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Immunology

Amino-terminal alteration of the HLA-A*0201-restricted human immunodeficiency virus pol peptide increases complex stability and *in vitro* immunogenicity

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ABSTRACT **Initial studies suggested that major histocompatibility complex class I-restricted viral epitopes could be predicted by the presence of particular residues termed anchors. However, recent studies showed that nonanchor positions of the epitopes are also significant for class I binding and recognition by cytotoxic T lymphocytes (CTLs). We investigated if changing nonanchor amino acids could increase class I affinity, complex stability, and T-cell recognition of a natural viral epitope. This concept was tested by using the HLA-A*0201-restricted human immunodeficiency virus type 1 epitope from reverse transcriptase (pol). Position 1 (P1) amino acid substitutions were emphasized because P1 alterations may not alter the T-cell receptor interaction. The peptide with the P1 substitution of tyrosine for isoleucine (I1Y) showed a binding affinity for HLA-A*0201 similar to that of the wild-type pol peptide in a cell lysate assembly assay. Surprisingly, I1Y significantly increased the HLA-A*0201–peptide complex stability at the cell surface. I1Y sensitized HLA-A*0201-expressing target cells for wild-type pol-specific CTL lysis as well as wild-type pol. Peripheral blood lymphocytes from three HLA-A2 HIV-seropositive individuals were stimulated *in vitro* with I1Y and wild-type pol. I1Y stimulated a higher wild-type pol-specific CTL response than wild-type pol in all three donors. Thus, I1Y may be an "improved" epitope for use as a CTL-based human immunodeficiency virus vaccine component. The design of improved epitopes has important ramifications for prophylaxis and therapeutic vaccine development.**

$CD8^+$ cytotoxic T lymphocytes (CTLs) play a major role in the clearance of viruses (1). The T-cell receptor (TCR) expressed by the CTL recognizes a trimolecular complex consisting of a major histocompatibility complex (MHC) class I heavy chain, β_2-microglobulin, and a peptide of endogenous origin (2). The peptide is usually 8–10 amino acids in length and interacts with the class I molecule's binding pockets. Peptides restricted to a specific class I molecule contain predominate amino acids, termed anchors, at particular positions (2). However, presence of favored residues at the anchor positions does not necessarily confer high affinity (3–5).

Peptides which bind to the same MHC molecule have a range of affinities (4, 5). Among high-affinity HLA-A*0201-restricted peptides, particular amino acids were overrepresented at nonanchor positions (4). Our mutational analyses of HLA-A*0201 demonstrated that mutations in any of the MHC binding pockets can have a dramatic effect on both peptide binding and CTL recognition (6–9). These data show that a peptide's nonanchor positions contribute significantly to its free energy of binding. Improvement of a known HLA-A*0201 epitope's affinity may be possible by changing nonanchor amino acids, thereby enhancing the interaction between the peptide and the heavy chain.

The ability to improve a natural epitope through nonanchor substitutions was tested by using a human immunodeficiency virus (HIV)-specific CTL response. The HLA-A*0201-restricted HIV-1 polymerase (pol) epitope (10) was investigated due to (*i*) its high sequence conservation among HIV-1 strains (95.1%) (ref. 11 and J.A.F., unpublished data), (*ii*) its relatively low affinity for HLA-A*0201 (4, 5), and (*iii*) the frequent observation of a CTL response to this epitope among HIV-1-positive individuals (12). Improvement of this epitope for immunotherapy is beneficial, since HLA-A2 is the most common human class I allele worldwide (13).

We focused on position 1 (P1) amino acid alterations due to its interaction with both MHC and TCR. Structural studies of five peptides bound to HLA-A*0201 showed that the orientation of the P1 side chains was very similar (14). The majority of the P1 residue was buried within the class I cleft. Therefore, alterations of the amino-terminal side chain may allow for higher affinity between the peptide and the heavy chain without altering TCR interaction. Our analysis of P1-substituted peptides bound to HLA-B27 subtypes supports this concept (15). Peptides with increased affinity may be able to enhance stimulation of natural epitope-specific CTL. Such "improved" peptides may be useful for CTL-based immunotherapy and prophylaxis.

MATERIALS AND METHODS

Cell Lines. T2 cells are defective in antigen presentation (16). T2 cells were maintained in RPMI medium 1640 (Mediatech, Washington, DC) containing 5% calf serum (HyClone), and 2 mM L-glutamine (GIBCO). C1R-A2 and C1R-neo cells are derivatives of the HLA-A null human B-lymphoid cell line Hmy2.C1R (C1R), transfected with HLA-A*0201 and pSV2-neo or pSV2-neo alone, respectively (9). The C1R transfectants were maintained in the same media supplemented with G418 (GIBCO) at 400 μg/ml.

Peptides. Peptides were synthesized by the University of North Carolina/Program in Molecular Biology and Biotechnology Micro Protein Chemistry Facility, using fluorenylmethoxycarbonyl (Fmoc) chemistry. The HLA-A*0201-restricted HIV-1 pol epitope corresponds to amino acid residues 476–484 of the HIV-1 reverse transcriptase (ILKEPVHGV) (10). The HLA-A*0201-restricted HIV-1 p17 gag epitope (amino acid residues 77–85: SLYNTVATL) is used as a control (17). Sequences of the substituted peptides are shown in Table 1. The peptides were purified by HPLC, and peptide concentration was determined by amino acid analysis at the Protein Chemistry Laboratory at the University of North Carolina at

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Abbreviations: MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; HIV, human immunodeficiency virus; TCR, T-cell receptor; P1, position 1; PBL, peripheral blood lymphocyte; WT, wild type; VV, vaccinia virus; EBV, Epstein-Barr virus.

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Table 1. pol substitution peptide sequences

Abbrev.	P1	P2	P3	P4	P5	P6	P7	P8	P9
WT	I	L	K	E	P	V	H	G	V
I1A	**A**	L	K	E	P	V	H	G	V
I1F	**F**	L	K	E	P	V	H	G	V
I1G	**G**	L	K	E	P	V	H	G	V
I1H	**H**	L	K	E	P	V	H	G	V
I1K	**K**	L	K	E	P	V	H	G	V
I1L	**L**	L	K	E	P	V	H	G	V
I1M	**M**	L	K	E	P	V	H	G	V
I1R	**R**	L	K	E	P	V	H	G	V
I1V	**V**	L	K	E	P	V	H	G	V
I1W	**W**	L	K	E	P	V	H	G	V
I1Y	**Y**	L	K	E	P	V	H	G	V
K3F	I	L	**F**	E	P	V	H	G	V
K3Y	I	L	**Y**	E	P	V	H	G	V
K3W	I	L	**W**	E	P	V	H	G	V
E4D*	I	L	K	**D**	P	V	H	G	V
E4C	I	L	K	**C**	P	V	H	G	V
E4S	I	L	K	**S**	P	V	H	G	V
E4T	I	L	K	**T**	P	V	H	G	V
P5Y	I	L	K	E	**Y**	V	H	G	V
V6Y	I	L	K	E	P	**Y**	H	G	V
H7A	I	L	K	E	P	V	**A**	G	V
G8E*	I	L	K	E	P	V	H	**E**	V
G8F	I	L	K	E	P	V	H	**F**	V

Abbreviations for the substitution peptides use the single-letter code and conform to the following order: the wild-type (WT) amino acid, the position at which the substitution occurs, and finally the substituted amino acid. In the sequences, boldface type indicates the substitutions. Previous studies have shown positions 2 and 9 to be anchor residues (1).
*A natural variant of the pol epitope.

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Generation of HIV-Specific CTL Lines. HIV pol-specific CTL lines were generated from peripheral blood lymphocytes (PBLs) of HIV-seropositive HLA-A2-positive individuals (donors 25 and 32) as described previously (18, 19).

Cell Surface Stabilization Assay. The stabilization assay was performed as described (20). Briefly, T2 cells were suspended in AIM V serum-free medium (GIBCO) supplemented with 100 nM human β_2-microglobulin and peptide (Sigma). The peptide-pulsed cells were incubated overnight at 37°C. The cells were stained for conformationally correct HLA-A*0201 with the monoclonal antibody BB7.2 (21), followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody (Southern Biotechnology Associates). Cells were analyzed by flow cytometry (FACScan, Becton Dickinson). The mean channel for each peptide peak was determined on a linear scale by using CYCLOPS software (Cytomation).

Assembly Assay. Peptide binding was analyzed with the T2 assembly assay essentially as described (15) except the heat denaturation of the lysate prior to peptide addition was eliminated. The HLA-A*0201 complexes were immunoprecipitated with BB7.2 (21). Immunoprecipitates were analyzed by electrophoresis on a reducing SDS/12% polyacrylamide gel. The gels were exposed overnight to a PhosphorImager screen (Molecular Dynamics). The screen was analyzed and quantitated by IMAGE QUANT 3.3 (Molecular Dynamics). The volumes of the bands were quantitated, subtracting local background. The resulting pixel value is directly proportional to dpm and was plotted as described (15).

Complex Stability Assays. T2 cells were incubated with 100 μM peptide and human β_2-microglobulin as described for the cell surface stabilization assay. After the overnight incubation, the cells were incubated in RPMI medium 1640 containing 10% fetal calf serum and brefeldin A (Sigma) at 10 μg/ml to block the egress of new class I molecules. After a 1-hr incubation at 37°C in CO_2, the block of Golgi to cell surface egress was maintained in medium containing brefeldin A at 0.5 μg/ml. At the indicated time points, an aliquot was stained with BB7.2 (21) as described above. All antibodies and wash solutions contained brefeldin A at 0.5 μg/ml.

^{51}Cr Release Assay. Recognition of the substituted peptides by pol-specific CTLs was analyzed by a ^{51}Cr release assay as described (6). C1R-A2 or C1R-neo cells were used as targets. The cells were incubated with peptide for 1 hr at 37°C. pol-specific CTLs were added to each well at an effector-to-target ratio of 4:1 unless otherwise noted. The graphs shown for donor 25 were from a single set of experiments; however, the data for donor 32 are representative of at least two assays. For assays which measure CTL activity on vaccinia virus (VV)-infected targets, target cells were infected at a multiplicity of infection of 5 with VV-pol, VV-gag p17, or the negative control, VV-NP, which expresses the nucleoprotein from influenza virus. After 1.5 hr of infection at 37°C, the cells were washed twice, resuspended in medium, and incubated overnight for expression of the VV construct genes. The infected targets were labeled with ^{51}Cr as described above.

***In Vitro* Immunization.** Frozen PBLs from HIV-seropositive/HLA-A2-positive individuals (donors 4, 9, and 32) were thawed and stimulated weekly with autologous Epstein–Barr virus (EBV)-transformed B cells. These B cells were previously incubated with 10 μM peptide and irradiated [3000 rads (30 Gy)] as described for the generation of CTL lines. Four peptides were tested for production of pol-specific CTLs (WT pol, I1F, I1Y, and p17). Starting at day 14, the PBLs were analyzed for their CTL reactivity in a standard ^{51}Cr release assay. Peptide-pulsed targets were autologous B cells pulsed with wild-type pol or p17 peptides. For testing recognition of endogenously processed antigen, cells of an HLA-A2$^+$ B-cell line AR (HLA-A1,2; B8, 44; Cw5) were infected with VV constructs as described above.

Sequencing of HLA-A2 Subtypes. RNA was isolated from 4 × 10^6 cells, either EBV-transformed B cells or PBLs, using the Micro FastTrack RNA isolation kit (Invitrogen). The HLA-A2 RNA was then amplified by using the GeneAmp RNA PCR kit (Perkin–Elmer). The following antisense primer from exon 5 was used (5′-GCTCCAAAGAGAACCAGGCCAGCAATG-3′), coupled with the sense primer from exon 1 (5′-GCCGAGGATGGCCGTCATGGCGCCCCGAACC-3′). The amplification followed the supplier's instructions, except that the $MgCl_2$ concentration in the PCR reaction mixture was increased to 5 mM. The 894-bp product was purified by Wizard PCR Preps (Promega). DNA was sequenced at the University of North Carolina at Chapel Hill Automated DNA Sequencing Facility on a model 373A DNA Sequencer (Applied Biosystems).

RESULTS

Affinity Analysis of pol Position 1 Substitutions. Eleven P1 amino acid substitutions of WT pol were analyzed for cell surface stabilization of HLA-A*0201 (Table 1). This assay measures the stabilization of empty cell surface heavy chains or the exchange of peptide onto heavy chains (20). The Phe (I1F) and Tyr (I1Y) substitution peptides were the only ones able to stabilize cell surface HLA-A*0201 with an affinity similar to that of the WT pol (Fig. 1*A*). The lower affinity of the Trp substitution is representative of the remaining substitutions. High peptide affinity has been posited to be important for class I-restricted immune responses (22); therefore, the I1F

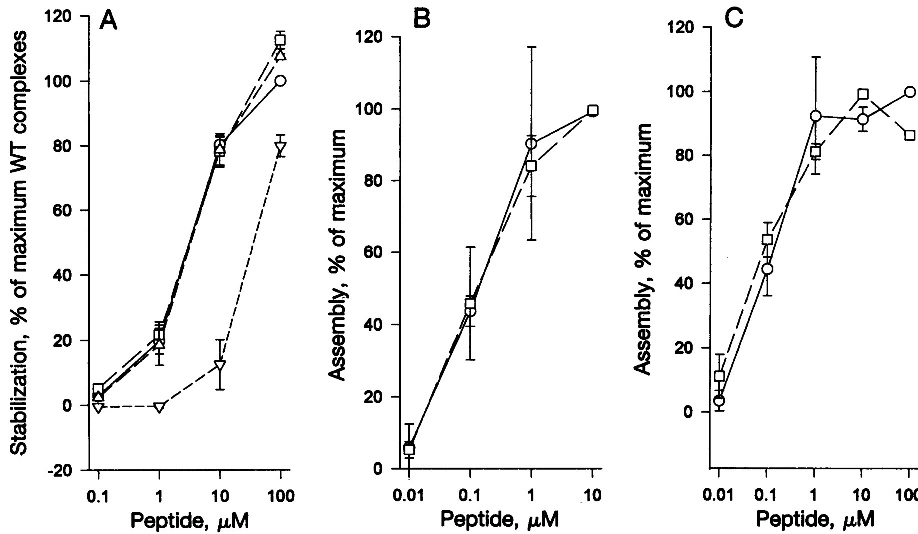

FIG. 1. Binding of P1 substitution pol peptides. All binding experiments used T2 cells (HLA-A2, -B51) (16). (*A*) Cell surface stabilization assay. Peptides are represented as follows: ○, WT; □, I1F; △, I1Y; and ▽, I1W. Error bars indicate SEM for three experiments. (*B* and *C*) T2 assembly assays. WT pol is indicated as ○ in both graphs. □ denotes I1F in *B* and I1Y in *C*. Error bars indicate SEM for three (*B*) or five (*C*) assays.

and I1Y substitutions were analyzed further. The kinetics of peptide binding to newly synthesized HLA-A*0201 was investigated in an *in vitro* assembly assay (15). In the assembly assay, the peptide concentration of half-maximal assembly represents the relative peptide affinity (15, 23). Both I1Y and I1F showed an affinity similar to that of WT pol (Fig. 1 *B* and *C*).

The half-lives of the peptide/class I complexes at 37°C were determined by complex stability assays. The stability of cell surface HLA-A2 was significantly increased by both the I1Y and I1F substitutions (Fig. 2). The half-life of the WT pol/heavy chain complex is approximately 10 hr, whereas both the I1F and I1Y substitutions increase the complex's half-life to approximately 30 hr. Treating the cells with brefeldin A showed that the number of conformationally correct HLA-A2 cannot be due to reloading of the peptide onto newly egressing molecules. Since the antibody is conformationally sensitive to the binding cleft (21), only class I molecules binding peptide were detected.

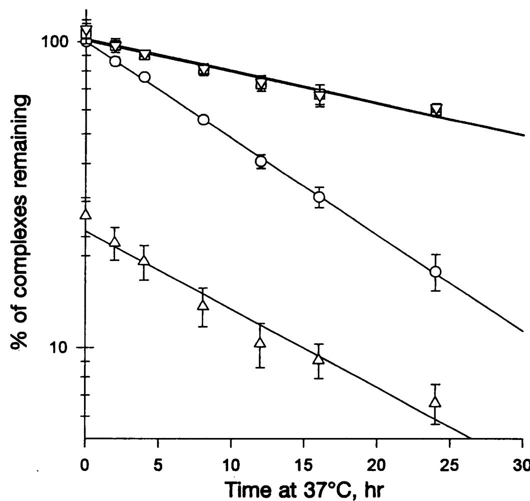

FIG. 2. Complex stability is increased with I1Y and I1F. A cell surface complex stability assay was conducted. After the peptide (○, WT pol; □, I1Y; ▽, I1F; △, none) had been loaded overnight, the cells were treated with brefeldin A to block the exit of new class I molecules. After 1 hr, the cells were maintained in medium containing a lower, less toxic, concentration of brefeldin A which maintained the egress block. At the indicated time points, cells were stained for conformationally correct HLA-A2 by indirect immunofluorescence using BB7.2. The mean channel fluorescence was determined for each peak. The error bars indicated SEM for three experiments. If only a symbol is apparent, the error is smaller than the symbol.

Effect of P1 Substitutions on WT pol-Specific CTL Recognition. Our mutational analysis of HLA-A*0201 showed differences between binding affinity and functional recognition

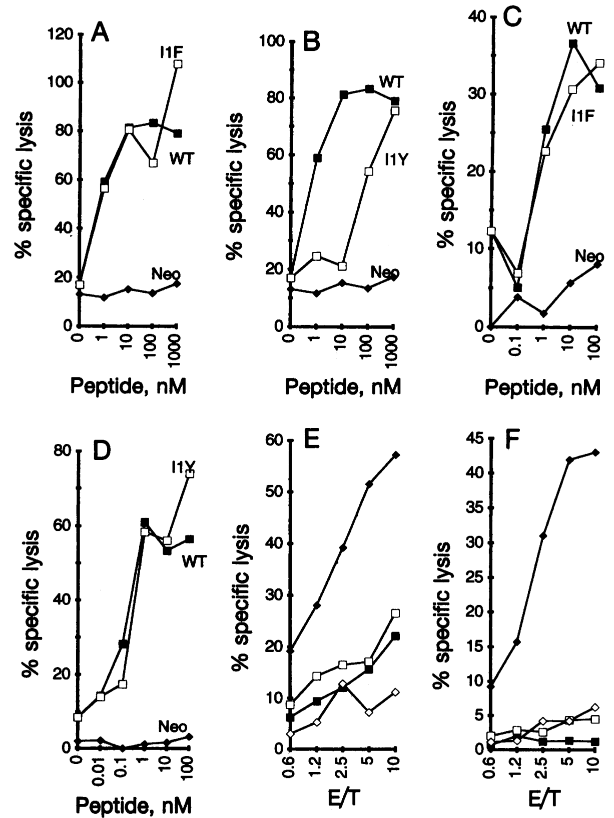

FIG. 3. CTL recognition of I1F and I1Y. Recognition of WT pol, I1F, and I1Y by pol-specific CTL was analyzed by a standard ^{51}Cr release assay. Each peptide concentration was analyzed in duplicate. (*A* and *B*) Recognition by donor 25 CTL line. (*C* and *D*) Recognition by donor 32 CTL line. The negative control, C1R-neo (Neo) was incubated with WT pol. Percent specific lysis of C1R-neo incubated with substituted peptides was similar to that of Neo (data not shown). In all panels WT pol recognition is denoted by ■, and Neo is represented as ◆. □ indicates I1F (*A* and *C*) and I1Y (*B* and *D*). Effector-to-target ratios (E/T) were as follows: 5 (*A–C*) and 3 (*D*). The CTL lines from donor 25 (*E*) and 32 (*F*) were assayed on VV-infected C1R-A2 or C1R-neo targets. All targets were infected at a multiplicity of infection of 5. For *E* and *F* the negative control of C1R-neo targets infected with VV-pol is noted as ◇. C1R-A2 cells infected with no virus (■), VV-pol (◆), and VV-gag p17 (□) are noted.

(6–9). CTL recognition of the substituted peptides was therefore analyzed with two independent WT pol-specific CTL lines. I1F was recognized like WT pol by both CTL lines (Fig. 3 *A* and *C*). Donor 32 CTLs recognized I1Y (Fig. 3*D*), while the CTL line from donor 25 showed reduced I1Y recognition (Fig. 3*B*). These CTL lines are capable of recognizing endogenous antigen (Fig. 3 *E* and *F*). The difference in I1Y recognition was not due to HLA-A2 subtype differences, since both donors have the HLA-A*0201 subtype as determined by DNA sequencing. The distinction in I1Y recognition suggests a difference in T-cell repertoires.

***In Vitro* Immunogenicity of I1Y and I1F.** The crucial question for generating "improved" epitopes is whether the substitution peptide can stimulate T cells which recognize the WT epitope. To address this question, PBLs were stimulated *in vitro* to determine if the substituted peptide could elicit a WT pol-specific CTL response. Since CTL generation from a naive individual's PBLs is difficult, HLA-A2^{+} PBLs were used from three HIV-1-seropositive donors: 4, 9, and 32. HIV-specific CTLs can often be detected directly from seropositive PBLs (12, 24); however, these donors exhibited minimal pol-specific CTL reactivity after a single *in vitro* stimulation. pol-specific CTL reactivity of WT pol peptide-pulsed, autologous EBV-transformed B cells was less than 9% for all three donors. Significant pol-specific CTL activity from these donors was not detectable after 2 weeks of *in vitro* stimulation with WT pol, suggesting low initial numbers of CTL precursors. Other donors, like donor 25, exhibited high CTL activity (25% specific lysis) after the initial week in culture. This high initial CTL activity to the WT epitope provides a high background for *in vitro* stimulation; thus, a believable increase over this high response would be difficult to detect. The low responders were specifically chosen to provide a large dynamic range for detecting improved peptide stimulation. PBLs were stimulated with each of the following peptides on autologous B cells: WT pol, I1F, I1Y, and p17.

Starting at day 14, the stimulated PBLs from donor 32 were analyzed for reactivity against autologous B cells pulsed with either WT pol or p17 (Fig. 4*A*). The substituted peptides were therefore tested for their ability to stimulate a WT pol-specific CTL response. While significant pol-specific reactivity was not seen in the WT pol-stimulated PBLs, I1Y-stimulated PBLs showed significantly higher WT pol-specific CTL reactivity at day 14 (Fig. 4*A*). Significant pol-specific CTL reactivity was not seen in I1F-stimulated PBLs (Fig. 4*A*). As a control, p17-stimulated PBLs from donor 32 showed p17-specific CTL reactivity and no pol-specific reactivity (Fig. 4*A*). At 4 weeks, a similar trend was apparent (data not shown).

Stimulation of PBLs from donors 4 and 9 with the I1Y peptide also resulted in higher pol-specific CTL reactivity at day 16 compared with PBLs stimulated with WT pol. For donor 4, the pol-specific CTL reactivity of WT pol-stimulated PBLs and I1Y-stimulated PBLs was 35% and 47%, respectively, at an effector-to-target ratio of 30 (Fig. 4*B*). The stimulated PBLs were also able to recognize the pol peptide when it was produced endogenously by a VV construct expressing pol (Fig. 4*C*). In donor 9, WT pol-stimulated PBLs and I1Y-stimulated PBLs showed pol-specific reactivity of 12% and 23%, respectively, at an effector-to-target ratio of 30 (data not shown). Unlike donor 32, donor 4 was stimulated for a WT pol-specific response by I1F. The difference in immunogenicity of I1F between the two donors may be due to HLA-A2 subtype or TCR repertoire differences. Thus, I1Y stimulated a WT pol-specific response better than WT pol from three donors' PBLs. These data suggest that I1Y is more immunogenic than the WT pol peptide *in vitro*. Donor differences in the amount of CTL stimulation by I1F and I1Y clearly demonstrate the role of T-cell repertoire on the availability of reactive T cells.

Changes at Other Nonanchor Positions Lower Binding and/or CTL Recognition. We tested 10 more substitutions at positions 3 through 8 which were based on amino acids frequently found at these positions in high-affinity HLA-A*0201 peptides (Table 1) (4). The two natural variants of the pol epitope, E4D and G8E, were also analyzed (11). All the position 3 substitutions resulted in higher affinity peptides which were not recognized by pol-specific CTLs (data not shown). Almost all of the position 4–8 substitutions displayed a similar phenotype: decreased binding in both assays and little to no recognition by pol-specific CTLs (data not shown). P5Y bound HLA-A*0201 like WT pol, and E4S was recognized by one CTL line despite a lower binding affinity. Interestingly, the natural variants bound HLA-A*0201 with an affinity similar to that of WT pol; however, the recognition was reduced or abolished (data not shown).

DISCUSSION

An epitope's affinity for an MHC molecule is believed to be an important determinant for an immune response. In addition

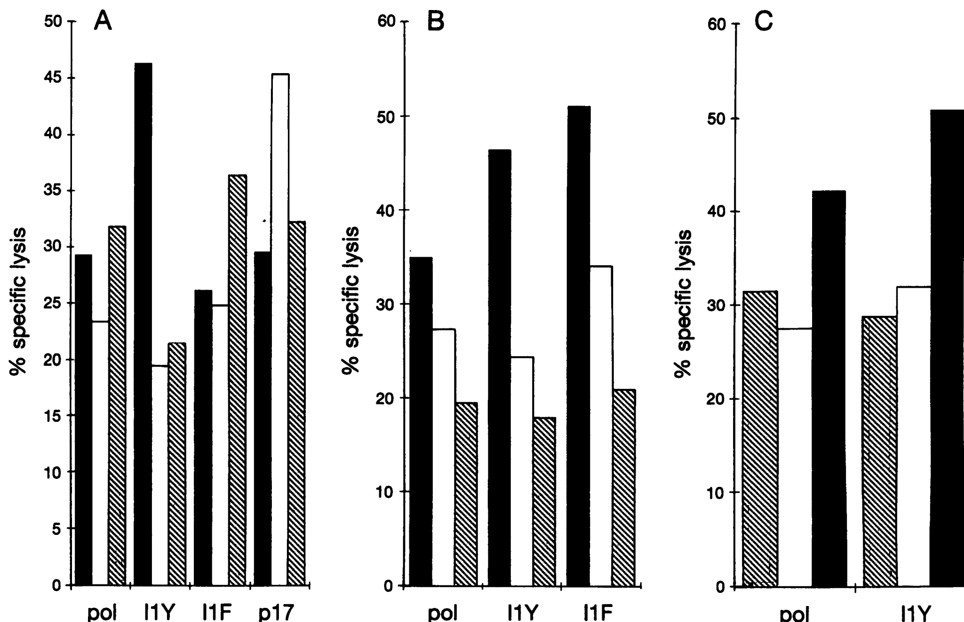

FIG. 4. Recognition of WT pol and p17 by *in vitro* stimulated PBLs. PBL from donor 32 (*A*) or donor 4 (*B*) were stimulated with autologous EBV-transformed B cells which were previously incubated with WT pol, I1F, I1Y, or p17 (only *A*). At day 14, the cells were tested for their recognition of autologous B cells incubated with WT pol (filled bars), p17 (open bars), or no peptide (hatched bars), in a standard ^{51}Cr release assay (6). Stimulated PBLs from donor 4 were also assayed for recognition of VV-infected targets (*C*). AR B-cell targets were infected as follows: no virus (hatched bars), VV expressing p17 gag (open bars), and VV expressing pol (filled bars). The *x*-axis shows the peptide used in stimulation. The effector-to-target ratio was 50 (*A*), 30 (*B*), or 12 (*C*).

to peptide binding, CTL recognition of foreign antigen is dependent on a productive interaction between the TCR and MHC/peptide complexes. Given a productive (nonantagonist) T-cell interaction, two models might describe the relationship between peptide affinity and T-cell response. One model suggests that a peptide's immunogenicity is proportional to its affinity for MHC. Alternatively, immune responses occur once a threshold peptide affinity or complex avidity is reached. In this second model, increasing the peptide affinity beyond this threshold would not enhance the specific immune response. Understanding which model is more accurate will lead to better developments in peptide therapeutics. The WT pol epitope must possess a binding affinity above the T-cell activation threshold, since many HLA-A2 HIV-positive individuals mount a response to this epitope (12). By improving the pol epitope's affinity, the relationship between complex stability and immunogenicity can be examined.

Given the importance of nonanchor amino acids in peptide affinity to MHC (3–5), improving the binding and recognition of the HIV-1 pol peptide was investigated by altering the nonanchor side chains. Since the majority of the P1 side chain is buried within the class I cleft (14), P1 is an excellent position to investigate increasing affinity without altering TCR interaction. Of the 11 P1 substitutions investigated, only 2 aromatic substitutions, I1Y and I1F, bound cell surface HLA-A*0201 as well as did WT pol (Fig. 1*A*). In the assembly assay, I1F and I1Y showed similar affinities to WT pol (Fig. 1 *B* and *C*).

Both I1Y and I1F displayed an approximately 3-fold increase in peptide–MHC complex half-lives at 37°C at the cell surface. This prolongation of the half-life should increase the peptide's cell surface concentration, thereby increasing the avidity of the cell–cell interaction. An epitope present at high cell surface concentration may be able to better stimulate peptide-specific T cells. Recent studies in MHC class I models have shown a correlation between peptide/MHC affinity and the ability to detect a response (22).

Since the P1 side chain's orientation is similar among several HLA-A*0201-restricted peptides (14), substitutions at P1 should not affect the overall peptide orientation. This idea is supported by the CTL recognition of I1F and I1Y (Fig. 3). CTL lines from two different donors were able to recognize these peptides. For the altered peptides to be effective as immunogens, they must be recognized by the host as the WT epitope. PBLs from HLA-A2, HIV-positive individuals were stimulated *in vitro* with WT pol, I1Y, and I1F. The resulting PBLs were analyzed for recognition of the WT pol peptide. I1Y was able to stimulate WT pol-specific CTLs better than WT pol from the PBLs of three seropositive donors (Fig. 4). These data suggest that I1Y is more immunogenic than WT pol, presumably due to the dramatic increase in the cell surface complex stability. However, differences between donors in the amount of CTL stimulation by I1F and I1Y clearly demonstrate the important role of T-cell repertoire on the availability of reactive T cells. In addition, the *in vitro* stimulated lymphocytes were able to recognize the pol peptide when it was produced endogenously by VV constructs (Fig. 4*C*). Thus, our stimulation conditions did not preferentially stimulate T cells with low-affinity receptors which recognize peptide-pulse, but not endogenously processed, antigen.

The *in vitro* immunization analysis was conducted on PBLs from seropositive donors. The question of previous T-cell priming is evident. Unlike many donors (12, 24), the three donors investigated all exhibited very low pol-specific reactivities even after 1 week of stimulation with WT pol peptide. This suggested low initial numbers of CTL precursors in these donors. It is unlikely that three independent donors were previously primed *in vivo* with I1F or I1Y, since the HLA-A2-restricted pol epitope is extremely conserved and neither the I1Y nor the I1F substitution has been reported in HIV-1 strains (11). Peptide immunization using I1Y may be able to boost or maintain pol-specific CTL activity in HIV-1-infected individuals. From these experiments, it is unclear if I1Y would stimulate CTLs in a naive individual that could cross-react with WT pol. In the HLA-A*0201/H-2 K^b transgenic mouse model, most high-affinity peptides tested were also highly immunogenic (22).

We have identified a peptide, I1Y, which has (*i*) increased complex stability for HLA-A*0201, (*ii*) maintains recognition by WT pol-specific CTL lines, and (*iii*) is more stimulatory for WT pol-specific CTLs *in vitro*. Thus, I1Y shows potential as a vaccine component. Optimizing peptides through amino acid substitution and immunogenicity analysis has significant therapeutic ramifications for infectious disease, tumor immunology, and autoimmunity.

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