An epitope in human immunodeficiency virus 1 reverse transcriptase recognized by both mouse and human cytotoxic T lymphocytes

(AIDS/vaccine/peptides/immune response genes)

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ABSTRACT   T-cell-mediated cytotoxicity may play an important role in control of infection by the human immunodeficiency virus (HIV). In this study, we have identified and characterized a relatively conserved epitope in the HIV-1 reverse transcriptase recognized by murine and human cytotoxic T cells. This epitope was identified using a murine antigen-specific CD8$^+$ class I major histocompatibility complex-restricted cytotoxic T-cell (CTL) line, a transfected fibroblast cell line expressing the HIV-1 pol gene, recombinant vaccinia viruses containing different truncated versions of the pol gene, and overlapping synthetic peptides. The optimal antigenic site was identified as residues 203–219 by synthesizing extended or truncated peptide analogs of the antigenic fragment. The optimal peptide was then tested for sensitization of autologous Epstein–Barr virus-transformed B-cell targets for killing by fresh human peripheral blood mononuclear cells. It was recognized by CTLs from several HIV-seropositive patients but not from any seronegative donor. Therefore, this peptide is a good candidate for inclusion in an AIDS vaccine. This study demonstrates that the same CTL epitope can be seen by murine and human CD8$^+$ CTLs, as previously demonstrated for epitopes recognized by CD4$^+$ helper T cells, and suggests the utility of screening for immunodominant CTL epitopes in mice prior to carrying out studies in humans.

Cytotoxic T lymphocytes (CTLs) have been found to mediate protection in vivo against certain virus-induced diseases, including some caused by retroviruses (1–3). This defense mechanism might be particularly relevant against the human immunodeficiency virus (HIV), which can spread through cell–cell contact and thus may not be accessible to antibody neutralization. Indeed, CD8$^+$ cells have been shown to inhibit growth of HIV or simian immunodeficiency virus in cells of infected patients or monkeys (4, 5). Efforts have thus been directed at studying the CTL response against different proteins of HIV. Human CD3$^+$ 8$^+$ CTLs specific for the envelope glycoprotein gp160 have been identified (6–12), and an immunodominant CTL epitope has been mapped in H-2^d mice to residues 315–329, a highly variable region of the envelope (13). However, immunizing with the whole envelope protein is not an ideal approach for several reasons. (i) The majority of the cytotoxic response assayed in vitro with fresh peripheral blood mononuclear cells (PBMCs) from HIV-seropositive individuals seems to be mediated by non-major histocompatibility complex (MHC)-restricted mechanisms [antibody-dependent cellular cytotoxicity (ADCC) or

natural killer cells (NK)] (14). (ii) The envelope is highly variable in sequence and CTL clones can distinguish different isolates of HIV (15, 16). (iii) There is evidence for antibody-dependent enhancement of HIV-1 infection, likely to be mediated by anti-envelope antibodies (17, 18). (iv) Immune responses to the HIV envelope have been suggested to contribute to immune deficiency: Uninfected CD4$^+$ T cells that bind gp120 may be killed by ADCC triggered by anti-envelope antibodies (19) or by CD4$^+$ CTLs specific for gp120 (20). Anti-gp120 antibodies can also inhibit CD4$^+$ T-cell function like anti-CD4 antibodies by binding to gp120, which binds to CD4 (21). Also, gp160 can elicit autoantibodies that crossreact with human class II MHC molecules and inhibit T-cell function (22). Finally, gp120 itself may inhibit T-cell function directly (19).

In contrast to the envelope glycoprotein, the internal proteins of HIV are more conserved and would be less likely to contribute to these deleterious effects. Moreover, in other viral models, internal proteins are the predominant targets of the CTL response (23–28). Responses have been found in HIV patients against the products of the gag, pol, nef, and vif genes (7, 9, 12, 29, 30). Because of its conservation and importance to viral function, the reverse transcriptase (RT) appears to be worth particular interest in this regard. We describe here our approach of using a murine model to identify evolutionarily conserved CTL determinants in RT and the relevance of this identification to human anti-HIV cytotoxic responses.

MATERIALS AND METHODS

Mice. C3H/HeJ (H-2^k) mice were obtained from The Jackson Laboratory.

Recombinant Vaccinia Viruses. v-pol (vCF-21, Fig. 1) (31) and v-pol-100 are recombinant vaccinia viruses containing the RT gene of human T-lymphotropic virus III (HTLV-III) subclone HXB.2 except for the last 22 residues (pol gene deduced amino acid sequence 168–706 for the insert, 168–728 for RT) (32). The truncated pol gene-recombinant vaccinia viruses (vCF 32, 33, 34, 35, and 37) (33) have been renamed

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; HIV, human immunodeficiency virus; RT, reverse transcriptase; L-pol, DAP3 fibroblast cell clone transfected with the HIV-1 RT gene; MHC, major histocompatibility complex; NK, natural killer cells; PBMC, peripheral blood mononuclear cell; v-pol, recombinant vaccinia virus containing the HIV-1 RT gene; v-ctrl, negative control recombinant vaccinia virus; HTLV, human T-lymphotropic virus.
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in this paper v-pol-100, -80, -67, -58, and -27, respectively, to indicate the proportional length of their inserts as a percentage of the v-pol-100 insert length (Fig. 1). These inserts all initiate at the 5' end of the original insert, corresponding to residue 168 of the amino acid sequence and to the N-terminal end of the processed protein. The negative control recombinant vaccinia virus v-ctrl (vSC-8) (34) contains the *Escherichia coli lacZ* gene as a control.

Transfectants. The transfectant L-pol was prepared using the pcEXV-3 vector containing the same RT-encoding insert as v-pol. pcEXV-3-pol was cotransfected with pSV2neo into DAP3 L cells using the $CaPO_4$ method (35), and clones were isolated after selection in G418. A single clone (RGT 1.10.7) having a high level of pol transcripts in RNA dot-blot analysis was selected for use in these experiments. The control L cells (L28) were prepared with pSV2neo alone.

Generation of a Murine CTL Line. The line Pol *a* was obtained from v-pol immunized (10^7 plaque-forming units, i.v.) C3H/HeJ female mouse spleen cells restimulated *in vitro* with mitomycin C-treated L-pol transfectants as in ref. 13 (5 × 10^6 spleen cells, 5 × 10^5 L-pol cells per well in 24-well plates) but in the presence of supplemented medium—i.e., complete T-cell medium containing 10% concanavalin A-stimulated rat spleen cell medium (T-cell Monoclone, Collaborative Research). The line has been maintained now for more than a year by weekly restimulations (0.3 × 10^6 line cells, 10^6 L-pol cells) and biweekly feeding with supplemented medium. It has been used for assays not earlier than 3 days after a T-cell Monoclone addition.

CTL Assay. The murine CTL assays were performed as in ref. 13 with the following differences: 10^6 L cells were infected with recombinant vaccinia viruses at a multiplicity of infection of 50 for 1 hr, washed, and then incubated overnight with ^{51}Cr; in other experiments, L cells were incubated overnight with peptides and with ^{51}Cr [0.3 × 10^6 cells, 0.05 mCi (1.85 MBq) per well in 24-well plates]. The target cells were then washed four times before use in the 6-hr assay (5000 target cells per well in round-bottom 96-well plates). In the human assays, lines derived from PBMCs of donors by Epstein–Barr virus (EBV) transformation were either pulsed overnight with 10 μM peptide or infected with recombinant vaccinia virus (multiplicity of infection = 100) for 1 hr and then washed. In both cases they were labeled overnight with 0.3 mCi (11.1 MBq) of ^{51}Cr and used as targets (5000 per well) the following day in a 6-hr ^{51}Cr release assay with unstimulated cryopreserved PBMCs from the same donor as effectors. The percentage specific release was calculated as 100× (experimental ^{51}Cr release − spontaneous release)/ (maximum release − spontaneous release).

Peptide Synthesis. A series of 15-residue peptides covering the fraction of RT sequence (HXB.2 subclone of HTLV-III/B) (32) that was expressed by the virus v-pol-27 was synthesized and purified as described (13, 36). Their molar concentration was determined by HPLC or by spectrophotometry. A series of analogs of peptide HP138 has been synthesized on an ABI automated synthesizer, cleaved by low HF procedure, desalted either by Bio-Gel P-4 or by reverse-phase (C_{18} Sep-Pak, Waters) chromatography, and purified to a single HPLC peak (C_{18} column, Waters). Amino acid analysis (performed by O. Bates and colleagues, University of California, Irvine) confirmed the expected sequences.

Patient Source and Clinical Evaluation. HIV$^+$ patients were obtained from Wilford Hall, U.S. Air Force Medical Center, Lackland Air Force Base. Individuals were diagnosed as being HIV infected if anti-HIV antibodies were demonstrated on two specimens tested by the HIV enzyme immunoassay (Abbott) and confirmed by Western blot analysis (Roche Biomedical Laboratories, Burlington, NC). Patients were classified according to the Walter Reed staging system (37).

Lymphocyte counts and T-cell subsets were determined using laser-based flow cytometry (Coulter epics profile) and OKT4A (anti-CD4) and OKT8 (anti-CD8) monoclonal antibodies (Ortho Diagnostics).

RESULTS

Generation of a Murine CTL Line (Pol *a*) Specific for the N-Terminal End of HIV-1 RT. The CTL line Pol *a* was derived from spleen cells of C3H/HeJ mice immunized with the recombinant vaccinia virus v-pol and restimulated *in vitro* with an H-2^k fibroblast line (L-pol) transfected with the same HIV-1 *pol* gene insert. This CTL line (Fig. 1) lysed specifically L-pol as well as untransfected H-2^k fibroblasts (L cells) infected by v-pol but not L cells infected by the control vaccinia virus v-ctrl. Therefore, the HIV-1 *pol* gene fragment expressed at least one CTL epitope recognized on H-2^k fibroblasts.

To localize the epitope(s), we first infected L cells with recombinant vaccinia viruses expressing truncated versions of the *pol* gene (Fig. 1) and used these cells as targets in a cytotoxicity assay with the Pol *a* CTL line. The targets infected with viruses containing from 100% to as little as 27% of the N-terminal part of the original insert were all lysed to a comparable extent (Fig. 1). This experiment indicated that an immunodominant epitope was present in the portion of the protein encoded by the shortest *pol* insert (27% of the original insert—i.e., residues 168–316). This area corresponds to the N-terminal end of RT. Other epitopes may have been present in the remainder of the sequence, but this portion by itself was able to induce maximal lysis.

Identification of the Immunodominant Epitope Recognized by the Line Pol *a*. To identify the epitope(s) contained in this area, we used 21 overlapping 15-amino acid peptides designated HP134 to HP154 covering residues 168–316. L cells were incubated overnight with ^{51}Cr together with peptides (20 μM), washed, and tested for lysis by Pol *a*. To reduce the number of different targets to be tested, we analyzed mixtures of two peptides at a time but avoided mixing overlapping peptides that might have a higher risk of inhibiting each other by sharing the same MHC binding site. Because preliminary experiments had suggested that peptide HP138
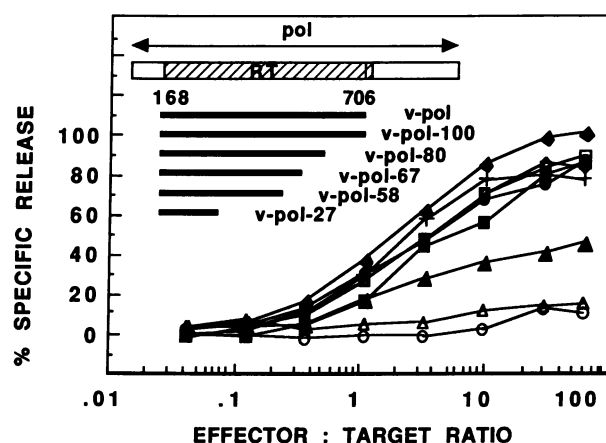

FIG. 1.   CTL line Pol *a* specificity for the N-terminal end of HIV-1 RT. (*Upper*) *pol* gene and the fragment inserted in the recombinant vaccinia virus v-pol and transfected into the fibroblast clone L-pol. The amino acid sequence is deduced from the nucleotide sequence (32). The recombinant viruses v-pol-100 to -27 contained 3'-truncated gene fragments as described in the text and in ref. 33. (*Lower*) Pol *a* line specific cytotoxicity on L-pol, L cells infected with v-pol or with truncated *pol* gene-recombinant vaccinia viruses. ○, L cells; ●, L cells plus v-pol; □, L cells plus v-pol-100; ■, L cells plus v-pol-80; ◇, L cells plus v-pol-67; ◆, L cells plus v-pol-58; +, L cells plus v-pol-27; △, L cells plus v-ctrl; ▲, L-pol.

was responsible for targeting the cytotoxic activity, HP138 was tested individually and was found to sensitize targets (Fig. 2). However, none of the other peptides was able to sensitize targets (Fig. 2), including HP137 and HP139 (that overlap HP138 by nine and five residues, respectively), even when tested individually (data not shown). HP138 (Cys-Thr-Glu-Met-Glu-Lys-Glu-Gly-Lys-Ile-Ser-Lys-Ile-Gly-Pro) corresponds to residues 205–219 of the HTLV-III/B strain sequence (32). It is not toxic by itself as determined by spontaneous lysis and cell recovery in the presence of peptide alone (data not shown). The line Pol *a* was class I MHC-restricted because it killed HP138-pulsed, class II MHC molecule-negative H-2^k L-cell fibroblasts but not 3T3 fibro-blasts (H-2^d) or EL4 thymoma cells (H-2^b). It was demonstrated to be CD4$^-$, CD8$^+$ by treatment with anti-CD8 or anti-CD4 monoclonal antibodies and complement (data not shown).

Definition of the Optimal Epitope. Because HP138 sensitized targets best at high concentrations (20 μM), we synthesized and purified a series of peptide analogs in the area of HP138 to determine whether activity could be improved by extending or shortening the peptide by a few residues. The addition of two amino acids (Fig. 3) on the N-terminal side of the sequence (peptide 203–219) induced a higher maximal specific release (>60% vs. 40%), and a 30-fold lower concentration was needed to obtain half-maximal activity (≈0.3 vs. 10 μM). In contrast, removal of residues 205 and 206 at the N terminus abrogated all activity (peptide 207–223 compared to 205–223). This result suggests that a critical determinant is at the N terminus of HP138. The addition of amino acids on the C-terminal end of 205–219 (peptide 205–223) also enhanced the activity (plateau at ≈60%, half-maximal lysis at ≈1 μM), but was not able to restore the loss of activity when residues 205 and 206 were missing in peptide 207–223. Peptide 199–223 contained the two residues 203 and 204 and
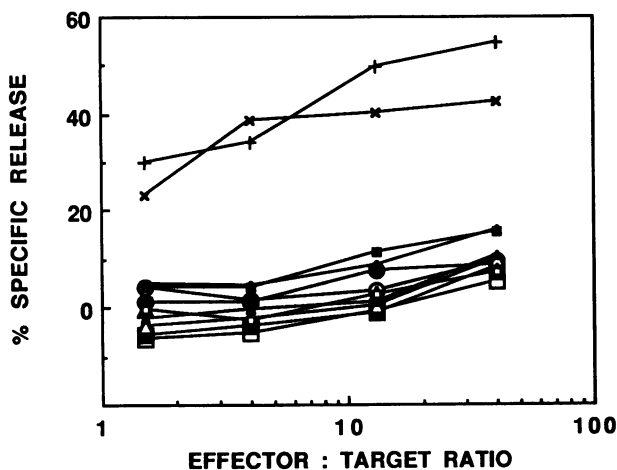
FIG. 2. CTL line Pol *a* specificity for peptide HP138. L cells were incubated overnight with peptides at a concentration of 20 μM and with ^{51}Cr as described in the text and then used as targets with different numbers of Pol *a* effector cells. The sequences of the peptides are as follows: HP134 (175–189), HP135 (185–199), HP136 (193–207), HP137 (199–213), HP138 (205–219), HP139 (215–229), HP140 (229–244), HP141 (238–252), HP142 (243–257), HP144 (265–279), HP145 (275–289), HP146 (283–297), HP147 (288–302), HP148 (300–314), HP149 (311–325), HP150 (315–329), HP151 (324–339), HP152 (329–343), HP154 (355–369). HP143 (255–269) and HP153 (344–359) could not be tested because they were insoluble. +, L-pol; ○, L cells; ●, L cells plus HP134 plus HP146; □, L cells plus HP135 plus HP147; ■, L cells plus HP136; △, L cells plus HP137 plus HP148; ×, L cells plus HP138; ▲, L cells plus HP139 plus HP149; □, L cells plus HP140 plus HP150; ■, L cells plus HP141 plus HP151; △, L cells plus HP142 plus HP152; ▲, L cells plus HP144 plus HP154; ◆, L cells plus HP145 plus HP155.
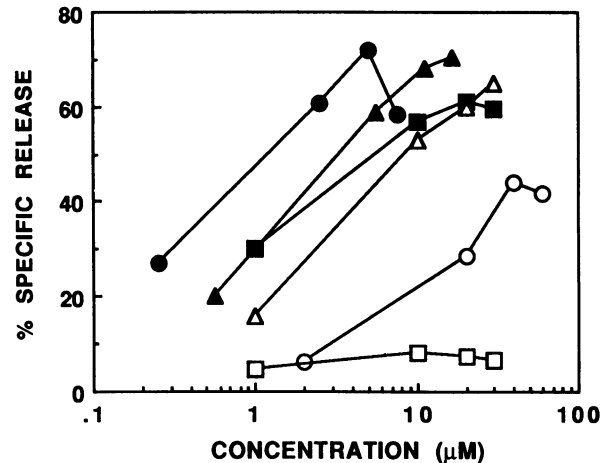
FIG. 3. Localization of the CTL epitope in the *pol* 199–223 area. Lysis by Pol *a* of peptide-pulsed L cells at E:T = 10:1. ○, Peptide 205–219; ●, peptide 203–219; □, peptide 207–223; ■, peptide 205–223; △, peptide 199–223; ▲, peptide 203–218. Peptide 205–219, which is the sequence of HP138, has been resynthesized along with its analogs.

the elongation on the C terminus that enhanced the activity in 205–223, but surprisingly its activity was not better than that of 205–223 (same maximal activity, half-maximal lysis at ≈2 μM), as if the sequence 199–202 was partly inhibiting recognition. Finally, the removal of the proline residue 219 did not modify the maximal activity of peptide 203–219 but increased slightly the concentration necessary for half-maximal lysis (≈1 μM, peptide 203–218).

Recognition of the Epitope by Human Cytotoxic Cells. Having identified an epitope for CD8 class I MHC-restricted CTLs in a mouse model, we wanted to know whether it would be recognized by cells from HIV-infected humans as well. PBMCs from two HIV seropositive and two seronegative individuals were tested, without restimulation *in vitro*, for the lysis of autologous EBV-transformed cells incubated overnight with peptide 205–219 or 203–219 (Fig. 4 *Upper*). The PBMCs from both patients were able to kill specifically targets preincubated with these two peptides but not targets preincubated with a control peptide or with medium only. Conversely, the PBMCs from both seronegative donors failed to kill the targets incubated with either peptide but did show some killing on autologous target cells infected with the control vaccinia virus (Fig. 4 *Lower*), as several other investigators have found in similar studies (6, 7, 9, 30). The significance of this response is not clear, because the vaccinia virus immunization history of the patients and the controls shown in Fig. 4 was not available to us. The two patients, but not the healthy controls, also showed a high cytotoxic activity against the targets infected with the pol-recombinant vaccinia virus (Fig. 4 *Lower*). For one of these two donors we were able to test the phenotype of the effector cells. The activity was blocked by anti-CD3 and anti-CD8, but not anti-CD4, monoclonal antibodies and complement and was genetically restricted (data not shown). Thus, these are conventional antigen-specific MHC-restricted CD8$^+$ CTLs. We have tested cells from 12 HIV seropositive patients and 5 control individuals (Fig. 5). The level of killing after incubation of the target cells with HP138, peptide 203–219, or peptide 205–219 was >10% in 5 of 12 patients, whereas it ranged from <0 to 3.2% in the seronegative donors. An arbitrary upper limit of 10% lysis was chosen to indicate positive CTL activity, based on this value being 3-fold above the maximum lysis obtained by PBMCs from HIV-seronegative control donors. At least 3 patients exhibited lysis considerably above this background. These data show

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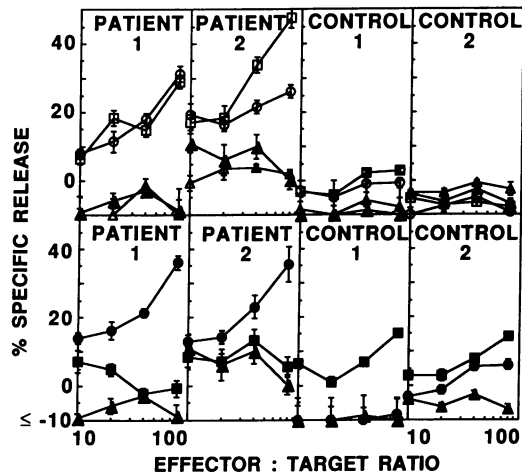*Proc. Natl. Acad. Sci. USA 87 (1990)* 2347

FIG. 4. Recognition of the immunodominant murine epitope of HIV-1 RT by PBMCs from two HIV-infected patients and two seronegative controls. EBV-transformed B cells derived from HIV-infected donors or HIV⁻ controls were used as targets. Aliquots of each individual's target cells were incubated overnight with a concentration of 10 μM peptide 203–219 (□), 205–219 (○) or 275–289 (△) [HP145, a control peptide without any activity in the murine tests as shown in Fig. 2 and not predicted to be a T-cell epitope by the AMPHI algorithm (38)]. Other aliquots of target cells were infected with v-pol (●) or with v-ctrl (■). The targets were then assayed with autologous PBMCs as effectors in a 6-hr ⁵¹Cr release assay. The error bars show the standard error of the mean of the triplicates. (*Upper*) Targets preincubated with peptides. (*Lower*) Targets infected with viruses. ▲, Medium only.

that this epitope, recognized in the context of one murine MHC haplotype, can also be recognized in association with one or several human MHC molecules. An attempt has been
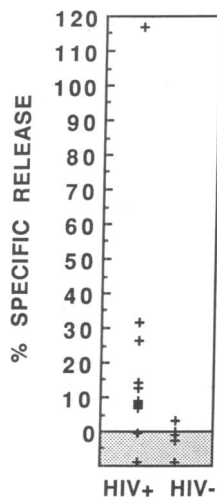

FIG. 5. Lysis of target cells incubated with peptide 205–219 by autologous PBMCs: Comparison of 12 seropositive patients (HIV⁺) and 5 seronegative controls (HIV⁻). Each point represents the % specific ⁵¹Cr release of peptide-incubated targets after a 6-hr assay in the presence of autologous PBMCs at the maximal effector:target ratio tested (80:1 or 40:1). For each HIV⁺ donor, control target cells either with no peptide or with control vaccinia virus were not lysed. Two additional patients who had % specific releases at 11% and 12%, respectively, were not included here because no adequate negative control was available. The following patients, indicated by the level of lysis obtained, have been HLA typed. 116.9%: HLA A 1,25; B 8,18; DR 2,3; DQ1. 14.4%: HLA A 2,29; B44; DR7. 8.4%: HLA A 2,3; B 7,41; Cw7; DR 2,4; DQ 1,3. 7.9%: HLA A 9,10; B 18,35; Cw4; DR2. 7.2%: HLA A 2,24; B 18,35; Dw4; DR 4,5; DQ 3. −0.4%: HLA A 2,3; B 7,35; Cw4; DR4; DRw6; DQ 1,3.

made to study a correlation between the HLA type and a response to the epitope, but, because of technical limitations (low amount of blood available, high nonspecific antibody binding on EBV-transformed lines, restricted availability of typing on HIV⁺ patient cells), only a few patients could be typed (Fig. 5). Within this group there did not appear to be a correlation of any HLA antigen and responsiveness to the HIV CTL epitope. The relevance of this epitope in HIV infection is underscored by the finding that it is recognized by CTL circulating *in vivo* in HIV-seropositive individuals, assayed using fresh peripheral blood, without any antigenic restimulation *in vitro*.

DISCUSSION

In this paper, we have identified a CTL epitope in the HIV-1 RT. This epitope is distinct from those recently reported by Walker *et al.* (33) to be recognized by individual clones from two infected patients. The cytotoxicity against this epitope was mediated by conventional CD3⁺ CD8⁺ antigen-specific MHC-restricted CTLs in the mouse and human. The sequence of this epitope (203–219) is much less variable than the epitope we had found in the HIV-1 envelope (13); it is conserved among all of the North American or European HIV-1 strains sequenced to date, although there are some variations in the African strains HIV-1/ELI and HIV-1/MAL and more in HIV-2 (two, three, and seven substitutions, respectively) (39). The epitope 203–219 is in a region highly conserved in evolution among RT genes of other viruses (32). For this reason, it is likely to be essential for RT activity and may therefore not tolerate substitutions required for escape from the immune system (15, 16).

The addition of two residues (203 and 204) enhanced dramatically the activity of the original peptide [cf. Kumar *et al.* (40)], whereas the analog missing residues 205 and 206 had no activity at all. Thus the sequence 203–206 seems essential for optimal activity of the peptide. It was also possible to enhance the activity by elongating the original peptide on the C-terminal side. Interestingly, a longer analog encompassing the N- and C-terminal extensions did not reach the same level of activity as the optimal epitope 203–219. The additional residues that it contains on its N-terminal side may include a hindering structure similar to those found in class II-restricted epitopes (41–43). The segment 203–219 was identified by the program AMPHI (38) to be able to fold as an amphipathic 3₁₀ helix, consistent with previously observed properties of T-cell epitopes (38).

Many class II-restricted T-cell epitopes have been identified, but in most cases they have not been studied in more than one species. We have identified several epitopes from HIV and malaria proteins seen by class II MHC-restricted proliferative T cells in mice, and every one of these tested with T cells from immune humans stimulated the human T cells as well (44–49). A similar case has been found for influenza (50, 51). However, such comparisons have not been made for epitopes recognized by class I MHC-restricted CTLs. In the present study we describe a class I MHC-restricted epitope recognized by mice immunized by a recombinant vaccinia virus and show that it is recognized by antigen-specific CTLs from HIV-infected patients. Concurrent studies indicate that the HIV-1 envelope epitope identified in recombinant vaccinia virus-immunized mice (13) is also recognized by class I MHC-restricted CTLs from HIV⁺ individuals (M.C., unpublished data) and by CTLs from recombinant vaccinia virus-immunized volunteers (52). To our knowledge, examples of common class I-restricted epitopes recognized by humans and mice have not been reported previously.

The most useful epitopes for a vaccine should be immunodominant—i.e., recognized by a majority of T-cell clones

in a bulk cell population. However, it is difficult to obtain sufficient quantities of blood from HIV-infected patients to screen large numbers of peptides on whole peripheral blood cell populations. Infected blood donors are usually unavailable for repeated blood drawing, and to allow time for production of EBV-transformed target cell lines, one must use as effectors cryopreserved cells with recoveries often of only 50%. In our experience, no more than 10–12 peptides, including controls, can be tested from a single bleed. Therefore, our approach, with class II as well as with class I MHC-restricted epitopes, has been to locate and characterize them first in mice and then to test them with human cells. The T-cell receptor and MHC molecule structures are very conserved between mice and humans (53, 54). In mice, the ability to pool cells from multiple genetically identical individuals removes restriction on cell numbers to test large numbers of peptides. Bulk cell lines can be grown with the specific stimulus of syngeneic transfected tumor cell lines. Congenic strains differing by their histocompatibility loci facilitate determination of the MHC restriction. In addition, one can immunize mice by various procedures, whereas in humans one is limited to studying cells from infected individuals, who may already have some degree of immune dysfunction (55). The initial identification of epitopes in mice allows effective use of limited quantities of blood from patients to test for the presence of cytotoxic cells *in vivo*, using fresh blood, without the need for restimulation *in vitro* that might bias the results. The current study demonstrates that the approach of locating and characterizing CTL epitopes in mice can lead to successful identification of peptides that may be useful in vaccines for humans.

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