

# Definition of Human Immunodeficiency Virus Type 1 gp120 and gp41 Cytotoxic T-Lymphocyte Epitopes and Their Restricting Major Histocompatibility Complex Class I Alleles in Simian-Human Immunodeficiency Virus-Infected Rhesus Monkeys

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**With the development of chimeric simian-human immunodeficiency virus (SHIV)-infected macaques as a model for assessing novel human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env)-based vaccine strategies for preventing HIV-1 infection in man, it will be important to determine HIV-1 Env-specific cytotoxic T-lymphocyte (CTL) responses in vaccinated and virus-infected monkeys. To facilitate performing such CTL studies, we have defined two HIV-1 Env CTL epitopes in SHIV-infected rhesus monkeys and characterized the major histocompatibility complex (MHC) class I alleles that bind these Env peptide fragments and present them to CTL. A 9-amino-acid (aa) fragment of HIV-1 gp41 (p6B, aa 553 to 561) is presented to CD8<sup>+</sup> CTLs of SHIV-infected animals by the rhesus monkey HLA-B homolog molecule Mamu-B\*12. An 8-aa HIV-1 gp120 peptide (p9CD, aa 117 to 124) represents a CTL epitope in rhesus monkeys restricted by the HLA-A homolog MHC allele Mamu-A\*08. This gp120 CTL epitope is fully conserved in all simian immunodeficiency virus, HIV-1, and HIV-2 isolates that have been sequenced to date and exhibits functional cross-reactivity. Screening of 14 unselected rhesus monkeys for expression of the two novel MHC class I alleles revealed the presence of each of the alleles in more than 40% of the animals. The characterization of the two HIV-1 Env CTL epitopes and their restricting MHC class I alleles will provide a basis for studying vaccine- and virus-elicited cytotoxic effector cell responses in rhesus monkeys.**

Growing evidence has implicated human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTLs) in controlling virus spread in infected individuals. CD8<sup>+</sup> T lymphocytes can inhibit HIV-1 replication in autologous CD4<sup>+</sup> T cells (28, 32). Containment of HIV-1 replication during the period of primary infection coincides temporally with the emergence of an AIDS virus-specific CTL response (12, 22, 23, 25). HIV-1-specific CTLs are present in large numbers in a diversity of anatomic compartments in infected individuals (1). Finally, emerging data suggest that a potent HIV-1-specific CTL response may prove important in maintaining a long-term nonprogressor clinical status in a subpopulation of infected individuals (9, 24). In view of the importance of HIV-1-specific CTL responses in containing the spread of HIV-1 in the infected individual, it will be crucial for an AIDS virus vaccine to elicit this effector T-cell response.

While the simian immunodeficiency virus (SIV)-infected macaque and the HIV-1-infected chimpanzee have provided powerful systems for exploring novel AIDS virus vaccine strategies, both models have proven to have significant limitations (15). Differences between SIV Env and HIV-1 Env in their neutralization determinants (3, 10) have made it difficult to assess Env-based vaccine strategies in the macaque model. An often low level of HIV-1 replication, an absence of pathogenicity, and high animal costs have limited the uses of the chimpanzee in AIDS vaccine research. The recent development of chimeric simian-human immunodeficiency viruses (SHIVs) (13, 16), constructed with an HIV-1 *env* gene on an SIV<sub>mac</sub> backbone, represents a potentially important advance

in the available models for assessing AIDS vaccine candidates (17). The infection of macaques with these chimeric viruses provides an important system to evaluate HIV-1 Env-based vaccine approaches.

The utility of an animal model for evaluating the vaccine induction of CTL responses is greatly enhanced by employing animals with defined CTL epitopes and characterized major histocompatibility complex (MHC) class I molecules. We have used rhesus monkeys with a shared *HLA-A* homolog allele and a defined SIV<sub>mac</sub> Gag CTL epitope (20) to facilitate characterizing the vaccine induction of CTL responses by using live vector (26, 35) and peptide (19, 36) immunization. To strengthen the SHIV/rhesus monkey system as a model for evaluating HIV-1 Env-based vaccine strategies, we have defined two HIV-1 Env CTL epitopes in SHIV-infected rhesus monkeys and characterized the novel MHC class I alleles that restrict these epitopes.

**Results. (i) Screening and fine mapping with synthetic peptides identify HIV-1 gp120 and gp41 CTL epitopes.** We have previously shown that SHIV-infected rhesus monkeys develop HIV-1 Env-specific cytolytic effector cell activity mediated by CD8<sup>+</sup>, MHC class I-restricted effector cells (29). To characterize this CTL response, monkeys were infected with 4 to 4,000 50% tissue culture infective doses of a nonpathogenic chimeric SHIV composed of SIV<sub>mac239</sub> expressing the HIV-1<sub>HXBc2</sub> *rev*, *tat*, *vpu*, and *env* genes (16). These animals developed HIV-1 Env-specific CTL responses (29). To define the epitopes recognized by these CTLs, 19- or 20-amino-acid (aa) peptides overlapping by 10 aa, spanning the entire HIV-1 gp120 (HIV-1<sub>IIIB</sub> sequence) or gp41 (HIV-1<sub>MN</sub> sequence), were mixed to yield a limited number of peptide pools, each containing 8 to 10 peptides. The peptide pools were used to initiate the expansion of cytolytic effector cells from peripheral

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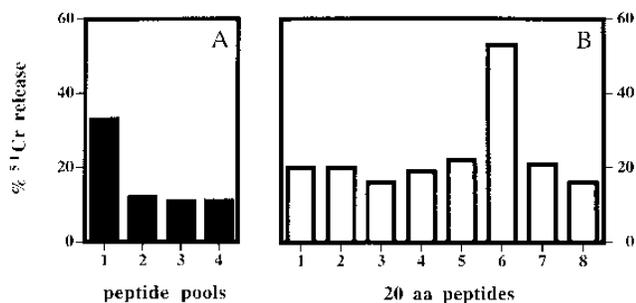


FIG. 1. Peptide screening defines a 20-aa HIV-1 gp41 CTL epitope in a SHIV-infected rhesus monkey. PBMC from Mm L28 were isolated from heparinized blood by Ficoll-diatrizoate density gradient centrifugation. One aliquot of the cells was cultured overnight at  $10^7$  cells per ml with gp41 peptide pools or single peptides at a concentration of  $10 \mu\text{g}$  per ml. The next day, the other aliquot of PBMC was treated for 1 h with  $50 \mu\text{g}$  of mitomycin C (Sigma, St. Louis, Mo.) per ml, washed, and incubated for a further 3 h with peptide pools or peptide at a concentration of  $50 \mu\text{g}$  per ml. Then, the peptide-pulsed cells were washed, resuspended in medium at  $10^7$  cells per ml, and added to the first aliquot of the cells. Two days later, and twice a week thereafter, half of the medium was replaced with medium containing 20 U of recombinant human interleukin-2 (Hoffman-La Roche, Nutley, N.J.) per ml. After 14 days of culture, the effector cells were assessed in CTL assays at an effector/target (E/T) ratio of 2.5:1. *Herpesvirus papio*-transformed B-LCL (31) were used as target cells in the  $^{51}\text{Cr}$  release assays (8). Cells ( $1.25 \times 10^5$ ) were labeled with  $20 \mu\text{Ci}$  of  $\text{Na}_2^{51}\text{Cr}_2\text{O}_4$  overnight. Simultaneously, the cells were incubated with peptide pools or peptide at a concentration of  $35 \mu\text{g}$  per ml. On the following day, the target cells were washed and added to the effector cells in 96-well round-bottom plates to give a final volume of  $200 \mu\text{l}$  per well. After 4 h of incubation,  $50 \mu\text{l}$  of supernatant was transferred to counting plates, and  $150 \mu\text{l}$  of scintillation fluid (OptiPhase SuperMix; Wallac, Gaithersburg, Md.) was added. The plates were sealed, agitated thoroughly, and analyzed in a 1450 microbeta liquid scintillation counter (Wallac). Cytotoxicity of the cultured cells was assessed on autologous B-LCL pulsed with the same peptide pool used for stimulation (A). Then cells stimulated with the first peptide pool were screened for lysis of target cells pulsed with the individual 20-aa peptides constituting peptide pool 1 (B).

blood mononuclear cells (PBMC) of the SHIV-infected monkey Mm L28. The resulting effector cells were assessed for cytolytic activity against autologous target B-lymphoblastoid cell lines (B-LCL) pulsed with the same peptide pools. While none of the gp120 peptide pools stimulated the expansion of effector cells from Mm L28 (data not shown), one of four pools containing gp41 peptides expanded effector cells that mediated specific reactivity with gp41 peptide-pulsed autologous B-LCL in a  $^{51}\text{Cr}$  release assay (Fig. 1A). These effector cells were then assessed for their recognition of the individual peptides constituting the pool. In fact, the effector cell activity was directed exclusively against the 20-aa gp41 peptide p6 (aa 550 to 569) (Fig. 1B). To determine the minimal peptide sequence recognized by these p6-specific effector cells, lysis of B-LCL pulsed with individual 9-aa peptides overlapping by 8 aa and spanning the 20-aa p6 was assessed (Table 1). The lytic activity of p6-stimulated effector cells was limited to target cells pulsed with peptide p6B (Table 1). These data, therefore, identify a rhesus monkey CTL epitope at aa 553 to 561 of the gp41 portion of the HIV-1 Env protein. (Amino acid numbering is based on the HIV-1<sub>HXBc2</sub> sequence [21].)

PBMC from a second SHIV-infected animal, Mm L3, were assessed similarly for effector cell specificity by using the same HIV-1 Env peptides. This analysis revealed a CTL epitope represented by the 19-aa gp120 peptide p9 (aa 112 to 130) (Table 2 and data not shown). The minimal sequence of this epitope was first narrowed down with overlapping peptides of different lengths and then finally defined with 9-aa peptides overlapping by 8 aa (Table 2). Two peptides, p9C and p9D, mediated target cell lysis. Therefore, the minimal CTL epitope p9CD is composed of 8 aa at positions 117 to 124 of HIV-1 gp120.

TABLE 1. HIV-1 gp41 p6-specific effector cells recognize the 9-aa peptide p6B

Peptide <sup>a</sup>	Sequence	% Lysis <sup>b</sup>
p6A	Q N N L L R A I E	10
<b>p6B</b>	<b>N N L L R A I E A</b>	<b>42</b>
p6C	N L L R A I E A Q	12
p6D	L L R A I E A Q Q	16
p6E	L R A I E A Q Q H	8
p6F	R A I E A Q Q H L	6
p6G	A I E A Q Q H L L	8
p6H	I E A Q Q H L L Q	8

<sup>a</sup> The peptide epitope p6B is printed in bold.

<sup>b</sup> PBMC from Mm L28 were stimulated with p6 and assessed for lysis of target cells pulsed with p6A-p6H at an E/T ratio of 5:1 in a  $^{51}\text{Cr}$  release assay.

(ii) Both the HIV-1 gp120 and gp41 CTL epitopes are well-conserved in primate immunodeficiency viruses. The sequences of both HIV-1 Env CTL epitopes were compared with those of other virus isolates (Fig. 2). Sequence alignments show that the p6B epitope is well-conserved among all HIV-1 isolates but not among viruses of the HIV-2/SIV group. Surprisingly, the p9CD epitope is absolutely conserved, with not a single amino acid change among the HIV-1 and the HIV-2/SIV isolates. Therefore, as predicted p9CD-stimulated PBMC from Mm L3 lysed target cells infected with recombinant vaccinia viruses expressing the *env* genes of HIV-1, HIV-2, and SIV<sub>mac</sub> (Fig. 3), indicating that the epitope p9CD is indeed functionally cross-reactive. As expected, p6B-specific effector cells from Mm L28 failed to lyse HIV-2 or SIV<sub>mac</sub> Env-expressing target cells (Fig. 3).

(iii) The effector cells specific for p6B and p9CD are CD8<sup>+</sup>, MHC class I-restricted. To determine the phenotype of the peptide-specific effector cells, p6B- or p9CD-stimulated PBMC from Mm L28 and Mm L3, respectively, were separated into CD8<sup>+</sup> lymphocyte-enriched or -depleted cells with CD8-specific immunomagnetic beads prior to assessment in a  $^{51}\text{Cr}$  release assay. The p6B- and the p9CD-specific cytotoxicity was mediated by the CD8<sup>+</sup>-enriched but not the CD8<sup>+</sup>-depleted cells (Fig. 4). These results indicate that the effector cells were CD8<sup>+</sup>, suggesting that their lytic activity should be restricted by MHC class I molecules. To verify this, peptide-stimulated PBMC from two SHIV-infected animals previously known to share MHC class I alleles were assessed for their ability to lyse a single peptide-pulsed target B-LCL. B-LCL generated from Mm L3 were lysed specifically by effector cells from Mm L3 and Mm 8A2 but not by effector cells from Mm L28 (Fig. 5).

TABLE 2. Peptide mapping defines an 8-aa HIV-1 gp120 CTL epitope

Peptide <sup>a</sup>	Sequence	% Lysis <sup>b</sup>
p9	W D Q S L <b>K P C V K L T P L C V S L K</b>	<b>57</b>
p.9	Q S L <b>K P C V K L T P L C V T</b>	<b>41</b>
p.13	C V K L T P L C I T M R	20
p9A	Q S L K P C V K L	15
p9B	S L K P C V K L T	15
<b>p9C</b>	<b>L K P C V K L T P</b>	<b>63</b>
<b>p9D</b>	<b>K P C V K L T P L</b>	<b>70</b>
p9E	P C V K L T P L C	13

<sup>a</sup> The optimally recognized peptides and the minimal 8-aa sequence are printed in bold.

<sup>b</sup> p9-stimulated PBMC from Mm L3 were assessed for peptide-specific cytotoxicity on autologous target cells pulsed with overlapping peptides at an E/T ratio of 2.5:1 in a  $^{51}\text{Cr}$  release assay.

Epitope p6B

HIV-1 <sub>HXBc2</sub>	-	Q	Q	Q	N	N	L	R	A	I	E	A	Q	Q	H	L	I	Q	L	T	-
HIV-1 <sub>SF-2</sub>	-	Q	Q	Q	N	N	L	R	A	I	E	A	Q	Q	H	L	I	Q	L	T	-
HIV-1 <sub>MAN</sub>	-	Q	Q	Q	N	N	L	R	A	I	E	A	Q	H	M	L	Q	L	T	-	
HIV-1 <sub>GCN</sub>	-	Q	Q	Q	N	N	L	R	A	I	K	A	Q	Q	H	L	L	Q	L	T	-
HIV-1 <sub>UG455</sub>	-	Q	Q	Q	S	N	L	R	A	I	E	A	Q	Q	I	L	L	Q	L	T	-
HIV-1 <sub>D687</sub>	-	Q	Q	Q	S	N	L	L	M	A	I	E	A	Q	I	L	L	Q	L	T	-
HIV-1 <sub>D747</sub>	-	Q	Q	Q	S	N	L	R	A	I	E	A	Q	Q	H	L	L	Q	L	T	-
HIV-1 <sub>MAL</sub>	-	Q	Q	Q	N	N	L	R	A	I	E	A	Q	Q	I	L	L	Q	L	T	-
HIV-1 <sub>ANT70</sub>	-	Q	Q	Q	D	N	L	R	A	I	E	A	Q	Q	Q	L	L	R	L	S	-
SIV <sub>mac25H</sub>	-	Q	Q	Q	<b>Q</b>	<b>Q</b>	<b>L</b>	<b>L</b>	<b>D</b>	<b>V</b>	<b>V</b>	<b>K</b>	<b>R</b>	Q	Q	E	L	L	R	I	T
HIV-2 <sub>R0D</sub>	-	Q	Q	Q	<b>Q</b>	<b>Q</b>	<b>L</b>	<b>L</b>	<b>D</b>	<b>V</b>	<b>V</b>	<b>K</b>	<b>R</b>	Q	Q	E	L	L	R	I	T
Amino acid (HXBc2)		550		551		551		551		561		561		561		561		561		561	569

Epitope p9CD

HIV-1 <sub>HXBc2</sub>	-	W	D	Q	S	L	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>I</b>	<i>C</i>	<i>V</i>	<i>S</i>	<i>L</i>	<i>K</i>	-
HIV-1 <sub>SF-2</sub>	-	W	D	Q	S	L	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>L</b>	<i>C</i>	<i>V</i>	<i>T</i>	<i>L</i>	<i>N</i>	-
HIV-1 <sub>MAN</sub>	-	W	D	Q	S	L	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>L</b>	<i>C</i>	<i>V</i>	<i>T</i>	<i>L</i>	<i>N</i>	-
HIV-1 <sub>GCN</sub>	-	W	D	E	S	L	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>L</b>	<i>C</i>	<i>V</i>	<i>T</i>	<i>L</i>	<i>N</i>	-
HIV-1 <sub>UG455</sub>	-	W	D	Q	S	L	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>L</b>	<i>C</i>	<i>V</i>	<i>T</i>	<i>L</i>	<i>D</i>	-
HIV-1 <sub>D687</sub>	-	W	D	Q	S	L	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>L</b>	<i>C</i>	<i>V</i>	<i>T</i>	<i>L</i>	<i>N</i>	-
HIV-1 <sub>D747</sub>	-	W	D	Q	S	L	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>L</b>	<i>C</i>	<i>V</i>	<i>T</i>	<i>L</i>	<i>H</i>	-
HIV-1 <sub>MAL</sub>	-	W	D	Q	S	L	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>L</b>	<i>C</i>	<i>V</i>	<i>T</i>	<i>L</i>	<i>N</i>	-
HIV-1 <sub>ANT70</sub>	-	W	D	Q	S	L	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>L</b>	<i>C</i>	<i>V</i>	<i>T</i>	<i>L</i>	<i>Q</i>	-
SIV <sub>mac25H</sub>	-	<i>F</i>	<i>E</i>	<i>T</i>	<i>S</i>	<i>I</i>	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>I</b>	<i>C</i>	<i>I</i>	<i>T</i>	<i>M</i>	<i>R</i>	-
HIV-2 <sub>R0D</sub>	-	<i>F</i>	<i>E</i>	<i>T</i>	<i>S</i>	<i>I</i>	<b>K</b>	<b>P</b>	<b>C</b>	<b>V</b>	<b>K</b>	<b>L</b>	<b>T</b>	<b>P</b>	<b>L</b>	<i>C</i>	<i>V</i>	<i>A</i>	<i>M</i>	<i>K</i>	-
Amino acid (HXBc2)		112		117		117		124		124		130		130		130		130		130	

FIG. 2. Amino acid sequence analysis of the epitopes p9CD and p6B reveals a high degree of conservation among HIV and SIV strains. Sequences corresponding to the peptides p9 and p6 were aligned. The minimal epitopes p9CD and p6B are printed in bold and highlighted by horizontal bars. Differences from the consensus sequence are printed in italics.

Similarly, B-LCL from Mm L28 were lysed by effector cells from Mm L28 and Mm 421-90 but not by effector cells from Mm L3 (Fig. 5). Therefore, these peptide-specific effector functions were restricted by MHC class I molecules.

(iv) p6B- and p9CD-specific cytotoxicity is restricted by novel rhesus monkey MHC class I molecules. Having deter-

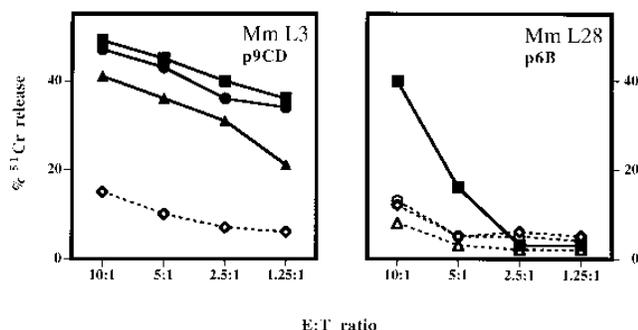


FIG. 3. CTLs from SHIV-infected rhesus monkeys that recognize p9CD, but not p6B, lyse HIV-1, HIV-2, and SIV<sub>mac</sub> Env-expressing target cells. p9CD- and p6B-stimulated PBMC from Mm L3 and Mm L28, respectively, were analyzed in a <sup>51</sup>Cr release assay at various E/T ratios. C1R cells transfected with the epitope restricting MHC clones 8A2-969 and L28-1087 were infected with wild-type vaccinia virus (diamonds) or recombinant vaccinia virus expressing the *env* genes of HIV-1 (vAbT 299) (25) (squares), SIV<sub>mac</sub> (vAbT 253) (34) (circles), or HIV-2 (30) (triangles) and used as target cells for effector cells from Mm L3 and Mm L28, respectively.

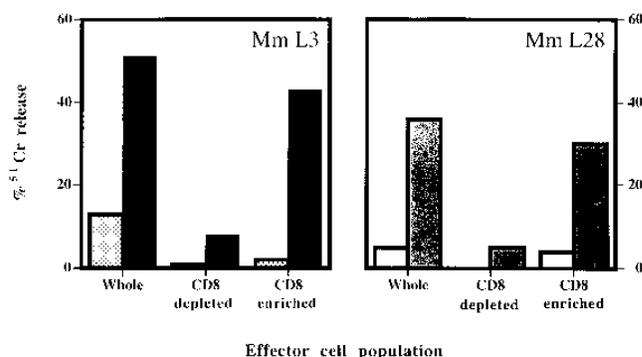


FIG. 4. p9CD- and p6B-specific cytotoxicity is mediated by CD8<sup>+</sup> cells. PBMC from Mm L3 and L28 were stimulated with p9CD and p6B, respectively. Prior to assessment in a <sup>51</sup>Cr release assay the effector cells were separated with CD8-specific immunomagnetic beads (Dynal, Lake Success, N.Y.) (30). The resulting cell populations were examined for lysis of autologous target B-LCL pulsed with p9B (dotted), p9CD (black), p6A (white), or p6B (grey) at an E/T ratio of 2.5:1.

mined that the peptide-specific effector cells recognize viral antigen in an MHC class I-dependent fashion, we sought to identify the MHC class I genes restricting this recognition. Rhesus monkey MHC class I genes were PCR amplified from cDNA obtained from B-LCL of Mm L28 and Mm 8A2. The genes were subcloned into the plasmid pcDNA3 to allow T7 promoter-directed and stably transfected mammalian cell line gene expression. A screening of the genes by in vitro translation and subsequent one-dimensional isoelectric focusing (1-D IEF) of the radiolabeled protein products revealed several clones with IEF bands matching those of the parental B-LCLs (data not shown). Individual clones were then transfected into C1R cells to generate stably expressing cell lines. 1-D IEF of radiolabeled cell lysates generated from these C1R transfectants showed that the cell lines expressed MHC class I molecules with pIs similar to those of molecules from the original B-LCL (Fig. 6A). In comparison to clones L28-1089 and 8A2-969, the clone L28-1087 was expressed only at low levels in both the parental B-LCL and in the transfected C1R cell line. The transfected C1R cells were then pulsed with peptide and used as target cells in <sup>51</sup>Cr release assays. Effector cells from Mm L28 specific for p6B lysed C1R cells expressing the MHC clone L28-1087, whereas a C1R cell line transfected with the

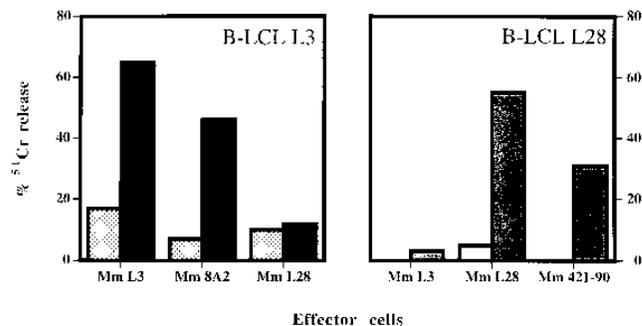


FIG. 5. p9CD- and p6B-specific cytotoxicity can be mediated by autologous and allogeneic effector cells. PBMC from Mm L3, Mm L28, and Mm 8A2 were stimulated with p9CD and assessed for cytotoxicity at an E/T ratio of 5:1 by using a B-LCL from Mm L3 pulsed with p9B (dotted) or p9CD (black). Similarly, p6B-stimulated PBMC from Mm L3, Mm L28, and Mm 421-90 were tested at an E/T ratio of 5:1 for lysis of B-LCL from Mm L28 pulsed with p6A (white) or p6B (grey).

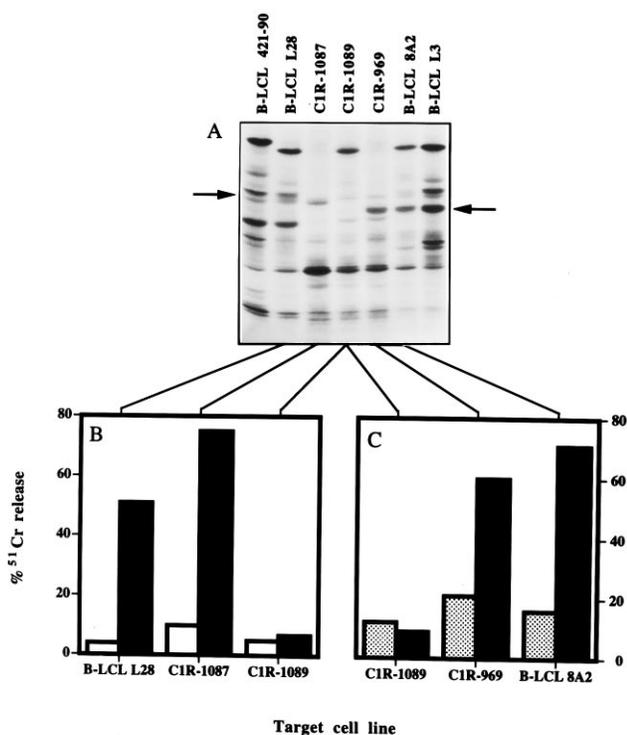


FIG. 6. Recognition of p9CD and p6B by CTL is restricted by two distinct rhesus monkey MHC class I gene products. mRNA was prepared from B-LCL from Mm L28 and Mm 8A2 with the QuickPrep Micro mRNA purification kit (Pharmacia Biotech, Piscataway, N.J.) according to the manufacturer's instructions. Then the mRNA was converted to single-stranded cDNA with an avian myeloblastosis virus reverse transcriptase-based system by using random primers following the manufacturer's protocol (Promega, Madison, Wis.). The cDNA served as a template for PCR amplification with primers specific for the leader peptide (5'-G CGC CTC GAG ATG ACG GTC ATG GCT CCC CGA ACC-3') and 3'-untranslated region (5'-GCG CAA GCT TAG TCC CAC ACA AGG CAG CTG-3') of primate MHC class I genes as described by Watkins et al. (33). The MHC clone L28-1087 was amplified with the 3'-untranslated region primer 5'-CCG CAA GCT TCT GGG GAG GAA ACA CAG GTC AGC ATG GGA AC-3' (14) with an annealing temperature of 59°C. Standard PCR with *pfu* polymerase and the supplied reaction buffer (Stratagene, La Jolla, Calif.) was performed with the following cycle conditions: 2 min at 94°C, 2 min at 37°C, and 4 min at 72°C for 30 cycles, followed by a final extension for 10 min. The PCR products were digested with *Hind*III and *Xho*I and ligated into the plasmid pcDNA3 (Invitrogen, San Diego, Calif.). The cloned PCR products were screened by in vitro translation. Radiolabeled MHC class I proteins were generated by transcription from the T7 promoter and translation in the presence of canine microsomal membranes (Promega) and [<sup>35</sup>S]methionine by using the TNT coupled reticulocyte lysate system (Promega). The MHC molecules were immunoprecipitated with an antiserum specific for free MHC class I heavy chains (18) and subjected to 1-D IEF as described by Gotch et al. (6). Clones yielding gene products focussing at a pI identical to those of certain molecules of interest from the parental B-LCL were then transfected into the *HLA-A*-deficient and *HLA-B35*-low human B-LCL HMy2.C1R (27) by electroporation (275 V, 1000 μF, 500 Ω) (Electroporator II; Invitrogen). The transfectants were selected and maintained in RPMI 1640 medium supplemented with fetal calf serum and containing 0.8 mg of G418 (Gibco, Gaithersburg, Md.) per ml. The expressed MHC class I molecules were characterized by metabolic labeling, immunoprecipitation, and 1-D IEF. The pI of the epitope restricting clones L28-1087 and 8A2-969 is indicated by arrows (A). Each of the transfected C1R cell lines were pulsed with p6A (white), p6B (black), p9B (dotted), or p9CD (black) and assessed for susceptibility to peptide-specific lysis by using p6B-stimulated effector cells from Mm L28 (B) and p9CD-stimulated PBMC from Mm L3 (C) at an E/T ratio of 2.5:1.

clone L28-1089 from the same monkey was resistant to lysis (Fig. 6C). Similarly, p9CD-specific effector cells from Mm L3 lysed C1R cells expressing the MHC clone 8A2-969 but not the clone L28-1089 (Fig. 6B). Thus, we had cloned and expressed

the rhesus monkey MHC class I genes restricting p6B- and p9CD-specific cytotoxicity in SHIV-infected animals.

To identify and characterize further the MHC class I clones from Mm L28 and Mm 8A2, their sequences were determined. The sequence analyses revealed that clone L28-1089 was identical to the previously described A locus allele *Mamu-A\*01*. Clone 8A2-969 is closely related to other primate MHC class I alleles of the A locus, and clone L28-1087 is closely related to alleles of the B locus (Fig. 7). Therefore, the new alleles have been named *Mamu-A\*08* and *Mamu-B\*12*, respectively, according to the recent description of new rhesus monkey MHC class I alleles (2) and following the nomenclature of Klein et al. (11).

To estimate the frequency of *Mamu-A\*08* and *Mamu-B\*12* in rhesus monkey populations, 14 randomly chosen animals at two different primate facilities were screened for the presence of the two novel MHC class I alleles. Analysis of B-LCL derived from the rhesus monkeys by 1-D IEF and using peptide-specific effector cells in <sup>51</sup>Cr release assays revealed that *Mamu-A\*08* was present in 5 of 8 screened monkeys and *Mamu-B\*12* was found in 6 of 14 screened animals.

**Discussion.** Novel rhesus monkey HIV-1 Env CTL epitopes were identified in these studies by a quick and convenient experimental procedure. Screening of effector cells for their ability to lyse target cells pulsed first with peptide pools and subsequently with individual peptides allowed the localization of CTL epitopes to 20 aa within 2 weeks. The epitopes were then fine mapped with 9-aa peptides. The sensitivity of the procedure was enhanced by using PBMC stimulated with pooled peptides to increase the relative number of specific CTLs in the effector cell populations. This effect is consistent with the observation that cocultivation of effector cells with fixed antigen-expressing stimulator cells, when compared with concanavalin A-stimulated PBMC, enhanced virus-specific cytotoxicity (29).

The newly defined CTL epitopes are located in conserved regions of HIV-1 Env. In particular, the p9CD gp120 epitope is completely conserved in all known HIV and SIV isolates. Moreover, rhesus monkey p9CD-specific effector cells lysed autologous target cells expressing SIV<sub>mac</sub>, HIV-1, or HIV-2 Env. Interestingly, an overlapping region of gp120 has been reported to contain a human *HLA-A2*-restricted HIV-1 Env CTL epitope (4). However, monkey effector cell function could not be shown with p9CD-sensitized human *HLA-A2*-positive target cells (data not shown), indicating that the two epitopes may only partially overlap. Other proteins of AIDS viruses have also been shown to exhibit clusters of CTL epitopes in different species. A 13-aa region of Gag is a target for CTL in SIV<sub>mac</sub>-infected cynomolgus monkeys and HIV-1-infected *HLA-B14*-positive humans (7). Additionally, the rhesus monkey SIV<sub>mac</sub> Gag CTL epitope p11C, restricted by *Mamu-A\*01* (20), also constitutes an *HLA-B53*-restricted CTL epitope in HIV-2-infected humans (5). With the definition of these immunogenic domains of AIDS viruses, an opportunity exists to evaluate the induction of identical epitope-specific CTLs in macaques and in human populations.

It has recently been shown that the MHC class I genes of rhesus monkeys are found in at least one A locus and two B loci (2). In contrast to humans, chimpanzees, and gorillas, there is no evidence for the existence of C locus genes in the rhesus monkey. The novel MHC class I genes described here, *Mamu-A\*08* (8A2-969) and *Mamu-B\*12* (L28-1087), have been assigned A and B locus genes on the basis of characteristic amino acid substitutions and the length of their cytoplasmic tails (2). Interestingly, *Mamu-B\*12* is expressed only to low levels in both rhesus monkey B-LCL and stably transfected

Mamu-A\*08

**Leader peptide**  
 CTC CTC CTG GTG CTC TCC GGG GCG CTG GCC CTG ACC CAG ACC TGG GCC  
 L L L V L S G A L A L T Q T W A  
 TCC CCG CCC GGC CCG GGG CAG CCC CCG TTC ATC TCC GTG GGC TAC GTG GAC GAC ACG CAG TTC GTG CCG TTC GAC ACG GGC CAG GAG  
 S R P R E P W Y V G Y V D D T Q F V R F D S D A E  
 AGT CCG AGA GAG CAG CCG CCG CCG CCG TGG GTG GAG CAG GAG GGG CCG GAG TAT TGG GAC CCG AAC ACA CCG ATC TAC AAG GCC CCG  
 S P R E E P R A P W V E Q E G P E Y W D R N T R I Y K A A  
 ACA CAG AAC TAC CCA GAG GGC CTG CAG AAC CTG CCG GGC TAC TAC AAC CAG ACG GAG GCC GCG TCC CAC ACC TAC CAG ACG ATG  
 T Q N Y R E G L Q N L R G Y Y N Q S E A G S H T Y Q T M  
 TAC GGC TGC GAC CTG GCG CCA GAC GGG CCG CTC CTC CCG GGG TAT GAC CAG TCC GCC TAC GAC GCG AGG GAT TAC ATC GCC CTG AAC  
 Y G C D L G P D G R L L R G Y D Q S A Y D G R D Y I A L N  
 GAG GAC CTG CCG TCC TGG ACC GCA GCG GAC ATG GCT GCT CAG AAC ACC CAG CCG AAG TGG GAG GCG GCG GGT GAG GCG GAG CCG TTC  
 E D L R S W T A A D M A A Q N T Q R K W E A A G E A E R F  
 AGA ACC TAC CTG GAG GGC GAG TGC GTG GAG TGG CTC CCG AGA TAT CTG GAG AAC GCG AAG GAG ACG CTG CAG CCG GCG GAC CCC  
 R T Y L E G E W L R R Y L E N G K E T L Q R A D P  
 CCG AAG ACA CAC GTG ACC CAC CAC CCC GTC TCT GAC CAT GAG GCC ACC CTG AGG TGC TGG GCC CTG GCG TTC TAC CCT GCG GAG ATC  
 P K T H V T H H P V S D H E A T L R C W A L G F V P A E I  
 ACA CTG ACC TGG CAG CCG GAT GGG GAG GAC CAA ACT CAG GAC ACG GAG CTC GTG GAG ACC AGG CCT GCA GGA GAT GGA ACC TTC CAG  
 T L T W Q R D G E D Q T Q D T E L V E T R P A G D A  
 AAG TGG GCG GCT GTG GTG GTG CCT TCT GGA AAG GAG CAG AGA TAC ACC TGT CAT GTG CAG CAT GAG GGT CTG CCG GAG CCC CTC ACC  
 K W A A V V V P S G K E Q R Y T C H V Q H E G L P E P L T  
**Transmembrane**  
 CTG AGA TGG GAG CCG TCT TCC CAG TCC ACC ATC CCG ATG GTG GGC ATC ATT GCT GGC CTG GPT CTC CTT GGA GCT GTG GTC ACT  
 L R W E P S S Q S T I P M V G I I A G L V L A G D A V V T  
**Cytoplasmic**  
 GGA GCT GTG GTC GCT GCT GTG ATG TGG AGG AGG AAG AGC TCA GAT AGA AAA GGA GGG AGC TAC TCT CAG GCT GCA AGC AGT GAC  
 G A V V A A V M W R R K S S D R K G G S Y S Q A A S S D  
 AGT GCC CAG GGC TCT GAT GTG TCT CTC ACG GCT TGT AAA GTG TGA  
 S A Q G S D V S L T A C K V \*

Mamu-B\*12

**Leader peptide**  
 CTC CTC CTG CTG CTC TCG GGG ACC CTG TCC CTG ACC GAG ACC TGG GCC  
 L L L L L S G T L S L T E T W A  
 GGC TCG CAC TCC ATG AGG TAT TTC AGC ACC GCA GTG  
 G S H S M R Y F S T A V  
 TCC CCG CCC GGC CCG CCG GAA CCC TGG TAT CTC GAA GTC GGC TAC GTG GAC GAC ACG CAG TTC GTG CCG TTC GAC AGC GAC GCC GAG  
 S R P R E P W Y V G Y V D D T Q F V R F D S D A E  
 AGT CCG AGG ATG GAG CCG CCG GCG CCG TGG GTG GAG CAG GAG GGG CCG GAG TAT TGG GAA GAG CAG ACA CCG AAC GCC AAG GCC AAC  
 S P R M E P R A P W V E Q E G P E Y W E E E T R N A K A N  
 GCA CAG ACT GAC GGA GTG AGC CTG GGG AAC CTG CCG CCG TAC TAC AAC CAG AGC GAG GGG GCG TCT CAC ACT GTC CAG ATA ATG  
 A Q T D R V S L G N L R R Y N Q S E G G S H T Y Q I M  
 TAC GGC TGC GAC CTG GGA CCC GAC GGG CCG CTC CTC CCG GGG TAT CAC CAG TTC GCC TAC GAC GGC AAG GAT TAC ATC GCC CTG AAC  
 Y G C D L G P D G R L L R G Y H Q F A Y D G K D Y I A L N  
 GAG GAC CTG CCG TCC TGG ACC GGC GCG GAC ATG CCG GCT CAG AAC ACC CAG CCG AAG TGG GAG GCG GAC CCF TAT CCG GAG CCG TTC  
 E D L R S W T A A D M A A Q N T Q R K W E G D R Y A A E R F  
 AGA GCG TGT CTG GAG GGC CCG TCC GTG GAG TGG CTC CCG AGA TAC CTG GAG AAC CCG AAG GAG ACG CTG CAG CCG GCG GAC CCC  
 R A Y L E G R C V E W L R R Y L E N G K E T L Q R A D P  
 CCA AAG ACA CAC GTG ACC CAC CAC CCC GTC TCT AAC CAT GAG GCC ACC CTG AGG TGC TGG GCC CTG GCG TTC TAC CCT GTG GAG ATC  
 P K T H V T H H P V S N H E A T L R C W A L G F Y P V E I  
 ACA CTG ACC TGG CAG CCG GAT GGG GAG GAC CAA ACT CAG GAC ACC TAC GTG GAG ACC AGG CCA GGA GGA GAT GGA ACC TTC CAG  
 T L T W Q R D G E D Q T Q D T E L V E T R P A G D A  
 AAG TGG GGA GCT GTG GTG GTG CCT TCT GGA GAA GAG CAG AGA TAC ACG TGC CAT GTG CAG CAT GAG GCG CTG CCA GAG CCC CTC ACC  
 K W G A V V V P S G E E Q R Y T C H V Q H E G L P E P L T  
**Transmembrane**  
 CTG AGA TGG GAG CCA TCT TCC CAG TCC ACC ATC CCG ATC GTG GGC ATC GPT GCT GGC CTG GCT GGC CTA GCA GPT GTG GTC ACC  
 L R W E P S S Q S T I P I V G I V A G L A G L A V V V T  
**Cytoplasmic**  
 GGA GCT GTG GTC GCT GCT GTG ATG TGG AGG AGG AAG AGC TCA GGT GGA AAA GGA GGG AGC TAC TCT CAG GCT GCG TCC AAC GAC  
 G A V V A A V M W R R K S S G G K G G S Y S Q A A S N D  
 AGT GCC CAG GGC TCT GAT GTG TCT CTC ACG GCT TGA  
 S A Q G S D V S L T A \*

FIG. 7. Sequencing of the MHC class I clones 8A2-969 and L28-1087 reveals two novel rhesus monkey alleles, *Mamu-A\*08* and *Mamu-B\*12*. The nucleotide and predicted amino acid sequences are shown for both genes. Three clones of each MHC class I molecule from different PCR amplifications were sequenced to exclude PCR-generated errors. The different protein domains are indicated above the sequences.

C1R cells. A similar low level of expression has been reported for other rhesus monkey B locus alleles (2). The demonstration that the weakly expressed *Mamu-B\*12* can restrict an HIV-1 Env CTL epitope indicates that these B locus alleles can present viral peptides to cytotoxic effector cells. The utility of the newly defined HIV-1 Env CTL epitopes will, in the end, be dependent on the frequency of *Mamu-A\*08* and *Mamu-B\*12* in rhesus monkey populations. Our limited

survey of randomly selected animals in two different colonies suggests that both alleles occur at fairly high frequencies. This should simplify the selection of monkeys for vaccine studies calling for CTL evaluation. Thus, the identification of two HIV-1 Env CTL epitopes and their restricting MHC class I alleles in SHIV-infected rhesus monkeys will facilitate the study of HIV-1 Env-specific CTL responses in nonhuman primates.

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