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Simian Immunodeficiency Virus Evades a Dominant Epitope-Specific Cytotoxic T Lymphocyte Response Through a Mutation Resulting in the Accelerated Dissociation of Viral Peptide and MHC Class I

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The ability of an AIDS virus to escape from immune containment by selective mutation away from recognition by CTL was explored in simian immunodeficiency virus of macaques (SIVmac)-infected rhesus monkeys. CTL recognition of a previously defined common viral mutation in an immunodominant SIVmac Gag epitope was evaluated. CTL were assessed for their ability to recognize a SIVmac Gag protein with a single residue (T → A) replacement in the minimal epitope peptide bound by the MHC class I molecule Mamu-A*01. SIVmac Gag-specific CTL lysed Mamu-A*01+ target cells infected with recombinant vaccinia virus expressing the wild-type but not the mutant Gag protein. In addition, CTL recognized the mutant epitope peptide less efficiently than the wild-type virus peptide. In studies to determine the mechanism by which the mutant virus evaded CTL recognition, this peptide was shown to bind Mamu-A*01 in a manner that was indistinguishable from the wild-type peptide. However, experiments in which an increasing duration of delay was introduced between peptide sensitization of target cells and the assessment of these cells as targets in killing assays suggest that the mutant peptide with a T → A replacement had a higher off-rate from Mamu-A*01 than the wild-type peptide did. Therefore, these findings suggest that AIDS viruses can evade virus-specific CTL responses through the accelerated dissociation of mutant peptide from MHC class I.

The Journal of Immunology, 2000, 164: 6474–6479.

A ccumulating evidence suggests that CTL play an important role in the immune containment of HIV replication. It has been shown that the emergence of virus-specific CTL is associated with a reduction of viremia during the acute infection of individuals with AIDS viruses (1–4). Moreover, high-frequency virus-specific CTL responses appear to contribute to a decrease in virus load or to a delay in disease progression in chronically HIV-1-infected persons (5–8). Nevertheless, viral replication is never fully controlled in most if not all infections. It has been proposed that this incomplete immune control of AIDS virus replication in infected individuals may be explained in part by the emergence of viral mutants capable of evading CTL recognition.

To date, HIV-1 mutants that can escape from CTL recognition have been found in only a limited number of infected persons (9–18). The difficulty in identifying such escape mutants may be attributed to the small number of well-defined dominant epitope-specific CTL responses in HIV-1-infected persons. Further studies are needed to characterize the mechanisms by which HIV-1 might escape from CTL and the role of viral escape mutants in HIV-1 disease progression.

The SIV-infected rhesus monkey provides an ideal model system in which to explore AIDS virus mutation to evade cell-mediated immune responses. Monkeys can be selected for study on the basis of their MHC class I alleles that will develop predictable, well-defined dominant SIV epitope-specific CTL responses. Moreover, these animals can be inoculated with a pathogenic virus isolate that has been molecularly characterized, facilitating a precise definition of the virus that initiates the infection. We previously addressed the possibility that SIVmac might escape by mutation from a CTL response in chronically infected rhesus monkeys. This study was done in monkeys expressing the MHC class I gene Mamu-A*01 that develop an immunodominant SIVmac Gag epitope-specific CTL response after simian immunodeficiency virus of macaques (SIVmac)4 infection (19). In that study, mutant virus encoding a change from threonine (T) to alanine (A) in the immunodominant Gag epitope (residues 181–189 of the Gag protein) emerged in two of three evaluated Mamu-A*01+ monkeys infected with SIVmac (20). Experiments in which 12 amino acid peptides (p11C, p11C2A) containing the immunodominant epitope (20) were used to sensitize target cells for lysis did not suggest that this T → A replacement would result in escape from the epitope-specific CTL recognition (20). However, it still remained possible that this mutant protein may not be properly processed in the intracellular MHC class I pathway for recognition by the epitope-specific CTL or that the mutant peptide may not form an optimally stable MHC class I peptide complex. Therefore, we
undertook further studies to assess the possibility that SIVmac iso-
lates with this T → A replacement might escape from the immu-
nodominant CTL response in Mamu-A*01+ rhesus monkeys.

Materials and Methods

Animals and virus

Rhesus monkeys (Macaca mulatta) were used in these studies. These an-
imals were maintained in accordance with the guidelines of the Committee
on Animals for Harvard Medical School and the Guide for the Care and
Use of Laboratory Animals (National Academy Press, 1996). All monkeys
were inoculated i.v. with SIVmac 251 strain, as described previously (20).
During the period of the studies, the monkeys were infected with SIVmac
for 2–5 years but showed no evidence of an AIDS-like syndrome.

Site-directed mutagenesis and generation of recombinant
vaccinia viruses

The plasmid pM40K containing the entire coding region of SIVmac 251
gag was engineered using site-directed mutagenesis to encode the T →
A replacement in the epitope-coding region (21, 22). This was done
following the instructions of the QuickChange site-directed mutagenesis
kit (Stratagene, La Jolla, CA). The sequence of oligonucleotides used to
create this change was 5′-CAC TGT CAG AAG GTG GGC CCC CCT
ATG ACA TTA ACT-3′. The mutated base G and the other two bases,
GCC, encoded the change in amino acid from threonine to alanine. The
sequence of plasmid DNA containing the desired substitution was con-
firmed by sequencing. A recombinant vaccinia virus expressing this T →
A replacement of SIVmac was then created through homologous recom-
bining using a host range selection system and the plasmid pM40K con-
taining the sequence encoding the single T → A replacement in the epitope
(23). The selected recombinant vaccinia viruses were amplified and titrated
using RK13 cells. vGag/182A-2 and vGag/182A-3 were viruses generated
by two clones of the corresponding mutated PK40 plasmids. As controls,
recombinant vaccinia viruses were also generated that expressed the wild-
type SIVmac gag or another SIVmac gag insert encoding a single amino
acid replacement 21 aa N-terminal to the epitope (216IV). These three recombinant vaccinia viruses were assessed for the expression of SIVmac Gag after infection of B lymphoblastoid cell line
(B-LCL) expressing the Mamu-A*01 gene. In the experiments using ef-
fectors from monkeys 597 and 403, the target B-LCL were infected
overnight with the recombinant vaccinia viruses. The infected B-LCL were
then divided into two aliquots, one for CTL assays and the other for 2′3S-
translabeling and SDS-PAGE characterization. The recombinant vaccinia
virus-infected B-LCL were assessed for viability by trypan blue exclusion
before CTL assays. Furthermore, immune fluorescence analysis showed
that SIVmac Gag protein was expressed in the B-LCL targets infected with
the recombinant viruses.

Cell lines

Rhesus monkey B-LCL were generated by incubating 105 Ficoll-diatrizo-
ate-isolated PBL in 100 μl of culture medium with 100 μl of S594 super-
natant. S594 is a cell line productively infected with the transforming ba-
boon Herpesvirus papio (20). The B-LCL were transformed and
maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supple-
mented with 1-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50
μg/ml), and 10% FCS (HyClone, Logan, UT).

Cytotoxicity assays

Rhesus monkey B-LCL immortalized with Herpesvirus papio served as
target cells. The B-LCL were incubated at a cell concentration of 1×
106/ml with recombinant vaccinia viruses carrying the SIVmac gag,
with a control (equine herpesvirus gH) gene at 10 PFU, or with decreasing
concentrations of synthetic peptides for 8 h at 37°C in a 5% CO2 humid-
ified atmosphere (20). CTL derived from PBL of SIVmac-infected mon-
keys were used as effector cells in a standard 51Cr-release assay performed
in U-bottom 96-well microtiter plates. 51Cr-labeled target cells were incu-
bated for 5 h with effector cells at different E:T ratios. Spontaneous release
varied from 10 to 20%. Specific release was calculated as ([experimental
release − spontaneous release] / spontaneous release) × 100.

Live-cell binding assays

Peptide binding to a C1R cell line stably transfected with Mamu-A*01 was
assessed as previously described (24). Cells were incubated overnight
at 26°C in the presence of 3 μg/ml human β2-microglobulin (β2m). The next
day cells were split into separate aliquots and incubated with 100,000 cpm
of the iodinated reference peptide ATPYDINQM and different concentra-
tions of the test peptides CTPYDINQM or CAPYDINQM at 20°C for 4 h.
Cell pellets were then spun down, washed, and the incorporated 125I was
measured by resuspension in Optiphase and counting in a gamma scintil-
lation counter. Percent inhibition of binding was calculated as [1 − (in-
corporated cpm in the presence of competitor peptide)/total incorporated
cpm in the absence of competitor peptide) × 100].

In vitro folding of Mamu-A*01/β2m with p11C,C-M or
p11C, C-M/2A

Different concentrations (100 μM, 10 μM, and 1 μM) of the wild-type
peptide p11C,C-M or the mutant peptide p11C,C-M/2A were added to the
fixed quantities of rhesus monkey Mamu-A*01 heavy chain and human
β2m as described previously (25). The same quantity of the 150-kDa pro-
tein alcohol dehydrogenase was added to each reaction as a control stan-
dard for determining the relative folding efficiency. Formation of the folded
43-kDa Mamu-A*01/peptide/β2m complex was monitored by gel filtration
on a TSK SWxl 3000 column ( Tosoh, Montgomeryville, PA)

CTL peptide-MHC class I stability assays

The kinetics of target cell-peptide sensitization for CTL-mediated lysis was
determined using a peptide-MHC class I stability assay as described by
Goulder et al. (10). Mamu-A*01+ target cells were pulsed with 20 μM of
the 9 aa wild-type or mutant peptide and washed twice with FCS-free
RPMI 1640 medium. In the CTL experiments other than those shown in
Fig. 6, peptide sensitization and chromium labeling of target cells were
done simultaneously, with an 8-h incubation before the addition of effector
cells. In optimizing CTL peptide-MHC class I stability assays, we found
that the specific lysis of targets incubated for 8 h with peptides was similar
to the specific lysis of target cells incubated for 2 h. Thus, the peptide-
loaded target cells were incubated for 0, 2, 16, or 18 h before addition of
CTL effector cells. The impact of this period of incubation on CTL lysis
was evaluated for the mutant peptide in comparison with the wild-type
peptide. An inverse correlation between target cell lysis and the incubation
time for a given peptide indicates an unstable peptide-Mamu-A*01

Sequence of SIVmac gag fragments containing the epitope-coding region

The virions and viroporins in six Mamu-A*01+ monkeys were assessed
for the mutation encoding the T → A replacement in the epitope
corresponding to p11C,C-M. To this end, plasma SIV RNA was isolated
and reverse-transcribed to cDNA (20); proviral DNA was extracted from
PBL of infected monkeys as described previously (20). The SIV cDNA and
proviral DNA were then characterized for mutations in the epitope-coding
region using PCR-based cloning and sequencing strategy (20). Up to 80
clones generated by PCR from each SIV cDNA or DNA sample were
analyzed. Sequence analyses showed that the mutation encoding the T →
A replacement in the epitope could be identified both in the plasma SIV
RNA and proviral DNA in PBL of the infected monkeys (data not shown).

Results

Target cells infected with a recombinant vaccinia virus
expressing a mutant Gag containing a T → A replacement in a dominant
CTL epitope were not lysed by Gag epitope-specific CTL

To determine whether virally expressed Gag containing the T → A
mutation would be endogenously processed and recognized by
epitope-specific CTL, we generated a recombinant vaccinia-SIV-
mac gag construct encoding this mutant Gag sequence. As controls,
recombinant vaccinia viruses were also generated that expressed the
wild-type SIVmac gag or a control mutant SIVmac gag encoding a single
amino acid replacement 21 aa N-terminal to the epitope-coding sequence
(vGag/161IV). These three recombinant vaccinia viruses were then assessed for recognition by Gag
epitope-specific CTL

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These results indicated that the mutant Gag containing the T→A replacement expressed in a recombinant vaccinia virus failed to be recognized by the CTL and suggested that processing or presentation of the mutant peptide was impaired.

A mutant peptide representing the optimal Gag epitope sensitized target cells less efficiently than an optimal wild-type Gag peptide for recognition by epitope-specific CTL.

We then sought to determine whether a peptide containing the T→A replacement could form a complex with Mamu-A*01 that would be recognized by the CTL and suggested that processing or presentation of the mutant peptide was impaired.

**FIGURE 1.** SDS-PAGE analysis of expression of SIVmac Gag proteins by recombinant vaccinia viruses. B-LCL infected overnight with recombinant vaccinia viruses were 35S-translabeled for 6 h and assessed for expression of SIVmac Gag protein by precipitating lysates with sera from two SIVmac-infected monkeys. The size of the precursor protein of SIVmac Gag (55 Kd) is indicated by an arrow. v249 is a recombinant vaccinia virus that expresses the control equine herpesvirus gH gene vGag/161V (simplified as v161V in Fig. 2) and expresses the full-length SIVmac Gag protein containing a single replacement (V) 21 aa N-terminal to the epitope. vGag/182A (simplified as v182A in Fig. 2) expresses the full-length SIVmac Gag protein containing a T→A replacement at the second position of the epitope (vGag/182A-2 and vGag/182A-3 were viruses generated from two clones of the corresponding mutated PK40 plasmids). vGag expresses the full-length wild-type SIVmac Gag protein.

**FIGURE 2.** CTL lyse B-LCL infected with a recombinant vaccinia virus expressing a wild-type but not a mutant SIVmac gag encoding a T→A replacement in a dominant epitope. A, Mamu-A*01 B-LCL infected with 10 PFU of the noted recombinant vaccinia viruses were assessed for lysis using varied E:T ratios as indicated. The effectors were PBL of SIVmac-infected Mamu-A*01 rhesus monkeys stimulated in vitro with Con A for 3 days and then expanded in IL-2-containing medium for an additional 3 days. B, Mamu-A*01 B-LCL infected with various PFU of recombinant vaccinia viruses were assessed for specific lysis by CTL using an 80:1 E:T ratio. Effector cells were prepared as described above.

**FIGURE 3.** Epitope-specific CTL inefficiently lyse Mamu-A*01 B-LCL pulsed with mutant p11C,C-M/2A. Peptide titration in sensitizing B-LCL for CTL lysis showed a marked decrease in CTL recognition of 9-aa mutant peptide. The E:T ratio used in the study of cells from monkey 403 was 50:1; the ratio for the study using cells from monkey 297 was 100:1. The sequences of wild-type and mutant peptides are as follows: p11C,C-M, CTPYDINQM; p11C,C-M/2A, CAPYDINQM; P11B (the control peptide), QALSEGCTPYDI.

2). These results indicated that the mutant Gag containing the T→A replacement expressed in a recombinant vaccinia virus failed to be recognized by the CTL and suggested that processing or presentation of the mutant peptide was impaired.

A mutant peptide representing the optimal Gag epitope sensitized target cells less efficiently than an optimal wild-type Gag peptide for recognition by epitope-specific CTL.

We then sought to determine whether a peptide containing the T→A replacement could form a complex with Mamu-A*01 that would be recognized by the CTL. In experiments using 12 aa peptides containing the epitope, differential CTL recognition of the mutant peptide with the T→A replacement and the wild-type peptide was not clear-cut. The concentration of mutant peptide required for sensitizing a target cell for CTL recognition appeared to be 10 times higher than the concentration needed for sensitizing targets with wild-type peptide (Refs. 20 and 24 and data not shown). However, the ability of the mutant peptide to evade CTL recognition was evident when two 9-aa peptides, which corresponded to the wild-type (p11C,C-M; CTPYDINQM) and the mutant (p11C,C-M/2A; CAPYDINQM) viral sequences, were used in CTL assays. Although target cells were sensitized for lysis by the wild-type p11C,C-M at peptide concentrations as low as 1 ng/ml, 1000-fold higher concentrations of mutant p11C,C-M/2A were required to sensitize target cells for comparable levels of epitope-specific lysis (Fig. 3). Thus, the results of experiments using optimal epitope peptides support the observation made in the study of CTL recognition of the mutant Gag in the vaccinia expression system. The mutant Gag containing a T→A replacement at the second position of the p11C,C-M epitope appears capable of escaping from CTL recognition.
The escape of the altered epitope from CTL recognition was not due to an inability of the mutant peptide to bind to Mamu-A*01.

We then sought to characterize the mechanism by which this mutant viral epitope escapes CTL recognition. We first determined whether the absence of CTL recognition of the T\(^3\)A mutant Gag could be attributed to an inability of the viral peptide to bind to the Mamu-A*01 molecule. Peptide-MHC class I binding studies were conducted using the two 9-aa peptides, which corresponded to the wild-type and the mutant sequences of the epitope (20). These two peptides were assessed for their relative ability to compete with the iodinated reference peptide (ATPYDINQM) for binding to Mamu-A*01-transfected C1R cells. The IC\(_{50}\) of binding for CTPYDINQM was 5 nm and for CAPYDINQM was 30 nm.

Interestingly, mutant p11C,C-M/2A was still able to bind quite efficiently to Mamu-A*01 expressed on the surface of cells. Its binding capacity may at most have been only slightly lower than that of the wild-type p11C,C-M (Fig. 4).

To confirm the results of this peptide binding experiment, we initiated small-scale in vitro folding reactions to assess the ability of the two 9-aa peptides to induce folding of the Mamu-A*01-β\(_{2m}\) complex at limiting peptide concentrations. Formation of the folded 43-kDa Mamu-A*01-peptide-β\(_{2m}\) complex was monitored by gel filtration. As shown in Fig. 5, the mutant and wild-type peptides were equally efficient in inducing the formation of a 43-kDa Mamu-A*01-peptide-β\(_{2m}\) complex.

These results indicated that the mutation containing the T\(^3\)A replacement did not significantly interfere with peptide-Mamu-A*01 binding. Therefore, these observations suggest that the absence of CTL recognition of the T\(^3\)A Gag mutant was not due to the inability of the viral peptide to bind to Mamu-A*01 expressed on the cell surface.

The mutant peptide with a T\(^3\)A replacement was unable to stabilize the peptide-Mamu-A*01 complex for recognition by the epitope-specific CTL.

Despite the preserved ability of mutant p11C,C-M/2A to bind Mamu-A*01, the T\(^3\)A replacement might allow the virus to escape CTL recognition by altering the stability of the peptide-MHC complex, increasing the off-rate of the bound peptide. To assess this possibility, we employed a peptide-MHC class I stability assay (Fig. 6). Mamu-A*01\(^+\) B-LCL were pulsed with 20 μM of wild-type p11C,C-M or mutant p11C,C-M/2A and were washed with FCS-free medium. The peptide-loaded cells were then incubated for 0, 2, 16, or 18 h before their use as targets for CTL effector cells. When compared with the wild-type peptide, the mutant peptide with the T\(^3\)A replacement exhibited a decreased

![FIGURE 4.](http://www.jimmunol.org/)

The wild-type and mutant peptides bind comparably to cell surface-expressed Mamu-A*01. The ability of the native sequence (CTPYDINQM; □), mutant sequence (CAPYDINQM; ○), and control sequence (p11B; ▲) peptides were assessed for their ability to compete with the iodinated reference peptide (ATPYDINQM) for binding to Mamu-A*01-transfected C1R cells. The IC\(_{50}\) of binding for CTPYDINQM was 5 nm and for CAPYDINQM was 30 nm.

![FIGURE 5.](http://www.jimmunol.org/)

Folding of Mamu-A*01 and human β\(_{2m}\) around the native p11C,C-M and the mutant p11C,C-M/2A are comparably efficient. Gel filtration profiles of soluble Mamu-A*01 monomers folded with human β\(_{2m}\) and either the wild-type p11C,C-M or the mutant p11C,C-M/2A are shown. The profile of the control alcohol dehydrogenase is also shown. The peptide concentrations (100 μM, 10 μM, and 1 μM) used in each reaction are indicated on the left.
ability over time to maintain a stable Mamu-A*01-peptide complex that was recognized by CTL. Epitope-specific CTL recognized wild-type peptide- and mutant peptide-loaded target cells equivalently when peptide-pulsed targets were incubated for 0 or 2 h before the assay. In contrast, 16 and 18 h after peptide pulsing, the mutant peptide-loaded targets were poorly recognized by the epitope-specific CTL, whereas the wild-type peptide-loaded targets remained susceptible to lysis. These results suggest that the T3A replacement in the epitope accelerates the dissociation of the peptide from Mamu-A*01 despite the preserved capacity of the peptide to bind Mamu-A*01.

Discussion
These experiments to characterize CTL recognition of recombinant vaccinia virus-expressed Gag, the impact of peptide concentration on target cell sensitization for CTL lysis, and the relative efficiency of peptides to maintain stable peptide/MHC class I complex suggest that the T → A replacement was most readily appreciated when using target cells prepared by vaccinia virus expression of the mutant Gag. The absence of CTL recognition of mutant peptides generated in the cell through processing of vaccinia virus-expressed Gag may mimic the naturally occurring events of CTL escape because viral peptides must be processed from endogenously synthesized proteins and presented by MHC class I molecules.

The present studies suggest that the absence of recognition of the mutant epitope by CTL was a result of the rapid dissociation of the Gag peptide from Mamu-A*01. The impact of the T → A replacement on peptide-MHC complex formation and viral escape from CTL recognition was most evident when the peptide-loaded target cells was incubated for a prolonged period of time before they were employed in the CTL assays. Despite its inability to maintain a stable complex with Mamu-A*01, mutant peptide exogenously maintained its ability to bind to Mamu-A*01. This may explain the finding that CTL recognition of the mutant peptide occurred when target cells were pulsed with high but not low concentrations of this protein fragment. A high concentration of the mutant peptide may maximize its association with MHC class I molecules, overcoming the increased off-rate of MHC-peptide interaction. The finding that the mutant peptide rapidly dissociates from Mamu-A*01 may also explain the complete abrogation of CTL recognition of the target cells infected with a recombinant vaccinia virus expressing the mutant Gag. Mutant peptides derived from the endogenous processing pathway may be particularly

FIGURE 6. CTL peptide/MHC stability assays showed that the T → A replacement accelerated the dissociation of the mutant peptide from Mamu-A*01. Mamu-A*01+ target cells were pulsed with 20 μM of the wild-type (■) and mutant (■) 9-aa peptides, washed twice to remove unloaded peptides, and then incubated at 37°C for increased numbers of hours before the addition of CTL effector cells. The loss of CTL recognition of target cells as the duration of time after peptide pulsing increased indicated the lack of stability of the peptide-Mamu-A*01 complex. Data were derived from up to three repeated experiments, with error bars indicating the variability among the CTL assays.


