Universal Epitopes for Human CD4+ Cells on Tetanus and Diphtheria Toxins

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Previous studies suggested that tetanus and diphtheria toxoids (TTD and DTD, respectively) contain “universal” epitopes for human CD4+ cells (residues 632–651 and 950–969 of TTD and 271–290, 321–350, 351–370, 411–430, and 431–450 of DTD). To investigate whether CD4+ cells of 100 randomly selected subjects recognized those sequences, the proliferation of CD4+ cell–enriched blood lymphocytes to TTD and DTD and individual synthetic universal epitopes was measured. CD4+ cells of 98 subjects recognized both toxoids, those of 1 subject only TTD, and those of 1 only DTD. The TTD peptides and DTD peptides 271–290 and 331–350 were recognized by ≳80% of the toxoid-sensitized subjects. The other DTD sequences were recognized by 63%–71% of subjects. DR-homozygous subjects recognized several universal epitopes less frequently than did DR-heterozygous subjects. The intensity of responses to the epitope correlated with that to TTD or DTD, consistent with recognition of the peptides by CD4+ cells specific for the cognate toxoid.

Studies on the recognition of antigens [1–6] and autoantigens [7–9] by human CD4+ T cells have suggested that some sequence regions of protein antigens might sensitize CD4+ cells of most or all subjects. The molecular basis for the existence of “universal” CD4+ cell epitopes is not known. Certain sequences may be especially effective at sensitizing CD4+ cells because they might be easily processed and released from the antigen [5, 10]. This, along with the promiscuous binding of human class II molecules to peptides [11–13], may result in their universal recognition. Also, the T cell receptors (TCRs) may cross-react with unrelated peptides, presented by the same or even by different class II molecules [14, 15]. Degenerate recognition by CD4+ cells sensitized by other proteins may also contribute to the existence of universal CD4+ cell epitopes.

Tetanus and diphtheria toxoids (TTD and DTD, respectively) are the key components of tetanus and diphtheria vaccines [16, 17] that are extensively used. The study of the human CD4+ cell response to TTD and DTD is useful to examine whether universal CD4+ cell epitopes exist on those antigens, to identify the structural properties that might relate to their universal recognition, and to determine whether the universal epitopes are recognized by CD4+ cells sensitized to the cognate toxoid or by cross-reacting CD4+ cells primed by another antigen.

Studies of small groups of healthy subjects [1, 10–14] have identified epitopes recognized by human CD4+ T helper cells on DTD and TTD. Some sequence regions were recognized by the CD4+ cells of most the subjects tested [1, 10–14]. They include 2 segments of the TTD sequence (residues 632–651 and 950–969 of the TTD precursor) and 5 segments of the DTD sequence (residues 271–290, 321–350, 351–370, 411–430, and 431–450) [1, 14]. Those findings suggested that those sequence regions may form universal epitopes for sensitization of human CD4+ cells. However, the small number of subjects tested (<8) did not allow firm conclusions on those matters. Also, all or most subjects used in those studies had been vaccinated against tetanus and diphtheria in the recent past. Recognition of those putative universal epitopes might be related to the hyperimmune condition to TTD and DTD.

In this study we investigated the frequency with which blood CD4+ cells of 100 randomly selected healthy subjects recognized the previously identified putative universal TTD and DTD epitope sequences [5, 6]. We used unselected blood CD4+ cells, rather than CD4+ cell lines enriched in antigen-specific T cells, to avoid misrepresentations of the CD4+ cell repertoire resulting from biased clonal propagation in vitro. We tested their proliferative response to TTD, DTD, and the individual peptide sequences previously found to be likely candidates as universal CD4+ cell epitopes in humans.

Subjects and Methods

Subjects. We studied 100 subjects, 62 men and 38 women 32–74 years old, designated with a code from 1 to 100. Their DRB1 alleles were determined experimentally, and the DRB3–5 alleles were deduced from their known association with the different
**DRB1** alleles (table 1). Three subjects (subsets 25, 59, and 65) were laboratory personnel who had received a tetanus-diphtheria vaccine 2–4 years before the testing. The others were randomly recruited from the blood donors of the American Red Cross in St. Paul, without knowing when they had received a vaccine boost.

**Peptide synthesis and characterization.** We synthesized [18] 9 peptides, 20 residues long. Eight peptides corresponded to putative universal CD4^+^ cell epitopes of TTD and DTD [5, 6], and 1 was a control unrelated sequence (residues 1–19 of the major intrinsic protein of bovine lens [19]). To test the response to the sequence DTD321–350, we used 2 overlapping peptides, corresponding to residues DTD321–340 and DTD331–350. The peptide codes include the letters TTD or DTD and numbers that refer to the position of the first and last peptide residues on the DTD and TTD sequences [20, 21]. Their sequences are as follows: TTD632–651, IDKISDVTIVPYIGPALNI; TTD950–969, NNFTVSWFLRVP-KVSASHEL; DTD271–290, PVFAGANYAAWAVNYVQVI; DTD321–340, VHHNTEEIVQSIASSLMLY; DTD331–350, QSI-IALSSLMVAQAIPLVGE; DTD351–370, VDIGFAAYNFVES-IINLFQV; DTD411–430, QGESGHDIKITAENTPLPIA; DTD431–450, GVLLPTIPGKLDVNKSHTHI.

We determined the amino acid composition of the peptides [22]. For all peptides, the results of the composition analysis corresponded to the expected theoretical values (data not shown). We verified the peptide sequence by mass spectrometry. For all peptides, the results of the composition analysis corresponded to the expected theoretical values (data not shown).

**CD8^+^ T cell depletion and proliferation assay.** We used peripheral blood lymphocytes (PBLs) depleted of CD8^+^ cells by use of mouse anti-CD8^+^ antibody (OKT8; Ortho, Raritan, NJ) and paramagnetic beads coated with goat antibody against mouse immunoglobulin (Advanced Magnetics, Cambridge, MA) [23]. Yields of the CD8^+^ cell-depleted, CD4^+^ cell-enriched PBLs were 45%–55% of the starting PBLs; they had <2.5% CD3^+^, CD8^+^ cells [5, 6, 20].

We tested the CD4^+^ cell-enriched PBLs in a 5-day proliferation assay [5–9] using sextuplet wells and the following antigens: phytohemagglutinin (10 µg/mL; Wellcome, London), interleukin (IL)–2 (10 U/mL; Lymphocult; Biotest Diagnostic, Dreieich, Germany), TTD or DTD (10 µg/mL; Connaught Laboratories, Swiftwater, PA), and the individual synthetic peptides (10 µg/mL). All peptides but DTD351–370 were used to test all subjects. Peptide DTD351–370 was used to test 89 subjects. We determined the basal growth rate from quadruplicate wells that contained CD4^+^ cell–enriched PBLs cultured without stimuli. The extent of cell proliferation was determined from the incorporation of [3H]thymidine.

**Results**

**Recognition of TTD and DTD.** Ninety-eight subjects recognized both TTD and DTD, 1 recognized only TTD, and 1 recognized only DTD. The subject who did not recognize TTD (subject 14) was homozygous for DR2, DRw51, whereas the subject who did not respond to DTD (subject 89) was DR heterozygous. The average SIs (n = 6) of cell cultures that responded significantly to TTD or DTD were reported in figure 1 as contour maps. Several subjects, including the 3 donors (subsets 25, 59, and 65) known to have received vaccine boosts ≤4 years before testing, responded to TTD and DTD strongly (SI >20).

**Recognition of putative universal TTD sequences.** All but 9 of the 99 subjects who responded to TTD responded significantly to 1 or both TTD peptides. Overall, 80% of them responded to TTD632–651 and 86% to TTD950–969 (table 2); 74% recognized both peptides. Most responses to the TTD
Figure 1. Contour maps that summarize response to diphtheria toxoid (DTD) and universal DTD peptide epitopes (bottom panel) and to tetanus toxoid (TTD) and universal TTD peptide epitopes (top panel) of CD4⁺ cell-enriched peripheral blood lymphocytes from 100 healthy subjects, as indicated at bottom of plots. Each vertical strip represents responses of 1 subject to DTD, DTD peptides, TTD, and individual TTD peptides, as indicated at right. Responses are expressed as mean stimulation indices (SIs; n = 6) of cell cultures that responded significantly to the antigen tested. SIs are represented by means of the color code shown at the top. For 11 subjects, we did not test responses to peptide DTD351-370; this is indicated with black dots.
peptides were lower than the responses of the same cells to the whole TTD molecule but within the same order of magnitude.

Twelve of the 13 DR-homozygous subjects responded to TTD. All but 1 recognized at least 1 TTD peptide; 9 recognized both peptides. The DR-homozygous subjects recognized peptide TTD632–651 with a frequency similar to that of the heterozygous subjects and peptide TTD950–969 with a slightly lower frequency (table 2).

The CD4⁺ cell–enriched PBLs of the only subject who did not respond to TTD responded only to peptide TTD632–651, and only to a minimal extent (SI 1.3). This was the lowest SI that we detected for peptide TTD632–651 among all of our subjects.

The average SIs (n = 6) of cell cultures that responded significantly to the individual TTD peptides are reported in figure 1A as a contour map, using the color code depicted in the figure. Each vertical strip represents the results obtained from 1 subject, as indicated at the bottom of the plot. The response to TTD, reported at the bottom of the plot, is followed by the response to the individual peptides, as indicated at the right of the plot.

To obtain clues about the specificity of the response to the universal epitope peptides, we compared, for each subject, the intensity of the CD4⁺ cell response to TTD with that of the responses to each of the TTD peptide epitopes. We used simple linear regression and the program EXCEL-ANOVA (Microsoft, Redmond, WA) to test whether the correlation between the log of the intensity of the response to TTD (measured as SI) and the log of the intensity of the response to the individual TTD peptides (measured as SI) is zero. For both peptides, the significance level was <1.1 × 10⁻⁶ (figure 2); this indicates that in each case the correlation was nonzero and that the intensity of the recognition of TTD correlated with that of the recognition of the TTD peptides. The correlation was especially good for peptide TTD632–651 (P < 2.4 × 10⁻⁸). Figure 2 (top) reports the results of these analyses. The plots of figure 2 also illustrate how most, but not all, of the TTD-sensitized subjects recognized the TTD epitopes.

**Recognition of putative universal DTD sequences.** All but 2 of the 99 subjects who responded to DTD responded significantly to at least 1 DTD peptide. Overall, 88% of them responded to DTD271–290; 82% responded to DTD331–350; 70%–71% responded to DTD321–340, DTD351–370, and DTD431–450; and 63% responded to peptide 411–430 (table 2). Peptides DTD321–340 and DTD331–350 overlap; it is possible that they include only 1 epitope. The frequency of recognition of the sequence region spanned by these 2 peptides (DTD321–350) was 87%. In most cases, the responses to the DTD peptides were lower than the response of the same subject to the whole TTD molecule but within the same order of magnitude.

All 13 DR-homozygous subjects responded to DTD. All of them recognized at least 2 DTD peptides; 7 of them recognized ≥4 DTD peptides. They recognized peptides DTD271–290, DTD411–430, and DTD431–450 with a frequency similar to that observed in the total population and in the heterozygous population (table 2). They recognized peptide DTD331–350 somewhat less frequently than did the heterozygous subjects (69% vs. 85%) and peptides DTD321–340 and DTD351–370 much less frequently than did the heterozygous population (46% vs. 74%–76%). The frequency of recognition of the sequence region spanning the overlapping peptides DTD321–340 and DTD331–350 was 77% among the DR-homozygous subjects and 88% among the heterozygous subjects.

The CD4⁺ cell–enriched PBLs of the 1 subject who did not respond to DTD responded only to peptide DTD331–350, and only to a minimal extent (SI 1.6); this was 1 of the 6 lowest SIs that we detected to this peptide.

The average SIs (n = 6) of cell cultures that responded significantly to the individual DTD peptides are reported in figure 1B as a contour map. Each vertical strip represents the results obtained from 1 subject, as indicated at the bottom of the plot. The response to DTD, reported at the bottom of the plot, is followed by the response to the individual peptides, as indicated at the right of the plot. In 11 subjects we did not test the response to peptide DTD351–370. This is indicated in figure 1B with black dots.

For each subject, we compared the intensity of the CD4⁺ cell response to DTD with that of the responses to each of the DTD peptide epitopes. We did this by testing, by means of simple linear regression and the program EXCEL-ANOVA, whether the correlation between the log of the intensity of the response to DTD (measured as SI) and the log of the intensity of the response to the individual DTD peptides (measured as SI) is zero. For each peptide, the significance level (P) was <.004, indicating that in each case the correlation was nonzero and the intensity of the recognition of DTD correlated to that of the recognition of the DTD peptides. The correlation was

| Table 2. Frequency of recognition (%) of diphtheria toxoid (DTD) and tetanus toxoid (TTD) epitope sequences by CD4⁺ cells of healthy subjects sensitized to the corresponding toxoid. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | DTD             |                 |                 |                 |                 |                 |                 |                 |                 |
| Total subjects  | 88              | 71              | 82              | 88              | 70              | 63              | 71              | 80              | 86              |
| DR heterozygous | 88              | 75              | 85              | 87              | 74              | 64              | 70              | 79              | 87              |
| DR homozygous   | 85              | 46              | 69              | 77              | 46              | 62              | 69              | 85              | 77              |
| * This sequence region includes overlapping epitope peptides DTD321–340 and DTD331–350. |
Figure 2. Correlation between the intensity of responses of CD4⁺ cell-enriched peripheral blood lymphocytes of different subjects to individual diphtheria toxoid (DTD) and tetanus toxoid (TTD) peptide epitopes, as indicated within each plot, and that to the relevant toxoid. The intensity of the responses is represented as log of stimulation index. Significance of correlation was tested by simple linear regression. $P$ values are indicated within each plot.
Figure 3. Relative crystallographic B factors and universal CD4+ cell epitopes. Because the size of B factors is a function of the order of atoms in a given crystal, absolute values obtained in different crystals cannot be compared directly. To normalize the B factors obtained from crystallographic studies of diphtheria toxoid (DTD), fragment Hc of tetanus toxoid (TTD), and the translocation domain of botulinum toxin (BTX), we divided the B factor of individual α carbons by the mean B factor of all α carbons in that protein or protein domain. We refer to this ratio as "relative B factor." Relative crystallographic B factors of Cα atoms are plotted as a function of the residue number for sequence segments of DTD, of the fragment Hc of TTD, and of the translocation domain of BTX that contain the epitopes studied here. In BTX, residues 623–643 are homologous to epitope TTD632–651. Residue numbers are indicated along the x axis; the location of universal epitope sequences is indicated by lines and related residue numbers. The crystallographic data used for DTD and fragment Hc of TTD were obtained from the Protein Data Bank (accession codes 1sgk and 1AF9, respectively); those for BTX were provided by R. C. Stevens (Department of Chemistry and Earnest Orlando Lawrence Berkeley National Laboratory, University of California, Berkeley).

Discussion

These results indicate that the TTD and DTD epitopes studied herein are frequently recognized by human CD4+ cells, irrespective of their HLA class II haplotype. Most subjects recognized both TTD epitope sequences and ≥3 DTD epitope
sequences. In most subjects, the responses to individual DTD and TTD epitopes, although lower than those to the whole DTD and TTD molecules, were of the same order of magnitude. This confirms our previous findings that the CD4\(^+\) cells recognizing these universal epitopes were a substantial fraction of the toxoid-specific CD4\(^+\) cells (8\%–29\% for TTD [6] and 28\%–51\% for DTD [5]).

The consistent correlation between the intensity of the responses to TTD or DTD and that of the responses to the epitope peptides (figure 2) indicates that peptide recognition was due at least in part to CD4\(^+\) cells that responded to the relevant toxoid. Also, the previous finding that CD4\(^+\) T cell lines propagated by stimulation with these peptides cross-reacted vigorously with the cognate toxoids indicated that they recognized epitopes resulting from processing of the toxoid molecules [24].

The CD4\(^+\) cells that respond to the universal epitope peptides may also include CD4\(^+\) cells originally sensitized to different antigens and sequences that are cross-reacting with the peptides we tested. A high level of cross-reactivity is an essential feature of the TCR [25, 26]. Recognition by a degenerate, cross-reactive TCR might be important in the initial priming to a new antigen, and epitope recognition by a degenerate TCR might eventually lead to preferential sensitization of CD4\(^+\) cells to the cognate antigens.

We selected the epitope sequences on the basis of our previous investigations of the human CD4\(^+\) cell repertoire on TTD and DTD [5, 6]. Other studies support the conclusion that the TTD peptides studied herein form universal CD4\(^+\) cell epitopes: peptide TTD632–651 includes the sequence TTD640–651, which another study found to be frequently recognized by PBLs of humans [13], and peptide TTD950–969 overlaps a previously described immunodominant TTD sequence (residues 947–967) [1, 2]. Among the DTD peptides, the sequences DTD271–290 and DTD321–350 are the best candidates for universal epitopes, because they are recognized by almost 90\% of the subjects we studied. The frequency of recognition of those DTD sequences and of the 2 TTD peptides was high also among the DR-homozygous subjects (77\%–85\%; see table 1). The immunodominance of these sequences might be related to the ability to bind to a broad population of class II molecules and to their preferential processing, as discussed below.

The universal DTD and TTD epitopes could be presented by each of the class II isotypes expressed by a given subject [2, 5]. The present results are consistent with a binding of some peptides to a variety of class II molecules: at least some of the peptides studied here (i.e., those recognized by \(\geq 80\%\) of the subjects [see table 2]) probably bind to a variety of class II molecules. Also, these universal epitopes might be presented by the DRB3-5 products, which are less polymorphic than the other class II alleles. Not all peptides studied herein may be universal class II ligands; some were recognized much less frequently by the DR-homozygous than by the DR-heterozygous subjects (table 2), suggesting that they have more stringent DR binding requirements. Also, the frequency of recognition of the different peptides by subjects who express certain alleles supports this possibility. For example, subjects who express DRw52 (as well as DR3, DR5, DR6, and DR8) but not DRw51 and DRw53 recognized peptides DTD271–290 and DTD331–350 with high frequency (\(\sim 80\%\) and 76\%, respectively [see figure 1]), whereas only 44\% of them responded to DTD321–340 or DTD411–430. The universal recognition of the epitopes studied herein might be due to both their promiscuous binding to class II molecules and the expression, especially by subjects heterozygous at the class II loci, of a variety of class II proteins.

A few subjects who responded to TTD or DTD recognized very few or none of the corresponding epitope peptides (figure 1). Other subjects expressing the same DR alleles responded well to several of the epitope peptides. Some negative results might be due to the low sensitivity of the assay we used.

The immunogenicity of a T epitope can be modulated by residues flanking the determinant [27]. Such effects might be related to structural properties that permit proteolytic cleavage. The crystal structures of DTD [28], of the receptor-binding fragment H of tetanus neurotoxin [29], and of botulinum neurotoxin type A [30], which is highly homologous to tetanus neurotoxin, have been solved. All of the sequence segments of DTD studied herein include, or are flanked by, residues forming relatively unstructured loops fully exposed to the solvent [5] and therefore easily accessible to the processing proteases.

The local mobility within a protein antigen might also predict T cell epitopes [10]. It might cause localized protease sensitivity and immunodominance of the sequence fragments released most easily. Crystallographic B factors are potential indicators of the motility of the atoms in the polypeptide chain: high B factors correspond to weaker electron density, which is usually a result of movements within the crystal protein lattice. We compared the sequence location of the universal epitopes we studied with the dynamic properties, as defined by the crystallographic B factors, of DTD; of the fragment H of TTD, which contains peptide TTD947–967; and of the translocation domain of botulinum toxin, which contains the sequence homologous to TTD622–651. We limited this analysis to the B factor of the \(\alpha\) carbons, because they should best reflect the motility of the peptide backbone. Sequence segments with high relative B factors were included in, and/or flanked, one or both ends of all the epitopes we studied, which in general aligned with “valleys” in the relative B factor values (figure 3). Epitope DTD271–290 is in a valley between a broad mobile region and a peak of moderately high mobility. The highest mobility peak in the DTD molecule includes a few carboxyl-terminal residues of the epitope region DTD321–350 and several additional residues flanking the carboxyl terminus. This peak of high B factors flanks and includes the amino terminus of epitope DTD350–370, and epitopes DTD411–430 and DTD431–450 are also in valleys between peaks of more elevated relative B
factors. In the H fragment of TTD, the epitope TTD950–969 is immediately preceded by a stretch of 10 residues (residues 939–948), the relative B factors of which are among the highest in this TTD domain, and it is followed by 2 clusters of residues (residues 968–970 and 981–986) that also have B factors substantially higher than the average in this crystal. In the translocation domain of botulinum toxin, the sequence region homologous to TTD632–651 (residues 623–643) is preceded by, and includes at its amino terminus, 7 residues (residues 621–627) that have very high relative B factors. This epitope sequence is followed by a stretch of 8 residues (residues 644–651) that have some of the highest relative B factors in this domain.

Universal T cell epitope sequences are useful as enhancers of the immune response to other antigens or haptens [31–34]. A pool of the epitope sequences investigated herein might be suitable as a universal enhancer of the human CD4+ response to poorly immunogenic antigens. These universal epitopes might also be used for the design of semisynthetic vaccines against tetanus and diphtheria, comprising a pool of the epitope peptides and small amounts of TTD and DTD necessary for stimulation of the B cells. Such vaccines would be even more effective than those presently available for stimulation of the CD4+ T helper cells and, because they would contain smaller amounts of bacterial products, would cause undesirable side effects with less frequency than do the tetanus and diphtheria vaccines presently available.

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