A Commonly Recognized Simian Immunodeficiency Virus Nef Epitope Presented to Cytotoxic T Lymphocytes of Indian-Origin Rhesus Monkeys by the Prevalent Major Histocompatibility Complex Class I Allele Mamu-A*02

SUZANNE ROBINSON, WILLIAM A. CHARINI, MICHAEL H. NEWBERG, MARCELO J. KURODA, CAROL I. LORD, AND NORMAN L. LETVIN*

Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

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The ability to monitor vaccine-elicited CD8+ cytotoxic T-lymphocyte (CTL) responses in simian immunodeficiency virus (SIV-)- and simian-human immunodeficiency virus (SHIV)-infected rhesus monkeys has been limited by our knowledge of viral epitopes predictably presented to those lymphocytes by common rhesus monkey MHC class I alleles. We now define an SIV and SHIV Nef CTL epitope (YTSGPGRYR) that is presented to CD8+ T lymphocytes by the common rhesus monkey MHC class I molecule Mamu-A*02. All seven infected Mamu-A*02+ monkeys evaluated demonstrated this response, and peptide-stimulated interferon gamma Elispot assays indicated that the response represents a large proportion of the entire CD8+ T-lymphocyte SIV- or SHIV-specific immune response of these animals. Knowledge of this epitope and MHC class I allele substantially increases the number of available rhesus monkeys that can be used for testing prototype HIV vaccines in this important animal model.

With compelling evidence that a virus-specific cytotoxic T-lymphocyte (CTL) response is of central importance in containing the spread of human immunodeficiency virus type 1 (HIV-1) in infected individuals (12, 13, 18, 23), there is a growing appreciation that an effective HIV-1 vaccine should elicit a high-frequency virus-specific CD8+ CTL population (14). Nonhuman primate models are playing a major role in the preclinical development of HIV-1 vaccine strategies. In particular, simian immunodeficiency virus (SIV)- and simian-human immunodeficiency virus (SHIV)-infected Indian-origin rhesus monkeys have become the most commonly used nonhuman primate models for assessing the immunogenicity and protective efficacy of prototype HIV-1 vaccines (3, 6, 9). It is therefore critical that vaccine-elicited SIV- and SHIV-specific CTL responses can be assessed in Indian-origin rhesus monkeys with quantitative precision and ease.

The detection of SIV- and SHIV-specific CTL responses in rhesus monkeys has been facilitated by our understanding of a dominant SIV Gag CTL epitope referred to as p11Cambi or p11C (CTPYDINQM) presented to CD8+ T lymphocyte populations by the common Indian-origin rhesus monkey MHC class I allele Mamu-A*01 (2, 15). Appropriately vaccinated and infected Mamu-A*01+ rhesus monkeys universally develop a high-frequency CTL response to this 9-amino-acid fragment of SIV Gag (7, 19, 20). The use of tetramer, Elispot interferon gamma production, and cytolytic assays based on an understanding of this peptide and MHC class I allele have allowed the assessment of CTL in this model system with a remarkable degree of reproducibility and quantitative precision. The obvious limitation of this technical approach has, however, been the fact that only approximately 20% of all Indian-origin rhesus monkeys express the Mamu-A*01 allele (11). Thus, it remains extremely difficult to quantitate SIV- and SHIV-specific CTL in the majority of available experimental animals. It would clearly be of enormous benefit to have knowledge of further CTL epitopes of SIV and/or SHIV that are reliably presented to CD8+ T lymphocytes by other common rhesus monkey MHC class I alleles. Such an understanding would allow the use of a larger proportion of available rhesus monkeys for studies in which the quantitation of SIV- and SHIV-specific CTL is important.

To explore the possibility that further predictable CTL epitopes might exist in this animal model, we confirmed the common expression of another MHC class I allele in Indian-origin rhesus monkeys. We then assessed SIV- and SHIV-infected rhesus monkeys for the presence of viral epitopes predicted to be presented to CD8+ T lymphocytes by this allele. In so doing we defined a novel Nef CTL epitope uniformly presented to immune cells by the MHC class I molecule Mamu-A*02.

MATERIALS AND METHODS

Animals. All animals used in this study were Indian-origin rhesus monkeys (Macaca mulatta). The monkeys had been shown to express Mamu-A*02 by PCR typing, as described below. Monkeys 19196, 483, and 833 also expressed Mamu-A*01. Monkeys 19196, 24195, and 16097 were chronically infected with SIV-mac251. Monkeys 26795, 14282, 18324, 19242, 483, and 833 were chronically infected with SHIV-89.6 or SHIV-89.6p (10, 17). 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 [Institute of Laboratory Animal Resources (U.S.) Committee on Care and Use of Laboratory Animals, 1996].

Cell lines and peptides. B-lymphoblastoid cell lines (B-LCL) were prepared from peripheral blood mononuclear cells as previously described (16, 25). Briefly, whole blood from rhesus monkeys was subjected to Ficoll-diatrizoate density gradient centrifugation, and cells were seeded into a 24-well plate at a
density of $5 \times 10^5$ per well. Cells were plated in the presence of supernatant from a cell line productively infected with the baboon herpesvirus Herpesvirus papio, and fetal calf serum was added every 2 to 3 days until transformed foci appeared (approximately 2 to 3 weeks).

Stably transfected cell lines expressing the Mamu-A*02 molecule were prepared as described previously (24). Briefly, HM2.C1R cells expressing human β2-m (21, 22) were transfected by electroporation using a Bio-Rad Gene Pulser II (0.25 kV, 950 μF) with plasmid pKG5-Mamu-A*02 (24) linearized at a unique Scfl site. G418 selection was begun on day 5 posttransfection at 0.8 mg/ml and was increased to 1.6 mg/ml on day 19. Cultures were screened for the presence of surface Mamu-A*02 by flow cytometry of cells labeled with the mouse anti-human immunoglobulin (Ig) monoclonal antibody W6/32 (American Type Culture Collection, 1:500 dilution). W6/32 binds to a conserved region of MHC class I α-chains. Labeled cells were stained with a fluorescein isothiocyanate-labeled goat anti-mouse IgG antibody. Two high-expressing cell populations were selected and cloned at limiting dilution.

Synthetic peptides were obtained from Quality Controlled Biochemicals (Hopkinton, Mass.), a division of Biosource.

**PCR typing of Mamu-A*02** rhesus monkeys. DNA was extracted from rhesus monkey peripheral blood lymphocytes (PBL) and amplified using allele-specific primers (11). DNA from monkeys that tested positive for the presence of the Mamu-A*02 allele by PCR was sequenced to confirm the identification. For screening, EDTA-preserved whole blood from monkeys was subjected to Ficoll-diatrizoate density gradient centrifugation to isolate leukocytes, and the washed cell pellets were resuspended in 200 μl of phosphate-buffered saline (PBS). DNA extraction was then carried out using a QIAamp blood kit (Qiagen Inc., Chatsworth, Calif.). PCR was performed on 500 ng of extracted DNA in a 50-μl reaction consisting of 60 nM Tris-HEC (pH 8.5), 15 mM ammonium sulfate, 1.5 mM MgCl2, 1 mM dNTP, and 0.25 mM each of the following sequencing primers: A*02/F4 (5′-GCT GCA CCA GGT GGG TCC A-3′) and A*02/R3 (5′-CAG CAC CTC AGG GTG GCC TCT-3′) were each used at a concentration of 150 nM each. The latter primer pair is specific for a conserved MHC class II sequence (based on the macaque homologue of HLA-DRB3) and was included in the PCR as an internal control. PCR was carried out using a Perkin-Elmer GeneAmp 9600 thermocycler (Perkin-Elmer Inc., Norwalk, Conn.). Samples were denatured for 2 min at 94°C and then cycled at 94°C for 30 s, 67°C for 30 s, and 72°C for 90 s, a total of 30 cycles. Potential Mamu-A*02-positive monkeys were identified by the presence of two bands on 1% agarose gels: the expected 260-bp MDRB product and a 1.3-kb product expected to be present in all samples tested. A*02 indicates the position of the 1.3-kb Mamu-A*02-specific amplification product. Based on the data shown, monkeys 14282, 18324, and T641 were tentatively identified as Mamu-A*02**, pending sequence confirmation.

**RESULTS**

To initiate these studies, a cohort of 100 Indian-origin rhesus monkeys were screened for their expression of the relatively common MHC class I allele Mamu-A*02 (24). This group of monkeys comprised animals from five distinct colonies. Screening was done by PCR amplification of cDNA derived from PBL of these animals (Fig. 1). Positive signals were detected in samples from 18 of these 100 monkeys, indicating that 18% of the animals expressed the Mamu-A*02 allele. DNA sequencing of the entire α2 domain coding region as well as 122 nucleotides of the region encoding the α1 domain of the Mamu-A*02 gene was carried out at a central core sequencing facility on an ABI-373 stretch (Perkin-Elmer). The following sequencing primers were used: A*02/Int2/R (5′-H11032/CCT GAA CTG TAA GAG CAG GAG GGG TCC A-3′) and A*02/Int3/R (5′-H11032/GCC CAG GGT GGG TCC A-3′) were each used at a concentration of 1.5 μM. The former primer pair is specific for HLA-DRB3 and was included in the PCR as an internal control. Sequencing of samples identified by PCR screening was carried out using ABI Amplitaq FS dye terminator (Perkin-Elmer Inc., Norwalk, Conn.). Samples were denatured for 2 min at 94°C and then cycled at 94°C for 30 s, 67°C for 30 s, and 72°C for 90 s, a total of 30 cycles. Potential Mamu-A*02-positive monkeys were identified by the presence of two bands on 1% agarose gels: the expected 260-bp MDRB product and a 1.3-kb Mamu-A*02-specific product. Sequencing of samples identified by PCR screening was carried out at a central core sequencing facility on an ABI-373 stretch DNA sequencing machine, using ABI Amplitaq FS dye terminator (Perkin-Elmer Inc., Norwalk, Conn.). The following sequencing primers were used: A*02F2/R1 (5′-TGG GAC CGG GAG ACA CGG AA-3′), A*02-In2F2/R1 (5′-CGG TTT CAT TTT GAG TCC AGG TAG TCT-3′), and A*02/Int3/R (5′-GAT TAT ACC CTT AAT GTG GCT-3′), A*02/Int3/R (5′-CAA CTG AAA ATG AAA CCG-3′), and A*02/R4 (5′-TCC AGG TAG GTG TTG-3′).

**CTL assays.** Lymphocytes were isolated from approximately 10 ml of monkey peripheral blood by Ficoll-diatrizoate gradient centrifugation. Cultures were started with 4 × 104 to 5 × 105 PBL per ml in RPMI-1640 containing 12% heat-inactivated fetal calf serum and antibiotics. Peptides were added at various concentrations. On day 3 of culture, 20 U of recombinant human interleukin-2 was added. On days 11 to 14 of culture, the lymphocytes were centrifuged over a Ficoll gradient, washed, resuspended at 10⁸ per ml in PBS with 2018 ROBINSON ET AL. J. VIROL.
Mamu-A*02 molecule was carried out on the PCR-amplified products from all of the putative Mamu-A*02/H11001 rhesus monkeys. Fifteen animals had sequences identical to the previously published cDNA sequence for this allele. Three monkeys had a single nucleotide change resulting in a predicted amino acid substitution of threonine for alanine at residue 85 (S) of the protein product. In the experiments described in the present report, lymphocytes were evaluated from monkeys expressing both Mamu-A*02 sequences. cDNAs extracted from PBL from a second cohort of 123 Indian-origin rhesus monkeys were similarly screened, and 35 were found to have a PCR-amplified Mamu-A*02 product, indicating an allele frequency of 28% in this population. The PCR-amplified products from the cDNAs of this cohort of monkeys were not sequenced. The high frequency of this MHC class I allele among captive Indian-origin rhesus monkeys suggested that Mamu-A*02 would be a useful molecule for further studies.

We next sought to determine whether a Mamu-A*02-presented Nef CTL epitope might be defined. To initiate this evaluation, PBL from a Mamu-A*02/Mamu-A*01/SIVmac-infected rhesus monkey were stimulated in vitro with distinct pools of 25-amino-acid peptides that spanned SIVmac239 Nef, overlapping each other by 8-amino-acids. These peptide-stimulated PBL were then assessed for their ability to lyse Mamu-A*02/B-LCL pulsed with the same pool of peptides that had been used to stimulate them (Fig. 2A). Target cell lysis was detected by the PBL population stimulated with the peptide pool p198 to p200. To determine which of the peptides in that pool contained the epitope recognized by the effector cells, the same effectors were assessed for the ability to lyse Mamu-A*02/B-LCL pulsed with the same pool of peptides that had been used to stimulate them (Fig. 2B). Target cells were sensitized for lysis by p199, suggesting that a CTL epitope existed within that 25-amino-acid peptide fragment.

Studies were then done to map the precise CTL epitope contained within the 25-amino-acid p199 sequence. PBL from three Mamu-A*02/SHIV-infected rhesus monkeys were stimulated in vitro with the 13-amino-acid peptide p199 and assessed for their ability to lyse a single Mamu-A*02/B-LCL pulsed with p199 or two 13-amino-acid fragments of p199, p199S and p199R (Fig. 3 and 4A). The peptide-stimulated effector cells lysed the target cells pulsed with p199R and with the 9-amino-acid p199RY fragment. The minimal epitope recognized by these effector T lymphocytes was identified as a 9-amino-acid fragment within the 13-amino-acid peptide p199R.
ment. This observation suggested that p199RY may be the optimal epitope recognized by these effector T cells.

To confirm the fine specificity of these effector T-lymphocyte populations, a further study was done to assess the relative efficiency of target cell sensitization using five peptides, 8 to 10 amino acids in length, that covered this region of SIVmac Nef. PBL from four Mamu-A*02/H11001 rhesus monkeys previously infected with SIVmac or SHIV were stimulated in vitro with the 13-amino-acid peptide p199R and assessed for their ability to lyse a Mamu-A*02/H11001 target cell pulsed with limiting concentrations of each of these five peptides (Fig. 3 and 5). Consistently, both the 9-amino-acid peptide p199RY and the 10-amino-acid peptide p199DY were much more efficient than the other peptides at sensitizing target cells. Because p199RY was consistently slightly more efficient than p199DY at target cell sensitization, and because it is the shorter of the two peptides, we assume that the optimal Nef CTL epitope is p199RY, YTSGPGIRY.

To demonstrate that p199RY is indeed a CTL epitope, experiments were performed to define the phenotype of the effector cells responsible for the recognition of the peptide and the precise MHC class I molecule that binds and presents it to T lymphocytes. PBL from SHIV-infected Mamu-A*02/H11001 rhesus monkeys were stimulated in vitro with p199RY, fractionated into CD8/H11001 lymphocyte-enriched and CD8/H11001 lymphocyte-depleted populations, and assessed as effector cells for the recognition and lysis of p199RY-pulsed, autologous B-LCL (Fig. 6A). Target cell lysis was mediated by the CD8 lymphocyte-enriched but not the CD8 lymphocyte-depleted effector cells. Effector cells generated by p199RY stimulation of PBL from an SHIV-infected Mamu-A*02/H11001 rhesus monkey were then evaluated for their ability to lyse untransfected or Mamu-A*02-transfected C1R cells pulsed with p199RY or the irrelevant control peptide p11C (Fig. 6B). Only the Mamu-A*02-transfected C1R cells pulsed with p199RY were lysed. Thus, the

FIG. 4. SIV Nef peptide-specific cytolytic activity of PBL from infected Mamu-A*02 rhesus monkeys is localized to the 9-amino-acid peptide p199RY. (A) PBL from 18324, 19242 (two SHIV-infected Mamu-A*01 Mamu-A*02 rhesus monkeys) and 19196 (an SIVmac251-infected Mamu-A*01 Mamu-A*02 rhesus monkey) were stimulated in vitro with p 199 (20 μg/ml). On day 12 of culture, the lymphocytes were assessed as effector cells in a standard 4-h ⁵¹Cr release CTL assay. Target cells were B-LCL made from monkey 19196 (used in experiments with effectors from monkey 19242 or monkey 19196) or monkey 24195 (Mamu-A*01 Mamu-A*02; used in experiments with effectors from 18324) that had been incubated overnight with p199 (20 μg/ml) or either of the 13-amino-acid peptides p199R or p199S. (B) PBL from monkeys 26795, 14282, and 19242 (three SHIV-infected Mamu-A*01 Mamu-A*02 rhesus monkeys) were stimulated in vitro with 5 μg of the SIV Nef 13-amino-acid peptide p199R per ml. On days 11 and 12 of culture, the lymphocytes were assessed as effector cells in a standard 4-h ⁵¹Cr release CTL assay. Target cells were B-LCL made from monkey 24195 that had been incubated overnight with 5 μg/ml of either SIV Nef p199R or one of the SIV Nef 9-amino-acid peptides p199RD, p199RY, or p199RT.
9-amino-acid peptide p199RY is presented to CD8\(^+\) CTL by the rhesus monkey MHC class I molecule Mamu-A*02. Most viral CTL epitopes that have been defined in outbred populations of nonhuman primates and humans are nondominant (26). Thus, CTL specific for these viral epitopes can be detected in only a subpopulation of infected or vaccinated individuals who express the MHC class I molecule capable of presenting that peptide fragment (7). We reasoned that this newly defined Nef epitope would prove most useful in HIV vaccine development and AIDS pathogenesis studies if it were predictably recognized by CTL in a majority of immune Mamu-A*02\(^+\) rhesus monkeys. To determine whether the optimal epitope peptide p199RY is indeed predictably recognized, PBL from seven Mamu-A*02\(^+\) rhesus monkeys infected with SIVmac or SHIV were stimulated in vitro with p199RY and assessed for their ability to lyse Mamu-A*02\(^+\) B-LCL pulsed with p199RY or a control peptide (p199S). The effector cells generated from all seven of the infected Mamu-A*02\(^+\) rhesus monkeys lysed p199RY-pulsed Mamu-A*02\(^+\) target cells (Fig. 7).

Peptide-stimulated lymphocytes from two infected Mamu-A*01\(^+\) and Mamu-A*02\(^+\) rhesus monkeys were evaluated for interferon gamma production by Elispot assay to determine whether p199RY represents a dominant CTL epitope. Whether p199RY-specific T-cell responses elicited by all the various peptides was generated in response to the single 9-amino-acid Nef peptide p199RY. In fact, the magnitude of the p199RY-specific T-cell response was comparable to the response stimulated by the peptide pool (Gag 1) containing the p11C epitope.

**DISCUSSION**

Dominance is a relative rather than an absolute property of CTL epitopes. For example, while CTL specific for the p54 SIV Env epitope are readily detected in most SIV-infected Mamu-A*01\(^+\) rhesus monkeys and might therefore be considered dominant (8), the frequency of the circulating effectors that recognize this epitope in infected monkeys does not appear to be as high as those that are specific for p11C when assessed by tetramer staining (data not shown). Whether p199RY represents a dominant CTL epitope remains an open question. While the peptide Elispot experiment shown in Fig. 8 suggests that the T-cell response to p199RY may represent a sizable proportion of the entire virus-specific T-cell repertoire of these infected monkeys, the study was performed comparing responses to an optimal 9-amino-acid peptide with those to
infected monkeys, a phenomenon attributed to viral mutation away from CTL recognition. In that particular case, long-term monitoring of the Tat-specific CTL response would be of little utility. The observation in the present study that the p199RY-specific CTL response is readily detected in a cohort of chronically infected monkeys suggests that viral mutation at this epitope to escape CTL recognition is not occurring.

The most likely immediate utility of this newly defined epitope will be in expanding the number of available Indian-origin rhesus monkeys that can be used in studies in which precise quantitation of CTL responses can be performed. For example, in a cohort of 123 rhesus monkeys recently screened for expression of the Mamu-A*01 and Mamu-A*02 alleles, 29 were Mamu-A*01-only positive, 35 were Mamu-A*02-only positive, and 2 were positive for both alleles (data not shown). Thus, 54% of all the monkeys tested expressed at least one of these alleles. This newly defined epitope will clearly prove to be of great importance in monitoring CTL responses specific for SIV or SHIV in rhesus monkeys.

The utility of this defined epitope for AIDS investigators will be determined, in part, by the degree to which this particular Nef sequence is conserved among various SIV and SHIV isolates in common experimental use. In fact, this 9-amino-acid sequence is highly conserved among various SIVs and SHIVs. Therefore, the p199RY sequence will be of great importance in monitoring the Tat-specific CTL response in rhesus monkeys, a phenomenon attributed to viral mutation away from CTL recognition. In that particular case, long-term monitoring of the Tat-specific CTL response would be of little utility. The observation in the present study that the p199RY-specific CTL response is readily detected in a cohort of chronically infected monkeys suggests that viral mutation at this epitope to escape CTL recognition is not occurring.

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The utility of this defined epitope for AIDS investigators will be determined, in part, by the degree to which this particular Nef sequence is conserved among various SIV and SHIV isolates in common experimental use. In fact, this 9-amino-acid sequence is highly conserved among nef genes encoded by the SIV and SHIV viruses that are employed at this time in nonhuman primate research. SIVmac251, SIVmac239, SIVSHM4, and SIVmac32H nef genes encode the identical 9-amino-acid sequence used to construct the peptide employed in this study (YTSGPGIRY). All of the SHIV chimeras in use by various investigators incorporate the SIVmac239 nef gene and therefore are predicted to express this epitope. The SIV-MNE sequence has a single isoleucine-to-serine substitution at position 7. SIV-MB670 differs at two amino acids, a serine-to-proline substitution at position 3, and an isoleucine-to-glutamate substitution at position 7.

Extensive studies in numerous laboratories worldwide have led to the definition of no viral epitopes in HIV-1-infected
humans that are predictably recognized by CTL from all individuals expressing a shared MHC class I molecule, yet a far less concerted effort has led to the definition of a number of such predictable CTL epitopes in SIVmac-infected Indian-origin rhesus monkeys. While there is no obvious explanation for this disparity, two possibilities bear consideration. First, the variety of MHC class I molecules that can bind viral peptide fragments and present them to CD8+ CTL in rhesus monkeys is greater than in humans. It is thought that rhesus monkeys have at least two A and B loci (4). The greater potential number of MHC class I molecules that can bind viral peptide fragments and present them to CD8+ CTL in rhesus monkeys is greater than in humans. It is thought that rhesus monkeys have at least two A and B loci (4). The greater potential number of such molecules that can bind viral peptide fragments and present them to CD8+ CTL in rhesus monkeys is greater than in humans.

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FIG. 8. The interferon gamma response to p199RY by PBL from Mamu-A*02 SHIV-infected rhesus monkeys constitutes a significant portion of the response to all peptides spanning Env and Gag, as measured by Elispot analysis. PBL from 483 and 833 (two SHIV-infected Mamu-A*01 Mamu-A*02 rhesus monkeys) were added at 2 × 10^5 per well to 96-well plates that had been coated with a mouse anti-human interferon gamma antibody. Peptide or medium alone was added in triplicate wells and incubated at 37°C for 18 h. Peptides used in p199RY or p199RD as a negative control at 8 μg/ml; two pools containing a combined total of 50 overlapping 20-mers at 8 μg/ml each, representing the entire SIVmac239 Gag protein, and two pools containing a combined total of 85 overlapping 20-mers at 8 μg/ml each, representing the entire HIV-1 89.6P Env protein. Plates were sequentially washed and incubated with biotinylated polyclonal anti-human interferon gamma, streptavidin-alkaline phosphatase, and chromogenic substrate, as described in Materials and Methods. The number of spot-forming cells per 10^6 PBL is shown as the mean ± standard deviation of triplicate wells.
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