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A CD4$^+$ Cytotoxic T-Lymphocyte Clone to a Conserved Epitope on Human Immunodeficiency Virus Type 1 p24: Cytotoxic Activity and Secretion of Interleukin-2 and Interleukin-6

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A CD4$^+$ cytotoxic T-lymphocyte (CTL) clone, established from the peripheral blood of a human immunodeficiency virus (HIV)-seropositive donor, lysed autologous target cells that were infected with a recombinant vaccinia virus containing the *gag* gene of HIV type 1 and target cells pulsed with p24^{gag} construct expressed in *Escherichia coli*. The recognition of the HLA-DQ-restricted epitope by this clone was further defined by using overlapping synthetic peptides. The epitope recognized by this CD4$^+$ CTL clone (amino acids 140 to 148) overlaps with a CD8$^+$ epitope and is highly conserved among all isolates of HIV type 1 that have been sequenced. Production and secretion of lymphokines such as interleukin-2 and interleukin-6 after specific antigenic stimulation were demonstrated by this *gag*-specific CD4$^+$ CTL clone.

Virus-specific cytotoxic T lymphocytes (CTL) are generally considered to recognize viral antigens presented by class I major histocompatibility complex (MHC) molecules, but a number of studies have described CD4$^+$ CTL restricted by class II MHC alleles which are specific for target cells exposed to exogenous viral proteins. Several reports have described CD8$^+$ class I-restricted CTL in the peripheral blood lymphocytes of individuals with human immunodeficiency virus type 1 (HIV-1) infection (19); however, there have been no reports about viral antigens involved in HIV-specific class II-restricted CTL recognition. Although they were isolated in the cerebrospinal fluid (15), CD4$^+$ cytotoxic T cells in the peripheral blood lymphocytes of HIV-1 infected individuals have not been described. The only report of HIV-1-specific CD4$^+$ CTL in peripheral blood lymphocytes was in healthy, seronegative individuals following immunization with a recombinant HIV-1 gp160 subunit vaccine (11). Since class II-restricted CTL may form an important component of the immune response to HIV-1, it is important to define HIV viral epitopes that are presented by class II MHC molecules.

We previously reported a CD8$^+$ HIV-1 *gag*-specific cytotoxic T-cell clone which is restricted by a class I molecule, HLA-Cw3 (7). An epitope on p24, with the sequence QAISPR (amino acids [aa] 145 to 150), which is highly conserved among all of the HIV-1 isolates that have been sequenced, was recognized by this clone. We subsequently isolated a CD4$^+$ T-cell clone, designated 108.2, which recognized a region of p24 (aa 140 to 148) that overlaps with this CD8$^+$ epitope. This CD4$^+$ CTL clone secretes high levels of interleukin-2 (IL-2) and IL-6 but not IL-4 upon activation. This region of p24^{gag} is highly conserved on different HIV-1 isolates and it is capable of inducing both CD8$^+$ and CD4$^+$ CTL responses. Thus, this region of p24 may be of interest for purposes of HIV-1 peptide-based vaccine development.

Determination of CTL epitope on p24^{gag}. The preparation of target and effector cells for cytotoxicity assays and phenotypic analysis has been described previously (7). We had observed that this donor's peripheral blood mononuclear cells showed significant HIV-1 *gag*, *pol*, and envelope-specific killer cell activity in bulk culture experiments (7). After subcloning by limiting dilution, a CD3$^+$ CD4$^+$ CD8$^-$ CD16$^-$ CTL clone, 108.2, was isolated from a well seeded with 0.3 cell per well. This clone lysed vaccinia virus (VAC)/*gag*- but not VAC-, VAC/*pol*-, or VAC/*env*-infected target cells (data not shown). To localize the epitope on *gag*, we added recombinant p24 antigen to autologous B-LCL target cells in a cytotoxicity assay. As shown in Fig. 1, these target cells were lysed to a level similar to that of VAC/*gag*-infected targets. Lysis of K562 cells and VAC-infected or uninfected B-LCL cells was minimal.

To identify the epitope on p24 which induced this CTL response, we synthesized a series of 14 overlapping peptides derived from the p24 amino acid sequence of the human T-cell lymphotropic virus IIIB isolate. As seen in Table 1 (experiment A), convincing cytotoxic activity was observed only on target cells pulsed with peptide 2 (amino acid residues 143 to 172). Peptide 1, which overlaps peptide 2 by 20 amino acids, did not sensitize target cells for lysis.

A series of smaller peptides contained in the amino acid sequence of peptide 2 were synthesized in order to define the core epitope recognized by this CD4$^+$ CTL clone (Table 1, experiment B). Target cells pulsed with peptide 90-17, which corresponds to amino acid residues 133 to 152, were lysed by this CTL clone. The removal of 13 amino acids from the N terminus (peptide 90-17A) abrogated all activity, while removal of 5 or 7 amino acid residues at the N-terminal side of the sequence (peptides 90-17B and 90-17C, respectively) did not change the specific lysis of target cells. In addition, the removal of four amino acid residues (149 to 152) at the C terminus (peptides 90-18, 90-18A, and 90-18B) did not alter recognition by the clone. Therefore, the epitope recognized by this CD4$^+$ CTL clone lies within residues 140 to 148 (GQMVHQAIS) of the p24 region of *gag*. The sequence of this epitope is highly conserved among all of the 18 North American or European strains sequenced to date, although there is a conservative amino acid substitution at position

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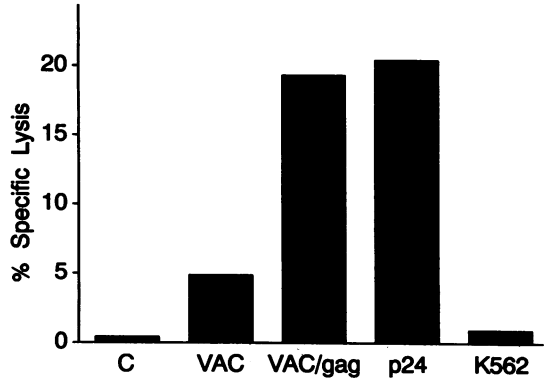

FIG. 1. Specificity of clone 108.2 for HIV-1 p24 protein. The CTL clone was tested for specific lysis of autologous B-LCL cells that were uninfected or expressing wild-type VAC, recombinant *gag* (VAC/*gag*), or p24 protein. K562 cells were used as another target cell. The CTL assay was performed in triplicate at an effector/target ratio of 2.5:1.

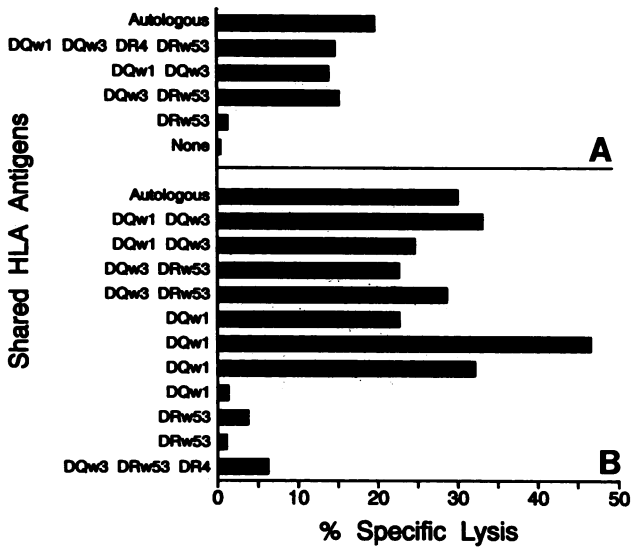

FIG. 2. MHC restriction of clone 108.2. Autologous and allogeneic targets matched at one or more HLA loci as indicated were infected with VAC/*gag* (A) or pulsed for 1 h with peptide 90-18B (B). Percent specific lysis was determined at an effector/target ratio of 3:1. The HLA class II alleles of the donor are DQw1, DQw3, DR4, DR10, and DRw53.

143 (isoleucine for valine) and residue 146 (proline for alanine) in the African isolates HIV-1 MAL and OYI, respectively.

We previously demonstrated that this region of p24 contains a class I HLA-Cw3-restricted epitope (7). Our results confirm previous observations that similar peptides can be recognized in a class I- and class II-restricted manner. Other investigators have described an overall similarity for structures of class I and class II MHC molecules (2, 5). The use of similar T-cell receptors by class I- and class II-restricted T cells (8, 14) suggests that antigen recognition mechanisms are similar in the two systems. Perkins et al. (12) reported that seven individual influenza virus peptides were able to stimulate both class I- and class II-restricted T-cell responses. Recently, Takahashi et al. observed that different sites located on the V3 loop of the HIV-1 *env* protein stimulated class I $H\text{-}2^d$-restricted $CD8^+$ CTL and induced T-cell help for itself (17). Our results indicate that the epitope which we previously defined as a class I-restricted epitope on $p24^{gag}$ overlaps with this class II-restricted response. Thus, this region (aa 140 to 150) of p24 may be a candidate for inclusion in an AIDS subunit vaccine.

HLA restriction of cytolysis by CTL clone 108.2. The $CD4^+$ *gag*-specific clone was tested for its cytolytic activity by using as target cells allogeneic B-LCL cells infected with VAC/*gag* matched at one or more HLA class II loci or unmatched as indicated in Fig. 2. The HLA class II alleles of the donor of clone 108.2 are DR4, DR10, DQw1, DQw3, and DRw53. We observed lysis of allogeneic target cells that shared DQw1 or DQw3 (Fig. 2A). This observation was confirmed in another experiment (Fig. 2B) by using other allogeneic targets pulsed with the core peptide, which also demonstrated recognition of the peptide in association with DQw1 or DQw3. In addition, we observed the inability of this CTL clone to lyse two HLA-matched allogeneic targets sharing DQw1 or DQw3 although it successfully lysed other allogeneic targets with DQw1 and DQw3 in the same assay. There is a high degree of structural homology among DQ

TABLE 1. Lysis of peptide-pulsed target cells by $CD4^+$ clone 108.2

Expt	Peptide no.	Residues[a]	Amino acid sequence	% Specific lysis[b]
A	1	133–162		1.9
	2	143–172		25.6
	3	153–182		5.1
	4	173–202		1.7
	5-14	193–363		0
B	2	143–172		18.5
	90-17	133–152	PIVQNIQGQMVHQAISPRTL	15.1
	90-17A	146–152	AISPRTL	−3.4
	90-17B	138–152	IQGQMVHQAISPRTL	16.6
	90-17C	140–152	GQMVHQAISPRTL	20.1
	90-18	133–148	PIVQNIQGQMVHQAIS	14.3
	90-18A	138–148	IQGQMVHQAIS	14.7
	90-18B	140–148	GQMVHQAIS	16.8

[a] Residue numbering for the $p24^{gag}$ sequence is based on the HIV-1 (IIIB strain) sequence.

[b] Autologous B-LCL cells were pulsed with peptides at a final concentration of 50 μg/ml for 1 h before being labeled with ^{51}Cr and used as targets for clone 108.2. Percent specific lysis was determined at an effector/target ratio of 3:1.

TABLE 2. Lymphokine production[a] by CD4+ CTL clone 108.2 in response to purified p24 protein, peptide antigen, and 12F6

Stimulant[b]	IL-2 (pg/ml)[c]	IL-6 (pg/ml)	IL-4 (ng/ml)[c]	IFN-γ (pg/ml)	TNF-α (pg/ml)
p24 protein	720	1,650	ND	2,588	176
Peptide 1 (aa 140–152)	700	1,963	ND	3,294	238
90-18B (aa 140–148)	754	1,438	ND	2,711	241
90-25 (aa 141–152)	660	1,422	ND	3,671	241
12F6[d]	390	3,390	ND	7,376	662
Peptide 14 (aa 334–363)	44	514	ND	2,555	204
None	22	404	ND	3,120	275

[a] Supernatants were harvested 3 days after the onset of the stimulation.
[b] The autologous B-LCL cells used as antigen-presenting cells, after 8 h of incubation with different peptides or overnight pulsing with p24 protein at concentrations of 100 μg/ml, had been irradiated with 5,000 rad.
[c] IL-4 levels of <0.022 ng/ml were not detected (ND).
[d] Anti-CD3 MAb, used at a final dilution of 0.1 μg/ml.

alleles (9). Therefore, this CTL clone probably recognizes this epitope on a conserved site on DQw1 and DQw3 which is present on most cells which serologically type as DQw1 or DQw3. From the known frequencies of DQ alleles in different individuals (1), we would expect that 55% of Caucasian, 63% of African, and 67% of Asian individuals would be capable of presenting this CD4+ epitope (aa 140 to 148) contained in HIV-1 $p24^{gag}$.

Lymphokine production by this CD4+ CTL clone. T cells, after antigenic stimulation, secrete lymphokines that are major determinants of the function of lymphocytes. To better define its biologic properties, in addition to cytotoxic function, this CD4+ clone was examined for lymphokine production after stimulation with purified p24 protein, the appropriate peptide antigen, or an anti-CD3 monoclonal antibody (MAb), 12F6. Irradiated (5,000 rads) autologous B-LCL cells were incubated with different peptide antigens at a concentration of 100 μg/ml for 8 h at 37°C or pulsed overnight with 100 μg of purified recombinant p24 protein per ml, then washed twice, resuspended in RPMI containing 10% fetal calf serum, and added to individual wells of microtiter plates (100 μl per well) containing 3×10^4 cells. The T-cell clone was then added (4×10^4 per well) at 100 μl per well. After 3 days of incubation at 37°C, 0.1 ml of supernatant fluid was collected from each well and stored at −70°C. Supernatants were tested for the presence of IL-2, IL-6, tumor necrosis factor alpha (TNF-α), IL-4, and gamma interferon (IFN-γ) activity by using commercially available enzyme-linked immunosorbent assay kits (Biokine IL-2, IL-6, and TNF test kits from T Cell Sciences, Cambridge, Mass.; Intertest-4 and Intertest-γ from Genzyme Corporation, Boston, Mass.). Table 2 shows that the clone produced large amounts of IL-2 and IL-6 but no IL-4 in response to stimulation by purified p24 protein or peptides which contained the epitope (peptides 1, 90-18B, and 90-25), or the anti-CD3 MAb 12F6. Stimulation with the p24 protein or peptide resulted in higher production of IL-2 than did stimulation with the anti-CD3 MAb, while the reverse was true in terms of IL-6 secretion. This contrasts with the low levels of IL-2 and IL-6 detected in the absence of a stimulatory signal. The culture supernatant from all wells including those containing an irrelevant peptide yielded high levels of IFN-γ and TNF-α. However, stimulation with 12F6 resulted in a twofold increase in the secretion of these lymphokines. This clone, therefore, cannot be readily classified as either a Th-1 or Th-2 type of clone as described for the murine system, in which activated T-helper (Th-1) clones secrete IL-2, IFN-γ, and lymphotoxin, whereas Th-2 clones secrete IL-4, IL-5, IL-6, and IL-10 (16). IL-6 is known to induce B-cell differentiation and antibody production, and it has also been reported to activate and enhance CTL responses of CD8+ cells that are able to secrete IL-2 (18). The role of IL-6 in T-cell activation stems from its ability to activate cells from G_0 to an early stage in G_1 in which they become blastic and express IL-2 receptors. This leads to increased IL-2 responsiveness which further moves the cells towards the S phase, causing proliferation of these T cells. This induction of IL-2 receptors has been observed with human T cells through the synergistic actions of IL-6 and IL-2 (18).

The biological role of CD4+ CTL in HIV-1-infected individuals is not known. It is reasonable to assume that CD4+ CTL responses are induced by HIV-1-infected cells which express relatively large amounts of MHC class II antigens, such as macrophages, B cells, and activated T cells, whereas resting CD4+ T cells, which express mainly MHC class I antigens, would induce CD8+ but not CD4+ CTL responses. CD4+ CTL responses have been reported for other viral systems, such as measles virus (3), herpes simplex virus (20), dengue virus (6), and influenza virus (4); therefore, CD4+ CTLs may play a role in vivo by killing virus-infected MHC class II-bearing target cells. The relative lack of detection of HIV-1-specific CD4+ CTL compared with CD8+ CTL may be due in part to the lower level of lysis in vitro generally caused by CD4+ CTL or to methods we and others use for detecting HIV-1 virus-specific CTL (7, 19). The level of lysis by CD4+ and CD8+ CTL is variable. Dengue virus CD4+ CTL clones (6) varied in their level of lytic activity, and some CD4+ clones had a moderate level of lysis similar to that caused by this HIV-1 CD4+ CTL clone. Our results suggest that CD4+ CTL are part of the effector population in HIV-infected individuals, by virtue of their cytotoxic activity and lymphokine secretion. These CTL may also contribute to the immunodeficient state by killing infected cells. It is critically important to determine the reasons why such CTL responses are unable to completely eliminate infected cells in individuals infected with HIV-1 or other lentiviruses, unlike with other virus infections (10, 13).

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