Recognition of a Highly Conserved Region of Human Immunodeficiency Virus Type 1 gp120 by an HLA-Cw4-Restricted Cytotoxic T-Lymphocyte Clone

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Human immunodeficiency virus type 1 (HIV-1) isolates exhibit extensive sequence variation, particularly in the gp120 subunit of the envelope glycoprotein, and the degree of this variation has raised questions as to whether conserved regions of the HIV-1 envelope can be recognized by the host immune response. A CD8⁺ cytotoxic T-lymphocyte (CTL) clone specific for the HIV-1 envelope was derived by culturing peripheral blood mononuclear cells from an HIV-1 seropositive subject in the presence of a CD3-specific monoclonal antibody, interleukin-2, and irradiated allogeneic peripheral blood mononuclear cells. Lysis of target cells was restricted by an HLA-C molecule, Cw4, which has not been previously shown to present viral antigen to CTL. Mapping of the specificity of this CTL clone by using synthetic HIV-1 peptides localized the epitope to an 8-amino-acid region of gp120 (amino acids 376 to 383) which is conserved among approximately 90% of sequenced viral isolates. Examination of the recognition of variant peptides by this CTL clone demonstrated that a single, nonconservative amino acid substitution within the 8-amino-acid minimal epitope could abrogate lysis of targets incubated with the variant peptide. The identification of a CTL epitope in a highly conserved region of gp120 documents the ability of cellular immune responses of infected persons to respond to relatively invariant portions of this highly variable envelope glycoprotein. However, the ability of even a single-amino-acid change in gp120 to abolish lysis by CTL supports the hypothesis that sequence variation in HIV-1 may serve as a mechanism of immune escape. In addition, the identification of an HLA-C molecule presenting viral antigen to CTL supports a functional role for these molecules.

The natural history of infection with human immunodeficiency virus type 1 (HIV-1) is characterized by a prolonged asymptomatic phase which typically lasts 5 to 10 years (26). During this asymptomatic phase, viral replication persists at a low level in most infected individuals despite the presence of both humoral and cellular immune responses (7, 16). Ultimately, CD4⁺ T lymphocytes decline in number and viral replication increases, resulting in profound immunodeficiency. The mechanisms utilized by HIV-1 to persist in the presence of, and to ultimately escape, a relatively vigorous immune response remain poorly understood. Precise characterization of the recognition of HIV-1 by host immune responses should facilitate delineation of the mechanisms of immune escape and the design of a vaccine.

Cytotoxic T lymphocytes (CTL) are an important host defense against many viral infections and are likely to play an important role in the control of HIV-1 replication. In mice, adoptive transfer experiments have demonstrated that CTL mediate protective immunity against lymphocytic choriomeningitis virus (53), herpes simplex virus (40), and influenza virus (52). In humans, evidence for a protective role of CTL against viral infection arises from the observations of a positive correlation between virus-specific CTL activity and recovery from infection with cytomegalovirus (36) and influenza virus (28). With regard to HIV-1, vigorous HLA class I-restricted CTL responses against multiple HIV-1 proteins, including the envelope glycoprotein gp160, have been described (19, 20, 22, 23, 33, 48, 50). However, the repertoire of CTL epitopes recognized in gp160 has not

been completely defined. Although several envelope peptides recognized by CTL have been identified by using polyclonal effector cells (6, 8, 25, 35), many of the epitopes recognized by these bulk cultures have not been precisely characterized. Only a subset of the reported epitopes in gp160 have been identified by using CTL clones (9, 15, 19, 41, 44, 45), and the majority of these are in gp41.

To gain a better understanding of the recognition of HIV-1 by human cellular immune responses, we have characterized CTL epitopes which are recognized by lymphocyte clones and unstimulated peripheral blood mononuclear cells (PBMC) derived from HIV-1 seropositive subjects (19, 20, 49). The specificity of these CTL, which are derived without in vitro stimulation with viral antigen, are likely to accurately reflect the specificity of CTL in vivo. In this report, we describe an HLA-Cw4-restricted CTL epitope in a highly conserved region of the external envelope glycoprotein of HIV-1, gp120. Fine mapping with synthetic peptides localized the specificity of this clone to an 8-amino-acid (8-aa) epitope. Analysis of amino acid substitutions within this epitope demonstrated that a single substitution could abolish recognition. Although the existence of CTL epitopes in highly conserved regions of gp120 should facilitate the design of vaccines against HIV-1, the ability of single-amino-acid changes to abolish recognition underscores the potential impact of sequence variation on the ability of HIV-1 to escape host immune responses.

MATERIALS AND METHODS

Subject. An HIV-1 seropositive subject (010-035i) known to have significant envelope-specific CTL activity was se-

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lected for study (19, 20, 48, 49). The subject gave written informed consent, and the study was approved by the Massachusetts General Hospital Human Studies Committee. At the time that blood was obtained for this study, the subject was asymptomatic with 145 CD4^+ lymphocytes per mm^3 . This subject was referred to as subject 63 in a previous report from this laboratory (49).

Cell lines. Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCL) were established and maintained as described previously (48) in RPMI 1640 (Sigma, St. Louis, Mo.) containing 20% (vol/vol) heat-inactivated fetal calf serum (Sigma). RPMI 1640 used for all cell lines was supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (10 mM). Allogeneic B-LCL were also obtained from the American Society for Histocompatibility and Immunogenetics B-cell line repository. All B-LCL were found to be free of mycoplasma infection by standard culture techniques.

HLA typing. HLA typing was performed by the Massachusetts General Hospital Tissue Typing Laboratory with a standard lymphocytotoxicity assay.

Recombinant vaccinia viruses. Preparation of recombinant vaccinia viruses expressing serial truncations of the HIV-1 envelope glycoprotein has been previously described (11). In brief, a series of truncated envelope genes was prepared from the BH8 molecular clone of HIV-1 (37) by digestion at unique restriction enzyme sites, and recombinant vaccinia viruses were generated by using homologous recombination. The recombinant vaccinia virus vAbt141 expressing the full-length p55 gag protein (24) was kindly provided by Gail Mazzara and Dennis Panicali (Therion Biologics, Inc., Cambridge, Mass.). Recombinant vaccinia viruses expressing the HIV-1 reverse transcriptase (RT) (VCF21) (50) and the control *lacZ* (VSC8) genes were also used in these studies. Stocks of recombinant vaccinia viruses were adjusted to approximately 10^9 PFU/ml, stored in aliquots at -80°C , and thawed immediately prior to use.

Synthetic HIV-1 peptides. Synthetic peptides corresponding to the HIV-1 envelope PV22 sequence (29) were synthesized by Multiple Peptide Systems (San Diego, Calif.) by using *t*-butoxycarbonyl NH_2 -protected amino acids and 4-methylbenzhydrylamine resin (17). Peptides were cleaved from the resin with anhydrous hydrogen fluoride, washed with ether, extracted with 10% acetic acid, and evaluated for purity by reverse-phase analytical high-performance liquid chromatography. Peptides consisted of a series of peptides 25 aa in length and overlapping by 8 aa and were synthesized as COOH-terminal amides, unless otherwise noted. All amino acids are numbered as indicated for the HXB2R sequence (30). Additional smaller peptides (8 to 15 aa) were synthesized for fine mapping by using a technique similar to that described above (2). Peptide 116J was synthesized as a COOH-terminal acid by using fluorenylmethoxycarbonyl-based chemistry (Research Genetics, Huntsville, Ala.). Lyophilized peptides were reconstituted at 2 mg/ml in sterile distilled water with 10% dimethyl sulfoxide (Sigma) with or without 1 mM dithiothreitol (Sigma).

Isolation of an HIV-1 envelope-specific CTL clone. CTL clones were isolated and maintained as described previously (19, 20, 49). Briefly, PBMC were obtained by separation on a Ficoll-sodium diatrizoate cushion (Histopaque-1077; Sigma) and were incubated at 50 or 25 cells per well in 96-well plates with 200 μl of feeder cell solution containing 10^6 irradiated allogeneic PBMC per ml from HIV-1 seronegative subjects in RPMI 1640 with 10% heat-inactivated fetal

calf serum (R10) supplemented with 100 U of human recombinant interleukin-2 (rIL-2) (Hoffmann-La Roche, Nutley, N.J.) per ml. The CD3-specific monoclonal antibody (MAb) 12F6 (51) was added at 0.1 $\mu\text{g}/\text{ml}$ as a stimulus to T-cell proliferation. After 2 to 3 weeks, cells from wells demonstrating growth were then transferred to 24-well plates and restimulated by adding 1 ml of rIL-2-containing medium with 10^6 irradiated allogeneic PBMC per ml and anti-CD3 MAb at 0.1 $\mu\text{g}/\text{ml}$. Approximately 2 weeks later, clones were screened for CTL activity against a panel of autologous target cells infected with recombinant vaccinia virus expressing either the control *lacZ* or the HIV-1 envelope, RT, or *gag* genes. Clones exhibiting envelope-specific CTL activity were then restimulated every 10 to 14 days with anti-CD3 MAb and irradiated allogeneic PBMC.

Flow cytometry analysis. Cells to be analyzed were incubated with a fluorescent probe-conjugated anti-CD4 MAb, anti-CD8 MAb, or a similarly labelled control MAb (Coulter Electronics, Hialeah, Fla.) and analyzed with a FACScan flow cytometer (Becton Dickinson and Co., Mountain View, Calif.) as previously described (20).

Cytotoxicity assay. Target cells consisted of B-LCL infected with recombinant vaccinia viruses or preincubated with synthetic HIV-1 peptides. Vaccinia virus-infected targets were prepared by incubating 2.5×10^6 to 10×10^6 B-LCL in log-phase growth with recombinant vaccinia virus at 1 to 2 PFU per cell for approximately 16 h at 37°C . Cells were then labelled with 100 μCi of $\text{Na}_2^{51}\text{CrO}_4$ (New England Nuclear, North Billerica, Mass.) for 60 min and washed three times with RPMI with 10% fetal calf serum. Peptide-sensitized targets were obtained by incubating 2×10^6 to 3×10^6 B-LCL with peptide for 60 min during ^{51}Cr labelling. Cytolytic activity was determined in a standard ^{51}Cr -release assay (48) using U-bottom microtiter plates containing 10^4 targets per well. Plates were incubated in a humidified incubator at 37°C for 4 h. All assays were performed in duplicate. Supernatants were then harvested and counted on a Cobra gamma counter (Packard Instrument Company, Meriden, Conn.), and percent lysis was determined from the formula $\% \text{ lysis} = 100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$. Maximum release was determined by lysis of targets in water or detergent (1% Triton X-100; Sigma). Spontaneous release was less than 30% of maximal release for all reported assays.

RESULTS

Isolation of an HIV-1 envelope-specific CTL clone. PBMC were cloned in the presence of a CD3-specific MAb, rIL-2, and irradiated PBMC obtained from HIV-1 seronegative donors. Clones obtained in this manner were then selected on the basis of lysis of autologous B-LCL infected with recombinant vaccinia virus expressing HIV-1 genes. One of these clones, 35J27, was highly specific for the envelope glycoprotein (Fig. 1) and exhibited vigorous lysis of target cells at effector cell/target cell (E/T) ratios as low as 1:1. Analysis by flow cytometry demonstrated that this clone was $\text{CD4}^- \text{CD8}^+$ (data not shown).

The envelope-specific CTL clone 35J27 is restricted by the class I HLA-Cw4 allele. Lysis of virus-infected target cells by human CD8^+ CTL is dependent on the recognition of a trimolecular complex of a specific HLA class I molecule, a processed viral peptide, and β_2 -microglobulin. To determine the HLA molecule responsible for presenting viral antigen to clone 35J27, we examined the lysis of a panel of allogeneic

FIG. 1. Recognition of target cells expressing HIV-1 proteins by an envelope-specific CTL clone. Target cells consisted of autologous B-LCL which were infected with recombinant vaccinia viruses expressing either the control *lacZ* (*lac*), HIV-1 p55^{gag}, RT, or envelope (*Env*) genes. The E/T ratio was 10:1.

target cells expressing viral antigen which were matched at one or more HLA class I alleles. Initial testing of this CTL clone against allogeneic target cells infected with recombinant vaccinia viruses expressing either gp160 or *lacZ* control suggested restriction by either HLA-A11 or Cw4 (Fig. 2A). Analysis of lysis by clone 35J27 against a larger panel of allogeneic target cells matched at either HLA-A11, Cw4, or both alleles demonstrated antigen-specific lysis of targets which expressed HLA-Cw4 but not HLA-A11 alone (Fig. 2B). As a control, an HLA-A11-restricted CTL clone, also

derived from this subject (49) and specific for the HIV-1 RT, was tested against the same panel of allogeneic targets sensitized either with a peptide containing the CTL epitope recognized by this clone (peptide 49) or a control peptide. Lysis by the HLA-A11-restricted CTL clone 63D35 was observed against all the HLA-A11-expressing targets listed in Fig. 2B but none of the four A11⁻ Cw4⁺ cell lines (data not shown).

Fine mapping of the epitope specificity of CTL clone 35J27. To determine the specific epitope recognized by clone 35J27, we first examined the ability of this clone to lyse autologous target cells which had been infected with recombinant vaccinia virus vectors expressing serial truncations of gp160 (Fig. 3). Lysis was observed against target cells expressing the NH₂-terminal 393 aa of the envelope glycoprotein but not shorter truncations, localizing the epitope for this clone to the region spanning aa 287 to 393. The lower levels of lysis observed against the 393-aa envelope construct (vPE20) compared with those of constructs expressing longer envelope proteins were observed in multiple assays and could not be overcome by increasing the multiplicity of infection to 5 PFU per cell (data not shown). Lysis of target cells infected with vPE20 by another CTL clone specific for an epitope in the NH₂-terminal 204 aa of gp120 demonstrated no significant differences in lysis between this construct and other truncated envelope constructs (unpublished data). This observation suggests that the decreased lysis of targets infected with vPE20 by clone 35J27 was not due to poor envelope expression by this construct.

Epitope specificity was then defined by using B-LCL incubated with a series of overlapping peptides, 25 aa in length with an 8-aa overlap, which spanned aa 287 to 393 (Fig. 4). CTL clone 35J27 specifically recognized envelope peptide env/116 (aa 371 to 395, sequence IVTHSFNCGGEF

FIG. 2. CTL clone 35J27 restricted by the HLA-Cw4 molecule. (A) Lysis of allogeneic B-LCL expressing the HIV-1 envelope. Autologous or allogeneic target cells matched at indicated HLA alleles were infected with recombinant vaccinia viruses expressing either gp160 or the control *lacZ* gene and then incubated with effector cells at an E/T ratio of 3:1. Envelope-specific lysis was calculated for each target tested by subtracting the specific lysis of targets infected with the control vaccinia virus from the specific lysis of envelope-expressing targets. Lysis of control target cells was less than 6% for all B-LCL. (B). Lysis of peptide-sensitized B-LCL. Autologous or allogeneic B-LCL were sensitized with the appropriate envelope peptide, env/116 (see Fig. 4), or, as a control, the HIV-1 *gag* peptide p24/124 (LDIRQGPKPEFRDYVDRFYKTL). The E/T ratio was 2:1. Peptide-specific lysis was calculated by subtracting the lysis of B-LCL sensitized with the control HIV-1 *gag* peptide p24/124 from the lysis of peptide env/116-sensitized targets, which ranged from 0 to 13%.

FIG. 3. Epitope mapping of the specificity of clone 35J27 by using recombinant vaccinia viruses expressing serial truncations of the HIV-1 envelope. Autologous B-LCL were infected with recombinant vaccinia viruses expressing serial truncations of gp160 or the *lacZ* control. The amino acid length of HIV-1 envelope expressed by each construct is indicated. Clones were incubated with target cells at the indicated E/T ratios. Envelope-specific lysis was calculated for each target tested by subtracting the specific lysis of targets infected with the control vaccinia virus from the specific lysis of envelope-expressing targets.

FYCNSTQLFNSTW in single-letter amino acid code). Further fine mapping of the epitope specificity of this clone was performed by analyzing the recognition of shorter peptides spanning this region (Table 1). Serial truncations of single amino acids at either end of the 8-aa sequence FNCGGEFF (aa 376 to 383) led to an almost complete loss of CTL activity, whereas deletions outside of this core sequence had no significant effect on recognition. Target cells incubated with the peptide FNCGGEFF were efficiently lysed when incubated with peptide at 100 µg/ml (Table 1), and titration curves revealed effective sensitization of autologous target cells at concentrations between 5 µg/ml and 500 ng/ml (data not shown). Analysis of nonamers consisting of an additional amino acid on either end of the core sequence FNCGGEFF was limited by the poor in vitro growth of clone 35J27. The core epitope (aa 376 to 383) lies in a highly conserved region

of the C3 domain of gp120. The identification of an 8-aa epitope is consistent with recent reports which have identified naturally processed peptides of 8 or 9 aa in association with human and murine major histocompatibility complex class I molecules (12, 18, 39, 47).

Effects of sequence variation on recognition by the CTL clone 35J27. Given the relatively high degree of variability in gp160 (30), we wished to assess the ability of a CTL clone, which had been selected on the basis of recognition of envelope glycoprotein of one HIV-1 strain, to recognize other strains of HIV-1. Although the core epitope sequence, aa 376 to 383 (FNCGGEFF), is conserved in approximately 90% of sequenced HIV-1 isolates (30), amino acid substitutions have been observed at two positions within this region. In addition, one HIV-1 strain (HIV-1 JFL) exhibits an amino acid substitution immediately outside aa 376 to 383. Peptides

FIG. 4. Epitope mapping of the specificity of clone 35J27 by using synthetic peptides. The CTL clone 35J27 was incubated with autologous target cells at an E/T ratio of 3:1. Peptide-sensitized target cells were prepared by incubating autologous B-LCL with 100 µg of the indicated peptide (env/108 through env/117) per ml for 60 min during ⁵¹Cr labelling. The sequence of peptide env/116 is IVTHSFNCGGEFFYCNSTQLFNSTW (aa 371 to 395). Target cells were also infected with recombinant vaccinia virus expressing the control *lacZ* (*lac*) or HIV-1 envelope (*Env*).

TABLE 1. Fine mapping of the epitope specificity of the gp120-specific CTL clone 35J27

Peptide	Sequence ^a	% Specific lysis at E/T ratio of ^b :	
		3:1	1:1
GG05-40	SSGGDPEIVTHSFNC(GC)	19	3
GG05-43	PEIVTHSFNC <u>GGEFF</u> (GC)	85	57
GG05-46	<u>HSFNCGGEFF</u> YCNS(TGC)	74	54
GG05-49	GGEFFYCNS(TQLFNS)(GC)	10	0
116A	<u>HSFNCGGEFF</u> YC	80	73
116B	<u>SFNCGGEFF</u> YC	86	69
116C	<u>FNCGGEFF</u> YC	80	62
116D	NCGGEFFYC	8	3
116E	CGGEFFYC	16	10
116F	<u>HSFNCGGEFF</u> Y	85	83
116G	<u>HSFNCGGEFF</u>	85	65
116H	<u>HSFNCGGEF</u>	9	5
116I	<u>HSFNCGGE</u>	8	2
116J	<u>FNCGGEFF</u>	72	35

^a Amino acids enclosed in parentheses indicate peptide amino acids which do not correspond to the HIV-1 sequence. The 8-aa sequence common to all peptides which were able to sensitize targets for lysis is underlined.

^b The CTL clone 35J27 was incubated at the indicated E/T ratios with autologous B-LCL incubated with 100 µg of peptide per ml during ⁵¹Cr labelling.

were synthesized with amino acid substitutions corresponding to sequenced HIV-1 isolates and the HIV-2 ROD strain. A conservative amino acid substitution outside the core epitope (Ser to Thr at aa 375) corresponding to the HIV-1 JFL strain had no effect on recognition by the clone 35J27 (Fig. 5). In contrast, the nonconservative substitution Gly to Arg at aa 379 exhibited by several HIV-1 strains, including SF2, SF33, and MAL, abolished recognition by the CTL clone. No lysis of targets incubated with either the HIV-1 NDK or HIV-2 peptides was observed.

DISCUSSION

In this paper, we describe an HLA class I-restricted CTL epitope in a highly conserved region of gp120. A number of HIV-1 envelope peptides which are recognized by human

CTL have recently been described, including peptides which lie in gp120 and gp41 (6, 8, 9, 15, 19, 25, 35, 41, 45). However, the majority of these peptides were identified by using bulk PBMC, and in many instances, the precise CTL epitopes were not determined. Several CTL epitopes in the HIV-1 envelope have been defined by using CD8⁺ CTL clones from HIV-1 seropositive subjects, and remarkably, all of the epitopes characterized in this fashion which have been previously reported lie in gp41 (9, 19, 45). CD8⁺ CTL clones derived from HIV-1 seropositive subjects which recognize gp120 have been reported, but the epitope specificity of these clones was not defined with synthetic peptides (11). One epitope in gp120 has been identified by using a CD4⁺ clone derived by the in vitro stimulation of PBMC from an HIV-1 seronegative donor with viral antigen (41). Several potential factors may account for the relative paucity of epitopes in gp120 identified by using CTL clones derived from HIV-1 seropositive subjects, including technical differences between the epitopes identified by using bulk PBMC and those identified by using CTL clones, the substantial sequence variation exhibited by gp120, and the possibility that gp120 is a relatively poor target antigen for CTL in infected humans. Of note is the fact that the HLA-Cw4-restricted CTL epitope described in this report overlaps with a peptide (KNCGGEFFYCNS) reported to be recognized by HLA-A2-restricted CTL (8, 35), although the peptide does not appear to contain a recently described HLA-A2-binding motif (13).

Characterization of HIV-1 T-cell epitopes by using CTL clones has several distinct advantages over other techniques which have been utilized to map epitopes. The ability to maintain these clones in long-term culture allows the precise characterization of the minimum amino acid sequences that make up CTL epitopes, exact demonstration of their HLA restriction, and detailed analysis of the effects of epitope sequence variation on recognition. In addition, because the methodology employed in this study allows the identification of CTL epitopes which may exist anywhere in the envelope protein, bias which may be introduced by screening CTL against only a subset of HIV-1 envelope peptides may be avoided.

The intensive study of HIV-1-specific CTL by multiple laboratories has revised some traditional concepts which have been derived from the study of CTL specific for other viruses. For instance, analysis of CTL specific for influenza

FIG. 5. Effect of natural sequence variation on recognition by a gp120-specific CTL clone. Autologous B-LCL were sensitized with the indicated concentrations of peptide (env/116, env/116 JFL, etc.) and incubated with the CTL clone 35J27 at an E/T ratio of 2:1. The index peptide corresponds to aa 374 to 385 of the HXB2 clone of HIV-1. The minimal epitope corresponding to aa 376 to 383 is underlined. Lysis against autologous B-LCL not sensitized with peptide ranged from 0 to 4% in these assays.

virus and lymphocytic choriomeningitis virus in inbred mice strains has suggested that the number of viral CTL epitopes was quite limited, in general consisting of one or two immunodominant epitopes (3, 4). Similar results were obtained by Takahashi et al., who identified a single immunodominant epitope in gp160 by using CD8⁺ CTL obtained from mice immunized with recombinant vaccinia virus expressing gp160 (44). In contrast, the accumulating data regarding HIV-1-specific CTL in humans suggest a remarkably heterogeneous response. More than 50 CTL epitopes in HIV-1 *gag*, *RT*, *nef*, and envelope have been described, and more are likely to be identified (32). Even within a single subject, these responses may be heterogeneous: CTL obtained from the subject studied in this report recognize at least 13 different epitopes, and seven CTL epitopes have been identified in another individual (19, 20, 49, and unpublished data). Moreover, the CTL epitopes identified to date are likely to underestimate the total breadth of the CTL response, because the detection of CTL in these studies utilizes target cells which express antigen derived from laboratory strains of HIV-1 and is therefore dependent on homology between the subject's viruses and the recombinant antigen expressed by the target cell.

The functional role of HLA-C molecules has been less well defined than that of the other classical human class I HLA molecules, HLA-A and -B. HLA-C molecules share a number of features with HLA-A and -B molecules, including sequence homology, a comparable degree of polymorphism, association with β_2 -microglobulin, and expression on the cell surface of lymphocytes (14, 21, 42, 43, 46). However, few data on the functional capacities of these molecules have accumulated. Dill et al. demonstrated that HLA-Cw3 when expressed in a transgenic mouse model could present viral antigens to murine CTL and stimulate an alloreactive response (10). Using a mutant cell line lacking HLA-A or -B molecules, Chen et al. have suggested that lysis by Epstein-Barr virus-specific CTL could be restricted by HLA-C molecules (5). Recently, Littau et al. have described a CD8⁺ HIV-1 *gag*-specific CTL clone which was restricted by HLA-Cw3 (27). The epitope described in this report is, to our knowledge, only the second instance of a viral CTL epitope presented by a defined HLA-C allele to human CTL and is the first identified as being presented by Cw4. It is not yet clear whether the identification of the HIV-1 CTL epitopes presented by HLA-C molecules is the result of a distinctive feature of presentation of HIV-1 antigens to CD8⁺ CTL or whether this merely reflects the increased scrutiny focused on HIV-1-specific immune responses. Several factors may have interfered with the ability to detect CTL restricted by HLA-C alleles in prior studies. Serological reagents used in routine serologic HLA typing may fail to identify some HLA-C alleles, leading to the designation Cw blank despite the expression of functional HLA-C gene products on the cell surface (21). In addition, some HLA-C alleles are closely linked to specific HLA-A and -B types (disequilibrium linkage) (46), and it is possible that CTL epitopes presented by HLA-C molecules may have been erroneously identified as being presented by HLA-A or -B alleles. In any case, the identification of two CTL epitopes which are presented by HLA-C alleles (27 and this report) supports a functional role for these molecules in the presentation of viral antigen to CTL.

The substantial sequence variation exhibited by HIV-1 has important implications for our understanding of AIDS pathogenesis, as well as for efforts to develop an AIDS vaccine. Data from several laboratories have illustrated the effects of

sequence variation in producing HIV-1 mutants which are not recognized by either antibodies (31, 38) or CTL (34). Similarly, because the majority of HIV-1 candidate vaccines currently under evaluation include gp160, the viral protein that exhibits the most extensive sequence variation, efforts to design vaccines are hampered by sequence variation. Increasing evidence suggests that cellular immune responses are able to recognize relatively conserved regions of both gp120 and gp41 (1, 6, 8, 9, 15, 19, 25, 35, 41, 45). However, the exquisite sensitivity of CTL to sequence variation occurring within an epitope demonstrates that sequence variation even within conserved regions may produce variants which are not recognized by the immune system. Thus, vaccines should be designed in an effort to induce immune responses against multiple conserved epitopes, so as to minimize the consequences of variation in any one site.

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ADDENDUM IN PROOF

Since the submission of this article, a report which documents the presentation of an Epstein-Barr virus antigen by HLA-Cw7 to CD8⁺ CTL has been published (D. J. Schendel, C. Reinhardt, P. J. Nelson, B. Maget, L. Pullen, G. W. Bornkamm, and A. Steinle, *J. Immunol.* **149**:2406-2414, 1992).

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