Clonally Diverse CTL Response to a Dominant Viral Epitope Recognizes Potential Epitope Variants


*J Immunol* 2001; 167:4996-5003; doi: 10.4049/jimmunol.167.9.4996

http://www.jimmunol.org/content/167/9/4996

**References**

This article cites 27 articles, 17 of which you can access for free at: http://www.jimmunol.org/content/167/9/4996.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
RNA viruses undergo rapid sequence variation as the result of error-prone RNA replication mechanisms. When viable mutations arise in RNA regions encoding B or T cell epitopes, mutant viruses that can evade immune detection may be selected. In the carefully studied CTL response to the Gag p11C(C-M) epitope in SIVmac-infected Mamu-A*01+ rhesus monkeys, it has been shown that CTL recognition of that epitope can occur even in the face of accruing mutations. To explore the underlying mechanism for this breadth of recognition, we have constructed Mamu-A*01 tetramers which discriminate T cells specific for epitope variants. Using these reagents we have defined discrete subsets of p11C(C-M)-specific T cells that cross-react with cells presenting variant peptides. We have found that individual Mamu-A*01+ monkeys differ functionally in their ability to recognize epitope variants despite consistently strong recognition of the p11C(C-M) epitope. This functional difference is accounted for by the relative number of variant-specific T cells and by differences in the functionally relevant TCR repertoire of the infected monkeys. We have also found that monkeys immunized with DNA vaccine constructs encoding only the wild-type epitope sequence develop p11C(C-M)-specific CTL cross-reactive with variant peptides. Thus, cross-reactive CTL do not merely arise secondary to the emergence and immune presentation of viral CTL escape mutants but rather arise de novo following priming with a dominant epitope peptide sequence. Taken together, our results support the concept that the CTL response to a dominant viral epitope, although highly focused, can be clonally diverse and recognize potential epitope variants. The Journal of Immunology, 2001, 167: 4996–5003.

H
uman immunodeficiency virus and SIV infections of man and nonhuman primates are characterized by intense, persistent viral replication with accruing mutations of the viruses that permit their survival in the face of ongoing immune responses. The infected host, in turn, generates immune responses that must continuously adapt to this viral sequence variation. Because CTL play a central role in containing HIV and SIV replication, it is important to understand the mechanisms used by these effector T lymphocytes to deal with newly generated viral variants.

SIVmac-infected rhesus monkeys provide a valuable system in which to explore CTL immunity to AIDS viruses. Monkeys expressing the MHC class I allele Mamu-A*01 (1) develop a reproducible dominant Gag-specific CTL response following infection with SIVmac. This Mamu-A*01-restricted CTL response is directed against a single 9-aa Gag epitope, CTPYDINQM, known as p11C(C-M) (2). Exploring the ramifications of variability at that epitope for CTL recognition, Shen et al. (3) observed that synthetic peptides containing single amino acid substitutions at positions 2, 5, 7, 8, and 9 were similar in their ability to sensitize B-lymphoblastoid cell line (B-LCL) targets for CTL lysis. In contrast, substitution of the amino acids at positions 4 or 6 resulted in a significant decrease in target sensitization, and peptides substituted at position 3, the anchor residue, were ineffective at sensitizing targets. These data suggested that considerable variability in this epitope sequence is tolerated without loss of CTL recognition.

Studies were initiated to investigate further the capacity of CTL to respond to variations in the p11C(C-M) sequence. In pursuing this work, we have found that subtle differences in the fine specificity of the CTL response allow cross-recognition of peptide variants that have not previously been presented to the immune system. This finding suggests that epitope selection evolves to permit recognition of a considerable degree of viral sequence variation.

Materials and Methods

Monkeys

Rhesus monkeys used in this study were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee for Harvard Medical School and the Guide for the Care and Use of Laboratory Animals (4). Monkeys were screened for the presence of the Mamu-A*01 allele using a PCR-based technique as previously described (5, 6). DNA sequence analysis was performed on all potential positive samples to confirm identity with the established prototype Mamu-A*01 sequence (1).

DNA immunizations and viruses

Some of the monkeys used in the current study were part of a larger study of CTL responses to plasmid DNA vaccination with or without cytokine augmentation. Details of the DNA vaccination methods have been published elsewhere (7, 8). Briefly, monkeys were immunized i.m. in the quadriceps and/or deltoids with 4–5 mg per injection site of CsCl-purified plasmid DNA using a needleless, CO2-powered Biojector 2000 delivery device.
(Bioject, Portland, OR). Each injection consisted of either the single plasmid, pVLR-SIVgag (7–9), encoding a codon-optimized SIVmac239 gag, or a combination of plasmids including pVLR-SIVgag and pVJR-HIVenv (encoding a codon-optimized HIV1 89.6) (10, 11) with or without a plasmid encoding Ig-linked IL-2 (12, 13). Monkeys used in the plasmid ± cytokine study (7) were subsequently challenged with the SIV/HIV chimeric virus SHIV89.6P. Data shown in the current study as examples of “DNA-vaccinated monkeys” are from studies done before viral challenge of these monkeys. Data used in the current study as examples of “chronically SIV-infected monkeys” are from rhesus monkeys infected with SIVmac251 for greater than 1 year before study.

Abs, tetramers, and peptides

PE-conjugated mouse anti-human TCR Vβ23 Ab (clone AF23) was obtained from Beckman-Coulter Immunotech (Marseille, France) and used at a concentration of 0.5 μg/100 μl sample. FTTC-conjugated mouse anti-human Vβ3.1 Ab (clone 8F10) was obtained from Endogen (Woburn, MA) and used at a concentration of 1 μg/100 μl sample. The following mAbs were also used: PE-Texas Red-conjugated anti-CD8α (clone 7PT3F9; Beckman Coulter), PE-Texas Red-conjugated anti-CD8αβ (Beckman Coulter), FTTC-conjugated anti-CD3 (clone FN18; BioSource International, Camarillo, CA), or FTTC-conjugated anti-CD3 (clone SP34; BD PharMingen, San Diego, CA). Tetrameric complexes of Mamu-A*01 bound to peptides p11C(C-M), mp4, mp6, or p54AS were prepared as described previously (14) using fluorescently labeled streptavidin conjugates of PE or allophycocyanin from Prozyme (San Leandro, CA) or PerCP (a gift of Holden Maecker, BD Biosciences, Mountain View, CA). Peptides, synthesized as nonamers and purified to 90% homogeneity, were obtained from QCB/Biosource (Hopkinton, MA). Lyophilized peptides were dissolved in a minimum volume of DMSO (Sigma-Aldrich, St. Louis, MO) and diluted to a stock peptide concentration of 15 mg/ml in water containing 5 mM DTT (Sigma-Aldrich), then frozen at −70°C in aliquots.

Before use, peptides were diluted to working concentration in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with glutamine, 12% FCS, penicillin, streptomycin, and gentamicin.

Cytotoxicity assays

PBMC from Mamu-A*01+ rhesus monkeys were seeded on day 0 in 12-well plates at a density of 7 million cells/well and grown for 3 days in RPMI 1640 medium supplemented with glutamine, 25 mM HEPES (Biochemicals, San Diego, CA), 12% FCS, penicillin, streptomycin, and gentamicin, and the appropriate stimulating peptide. We have found that the concentration of p11C(C-M) peptide which results in half-maximal stimulation and target sensitization is 30–50 ng/ml (data not shown). The mp4 and mp6 peptides both bind Mamu-A*01 with 3.5- to 5-fold lower affinity than p11C(C-M) and require an −1 log higher concentration to sensitize targets (data not shown). However, both are comparable to p11C(C-M) in the concentration required for half-maximal stimulation (data not shown).

Flow cytometry

PBMC cultured in the presence of peptide and IL-2 were harvested on days 10–14 and separated over a Ficoll layer (Ficoll-Paque Plus; Amersham Pharmacia Biotech, Uppsala, Sweden). Cultured cells or whole-blood specimens were stained with mp4, mp6, or mock tetramer for 15 min at room temperature followed by addition of the second tetramer (p11C(C-M) or mock) for 15 min at room temperature. Cells were then stained with a mixture of anti-CD3 and anti-CD8 Abs for 15 min. Cultured cells were fixed in 1% formaldehyde. Whole-blood specimens were lysed using a Coulter Immunoprep reagent system and a Q-prep workstation (Beckman Coulter) before fixing in 1.5% formaldehyde. Samples were analyzed on a Coulter EPICS XL-MCL or a FACScaliber flow cytometer (BD Biosciences).

Results

Infection of rhesus monkeys by SIVmac elicits CTL that recognize diverse mutant sequences in a single epitope

PBMC from Mamu-A*01+ rhesus monkeys chronically infected with SIVmac were assessed for functional cytotoxicity directed against the dominant Mamu-A*01-restricted Gag epitope p11C(C-M) (CTYPDINQM), or two peptide variants, each differing from p11C(C-M) by a single amino acid. As shown for one monkey in Fig. 1A, PBMC stimulated with p11C(C-M) effectively lysed autologous target cells pulsed with p11C(C-M) or either of the two variant peptides, mp4 (CTPDIQNM) or mp6 (CTPYDYNQM). In contrast, mp4-stimulated effectors mediated minimal cytotoxicity when tested against mp6-pulsed targets, although these effectors readily lysed mp4- or p11C(C-M)-sensitized targets. Similarly, mp6-stimulated effectors mediated minimal cytotoxicity when tested against mp4-pulsed targets, but readily lysed mp6- and p11C(C-M)-sensitized targets.

Results

Infection of rhesus monkeys by SIVmac elicits CTL that recognize diverse mutant sequences in a single epitope

PBMC from Mamu-A*01+ rhesus monkeys chronically infected with SIVmac were assessed for functional cytotoxicity directed against the dominant Mamu-A*01-restricted Gag epitope p11C(C-M) (CTYPDINQM), or two peptide variants, each differing from p11C(C-M) by a single amino acid. As shown for one monkey in Fig. 1A, PBMC stimulated with p11C(C-M) effectively lysed autologous target cells pulsed with p11C(C-M) or either of the two variant peptides, mp4 (CTPDIQNM) or mp6 (CTPYDYNQM). In contrast, mp4-stimulated effectors mediated minimal cytotoxicity when tested against mp6-pulsed targets, although these effectors readily lysed mp4- or p11C(C-M)-sensitized targets. Similarly, mp6-stimulated effectors mediated minimal cytotoxicity when tested against mp4-pulsed targets, but readily lysed mp6- and p11C(C-M)-sensitized targets.

Flow cytometry

PBMC cultured in the presence of peptide and IL-2 were harvested on days 10–14 and separated over a Ficoll layer (Ficoll-Paque Plus; Amersham Pharmacia Biotech, Uppsala, Sweden). Cultured cells or whole-blood specimens were stained with mp4, mp6, or mock tetramer for 15 min at room temperature followed by addition of the second tetramer (p11C(C-M) or mock) for 15 min at room temperature. Cells were then stained with a mixture of anti-CD3 and anti-CD8 Abs for 15 min. Cultured cells were fixed in 1% formaldehyde. Whole-blood specimens were lysed using a Coulter Immunoprep reagent system and a Q-prep workstation (Beckman Coulter) before fixing in 1.5% formaldehyde. Samples were analyzed on a Coulter EPICS XL-MCL or a FACScaliber flow cytometer (BD Biosciences).

Results

Infection of rhesus monkeys by SIVmac elicits CTL that recognize diverse mutant sequences in a single epitope

PBMC from Mamu-A*01+ rhesus monkeys chronically infected with SIVmac were assessed for functional cytotoxicity directed against the dominant Mamu-A*01-restricted Gag epitope p11C(C-M) (CTYPDINQM), or two peptide variants, each differing from p11C(C-M) by a single amino acid. As shown for one monkey in Fig. 1A, PBMC stimulated with p11C(C-M) effectively lysed autologous target cells pulsed with p11C(C-M) or either of the two variant peptides, mp4 (CTPDIQNM) or mp6 (CTPYDYNQM). In contrast, mp4-stimulated effectors mediated minimal cytotoxicity when tested against mp6-pulsed targets, although these effectors readily lysed mp4- or p11C(C-M)-sensitized targets. Similarly, mp6-stimulated effectors mediated minimal cytotoxicity when tested against mp4-pulsed targets, but readily lysed mp6- and p11C(C-M)-sensitized targets.

Flow cytometry

PBMC cultured in the presence of peptide and IL-2 were harvested on days 10–14 and separated over a Ficoll layer (Ficoll-Paque Plus; Amersham Pharmacia Biotech, Uppsala, Sweden). Cultured cells or whole-blood specimens were stained with mp4, mp6, or mock tetramer for 15 min at room temperature followed by addition of the second tetramer (p11C(C-M) or mock) for 15 min at room temperature. Cells were then stained with a mixture of anti-CD3 and anti-CD8 Abs for 15 min. Cultured cells were fixed in 1% formaldehyde. Whole-blood specimens were lysed using a Coulter Immunoprep reagent system and a Q-prep workstation (Beckman Coulter) before fixing in 1.5% formaldehyde. Samples were analyzed on a Coulter EPICS XL-MCL or a FACScaliber flow cytometer (BD Biosciences).
Interestingly, a somewhat different result was obtained in similarly performed studies of PBMC from two other SIVmac-infected *Mamu-A*01 rhesus monkeys (Fig. 1, B and C). Effectors stimulated with p11C(C-M) preferentially lysed mp6- or p11C(C-M)-sensitized targets and exhibited only weak to moderate cytotoxic activity for mp4-sensitized targets. However, in both of these cases, stimulation of PBMC with mp4 expanded effector populations that mediated moderate lysis of mp+ and p11C(C-M)-sensitized targets and minimal lysis of mp6-sensitized targets. Similarly, mp6 stimulation of PBMC expanded effector populations that mediated lysis of mp6- and p11C(C-M)-sensitized targets but not mp4-sensitized targets. These data support the notion that the dominant p11C(C-M)-directed CTL response of monkeys infected with SIV can be functionally subdivided into components with distinct fine specificities.

**MHC class I tetramers can be used to distinguish subpopulations of epitope-specific CD8\(^+\) T cells with differing fine specificities**

To determine whether MHC class I/peptide tetramers might discriminate CD8\(^+\) T cells with specificities for closely related epitopes, peptide/Mamu-A*01 tetramers were constructed using the nonamer p11C(C-M), mp4, and mp6 peptides. PBMC from SIVmac-infected *Mamu-A*01 rhesus monkeys were stimulated with p11C(C-M) and stained with the p11C(C-M)-, mp4-, or mp6-tetramers. As a control, a tetramer was used that was constructed with Mamu-A*01 and the unrelated Mamu-A*01-restricted peptide p54AS (TVPWPNASL) (15, 16). As shown in Fig. 2A, p11C(C-M)-stimulated cells (Fig. 2A, filled bars) were readily detected with the mp4 tetramer (Fig. 2A, left) and with the p11C(C-M) tetramer (Fig. 2A, middle). Neither tetramer detected p54AS-stimulated cells, but p54AS-stimulated cells were detected by the control p54AS tetramer (Fig. 2A, hatched bar, right). Thus, both the p11C(C-M) and the mp4 tetramers could discriminate CD8\(^+\) T cells with specificity for an unrelated Mamu-A*01-restricted peptide. As shown in Fig. 2B, the mp6 and p11C(C-M) tetramers could also discriminate cells from a second monkey with specificity for the unrelated Mamu-A*01-restricted epitope.

To assess the ability of CD8\(^+\) T cells to recognize minor variations in an epitope amino acid sequence, we sought to determine whether the mp4 and mp6 tetramers would bind mutually exclusive CD8\(^+\) T cell populations. PBMC from an SIVmac-infected Mamu-A*01 rhesus monkey (monkey 403) were stimulated in vitro with mp4, mp6, or p11C(C-M) and stained with the mp4 or mp6 tetramers. As shown in Fig. 3, cells stimulated with mp4 stained exclusively with the mp4 tetramer, while cells stimulated with mp6 stained exclusively with the mp6 tetramer. In contrast, p11C(C-M)-stimulated cells stained with both tetramers. Importantly, cells stimulated in vitro with mp4, mp6, or p11C(C-M) all stained with the p11C(C-M) tetramer (data not shown). These data are in agreement with the functional CTL data showing that CD8\(^+\) T cells can discriminate between closely related peptides presented by the Mamu-A*01 molecule (Fig. 1A).

We also examined the mp4- and mp6-specific CD8\(^+\) T cell responses of several other SIVmac-infected *Mamu-A*01 rhesus monkeys. In studies done using fresh whole-blood staining, the fraction of CD3\(^+\)CD8\(^+\) lymphocytes that stained with the p11C(C-M) tetramer (Fig. 4A, filled bars) varied between 0.22 and 9.9%. Interestingly, in monkeys 138 and 191, and to a lesser extent in monkey 94, unstimulated lymphocytes stained strongly with the mp4 tetramer (Fig. 4A, hatched bars) but only weakly with the mp4 tetramer (Fig. 4A, open bars). In unstimulated lymphocytes of monkeys 403 and 348, the opposite pattern was observed (Fig. 4A). Because the total number of tetramer-staining cells in fresh whole blood is generally low, PBMC from the same monkeys were similarly assessed after these cells were expanded by stimulation in vitro with p11C(C-M). As shown in Fig. 4B, the fraction of the cultured CD3\(^+\)CD8\(^+\) lymphocytes that stained with the p11C(C-M) tetramer (Fig. 4B, filled bars) varied between 15 and 82%. As in the studies of the freshly obtained whole blood, a larger proportion of the cultured lymphocytes from monkeys 138, 191,
and 94, as well as from the additional monkey 253, stained with the mp6 tetramer (Fig. 4B, hatched bars) than with the mp4 tetramer (Fig. 4B, open bars), while in cultured cells from monkeys 403 and 348, mp4 tetramer-binding cells outnumbered the mp6 tetramer-binding cells. Thus, consistent with the functional CTL studies (Fig. 1), these tetramer studies indicate that the p11C(C-M)-specific CD8+ T cell response of the Mamu-A*01+ SIVmac-infected monkeys appears to be biased toward mp4 in some animals and toward mp6 in others.

The bias in mutant peptide specificity is not a result of viral sequence variation

One possible explanation for the preferential staining of CD8+ T lymphocytes in fresh blood by the mp6 or mp4 tetramers and the preferential expansion of mp6- or mp4-specific CTL after in vitro stimulation with p11C(C-M) is that SIVmac variants expressing mutations in the p11C(C-M) epitope evolve in vivo, and that these viral variants, in turn, cause preferential expansion of selected CTL subpopulations. If this hypothesis were correct, Mamu-A*01+ rhesus monkeys immunized with a nonreplicating wild-type SIVmac Gag immunogen would not be expected to exhibit a bias in mp4 or mp6 recognition. We examined the p11C(C-M)-, mp4-, and mp6-specific responses of seven Mamu-A*01+ rhesus monkeys that were immunized with a plasmid DNA vaccine expressing a codon-optimized SIVmac239 gag gene. These animals were being used in a study assessing the immunity to SIV induced by cytokine-augmented plasmid DNA vaccination (13). The p11C(C-M)-specific CD8+ T cell responses of these animals, as measured by fresh-blood tetramer staining, were too low (0.0–1.2% of CD3+CD8+ cells, Fig. 5A) to allow reliable quantitation of the unstimulated mp4 and mp6 tetramer-binding cell populations (data not shown). However, after in vitro stimulation with p11C(C-M), 16–71% of the CD8+ peripheral blood T cells from these animals could be stained with p11C(C-M) tetramer. As shown in Fig. 5A, the fraction of p11C(C-M) tetramer-binding CD8+ T cells detected after in vitro expansion correlated closely with the fraction of tetramer-binding CD8+ T cells detected in fresh blood. When these p11C(C-M)-stimulated lymphocytes were stained with the mp4 and mp6 tetramers, biases in the mp4 and mp6 tetramer binding were detected. As shown in Fig. 5B, a substantial percentage of the p11C(C-M)-stimulated PBMC from monkeys 483, 728, 811, 820, and 833 stained with the mp6 tetramer but not with the mp4 tetramer. CD8+ T cells from monkey 893 cells stained with the mp4 but not the mp6 tetramer. In the CD8+ T cells from monkey 702, staining with the mp4 and mp6 tetramers was approximately equal. Thus, the CD8+ T cell response elicited by the p11C(C-M) sequence is usually biased toward either mp4 or mp6, and the establishment of this bias does not depend upon the evolution of the epitope sequence.

mp4- and mp6-specific CD8+ T cells represent subsets of the p11C(C-M)-specific CD8+ T cell population

Based on the above observations, we hypothesized that the mp4- and mp6-specific CD8+ T cells detected in tetramer assays and in functional CTL assays were mono- or oligoclonal subsets of the polyclonal population of p11C(C-M)-specific CD8+ T cells. To test this hypothesis, we sought to perform simultaneous tetramer staining with the p11C(C-M) tetramer and either the mp4 or mp6 tetramer. All of these tetramers were predicted to bind to the same target, the TCR, with perhaps different affinities and avidities. Therefore, it was necessary first to establish empirically the sub-saturating concentrations of each tetramer for use in staining these lymphocytes to maximize the mean fluorescence intensity per cell of the tetramer-binding lymphocytes, while at the same time avoiding the use of concentrations of a tetramer that were so high as to inhibit competitively the binding of the other tetramer. The concentration of p11C(C-M) tetramer used for staining in this study resulted in a ≥0.3-log decrease in mutant peptide tetramer mean fluorescence intensity compared with cells stained with mutant peptide tetramer alone. Similarly, the concentration of mutant peptide tetramers used for staining in this study resulted in a ≥0.15-log decrease in p11C(C-M) tetramer mean fluorescence intensity compared with cells stained with mutant peptide tetramer alone. It is likely that, at the concentrations of tetramers used, only a fraction of the available binding sites were occupied. However, staining with higher concentrations of tetramers did result in

**FIGURE 4.** Tetramer staining of CD8+ T lymphocytes from SIVmac-infected rhesus monkeys. Whole fresh blood (A) or cultured PBMC stimulated in vitro with p11C(C-M) (B) were stained with the p11C(C-M) tetramer (■), the mp4 tetramer (□), or the mp6 tetramer (●). Shown are the fractions of CD8+CD3+ lymphocytes that bound tetramer. Numbers on the x-axis refer to individual rhesus monkeys.
significant competition for binding (data not shown). These results support the notion that all three tetramers were binding to a common receptor site.

A representative example of double tetramer staining of p11C(C-M)-stimulated cultured PBMC from an SIVmac-infected Mamu-A*01猴 is shown in Fig. 6. As predicted, the p11C(C-M) tetramer-binding CD8\(^+\) T lymphocytes could be subdivided into mp4 tetramer-positive and mp4 tetramer-negative subsets (Fig. 6A), as well as into mp6 tetramer-positive and mp6 tetramer-negative subsets (Fig. 6B). Importantly, we did not identify any mp4-specific or mp6-specific CD8\(^+\) T cells that did not also stain with the p11C(C-M) tetramer.

We analyzed PBMC of the six SIVmac-infected and seven DNA-vaccinated Mamu-A*01猴 rhesus monkeys using double tetramer staining. The mp6 tetramer-positive cells constituted a large fraction of the CD8\(^+\) p11C(C-M) tetramer-binding T cells in the monkeys previously identified as having a bias toward mp6 specificity in their T cell repertoire (Fig. 7). Similarly, in the PBMC of the three animals previously identified as having a high frequency mp4-specific CD8\(^+\) T cell response, the majority of p11C(C-M) tetramer-binding CD8\(^+\) T lymphocytes also bound mp4 tetramer. Taken together, these data show that the CD8\(^+\) T cell response to p11C(C-M) in both SIVmac-infected and plasmid DNA-immunized Mamu-A*01猴 rhesus monkeys is functionally polyclonal, and subtle differences in the fine specificity of the p11C(C-M)-specific CD8\(^+\) T cells can be detected by both functional and tetramer analyses.

\textit{p11C(C-M)}-specific CD8\(^+\) T lymphocytes that recognize mp4 use different TCR-\(\beta\) variable regions than those \textit{p11C(C-M)}-specific CD8\(^+\) T lymphocytes that recognize mp6

We reasoned that these differences in fine specificity may result from differences in the TCRs expressed by the different p11C(C-M)-specific lymphocyte subpopulations. To demonstrate differences in the TCRs of these cells, we used a panel of human TCR-specific mAbs that cross-react with epitopes on rhesus monkey T cells (W. A. Charini, J. E. Schmitz, and A. Necker, unpublished observations; data available on-line at http://research.bidmc.harvard.edu/V_path/v_pathogens.asp) for these studies. Fresh peripheral blood was screened for the presence of CD8\(^+\) T cells that stained with both the p11C(C-M) tetramer and one of several V\(\beta\)-specific Abs. PBMC from all 13 monkeys described in this study bound the p11C(C-M) tetramer and each of the V\(\beta\) Abs tested. In

\textbf{FIGURE 5.} Tetramer staining of CD8\(^+\) T lymphocytes from SIVmac gag DNA vaccinated rhesus monkeys. A, Whole fresh blood (left axis, ■) or cultured PBMC stimulated in vitro with p11C(C-M) (right axis, □) was stained with the p11C(C-M) tetramer. Shown are the fractions of CD8\(^+\) CD3\(^+\) lymphocytes that bound tetramer. (Note difference in scale between left and right axes.) Numbers on the x-axis refer to individual rhesus monkeys. B, Cultured PBMC restimulated in vitro with peptide p11C(C-M) were stained with the p11C(C-M) tetramer (■), the mp4 tetramer (□), or the mp6 tetramer (△). Data represent the fraction of CD8\(^+\) CD3\(^+\) lymphocytes which bound tetramer. Monkeys were immunized with a DNA expression plasmid containing the SIVmac239 Gag coding region.

\textbf{FIGURE 6.} Double tetramer staining. PBMC from rhesus monkey 348 were cultured in vitro for 10–14 days in the presence of IL-2 and p11C(C-M) before staining. Dot plots are gated on CD3\(^+\)CD8\(^+\) lymphocytes. A, Stained with mp4 tetramer and p11C(C-M) tetramer. B, Stained with mp6 tetramer and p11C(C-M) tetramer. Numbers refer to percentages of gated cells in each quadrant.
two monkeys, a small subset of PBMC were observed to stain with both the p11C(C-M) tetramer and one of the Vβ-specific mAbs, simultaneously. PBMCs from these two monkeys were then stimulated in vitro with mp4 or mp6 peptides and then stained with the corresponding Vβ Ab together with either the mp4 or mp6 tetramers. As shown in Table I, 78% of the mp6 tetramer-staining PBMC of monkey 253 stained with anti-Vβ23, whereas only 8.1% of the mp4 tetramer-staining cells stained with the same mAb. This finding indicates a preference for Vβ23 usage among CD8+ T cells specific for mp6 in this animal. In PBMC of monkey 712, a significant proportion (14%) of the mp6 tetramer-binding, but less than 1% of the mp4 tetramer-binding, CD8+ T lymphocytes stained with the anti-Vβ3.1 mAb, indicating a preference for Vβ3.1 usage among some, but not all, CD8+ T cells specific for mp6 in this monkey. Moreover, these data indicate that the mp6 tetramer-specific T cells can be further subdivided into at least two subfractions based on anti-Vβ3.1 staining. With these reagents it is thus possible not only to demonstrate the polyclonality of the p11C(C-M)-specific CTL response, but also to begin to ascribe functional differences in specificity to individual clonal subpopulations.

Discussion
In this study we have found that the CTL response of Mamu-A*01+ rhesus monkeys to a well-characterized dominant epitope of SIVmac, p11C(C-M), is quite heterogeneous. Both functional (chromium release) and structural (tetramer binding) assays point to a striking clonal diversity of the p11C(C-M)-specific CTL population. This CTL population appears to be comprised of cells with overlapping yet distinguishable specificities for closely related peptide Ags. Using two single-amino acid variants of p11C(C-M), mp4 and mp6, we have been able to dissect the fine specificity of the p11C(C-M)-specific CTL response. We find that the relative percentages of CD8+ T cells with distinct fine specificities can vary from monkey to monkey, but that the response is most often dominated by T cells with one or the other fine specificity. We also find that establishment of this type of clonal bias is not an adaptive response to viral antigenic variation, because it arises in monkeys that have been immunized with a plasmid DNA vaccine that expresses a single protein sequence.

Although the three-dimensional structures of the TCR/peptide/MHC class I complex have been solved by x-ray crystallography for a small number of human and murine complexes (17–19), no data are yet available for Mamu-A*01/p11C(C-M) or its TCR ligands. Indirect evidence from functional assays using substituted peptides (2, 3) and peptide elution and peptide binding assays (2, 20) suggests that the proline at position three of the p11C(C-M) sequence interacts with the Mamu-A*01 molecule and serves as an anchor residue for the epitope peptide. This possibility is consistent with the finding that all known Mamu-A*01-binding peptides contain prolines at position three (20). Other epitope peptide requirements for binding to Mamu-A*01 inferred from a comparative analysis of Mamu-A*01-binding peptides and direct testing of peptides both in whole cell binding assays and intracellular IFN-γ assays include the preference for a small residue (T or S) at position two and a hydrophobic or aromatic residue (I, L, V, M, or F) at the C terminus (2, 20). Thus, side chains of the amino acids at positions 2 and 3, and the C terminus of the peptide are predicted to interact with the Mamu-A*01 molecule, while the remaining amino acid side chains, including those of the amino acids at positions 4 and 6, are likely to interact with the TCR.

Understanding the clonal diversity of the CTL response is particularly important for vaccine design. A clonally diverse response to a viral epitope increases the likelihood that multiple divergent strains of AIDS viruses may be recognized by CTL elicited

Table I. TCR Vβ expression by mutant peptide-specific CD8+ T cells

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Anti-TCR Ab</th>
<th>mp4+ Fraction (%)</th>
<th>mp6+ Fraction (%)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>253</td>
<td>Vβ 3.1</td>
<td>8.1</td>
<td>78</td>
<td>9.6</td>
</tr>
<tr>
<td>712</td>
<td>Vβ 23</td>
<td>0.9</td>
<td>14</td>
<td>15</td>
</tr>
</tbody>
</table>

*Numbers represent the percentage of tetramer-binding CD8+ T cells that bound the corresponding anti-Vβ Ab, or the ratio of mp6+ Vβ+ cells to mp6+ Vβ+ T cells.
through immunization with only a single consensus epitope sequence. Moreover, the more diverse the clonal response of T cells in an infected individual, the fewer the possibilities for viral escape through variation of the epitope sequence.

The polyclonality of the CTL response to a single peptide epitope is not unexpected and has, in fact, previously been demonstrated for p11C(C-M) (21). Several studies have also demonstrated that individual T cell clones can recognize MHC complexed with any of a number of related peptides. Such TCR/peptide/MHC class I interactions can vary in their binding affinities and in the type of T cell signaling that results. Some peptides are strong activators of T cells, whereas others act as weak T cell agonists or even as T cell antagonists (22, 23). Given the promiscuity of TCR contacts with peptide/MHC class I complexes, it is not surprising that different clonal populations of T cells overlap in their recognition of particular MHC/peptide class I complexes.

It is well established that Ab responses are polyclonal. Indeed, polyclonality is a critical prerequisite for affinity maturation. Competition among B cells for Ag binding results in the positive selection of those B cell clones with the highest affinity for Ag. Recent studies suggest that an analogous form of competition may occur among T cells to create a highly focused secondary CTL response (24–26). Haanen et al. (26) have demonstrated that when mice are sequentially infected with strains of influenza differing by two amino acids in the region of a dominant CTL epitope they develop CTL capable of recognizing both viral variants, and these T cells express a limited subset of Vβ gene segments. However, infection of mice with both influenza variants, simultaneously, did not elicit a population of dual-specific T cells, but, instead, distinct populations of strain-specific T cells. These results provide evidence that fine-tuning of the CTL response occurs following secondary exposure to epitope variants by selective stimulation of a subset of memory T cell clones. Indeed, Kedel et al. (25) have provided direct evidence that competition among T cells recognizing unrelated peptides restricted by the same MHC class I molecule occurs at the level of access to the APC. Such a mechanism may also underlie the selection of T cell clones recognizing pairs of closely related peptides.

The results of the present study suggest that a dominant epitope-specific CTL response is heterogeneous enough to include clones that recognize that epitope as well as multiple variants of that epitope. The extent of the heterogeneity of the responses that have been characterized in detail using T cell clones or PBMC from immunized or virus-infected animals may, in fact, represent only a small fraction of the epitopes that can be recognized by these populations of effector cells. Using only two variant peptide epitopes, we were able to discriminate four subtypes of CTL: those with specificity for p11C(C-M) and mp4 but not mp6; those with specificity for p11C(M-C) and mp6 but not mp4; those with specificity for mp4, mp6, and p11C(C-M) (in most animals, a small fraction of cells; data not shown); and those with specificity for p11C(M-C) but neither mp4 nor mp6 (data not shown). The relative representation of cells of each subtype varied from animal to animal and depended on the nature of the immunogen and the secondary stimulation used. If the present studies had been done with more than two variant peptides, we would likely have defined an even larger number of variant recognition patterns.

Indeed, each CTL clone can be defined functionally on the basis of its response to the immunizing and variant sequences. In responding strongly to some variants and weakly to others, each clone will have a unique profile. The ability of an individual’s immune system to recognize epitope variants is dependent on each CTL clone’s contribution to the total response. That clone’s contribution to the immune response is a function of both the fractional representation of the individual clone in the total epitope-specific response and the clone’s ability to recognize variant sequences. Thus, if an individual is immunized with a particular epitope sequence, a polyclonal CTL response emerges that includes clones with specificities for epitope variants that the individual’s immune system has not yet encountered.

When monkeys are immunized with an SIVmac gag DNA vaccine, they develop p11C(C-M)-specific CTL responses with the same heterogeneous fine specificity seen in the p11C(C-M)-specific CTL of chronically SIVmac-infected monkeys. Because the immunogen in these DNA-vaccinated monkeys is nonreplicating and, therefore, not subject to mutational selective pressure in the region encoding the p11C(C-M) epitope, we can be confident that the immunized monkeys have only been exposed to the single wild-type p11C(C-M) sequence, CTPYDINQM. Nevertheless, the vaccine-elicited CTL response includes cell clones that are cross-reactive with p11C(C-M) variants to which the monkeys have not been specifically primed. This observation suggests that cell clones exist in this population of CTL that are reactive with numerous other peptides differing from the wild-type sequence by one or multiple amino acids.

Following primary immunization, exposure of PBL to a peptide variant in vitro results in an expansion of CTL reactive with that particular amino acid sequence. Indeed, a focusing of the CTL population like that described by Haanen et al. (26) can be seen in vitro when PBMC from SIVmac-infected (Figs. 1 and 3, and Table I) or DNA-immunized (data not shown) monkeys are cultivated in the presence of a variant epitope peptide. In vitro stimulation with mp4 expands a population of p11C(C-M)- and mp4/p11C(C-M)-specific CD8+ T cells that exhibits minimal cross-reactivity with mp6. Similarly, with mp6 stimulation, a population of p11C(C-M)- and mp6/p11C(C-M)-specific CD8+ T cells is selectively expanded that exhibits minimal cross-reactivity with mp4.

The present study supports the conclusions of Buseyne et al. (27) and Buseyne and Rivière (28) that recognition of several naturally occurring variants of sequences of an HIV CTL epitope can occur through a single TCR. Those authors also demonstrated that, in certain circumstances, promiscuous CTL recognition of exogenous peptides presented by allogeneic TCR can occur. These findings indicate a remarkable tolerance of the TCR for variations of its ligand, both the peptide component and the MHC component.

In conclusion, priming a CTL response to a dominant epitope simultaneously results in priming to variants of the dominant peptide sequence. Subsequent exposure to the original epitope sequence or one of its variants can result in a focusing of the CTL response with a relative increase in the representation of previously minor epitope-specific CTL populations. In this way, the immune response “anticipates” viral sequence variation. Such a mechanism places severe restrictions on the types of mutations that can lead to loss of CTL recognition.

Acknowledgments
We thank Ramu Subbramanian, Keith Reimann, Dan Barouch, and Tomas Hanke for helpful discussions and Darci Gorgone and Christi Nickerson for technical assistance.

References