Escape in One of Two Cytotoxic T-Lymphocyte Epitopes Bound by a High-Frequency Major Histocompatibility Complex Class I Molecule, Mamu-A*02: A Paradigm for Virus Evolution and Persistence?


Wisconsin Regional Primate Research Center and Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, Wisconsin 53715; Laboratory of Histocompatibility/Molecular Diagnostics, University of Wisconsin Hospital and Clinics, Madison, Wisconsin 53792; Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190; Epimmune, Inc., San Diego, California 92121; and Department of Biological Sciences, University of South Carolina, Columbia, South Carolina 29208

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It is now accepted that an effective vaccine against AIDS must include effective cytotoxic-T-lymphocyte (CTL) responses. The simian immunodeficiency virus (SIV)-infected rhesus macaque is the best available animal model for AIDS, but analysis of macaque CTL responses has hitherto focused mainly on epitopes bound by a single major histocompatibility complex (MHC) class I molecule, Mamu-A*01. The availability of Mamu-A*01-positive macaques for vaccine studies is therefore severely limited. Furthermore, it is becoming clear that different CTL responses are able to control immunodeficiency virus replication with varying success, making it a priority to identify and analyze CTL responses restricted by common MHC class I molecules other than Mamu-A*01. Here we describe two novel epitopes derived from SIV, one from Gag (Gag181-189 CM9), and one from the Nef protein (Nef159-167 YY9). Both epitopes are bound by the common macaque MHC class I molecule, Mamu-A*02. The sequences of these two epitopes are consistent with the molecule’s peptide-binding motif, which we have defined by elution of natural ligands from Mamu-A*02. Strikingly, we found evidence for the selection of escape variant viruses by CTL specific for Nef159-167 YY9 in 6 of 6 Mamu-A*02-positive animals. In contrast, viral sequences encoding the Gag181-189 CM9 epitope remained intact in each animal. This situation is reminiscent of Mamu-A*01-restricted CTL that recognize Tat28-35 SL8, which reproducibly selects for escape variants during acute infection, and Gag181-189 CM9, which does not. Differential selection by CTL may therefore be a paradigm of immunodeficiency virus infection.

As the search for a safe and effective vaccine against AIDS enters its third decade, the pandemic continues, with over 40 million human immunodeficiency virus (HIV)-infected individuals worldwide as of the end of 2001 (36). The need for a prophylactic vaccine, and thus for a clear understanding of effective antiviral immune responses, is increasing in urgency. There is much evidence that cellular immune responses play a major role in controlling HIV and simian immunodeficiency virus (SIV) infection (19), and it is therefore logical that candidate vaccines target cytotoxic-T-lymphocyte (CTL) responses. However, what constitutes an effective cellular immune response is far from clear. Studies in the SIV-infected rhesus macaque have proven to be invaluable for the analysis of immunodeficiency virus pathogenesis, cellular and humoral immune responses, and viral evolution. Many insights into AIDS pathogenesis drawn from the study of SIV-infected rhesus macaques could not have been gleaned from studies of HIV-infected humans, so the development of this model remains a priority.

Most studies of CTL responses against SIV have involved macaques that express the common major histocompatibility complex (MHC) class I molecule, Mamu-A*01. These studies have been fundamental to our understanding of antiviral CTL responses. Crucially, the identification the peptide-binding motif (4) of Mamu-A*01 allowed for the comprehensive screening of the viral genome for possible CTL epitopes, and resulted in the identification of multiple Mamu-A*01-restricted epitopes (2). Knowledge of minimal optimal epitopes has also enabled the synthesis of Mamu-A*01 tetrameric reagents specific for CTL that recognize these epitopes (16). Tetramers that bind CTL specific for the immunodominant epitope Gag181-189 CM9 have been used routinely as a benchmark measurement of immune responses in SIV-infected rhesus macaques and have facilitated analysis of the impact of CTL on viremia (9, 17, 25, 32). However, since investigators have focused on analysis of the Mamu-A*01-restricted CTL for which reagents were available, there is a paucity both of Mamu-A*01-positive animals available for research and of information regarding immune responses restricted by other MHC class I molecules.
Identification of minimal optimal CTL epitopes has also enabled us and others to demonstrate the effects of selective pressure exerted by particular CTL on SIV. Strikingly, we described rapid escape in the Mamu-A*01-restricted CTL epitope Tat_{28-35} SLS (3) during the first 4 weeks of infection with the molecularly cloned virus SIVmac239. One unresolved question arising from CTL escape studies, however, is why some high-frequency CTL responses reproducibly select for escape variants and some do not. Most recently, we showed that, whereas the previously characterized Mamu-A*01-restricted CTL recognizing Tat_{28-35} SL8 and Gag_{181-189} CM9 were present at similar frequencies during acute infection (21), only Tat_{28-35} SLS-specific CTL are capable of rapidly selecting for escape variant virus. It was, therefore, of interest to determine whether this pattern of differential escape was recapitulated in epitopes bound by common macaque MHC class I alleles other than Mamu-A*01. Such information would not only shed light on the kinetics of viral evolution within a host but also expand the pool of animals available for vaccine and pathogenesis studies.

In the present work, then, we characterized SIV epitopes bound by the common Indian macaque MHC class I molecule, Mamu-A*02. We found high levels of Mamu-A*02-restricted CTL in vaccinated and naïve macaques directed against an epitope in Gag (Gag_{71-79} YY9) and one in Nef (Nef_{159-167} YY9). The amino acid sequences of these epitopes were consistent with the Mamu-A*02 peptide binding motif, which we identified by elution of natural ligands from the Mamu-A*02 molecule. Furthermore, we observed that Nef_{159-167} YY9-specific CTL selected for escape variants of SIVmac239 in six of six Mamu-A*02-positive macaques, while we detected no evidence of escape from Gag_{71-79} YY9-specific CTL.

### MATERIALS AND METHODS

**Animals.** Rhesus macaques (*Macaca mulatta*) were maintained in accordance with the NIH Guide to the Care and Use of Laboratory Animals and with the approval of the University of Wisconsin Research Animal Resource Center review committee.

**Peptides.** Overlapping peptides (20-mers, 15-mers, 10-mers, 9-mers, and 8-mers) were synthesized by Chiron (Raleigh, N.C.), the Natural and Medical Sciences Institute (University of Tübingen, Tübingen, Germany) or by the Biotechnology Center (University of Wisconsin-Madison) based on SIVmac239 protein sequences, with the exception of Pol peptides, which corresponded to the SIVmac251 sequence. Lyophilized peptides were resuspended in phosphate-buffered saline (PBS) with 10% dimethyl sulfoxide (Sigma). Consecutive 20-mer, 15-mer, and 9-mer peptides overlap by 10, 11, or 8 amino acids, respectively. Pools of peptides contained 10 peptides, each at a final concentration of 1 mg/ml.

**PBMC.** Peripheral blood mononuclear cells (PBMC) were separated from whole heparin- or EDTA-preserved blood by Ficoll-diатrizoate (Histopaque; Sigma, St. Louis, Mo.) density gradient centrifugation. The PBMC were either used immediately or stored at −180°C in liquid nitrogen. PBMC were cultured in R-15 medium (RPMI 1640 supplemented with 15% fetal calf serum, 2 mM l-glutamine, 25 mM HEPES, 25 μM 2-mercaptoethanol, 50 μg of streptomycin/ml, 50 U of penicillin/ml) supplemented with 20 to 100 U of recombinant interleukin-2 (IL-2; Proleukin; Chiron Therapeutica, Emeryville, Calif./mil) and 10 ng/ml γ-interferon (IFN-γ; Roche Diagnostics, Nutley, N.J.). Trypan blue- and acridine orange-stained cells were counted in a hemocytometer (Coulter Electronics, Hialeah, Fla.) and results, therefore, in the intracellular accumulation of 10 μg/ml, as reported in detail elsewhere (13). The sequence of each peptide was determined by the suppliers.

**Peptide-specific T-cell lines.** In order to generate peptide-specific T-cell lines, fresh or thawed PBMC were stimulated in vitro with peptide-pulsed, autologous B-LCL as stimulator cells. Briefly, stimulator cells were generated by incubation of 5 × 10^7 autologous B-LCL with 0.1 to 0.5 μM peptide at 37°C in a humidified atmosphere with 5% CO_2. Autologous, the cells were washed with 0.005 to 5,000 C in MACS buffer (FACS buffer, 2 mM EDTA) in a total volume of 200 μl/ml were added to the cell culture. After 2 days, 20 U of recombinant IL-2 (Proleukin)/ml was added, and the cells were fed every second day with R-15 medium containing 100 U of IL-2/ml. At day 7, CD8-positive cells were enriched using the MiniMACS system (Miltenyi Biotech, Auburn, Calif.). Briefly, live lymphocytes from the 7 day old in vitro-stimulated culture were purified (Ficoll-diатrizoate, Histopaque; Sigma), washed twice with fluorescent-activated cell sorting (FACS) buffer (PBS, 2% fetal bovine serum), and incubated with 6 μl of anti-CD8-phycocerythrin (PE; Immunotech, West-brook, Maine) for 30 min at 6°C in MACS buffer (FACS buffer, 2 mM EDTA). CD8-positive cells were then incubated with anti-PE-labeled beads (Miltenyi) and enriched by using MS columns (Miltenyi) for MiniMACS according to the manufacturer’s protocol. These CD8-positive cells were again stimulated in vitro by using peptide-pulsed, autologous B-LCL as stimulator cells. After a total of 14 days of in vitro stimulation, the cells were used as effectors in the intracellular cytokine staining (ICS [see below]) assay to test for peptide-specific cells.

**DNA/MVA vaccinations and intrarectal SIVmac239 challenge.** Animal 87082 was immunized 10 times with DNA by using the PowderJectXR1 device (Pow-derJect Vaccines, Inc., Madison, Wis.) at intervals of 4 to 9 weeks, as described elsewhere (14). Briefly, four vaccine plasmids were coadministered, representing all open reading frames (ORFs) of SIV. SIV gag, pol, env, vif, vpr, vpu, tat, and rev were encoded by pSIV17E-Fr gag-pol-env; SIV nef was encoded by pSIVNef-TPA and pSIVNeF, and SIV rev was encoded by pSIVRev. The sequence of each ORF was derived from the macrophage-tropic clone SIVmac17E-Fred except for rev, which was derived from SIVmac239 (29, 33). SIVmac17E-Fred shares significant sequence identity with SIVmac239 (3, 33). The construction of these DNA vectors has been described elsewhere (14). Approximately 1 year after the last DNA vaccination, animal 87082 was inoculated twice with recombinant modified vaccinia Ankara (MVA [7, 10, 20]) vectors within a 13-week interval (14). The animal received 10^6 infectious units of each MVA vector, which together encode the gag-pol, env, nef, rev, and tat genes of SIVmac5 (31). No MVA was available that expressed vif, vpr, or vpu. MVA vectors were delivered intradermally and intrarectally (14). No side effects or lesions were found associated with the inoculations. At 9 weeks after the last MVA boost, animal 87082 was challenged intrarectally with the molecularly cloned virus SIVmac239 nef-open (29) by using a dose of 9 ng of p27, or ca. 10 intrarectal 50% monkey infectious doses (23), as described elsewhere (14). All other Mamu-A*02** animals investigated in this study were naïve animals challenged with the same dose and virus, by the same route, as 87082.

**ICS of fresh PBMC.** A total of 10^6 freshly isolated PBMC were incubated with each of the polyepitope-conjugated B (10 μg/ml; Sigma, St. Louis, Mo.) as a positive control, or pools of 10-15- or 20-mer peptides, together with 0.5 μg of anti-CD28 (clone L293) and 0.5 μg of anti-CD49d (clone 9F10; BD Pharmingen, San Diego, Calif.) in a total volume of 200 μl of R-10 medium. For functional avidity assays, minimal optimal peptides were tested in dilutions ranging from 0.005 to 5,000 μg/ml, as reported in detail elsewhere (26). Anti-CD28 and anti-CD49d antibodies were added to provide optimal costimulation (27). After 1.5 h at 37°C, 10 μg/ml of brefeldin A was added and the cells were incubated for another 5 h at 37°C. Brefeldin A inhibits the export of proteins from the endoplasmic reticulum and results, therefore, in the intracellular accumulation of cytokines, which would otherwise be secreted. Cells were washed twice with 1 ml of FACS buffer and then stained with 6 μl of anti-CD4-PerCP (clone SK1; Becton Dickinson, San Diego, Calif.) and 4 μl of anti-CD4-APC (clone SK3; Becton Dickinson) in 100 μl of FACS buffer for 40 min. After two washes with 1 ml of FACS buffer, the cells were fixed with 2% paraformaldehyde in PBS overnight at 4°C. The cells were then washed once with FACS buffer, treated with permeabilization buffer (0.1% saponin in FACS buffer) for 10 min at room temperature, washed once more with 0.1% saponin buffer, and resuspended in 100 μl of 0.1% saponin buffer. Then, 1 μl of anti-human gamma interferon (IFN-γ)/fluorescein isothiocyanate isononanoyl monoclonal antibody (clone 4S.B3; BD Pharmingen) and either 6 μl of anti-CD69-PE (clone L78; Becton Dickinson) or 1 μl of anti-human tumor necrosis factor alpha (TNF-α)/PE monoclonal antibody (clone MAb11; Pharmingen) was added. After 50 min of incubation at room temperature, the cells were washed twice with 0.1% saponin buffer and resuspended in 100 μl of 0.1% saponin buffer fixed with 2% paraformaldehyde. Samples were stored overnight at −20°C. Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson) and
analyzed with FlowJo software (Treestar). The background level of IFN-γ staining in PBMC (induced by a control influenza peptide SNEGSGYFFG) varied among animals but was typically <0.05% in the CD8+ lymphocytes and <0.02% in the CD4+ lymphocytes. The only samples considered positive were those in which >0.5% of CD8+ or CD4+ lymphocytes were stained or in which there was a distinct population of brightly IFN-γ-positive cells also positive for CD69 or TNF-α.

ICS of T-cell lines for fine mapping, MHC restriction analysis, and testing of mutant peptides. When T-cell lines were analyzed by ICS, the method described above (for fresh PBMC) was modified so that 10^5 B-LCL were used instead of autologous PBMC. The background level of IFN-γ staining induced by the control peptide SNEGSGYFFG was usually <0.5% and was subtracted from all values. To determine the restricting MHC class I allele, heterologous B-LCL from MHC defined animals were initially pulsed with the epitope peptide, washed after 1.5 h of incubation at 37°C, and used as stimulus for peptide-specific T-cell lines. Afterward, transfers expressing only one of the potential MHC class I alleles were used as stimulator of the T-cell lines. The only samples considered positive were those in which >0.5% of CD8+ or CD4+ lymphocytes were stained or in which there was a distinct population of brightly IFN-γ-positive cells also positive for CD69 or TNF-α.

Mixed-base sequence detection. The amplified viral cDNA was purified using the Qiagen PCR purification kit. Approximately 150 ng of purified, amplified cDNA was used as a template for each direct sequencing reaction. Both strands of the CTL epitope-spanning portion (ca. 400 bp) of each amplicon were sequenced by using BigDye chemistry (Applied Biosystems, Foster City, Calif.). For the Mamu-A*02-restricted Nef CTL epitope, the sense oligonucleotide was 5′-[TCATGAGAAAACCACGGCTG]-3′ and the antisense oligonucleotide was 5′-[CTTCTCTGGTGTCTATG]-3′. For the Mamu-A*02-restricted Gag CTL epitope, the sense oligonucleotide was 5′-[GCGATCGGCGACTG]-3′ and the antisense oligonucleotide was 5′-[CCGAATGACGCTACTG]-3′. Most sequencing was performed using the ABI 377 automated DNA sequencer (Applied Biosystems). The amplified DNA was sequenced by the Qiagen PCR purification kit. Approximately 150 ng of purified, amplified cDNA was used as a template for each direct sequencing reaction. 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tang was collected. Approximately 50 mg of molecule was purified on W6/32 affinity columns and acid denatured to elute bound peptide. Peptides were separated from free heavy- and light-chain by passage through a 3-K_2O cationic-stirred cell (Millipore, Bedford, Mass.). One-tenth of the stirred-cell flow through was used for automated Edman sequencing on an ABI 492A pulsed-phase protein sequencer (Perkin-Elmer Applied Biosystems Division, Norwalk, Conn.). A peptide motif was compiled by using resultant data and previously described methods (6). The remaining peptide was fractionated by reversed-phase high-pressure liquid chromatography prior to tandem mass spectrometry (MS/MS sequencing on a Q-STAR quadrupole time-of-flight mass spectrometer (Sciex, Applied Biosystems, Foster City, Calif.). For assays of SIV-derived peptide binding to Mamu-A*02, the molecule was purified from 721.221 transfected supernatants by affinity chromatography with monoclonal antibody W6/32, as described previously (34). SIV-derived peptides were then tested for their affinities to purified Mamu-A*02 molecules in standard competition assays. Affinities of SIV-derived peptides for Mamu-A*02 are expressed as the concentration of unlabeled SIV peptide required to reduce binding of a labeled reference peptide to 50% of its maximum level; this is the 50% inhibitory concentration.

RESULTS

Mamu-A*02 is expressed at a high frequency in rhesus macaques of Indian descent. Since Mamu-A*02 was reported to bind an epitope from SIVmac251 Env (39), we designed a PCR-SSP-based method for detection of this allele. We determined that this PCR-SSP reaction was robust and yet specific for the Mamu-A*02 allele (data not shown). The PCR amplicon from eight animals was sequenced and corresponded to the published sequence of Mamu-A*02 (data not shown). We used the assay to determine the frequency of this allele in rhesus macaques of Indian descent (Table 1). Mamu-A*02 was present in approximately one-fifth (19%) of the 992 animals that were typed. This allele is thus almost as common as Mamu-A*01 (15). About one of six Mamu-A*02-positive animals in our colony was also positive for Mamu-A*01 (Table 1).

Identification of SIV-derived epitopes recognized by CD8-positive lymphocytes in fresh PBMC from a Mamu-A*02-positive animal. The Mamu-A*02-positive animal 87082 had been immunized against SIVmac Gag, Pol, Env, Nef, Tat, and Rev proteins as described above. At 1 week after the rMVA boost, a time point at which levels of vaccine-induced CTL are expected to be high, fresh PBMC were isolated and tested in the ICS assay for the presence of SIV-specific CD8+ lymphocytes. Pools of peptides (10 overlapping peptides per pool) spanning the SIV proteins Gag, Pol, Env, Nef, Tat, and Rev were used as stimuli in these assays (14). We identified three peptide pools that induced IFN-γ production in the CD8+ lymphocyte population in this animal (Gag pool B, Nef pool C, and Nef pool D; Fig. 1A and C). If a pool elicited an IFN-γ response, its 10 constituent peptides were tested individually with fresh PBMC isolated at week 2 post-rMVA. The individual 15-mer peptides Gag 17 and 18 and Nefs 63 and 64 were recognized by CD8+ lymphocytes from animal 87082 (Fig. 1B and D). The peptide recognized in Nef pool C was also identified (results not shown).

Fine mapping of the CTL epitopes contained in peptide Gag 17 and Nef 64 by using in vitro-stimulated T-cell lines. The optimal length of peptides bound by MHC class I molecules is between 8 and 10 amino acids (11, 18, 28). To fine-map the newly identified epitopes, T-cell lines were generated by using the 15mer peptides Gag 17 and Nef 64. Then, overlapping 9mers were tested by ICS to define further the region of the epitope. Thereafter, dilutions of peptides of various lengths were tested by ICS to determine which peptide elicited optimal stimulation of the T-cell line. The 9mer peptide Gag 17-4 (GSENLKLSSLY; Gag71-79 GY9) effectively stimulated Gag 17-specific T cells at lower concentrations than all other peptides (Fig. 2A). Similarly, the 9mer peptide Nef 139 (YTSVPGRHY; Nef139-147 YY9) stimulated the Nef 64-specific T cells optimally (Fig. 2B). The fine specificity of Nef 139-147 YY9-specific T cells was also confirmed with a T-cell line from a naive animal infected with SIV (animal 95084; data not shown). We therefore conclude that Gag71-79 GY9 and Nef139-147 YY9 represent the minimal optimal CTL epitope recognized in vivo. The amino acid sequences of each epitope are conserved among SIVmac17E-Fred, SIVmac239, SIVmac251, and SIVmac32H. Identification of the restricting MHC class I molecule by using heterologous B-LCL from MHC class I-defined animals and MHC class I transferents. After definition of these two new CTL epitopes, we determined the restricting MHC class I molecules. A family of MHC-defined animals were screened to determine whether the restricting MHC class I molecule was expressed by any of these MHC-typed animals. B-LCL from these animals were pulsed with the appropriate epitope-containing peptide and were used in the ICS assay, together with the peptide-specific T-cell lines from animal 87082. B-LCL from two of these MHC-defined animals (animals 83098 and 90135) were able to present both the Gag 17 and the Nef 64 epitopes to the corresponding T-cell lines from animal 87082; these were the only two animals in the family that expressed Mamu-A*02 (data not shown). To confirm that Mamu-A*02 was the restricting allele for these two new CD8 epitopes, a 721.221-derived cell line expressing Mamu-A*02 was pulsed with the appropriate peptide and used in the ICS assay for the peptide-specific T-cell lines from animal 87082 (Fig. 3). Both epitopes were indeed restricted by Mamu-A*02 (Fig. 3).

Mamu-A*02 binds peptides with a threonine or serine at position 2 and a hydrophobic position 9 anchor. We next determined that the Nef- and Gag-derived epitopes were similar to peptides naturally bound by Mamu-A*02. Endogenously loaded peptides were eluted from Mamu-A*02 molecules and subjected to pooled Edman sequencing. Compilation of a motif from raw data (Fig. 4A) revealed a strong preference for the amino acids threonine (T) or serine (S) at position 2 (P2) of the peptides bound by this molecule. The C termini of bound peptides were composed of a hydrophobic tyrosine (Y) or, to a lesser extent, phenylalanine (F). In order to characterize further the peptides bound by this molecule, individual ligands...
Seven ligands were sequenced and are reported in Fig. 4B. Peptide sequences obtained by MS/MS reiterate the importance of the P2 and P9 anchors for peptide binding; each ligand possesses either a T or S at its second position, and each terminates with a hydrophobic residue. Comparison of endogenously loaded peptides with the Mamu-A*02-restricted SIV epitopes (Fig. 4B) indeed reveals a similar sequence of amino acids, particularly at the P2 and P9 positions.

**T cells specific for Gag 17 and Nef 64 have cytotoxic activity.** To investigate whether the T cells that recognize the two Mamu-A*02-restricted epitopes possess cytolytic activity, the peptide-specific T-cell lines were also tested in standard chromium-release CTL assays. Peptide-pulsed or rMVA-infected, autologous B-LCL, as well as peptide-pulsed Mamu-A*02-transfected 721.221 cells, were specifically lysed by the peptide-specific T-cell lines (data not shown).

**Nef159-167 YY9-specific CTL select for escape mutations, but Gag71-79 GY9-specific CTL do not.** Having defined the minimal optimal Mamu-A*02-restricted epitopes, we sought to determine whether CTL recognizing these epitopes selected for escape in SIVmac239. We therefore amplified regions of the gag and nef genes from vRNA in plasma isolated from 6 Mamu-A*02-positive animals during the acute (4 to 16 weeks postinfection) and chronic (30 to 67 weeks postinfection) phases. Regions of each amplicon (~400 bp) surrounding the epitope were directly sequenced. In each acute-phase sample, we observed at least one site of nonsynonymous mixed-base heterogeneity in the region encoding the Nef159-167 YY9 epitope, whereas Gag71-79 GY9 epitope sequences remained intact (Fig. 5). In the chronic phase of infection, direct sequencing showed at least one complete amino acid replacement in the Nef159-167 YY9 epitope of each animal sequenced, whereas no substitutions were detected in the Gag71-79 GY9 epitope (data not shown). We confirmed the presence of CTL against both Mamu-A*02-restricted epitopes by generating T-cell lines from four of these animals (animals 87082, 97086, 95084, and 96020) by using thawed PBMC (not shown).

We next examined the evolution of the Nef159-167 YY9 epitope more thoroughly by sequencing individually cloned nef amplicons. At least 10 independently cloned amplicons were prepared from chronic-phase vRNA for each Mamu-A*02-positive animal. Cloned amplicons each contained at least one variant codon in the Nef159-167 YY9 sequence, whereas relatively few nonsynonymous substitutions were detected in surrounding regions (Fig. 6). Clone sequences were also subjected

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**FIG. 2.** Definition of minimal optimal CD8-positive T-cell epitopes. T-cell lines specific for Gag 17 and Nef 64 were generated and tested in ICS against a range of concentrations of overlapping peptides in order to determine which peptide results in maximal stimulation of the T-cell line. The two 9mer peptides, Gag 17-4 (GSENLSLY; Gag71-79 GY9) and Nef 139 (YTSGPGIRY; Nef159-167 YY9), optimally stimulate the Gag 17-specific (A) or the Nef 64-specific T cells (B).
to dN/dS analysis, according to the method of Nei and Gojobori (24). dN exceeded dS within the Nef 159-167 YY9 epitope sequence (P < 0.005; Table 2), whereas dS was greater than dN outside the epitope region (P < 0.05). These results provide strong evidence that substitutions within the epitope were the result of positive selection by CTL.

**CTL recognition is diminished by Nef<sub>159-167</sub> YY9 peptide variants.** We next synthesized peptides encoded by Nef<sub>159-167</sub> YY9 escape variants and tested them in Mamu-A*02 binding assays and in serial dilutions with Nef<sub>159-167</sub> YY9-specific CTL. The only peptide variant that significantly reduced binding to Mamu-A*02, since this variant is the only peptide tested in which an anchor position substitution introduces a residue not tolerated by the molecule. Interestingly, we also found that a previously de-

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**FIG. 3. Mamu-A*02 restricts SIV-derived CTL epitopes.** Cells of the MHC class I-negative human B-lymphoblastoid cell line 721.221, transfected with Mamu-A*02 (Mamu-A*02.221) or Mamu-A*04 (Mamu-A*04.221), were pulsed with the appropriate peptide and used in the ICS assay, together with peptide-specific T-cell lines from animal 87082. Both T-cell lines specific for Gag<sub>71-79</sub> GY9 (A) and for Nef<sub>159-167</sub> YY9 (B) were stimulated by Mamu-A*02-transferents pulsed with the relevant peptide but not by Mamu-A*04 transferents.
scribed epitope derived from SIV Env (YNLTMKR [39]) and reported to be bound by Mamu-A*02, failed to bind appreciably to this MHC class I molecule. Again, this finding is consistent with the Mamu-A*02 binding motif, since this epitope lacks the preferred residues at anchor positions 2 and 9. We were also unable to culture CTL that recognized this peptide from any of our Mamu-A*02-positive animals infected with SIVmac239 (not shown).

On the other hand, nearly all Nef159-167 YY9 variants diminished recognition by CTL raised against the wild-type peptide, 

![A] Pooled-peptide motif

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**dominant residues**

**strong residues**

**weak residues**

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![B] Individual ligand sequences

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**source protein**

- Ovarian carcinoma immunoreactive antigen (121-129)
- Calponin 3 (253-261)
- Alpha globin (119-127)
- Eukaryotic elongation factor 1-alpha (21-29)
- Stearoyl co-A desaturase (20-28)
- Splicing factor similar to dnaJ (57-65)
- Hypothetical protein FLJ10143 (183-191)

**Previously reported Env epitope**

- Nef<sub>57-165</sub> YY9
- Gag<sub>71-79</sub> GY9

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**FIG. 4.** Pooled motif and individual ligand sequences for Mamu-A*02. (A) Peptides eluted from Mamu-A*02 were pooled and subjected to Edman sequencing. The resultant data were analyzed as previously described (6) to generate a peptide motif based on the fold increase in picomoles over the prior round of sequencing. Dominant residues (in boldface) exhibited a ≥3.5-fold increase over the prior round; strong residues (in blue) exhibited a 2.5- to 3.5-fold increase; weak residues (in green) exhibited a 2.0- to 2.5-fold increase. (B) Individual ligand sequences were derived from MS/MS sequencing of selected ions. The two new Gag and Nef SIV epitopes and the previously described Env epitope (39) are listed below the naturally loaded ligands.
although the degree of reduction varied among animals (Fig. 7). One variant detected in animal 97086, YTSGPGIRS, was poorly recognized in this animal but was recognized nearly as well as, or even better than, the wild-type peptide in animals 95084 and 87082, although this peptide binds very poorly to Mamu-A*02 in vitro (see Table 3). Interestingly, this variant sequence was not detected in any other animals. The most frequent variant detected in animal 95084 (YTSGPGIRF, 15 of 16 clones) demonstrated the greatest reduction in recognition by CTL from this animal (Fig. 7). All other variant peptides demonstrated only slight reduction in recognition by CTL from animal 95084, even when excess peptide was washed from the B-LCL before the were used in the assay (results not shown). All tested variant peptides were poorly recognized by CTL from animal 96020 (Fig. 7).

FIG. 7. Amino acid variation accumulates in the Mamu-A*02-restricted Nef\textsubscript{159-167} YY9 epitope during acute infection with SIVmac239. Virus isolated from Mamu-A*02-positive monkeys 4 to 16 weeks after infection with SIVmac239 was analyzed by direct sequencing of viral amplicons spanning the SIV genes \textit{nef} and \textit{gag}. The Mamu-A*02-restricted Nef\textsubscript{159-167} YY9 epitope accumulated amino acid variation in all animals analyzed, whereas the second Mamu-A*02-restricted epitope Gag\textsubscript{71-79} GY9 did not. Sites of mixed-base heterogeneity are shown in black; codons containing these sites are boxed, with the encoded amino acid(s) indicated in black.
caques, we found that viruses from 19 of 21 animals showed evidence of escape from CTL responses within the first 4 weeks of infection, leading us to hypothesize that acute-phase CTL escape is a hallmark of SIV infection (26). In that study, we showed that CTL responses that rapidly selected for escape mutant viruses were capable of recognizing lower concentrations of peptide than those CTL that did not select for acute-phase escape. CTL that are sensitized by low levels of peptide have been termed high "functional avidity" CTL (1, 8, 35); it is thought that such CTL may be more effective at elimin-
ing virus-infected cells than CTL that require more peptide for activation (low "functional avidity"). We determined that Nef159-167 YY9-specific CTL were of high "functional avidity" and capable of 50% maximal IFN-γ production in the ICS assay when stimulated with peptide at a concentration of 4.8 μg/ml (26). Since high-avidity, Nef159-167 YY9-specific CTL selected for escape variant viruses but Gag71-79 GY9-specific CTL did not, we sought to measure the functional avidity of CTL recognizing the latter epitope. Fresh PBMC from animal 87082 obtained at 2 and 3 weeks postinfection yielded 50% maximal IFN-γ production when stimulated with 12.2 μg of peptide/ml and are thus of lower functional avidity than CTL that recognize Nef159-167 YY9 (data not shown).

**DISCUSSION**

We have identified two SIV-derived epitopes, Nef159-167 YY9 and Gag71-79 GY9, restricted by a common rhesus macaque MHC class I molecule, Mamu-A*02. We developed a robust, sequence-specific DNA amplification technique allowing for rapid typing of macaques for the Mamu-A*02 allele and found it to be common in captive-bred Indian rhesus macaques, expressed by 19% of 992 animals screened. We also defined the peptide binding motif of the Mamu-A*02 molecule by sequencing of eluted natural ligands; the amino acid sequences of both epitopes fit this motif exactly. Knowledge of the motif will make it possible to screen proteins from SIV and other pathogens to identify more potential epitopes restricted by Mamu-A*02. This method has proven successful for Mamu-A*01 and Mamu-B*17 and has resulted in the identification of 14 Mamu-A*01-restricted and 16 Mamu-B*17-restricted epitopes (2, 22).

We were also interested in determining whether CTL that recognized either of these two new epitopes were capable of exerting selective pressure on SIV. Direct sequencing of viral amplicons showed that the Nef159-167 YY9 epitope sequence accumulated nucleotide substitutions beginning in the acute phase of infection and that variant codons appeared fixed in viruses sequenced during chronic infection. Meanwhile, we detected no nucleotide substitutions in the Gag71-79 GY9 epitope, even in chronic infection. Moreover, we found the rate of nonsynonymous substitutions within the Nef159-167 YY9 epitope to be significantly elevated in comparison to surrounding sequences, indicating that the variation we detected was the result of positive selection. While most Nef159-167 YY9 variant peptides tested bound Mamu-A*02 as well as the index peptide, one variant, YTSGPGIRS, effectively prevented binding.

Still, T-cell lines generated by using wild-type Nef159-167 YY9 peptide showed a diminished capacity to recognize most variant peptides. We therefore conclude that Nef159-167 YY9-specific CTL selected for escape variant viruses, which altered either epitope binding to the cognate MHC class I molecule or recognition by T-cell receptors. CTL that recognized the novel epitope Gag71-79 GY9, however, did not select for variant viruses, even late in infection. Surprisingly, we also found that a previously described Mamu-A*02-restricted epitope (39) was not bound effectively by the Mamu-A*02 molecule. The sequence of this peptide did not fit the Mamu-A*02 binding motif, and we could not detect CTL directed against this epitope in any animal with a Mamu-A*02-positive infected macaque.

The Nef159-167 YY9 epitope was recently described in macaques chronically infected with SIVmac251 or SHIV by other researchers (30). Because significant numbers of Nef159-167 YY9-specific CTL were detected in chronic-phase PBMC from two animals infected with SHIV-89.6 or SHIV-89.6P, the authors of that study concluded that these CTL did not select for escape variant viruses. In striking contrast, we detected no wild-type sequences in the region encoding Nef159-167 YY9 in cloned viral amplicons obtained during chronic infection from five animals. Indeed, the results reported above and elsewhere (26) suggest that Nef159-167 YY9-specific CTL reproducibly select for escape variants during acute SIVmac239 infection of Mamu-A*02-positive animals.

Interestingly, the relationship between Nef159-167 YY9-specific CTL responses, which rapidly select for escape, and Gag71-79 GY9-specific responses, which do not, recapitulates the relationship between the immunodominant Mamu-A*01-restricted CTL responses directed against Tat28-35 SL8 (rapid escape) and Gag181-189 CM9 (slow escape). These phenomena suggest that, whereas CTL directed against different epitopes
may be present at similar levels, they exert differential selective pressure on the virus. This may be explained in part by a difference in “functional avidity” of CTL for their cognate epitopes, as we have recently argued elsewhere (26). With the identification of epitopes bound by another high-frequency macaque MHC class I molecule, one “high avidity” and one “low avidity,” we are presented with the opportunity to better define the mechanisms by which CTL select for escape variant viruses. In any case, these data, along with those we have reported elsewhere (26), suggest a model wherein acute-phase CTL responses facilitate control, but not clearance, of primary viremia. Escape from these effective CTL responses would then enable the virus to persist in the host, in the presence of other CTL responses that are less effective at controlling viral replication. Vaccine or immunotherapy regimens that preferentially stimulate CTL of “high functional avidity” may therefore induce immune responses that are particularly effective at elimination of virus-infected cells.

Knowledge of two high-frequency CTL responses restricted by the high-frequency Mamu-A*02 molecule will also help alleviate the pressure to use Mamu-A*01-positive animals for vaccine challenge experiments. An understanding of CTL responses restricted by MHC class I molecules other than Mamu-A*01 will enable us to elucidate interactions between SIV and the macaque immune system with greater breadth, thus increasing the utility of this model for HIV pathogenesis and immunity.

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