3 are underlined.

licity concerning these potential hazards of pertussis vaccines, vaccination acceptance fell to a level below 50% in Britain in the mid-1970s (28). There is interest, therefore, in developing a safer DPT vaccine.

The use of synthetic peptides as vaccines is currently being explored for a number of infectious diseases, including malaria, hepatitis B, and AIDS (29). One hazard of vaccines based particulary on discrete peptides may be the variability in the response among different individuals with different HLA types. In the present work we examined the MHC-restricted T cell responses to synthetic peptides corresponding to the subunit I of the pertussis toxin sequence. Of the 10 peptides, 9 elicited vigorous responses as detected by cellular proliferation and [^{3}H]thymidine uptake. It was important however, to identify a family of peptides derived from the pathogen that would be immunogenic for all the individuals to be vaccinated. A combination of two partially immunostimulatory peptides resulted in complete coverage for T cell responses for all the individuals in our panel. When broader groups are tested, it may be possible to achieve wide or complete coverage with a relatively small number of peptides.

The T cell response to various antigens has been shown to be restricted to certain HLA-DR, DQ, or DP types. Several examples demonstrate the association between specific HLA types and the immune response to viral (30), bacterial (31), parasitic (32), and even synthetic antigens. An example of such a correlation was recently provided by Brocke and colleagues (15), who demonstrated that the antigens HLA-DR 5 and DR 3 are differentially associated with proliferative responses to distinct peptides of the acetylcholine receptor molecule in myasthenia gravis patients. We therefore used different anti-class II mAbs to determine the contribution of the different

HLA class II molecules in the T cell response to PT peptides. It appears that it is possible to characterize responses to different peptides with some responses associated with HLA-DR, and some with HLA-DQ MHC determinants. Associations such as these could be due to the selective physical association of these peptides with a particular class II antigenic site (33, 34).

Using Brd Urd and light treatment to block proliferation due to alloreactivity, we demonstrated the requirement for at least partial genetic identity at the MHC class II level between APC and responder T lymphocytes. Interestingly, T cells from nonresponder donors for a particular epitope fail to respond to the relevant peptide even in the presence of histocompatible APC from a responder donor, and conversely, T cells from a responder donor will respond to the peptide in the presence of histocompatible APC from a nonresponder donor. These data clearly show the importance of the T cell genotype in the specificity of the response.

The association of the ADP-ribosyl transferase activity of PT with the immunomodulatory properties of *B. pertussis* suggested that ADP-ribosylase activity is necessary for optimal immunoprotection (11). The present data indicate that peptides of the S1 subunit of PT containing stretches distinct from Glu 140, a critical component of the ADP-ribosylase enzymatic site, are immunogenic in man. A recent study describes other peptides of the S1 subunit of PT, again distinct from Glu 140, that bind antisera from infants immunized with *B. pertussis* (35). Thus in man, optimal immunoprotection may not necessarily require an immune response to epitopes of PT including Glu 140 in the S1 subunit.

These results indicate that it might indeed be possible to construct a vaccine with a limited number of PT peptides that could cover a wide population. This raises an important issue. Potency testing for *B. pertussis* vaccine uses the intracerebral challenge test in CFW outbred mice (36, 37). This test has not proven useful for testing acellular *B. pertussis* vaccines or for peptides (38). We plan to see whether the peptides that elicit HLA-restricted T cell responses in man do indeed protect in the intracerebral challenge test, as well as ascertain whether these peptides block other PT reactions including lymphocytosis. If these peptides can evoke protective T and B cell responses in such assays, then it might be possible and acceptable to consider them as a candidate vaccine.

Summary

The S1 subunit of Pertussis toxin (PT) is responsible for the reactogenicity and in part the immunogenicity of Bordetella pertussis vaccine. The critical residues associated with the immunomodulatory effects of PT were located around Glu^{140} in the S1 subunit. In man, T cell responses to PT are directed at S1 peptides distinct from Glu^{140}. Two such epitopes, p64–75 and p151–161, are immunogenic in a panel of individuals covering a wide range of HLA genotypes. The response to PT peptides is HLA class II restricted. The response to p64–75 is blocked by an anti-HLA-DQ mAb, while that to p151–161 is blocked by an anti-HLA-DR mAb. These findings may allow for the development of a *B. pertussis* vaccine free from reactogenicity.

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