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References

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Epitope Enhancement of a CD4 HIV Epitope toward the Development of the Next Generation HIV Vaccine

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Virus-specific CD4+ T cell help and CD8+ cytotoxic T cell responses are critical for maintenance of effective immunity in chronic viral infections. The importance of CD4+ T cells has been documented in HIV infection. To investigate whether a stronger CD4+ T cell response can be induced by modifications to enhance the T1 epitope, the first CD4+ T cell epitope discovered in HIV-1-gp120, we developed a T1-specific CD4+ T cell line from a healthy volunteer immunized with a canarypox vector expressing gp120 and boosted with recombinant gp120. This T1-specific CD4+ T cell line was restricted to DR13, which is common in U.S. Caucasians and African-Americans and very frequent in Africans. Peptides with certain amino acid substitutions in key positions induced enhanced specific CD4+ T cell proliferative responses at lower peptide concentration than the original epitope. This relatively conserved CD4 epitope improved by the epitope enhancement strategy could be a component of a more effective second generation vaccine construct for HIV infection.


Materials and Methods

Synthetic peptide

Peptides were prepared in an automated multiple peptide synthesizer (Symphony; Protein Technologies) using F-moc chemistry. They were purified by reverse-phase HPLC, and their sequences were confirmed, where needed, on an automated sequencer (477A; Applied Biosystems) or by amino acid analysis. Peptide purity was determined by a single peak on reverse-phase HPLC, and by amino acid analysis. Where necessary, sequencing and/or mass spectrometry were also performed.

Immunized blood donor

The healthy HIV-negative volunteer, J022A304X, had been immunized previously with a canarypox virus vector expressing HIV gp120 as part of...
AVGE clinical protocol 022A. Human studies were approved by an appropriate institutional review board, and were conducted with the informed consent of the subject. Briefly, the blood donor was immunized with ALVAC (vCP205) at months 0 and 1 and was boosted with ALVAC and Chiron’s rgp120 at months 3, 6, 9, and 12. Chiron’s rgp120 has the T1 Ag of the SF2 strain of HIV-1, whose amino acid sequence is KQIMNWQEVKAMYA.

Monocyte-derived dendritic cell preparation

After thawing frozen stocks of PBMCs from the donor, 5 x 10^6 PBMCs were cultured with 1000 U/ml human GM-CSF and 1000 U/ml human IL-4 in a 6-well culture plate in RPMI 1640 medium containing 10% autologous plasma. Human GM-CSF and human IL-4 were added every other day to grow out dendritic cells. After pulsing with an appropriate concentration of peptide, 3 μg/ml soluble CD40-ligand (provided by E. Thomas, Immunex, Seattle, WA) was added to the culture at day 4 to mature the dendritic cells. Maturation of dendritic cells was confirmed by up-regulation of CD83 measured by flow cytometry. The next day, after being harvested, irradiated with 10,000 rad, and washed three times, the cultured cells enriched in monocyte-derived dendritic cells were used as an APC source for T cell culture development and maintenance. However, peptide pulsing was not done in the preparation of these APCs for proliferation assays; rather, soluble peptide was titrated in the wells with the APCs and T cells in the culture.

Generation of peptide-specific CD4+ T cell line

A total of 5 x 10^4/well freshly isolated PBMCs obtained from the healthy donor immunized with a canarypox virus vector expressing HIV gp120 and boosted with rgp120 was cultured with 3 μM T1 peptide in 96-well U-bottom plates in R2E (RPMI 1640:Eagle-Hanks’ amino acid medium = 1:1) medium containing 10% autologous plasma. On day 7, 1 x 10^5 cultured cells were restimulated with 8 x 10^5 irradiated monocyte-derived dendritic cells pulsed with 0.01 μM T1 peptide in a 48-well culture plate. One day after each restimulation, T cell growth factor (CELLKINES TCGF, as a source of natural human IL-2 from human lymphocytes; Zepmetrix) was added to a final concentration of 10%. Cell lines were maintained in R2E containing 10% autologous plasma, 1 mM sodium pyruvate, nonessential amino acids (Biofluids), 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. Autologous plasma was positive for anti-gp120 Abs, but such Abs generally do not affect presentation of peptide Ag bound to class II MHC molecules. After checking the Ag-specific response, the cell line (KT9) was restimulated every other week for maintenance using the same conditions as for its original establishment (with irradiated monocyte-derived dendritic cells pulsed with 0.01 μM T1 peptide).

Proliferation assay

T cell proliferation was assessed by culturing 5 x 10^4 KT9 cells with 1 x 10^5 autologous monocyte-derived dendritic cells with or without antigenic peptide in 200 μl of R2E medium containing 10% autologous plasma in 96-well U-bottom culture plates for 3 days. KT9 cells were used in a restimulating rest after 2 wk of rest, just before the next restimulation. (Proliferation assays were done on the same day as, and in parallel with, restimulation of the remaining cells of the line.) Cultures were pulsed with 1 μCi of [H]thymidine/well for the last 24 h. Plates were harvested and counted in a 1205 Betaplate counter (Wallac). All samples were analyzed in triplicate, and all experiments were performed at least twice with comparable results.

IFN-γ assay

A total of 4 x 10^3 KT9 cells and 4 x 10^4 monocyte-derived dendritic cells were cultured with or without peptide at 10 μM in 96-well U-bottom plate. Seventy-two hours later, each supernatant was collected, and IFN-γ in the culture supernatant was determined by ELISA kit (R&D Systems) according to the manufacturer’s instructions. All samples were analyzed in triplicate.

Results

The T1-specific CD4 T cell line is restricted to DR13

We developed a T1-specific CD4+ T cell line (KT9) from a healthy volunteer immunized with a canarypox virus vector expressing HIV gp120. KT9 was determined to be CD4+ and HLA-DR restricted in an inhibition assay using anti-CD4 or anti-HLA-DR, respectively (Fig. 1A). The HLA-DR haplotype of the volunteer was DRβ1*01 and DRβ1*13. Thus, we tested the HLA-DR restriction of this helper line using dendritic cells from the PBMCs of different volunteers sharing one or the other HLA-DR type. This helper line was restricted to DRβ1*13, which is common in U.S. Caucasians and is also one of the major HLA-DR haplotypes in Africa (21, 22) (Fig. 1B). It should also be noted that the lack of response to T1 with DR13-negative presenting cells clearly rules out a nonspecific mitogenic effect of the T1 peptide, and the lack of response to other peptides shown below in Figs. 2 and 3B clearly shows that the KT9 line is Ag specific.

Recruitment specificity of KT9 using single alanine-substituted analogues

In a set of experiments to define key functional residues in the T1 peptide, peptides were synthesized with single alanine substitution for each original amino acid, except using Ser in sites at which Ala was the natural residue (Table I). All peptides were tested in an epitope-specific proliferation assay (Fig. 2). Among the 14 alanine- or serine-substituted peptides, substitutions at the first five positions (peptides 28–40) did not have any effect on the T1-specific CD4 response. However, the next six substituted peptides (peptides 40–61, except 49) induced a significantly lower response. Furthermore, two alanine-substituted T1 peptides (peptides 64 and 67) showed a slightly better response than the original peptide. Based on the data of Fig. 2 and of the peptide sequence motif pattern of DRβ1*13-restricted peptides (22, 23), we hypothesized that the binding core motif of the T1 peptide in humans is WQEVKAMYA.

Recognition property of KT9 using substituted peptides in anchor positions

In general, a typical HLA class II-binding peptide has four anchor residues within a span of nine residues in positions 1, 4, 6, and 9 from the N-terminal end. In the case of the peptide sequences presented by DRβ1*13, hydrophobic residues are preferentially found at positions 1 and 4. A positively charged residue is frequently present at position 6. A small or hydrophobic residue is found at position 9 (22, 23). Thus, we next investigated the characteristics of the amino acid fitted to each anchor position of the postulated T1-binding core peptide using peptides substituted with amino acids having different chemical properties. First, we used peptides with a single amino acid substituted in each anchor position (Fig. 3A). In position 1 (tryptophan in the postulated T1 core peptide), alanine (small) and isoleucine (aliphatic)-substituted T1 made the response worse. However, a phenylalanine (aromatic) substitution did not reduce the response. This result suggested that an aromatic amino acid is required in position 1. In position 4, the alanine- and phenylalanine-substituted T1 elicited a poor response from KT9. However, the isoleucine substitution induced much better helper response. This result suggested position 4 as anchor should be an aliphatic amino acid and isoleucine could enhance the Ag-specific response of the T1-specific CD4+ T cell line. In contrast, in position 6, only a positively charged amino acid-substituted peptide induced a better T1-specific response. The positively charged arginine (R) at position 6 might be better binding to HLA-DRβ1*13 because its pK is higher (~12.5 for R vs ~10.5 for K). In position 9, all peptides substituted with small, aromatic, or aliphatic amino acids induced a better CD4 response than the original peptide. Also, in a titration assay, KT 9 stimulated with the substituted peptides that had induced the best response in each anchor position at 10 μM showed a better response than with the original peptide (Fig. 3B). It should be noted that peptides presented by HLA class II molecules usually need to be longer than the minimal 9-mer that spans the length of the HLA groove.
we do not know the optimal length, which could affect the magnitude of the response. Moreover, we tested and compared peptide-specific IFN-γ production of KT9 T cells between the wild-type T1 and the altered T1 peptide 53. The altered T1 peptide induced higher IFN-γ production by KT9 than the wild-type T1 (only peptide 53 was significantly different from the no-Ag control; \( p < 0.01 \)) (Fig. 3c). This result suggested the epitope-enhanced peptide could induce not only more active proliferation of the T1-specific CD4 T cells, but also greater CD4 T cell activation in a Th1 functional response. In other words, these results mean that a CD4 epitope does not always have the most optimal amino acid in each anchor position required for both binding to the HLA class II molecule and recognition by a CD4+ T cell. At the same time, it suggests that the peptides with a combination of substitutions in anchor positions might be able to induce a much better CD4+ T cell response as a vaccine construct.

**Antigenic potency of the multiply substituted T1 peptide**

Each of three separate point mutations of the T1 peptide, V to I in position 4, K to R in position 6, and Y to A in position 9, respectively, enhanced the proliferative response of KT9 (Fig. 3). Thus, we combined all three mutations in the peptide 70 (Table I) and tested antigenic potency of this multiply substituted peptide in the proliferation assay (Fig. 4). The peptide concentration in which KT9 showed the peak proliferative response was shifted to a lower concentration when using this triple combinational peptide.
than the original peptide T1, and the activity remained substantially higher than that of the wild-type peptide at a 10-fold lower concentration. Note that the high-dose inhibition of T cell proliferation also occurs at lower peptide concentration for the triple combinational peptide than for T1, again consistent with the shift in dose-response curve. These results suggested that the epitope-enhanced peptide that has the best binding capacity in each pocket in the HLA molecule could induce the best response of the wild-type specific CD4 T cell. Also, the multiple substitutions in anchor positions do not interfere with recognition by the wild-type specific human CD4 T cell line.

We attempted to assess and rank order the modified and wild-type peptides as specific stimuli of CD4 T cell proliferation in PBMCs of four HIV long-term nonprogressing individuals who had been typed as HLA-DR13 positive (data not shown). Unfortunately, using thymidine incorporation and CFSE dye dilution detection by flow cytometry, we detected no response to any of the peptides, including the enhanced substituted peptides, nor to the full-length IIIB gp120 envelope, in any of several repeated attempts with cells from the four donors. This is consistent with the published literature describing a rapid and profound loss of CD4 T cell.

**FIGURE 2.** Proliferation of KT9 against T1 variant peptides with alanine substitutions. The concentration of each substituted peptide was 10 µM. Each peptide number is listed in Table I. Error bars represent SEM of triplicate wells, and the experiment was performed twice with comparable results.

**FIGURE 3.** Proliferation and IFN-γ production of KT9 against peptides substituted in anchor positions. A, The concentration of each substituted peptide was 10 µM. Each peptide number is listed in Table I. The position of these residues in the original T1 peptide and the corresponding motif residue preferences (22, 23) are shown below. B, Titration of peptides 53, 59, and 67, and the original T1 peptide in a proliferation assay of KT9 T cells. Error bars represent SEM of triplicate wells, and the experiment was performed twice with comparable results. C, Comparison of peptide-specific IFN-γ production between the wild-type T1 and the altered T1 peptide 53.
proliferative response to HIV envelope even among long-term nonprogressors (24–29).

Discussion

The T1 Ag was the first CD4 helper epitope discovered in the HIV envelope protein (13, 16, 17). A CD4 response specific for this epitope could be observed in some HIV-seropositive individuals in the United States and healthy volunteers vaccinated with gp160 in Zaire, Africa (16, 17). These facts indicated that the T1 epitope is obviously presented by some relatively prevalent human HLA class II molecules. However, the actual class II restriction of T1-specific CD4+ T cells remained yet to be clarified. In this study, we developed a T1-specific human CD4+ T cell line (KT9) from a healthy Caucasian American volunteer immunized with a canarypox virus vector expressing gp120 and found that this CD4 helper line is restricted to DR1*13 (Fig. 1b). DR13 is one of the major haplotypes in Africa, where there are >25 million HIV-infected patients (30), and is also common in U.S. Caucasians as well as African-Americans (22). Thus, the T1 Ag could be a very useful CD4 epitope as an HIV vaccine component in North America and Africa. Because HIV epitopes may have evolved to evade the immune system, we applied epitope enhancement by sequence modification to increase the potency of this epitope.

To achieve a stronger CD4+ T cell response against this T1 epitope, we tested the proliferative response of KT9 against some amino acid-substituted variants of T1 peptide. Except for two cases, T1 peptides with alanine substituted in each position could not induce a stronger proliferative response from KT9 than the original T1 (Fig. 2). However, we could identify the likely binding core as WQEVGKAMY based on the alanine substitutions that diminished recognition, together with proliferation assay data using truncated peptides of T1 (data not shown). Most HLA-DR-binding core sequence motifs typically have four anchor residues. An aromatic amino acid and an aliphatic amino acid are preferably found in positions 1 and 4, respectively (Fig. 3). Although the substitution of phenylalanine for tryptophan in position 1 did not produce a better CD4 response, the substitution of isoleucine for valine in the T1 peptide increased proliferation of KT9 up to 2-fold over the original T1-stimulated response. Both valine and isoleucine belong to the same aliphatic branched chain amino acid group. This suggests that there might be a hierarchy for binding to pockets of HLA-DR molecules among amino acids in a same group. Position 6 is thought to be an allele-specific anchor (31). In the case of DR13, a positively charged amino acid is preferable. Also from our data (Fig. 3), a replacement of lysine by the positively charged amino acid with a higher pK, arginine, in position 6 enhanced the CD4 response. The replacement by this positively charged amino acid with a higher pK (12.5 vs 10.5 for lysine) in this allele-specific anchor position might be a broadly applicable method for epitope enhancement of CD4 epitopes presented by DR13. However, more confirmation using many different DR13-binding peptides would be required to confirm the generality of this approach. It is of interest that although the T1 sequence is relatively conserved, being in the CD4 binding site, the position 6 residue is essentially always K in clade B viruses, mostly R in

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The sequences of T1 and the various substituted analogs are given in single letter amino acid code. Numbers at the top of each column refer to positions in the HIV-1 IIIB gp160 protein. Dashes in each row indicate that the residue at that position in that peptide is the same as T1.

![FIGURE 4. Proliferation of KT9 with multiply substituted peptide (70) and the original T1 peptide. Data represent means of triplicate wells, and the experiment was performed twice with comparable results.](image-url)
clade C viruses, and either K, R, or Q in clade A viruses. All three of these alternatives can be accommodated in the DR13-binding motif.

In our previous paper, we showed that an epitope-enhanced HIV CTL epitope substituted with the appropriate amino acids in all class I MHC anchor positions can induce a better CTL response in vitro and in vivo, and further that the anchor-enhanced epitope-specific CTL can recognize the wild-type epitope with complete cross-reactivity (32). Other enhanced CTL epitopes from HIV-1 reverse-transcriptase binding better to a class II Molecule have been described (33). However, it is equally important to determine whether the epitope-enhancement strategy could magnify the activation of human HIV-specific CD4+ T cells recognizing a peptide binding to a human class II HLA molecule to help the expansion and maintenance for HIV-specific CTLs. Yet, there is little experience with epitope enhancement for peptides binding human class II HLA molecules.

The importance of improving the CD4+ T cell response as well as the CD8+ T cell response is becoming more apparent as more is known about the importance of CD4+ T cell help in inducing a long-lived memory CD8+ T cell response (5, 7–9, 34). In a mouse model using a T1 variant with higher affinity for a mouse class II HLA molecule, we found that this enhanced peptide induced not only a stronger CD4+ T cell response, but shifted the quality of the response toward Th1 by up-regulating CD40 ligand on the helper cells and polarizing dendritic cells to make IL-12, and thus shifting the cytokine profile of the helper cells and inducing a stronger CTL response to an attached CTL epitope (19, 20). The result was better protection against viral challenge in the mouse model. Thus, we tested whether this principle could be true for the epitope enhancement of this human HIV CD4 epitope using KT9.

The multiply substituted peptide 70 shifted the peak proliferative response of KT9 to lower concentrations (Fig. 4). An epitope-enhanced peptide also showed a stronger IFN-γ response (Fig. 3c). The result suggested that the epitope-enhanced HIV CD4 epitope substituted with appropriate combinations of anchor residues could also induce a stronger wild-type specific CD4+ T cell response. However, it will also be important to determine the portion of the CD4 T cell repertoire specific for the enhanced peptide that cross-reacts with the wild-type epitope, for example, immunizing humans with the enhanced peptide, or using HLA-DR13 transgenic mice, as described in our previous paper on enhancing an HLA class I-restricted epitope (26). Such an epitope-enhancement strategy could induce stronger CD4 responses in humans that might provide more effective CD4+ T cell-mediated help for CTL maintenance against HIV. A similar strategy could be applicable to any Ag for which an optimal CD4+ and CD8+ T cell response is desirable.

Our findings show that modifying this HIV CD4 epitope with some amino acid substitutions in anchor regions for epitope enhancement could magnify the T1-specific CD4 helper response compared with the original T1 peptide. Of course, enhanced antigenicity in vivo may not necessarily translate into enhanced immunogenicity in vivo, although it often does. This cannot be determined without a clinical trial, for which this study provides the preclinical data and motivation. Furthermore, the T1 peptide is just one of a number of HIV CD4 epitopes. This strategy would be more effective when applied to multiple conserved CD4 epitopes in HIV, as well as to HIV CTL epitopes, as we recently described (32). Also, these studies provide a rational strategy for the construction of enhanced epitopes that can be applied to build the next generation vaccines, applicable to all forms of vaccine, peptide, DNA, recombinant viral or bacterial vector, or live attenuated virus.

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