Emergence of Cytotoxic T Lymphocyte Escape Mutants following Antiretroviral Treatment Suspension in Rhesus Macaques Infected with SIVmac251

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INTRODUCTION

Potent antiretroviral therapy can control infection with HIV-1 in many patients by reducing the extent of ongoing viral replication in CD4+ T cells and other cell types in blood and lymphoid tissue. This reduction in HIV-1 replication associates with improved immune function (Auran et al., 1997) and decreased patient morbidity and mortality (Palella et al., 1998). Although combinations of antiretroviral drugs can suppress replication of HIV-1 for a long time, a long-lived reservoir of infectious virus remains (Chun et al., 1997; Finzi et al., 1999; Furtado et al., 1999; Wong et al., 1997), indicating the need for lasting treatment. The continued presence of this reservoir of HIV-1 represents a likely impediment to the enduring control of viral infection.

Structured treatment interruption (STI) of potent antiretroviral drug therapy has been explored as an alternative approach to manage patients with HIV-1 infection (Davey et al., 1999; Lisziewicz et al., 1999; Lori and Lisziewicz, 2001) that would enhance the HIV-1-specific immune response (Ortiz et al., 1999) and reduce drug-related toxicity. STI begun during early infection with HIV-1 may preserve the HIV-1-specific CD4+ and CD8+ T cell response and maintain immune control (Berrey et al., 2001; Markowitz et al., 1999; Rosenberg et al., 2000; Spiegel et al., 2000), whereas later initiation of STI may resurrect a dormant HIV-1-specific CD8+ T cell response (Davey et al., 1999; Ortiz et al., 1999). A clinical benefit of this approach is likely limited to those patients who start STI soon after their infection with HIV-1, however, an observation supported by the rhesus macaque model of AIDS (Hel et al., 2000; Lifson et al., 2000; Lori et al., 2000; Mori et al., 2000).

Despite evidence that STI can contain ongoing viral replication and preserve or augment the immune response in selected people in the early stage of HIV-1 infection (Berrey et al., 2001; Hel et al., 2000; Markowitz et al., 1999; Montefiori et al., 2001; Rosenberg et al., 2000), the fundamental mechanism underpinning the observed clinical benefits for these particular persons is unknown. In addition, it is not known to what extent failure of this approach represents virus escape from immune control, especially in the setting of increased antigenic variation and waned immunity found during long-standing HIV-1 infection (Casazza et al., 2001; Gray et al., 1999; Ögg et al., 1999). Therefore, we used the rhesus macaque model of AIDS to assess the underlying
mechanisms of virological failure after STI in macaques with the Mamu-A*01 haplotype that had had acute and long-standing infection with SIVmac251.

RESULTS

Viral rebound in plasma in macaques treated with ART during primary infection with SIVmac251

Six rhesus macaques were treated with the combination of DDI, d4T, and PMPA 15 days after an intravenous inoculation with SIVmac251. IL-2 was administered together with antiretroviral drug therapy. Before interruption of antiretroviral drug therapy, plasma levels of SIV RNA remained below the detectable limit. The details on the immunological response in these animals is reported elsewhere (I. Nacsa, Z. Hel, W.-P. Tsai, E. Tryniszewska, L. Giuliani, J. Altman, M. G. Lewis, P. Markham, D. Venzon, N. Bischofberger, S. M. Wolinsky, J. Tartaglia, K. A. Smith, and G. Franchini, unpublished data).

After interruption of antiretroviral drug therapy, the increase in the level of SIV RNA in plasma in three animals (macaques 683, 686, 706) occurred during the administration of IL-2 and was transient (Fig. 1A). After IL-2 suspension, two of these animals (macaques 686 and 706) had subsequent transient increases in plasma SIV RNA, whereas one macaque (animal 683) had a substantial increase in the level of SIV RNA in plasma associated with rapid progression to SIV disease and death (Fig. 1A). Three other animals (macaques 685, 680, 760) had a transient upsurge in the concentration of SIV RNA in plasma 10 to 20 days after the cessation of IL-2 treatment (Fig. 1B). All three of these macaques subsequently maintained levels of plasma SIV RNA below 2000 copies per 100 μl of plasma without antiretroviral drug treatment.

SIVmac Gag-specific CD8+ T cells in macaques treated during acute infection

We next sought an association between the rise and fall in the level of plasma viral RNA after STI and the expansion of SIV-specific CD8+ T cells. Staining of CD3+CD8+ T cells of blood in rhesus macaques with (macaques 680, 683, 685, 686, and 706) and without (macaque 760) the Mamu-A*01 haplotype with the Mamu-A*01-restricted SIV Gag181–189 CM9 tetramer (Allen et al., 1998; Kuroda et al., 1998) revealed an expansion of the SIV-specific CD8+ T cell population (up to 16%) in macaques 683, 686, and 706 that was associated with a 2 log fall in the level of plasma SIV RNA following the suspension of antiretroviral drug therapy (Fig. 1A). The increased frequency of SIV-specific CD8+ T cells in peripheral blood in macaque 680 was found in the absence of detectable viral RNA in plasma (Fig. 1B). As expected, the PBMC of the Mamu-A*01-negative macaque 760 did not stain with the Gag181–189 CM9 tetramer (Fig. 1B). Macaque 683 had an abrupt rise in the level of SIV RNA in plasma, despite the highest frequency (16%) of SIV-specific CD8+ T cells (Fig. 1A).

To determine the association of the loss of immune control with the appearance of nonsynonymous substitutions in the amino acid residues that likely affect binding to the MHC class I molecule Mamu-A*01, we assessed the extent of genetic diversity within the Mamu-A*01-restricted Gag181–189 CM9 epitope over time. We used DNA sequencing of PCR-product DNA amplified from plasma viral RNA at end-point dilution to assess the frequency of mutations coding for amino acid changes in the Mamu-A*01-restricted Gag181–189 CM9 epitope in the SIV gag gene.

DNA sequence analysis of the SIVmac251 viral stock used to infect all macaques studied here (Pal et al., 2001) showed the inferred wild-type Mamu-A*01-restricted Gag181–189 CM9 epitope sequence (CTPYDINQM) in 42 of 42 independent Gag clones (data not shown). The deduced amino acid sequence of the Mamu-A*01-restricted Gag181–189 CM9 epitope was determined for up to 11 independent clones from viral RNA in plasma beginning at day 15 of viral inoculation and thereafter for macaques with primary SIV infection, as indicated by arrows in Figs. 1A and 1B.

For macaque 683, few amino acid changes were observed in the Gag181–189 CM9 epitope at the time of primary infection with SIVmac251 (day 15), as well as at the first and second resurgences of virus (days 168 and 210) (Fig. 1C). By day 238, however, the T → A substitution in the second amino acid position of the Mamu-A*01-restricted Gag181–189 CM9 epitope was determined for up to 11 independent clones from viral RNA in plasma beginning at day 15 of viral inoculation and thereafter for macaques with primary SIV infection, as indicated by arrows in Figs. 1A–1C.

Viral rebound in plasma in ART-treated macaques with long-standing infection with SIVmac251

The combination of DDI, d4T, and PMPA with or without low dose of IL-2 was given for 5 months to four macaques infected with SIVmac251 for 11 months or more. Table 1 shows the level of SIV RNA in plasma (range, 8.8 × 10^5 to 9.2 × 10^6 copies per ml) and CD4+ T cell number in peripheral blood (range, 449 to 1415 cells per ml) before the start of treatment. Macaques 432 and 449 had previously received ALVAC-SIV-gag-pol-env
FIG. 1 Kinetics of virus-specific CD8\(^+\) T cell responses, plasma virus resurgence, and viral immune escape following STI in macaques treated in primary infection. A and B: Six macaques were infected by the intravenous route with SIVmac251. All animals became viremic and were treated with ART at day 15 from exposure to SIVmac251 (561) (Pal et al., 2001). These animals were treated with ART for 154 days and initiated on low-dose IL-2 treatment at day 70 and maintained until day 196. At day 196, all treatment was suspended. Macaques 683, 686, 685, and 760 were also vaccinated with NYVAC-SIV-gpe (J. Nacsa, Z. Hel, W.-P. Tsai, E. Tryniszewska, L. Giuliani, J. Altman, M. G. Lewis, P. Markham, D. Venzon, N. Bischofberger, S. M. Wolinsky, J. Tartaglia, K. A. Smith, and G. Franchini, unpublished data), whereas the remaining macaques were mock-vaccinated with NYVAC. Data from these macaques in which viral rebound was detected following weekly assessment of viremia are presented in Figs. 1A and 1B.

I represents the level of viremia over time. F represents frequency of CD3\(^+\)CD8\(^+\) Gag181–189 CM9-specific T cells in blood. \* refers to the Mamu-A*01-negative macaque (760) whose blood was used as a control for the specificity of tetramer staining. The arrows indicate the time of analysis of plasma RNA for the data presented in Fig. 1C.

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(ALVAC-SIV-gpe) (Pal et al., 2001); macaque 644 had received NYVAC-SIV-gag-pol-env (NYVAC-SIV-gpe), and macaque 642 had received NYVAC alone (Hel et al., 2000) and had sustained elevation in the concentration of SIV RNA in plasma for 11 months or more. During treatment with a potent combination of antiretroviral drugs augmented by low-dose IL-2, plasma levels of SIV RNA remained below detectable level. Cyclic interruptions of antiretroviral therapy were begun with (macaques 432 and 642) and without (macaques 449 and 644) overlapping IL-2 administration.

The level of SIV RNA in plasma in macaques 449 and 644 rose substantially (>10^6 copies per ml) after the first interruption of antiretroviral drug therapy and remained high thereafter, despite reinstitution of treatment (Fig. 2A). Macaque 449 developed clinical SIVmac disease progression and was euthanized because of AIDS-related complications. Likewise, the concentration of plasma SIV RNA in macaques 432 and 642 rose (>10^5 copies per ml) and then fell below the threshold in the interval between the beginning and end of the first STI. All four of these macaques subsequently maintained levels of plasma SIV RNA above 5 x 10^5 copies per milliliter without antiretroviral drug treatment (Fig. 2A). Before the interruption of treatment, we found no mutation coding for resistance to DDI, d4T, and PMPA in the reverse transcriptase gene of plasma viral RNA. After the first interruption of antiretroviral drug therapy, a mutation coding for resistance to PMPA (K65R) (Van Rompay et al., 1996) was found in the plasma virus reverse transcriptase gene of monkey 644, which associated with the burst of viral replication, loss of CD4^+ T cells, and progression to SIVmac disease. No drug-resistant mutations were found in the plasma viral RNA from the other rhesus macaques, indicating that STI did not usually select for drug resistance.

### SIVmac Gag-specific CD8^+ T cells in macaques after long-standing infection with SIVmac251

SIV-specific CD8^+ T cells staining with the immuno-dominant Mamu-A*01-restricted SIV Gag_181-189 CM9 tetramer related to the presence or absence of concurrent IL-2 administration. Before starting antiretroviral drug therapy, the frequency of CD8^+ T cells stained with the Mamu-A*01 peptide tetrameric complex for the major Mamu-A*01-restricted SIV Gag_181-189 CM9 in all four macaques ranged from 0.25 to 1.5% (Fig. 2B). In the absence of IL-2 (macaques 449 and 644), there was no change in the frequency of SIV-specific CD8^+ T cells staining with this Mamu-A*01 tetrameric molecule (Fig. 2A). In the presence of IL-2 (macaques 432 and 642), the frequency of SIV-specific CD8^+ T cells staining with the Mamu-A*01-restricted SIV Gag_181-189 CM9 tetramer was high, especially in macaque 432 (up to 40%) (Fig. 2A).

We assessed the genetic variation within the Mamu-A*01-restricted Gag_181-189 CM9 epitope by screening for mutations that accrued in amino acid residues critical for this epitope presentation (Sidney et al., 2000). Amino acid substitutions were principally limited to the anchor residue in the second position of this Mamu-A*01-restricted Gag_181-189 CM9 epitope sequence as deduced T → A, T → S, and S → C (macaque 644) substitutions that existed before or emerged immediately after the interruption of treatment (Fig. 2C). In macaque 432, 26 days after stopping antiretroviral drug therapy, a T → A substitution emerged in the second position of the Mamu-A*01-restricted Gag_181-189 CM9 epitope (Fig. 2C). In macaque 449, only 1 of the 10 clones obtained at the start of treatment (day 0) had the canonical Gag_181-189 CM9 epitope sequence. By the time of the first interruption of antiretroviral treatment, the T → A substitution became the predominant Gag_181-189 CM9 epitope virus in plasma (Figs. 2B, top panel, and 2C). Each of the amino acid changes in the second position of CTPY-DINQM has been shown to decrease or abrogate the binding capacity of the Gag_181-189 CM9 peptide to the Mamu-A*01 molecule (Sidney et al., 2000).

### DISCUSSION

In patients infected with HIV-1, long-lived reservoirs of infectious virus persist (Chun et al., 1997; Finzi et al., 1999; Furtado et al., 1999; Wong et al., 1997) despite...
treatment with combinations of antiretroviral drugs that suppress ongoing viral replication and reduce plasma viral RNA to undetectable levels. The persistent presence of reservoirs of HIV-1 has been the impetus for exploring alternative treatment strategies, including STI as a means to reduce drug-related toxicity and enhance the HIV-1-specific immune response (Davey et al., 1999; Lisziewicz et al., 1999; Ortiz et al., 1999). Clinical experi-

FIG. 2. Kinetics of virus-specific CD8⁺ T cell response, plasma virus resurgence, and viral immune escape in macaques with long-standing SIVmac251 infection. A: Level of plasma viremia over time (■) and frequency (●) of Gag₁₈₁-₁₈₉ CM9 tetramer-positive CD3⁺CD8⁺ T cells during STI and after IL-2 suspension in four Mamu-A*01-positive macaques. B: Percentage of Gag₁₈₁-₁₈₉ CM9 tetramer-positive CD3⁺CD8⁺ T cells in the blood of the four Mamu-A*01-positive macaques at the start of treatment. C: Analysis of the genetic composition of plasma virus with respect to the amino acid sequence of peptide Gag₁₈₁-₁₈₉ CM9 over time in macaques 449, 644, 642, and 432. A single letter amino acid code of the presumed amino acid sequence of the Gag₁₈₁-₁₈₉ CM9 epitope is presented. The number 0 refers to initiation of ART. The initiation of ART with respect to the time from infection for these macaques is summarized in Table 1.
ence with each of these alternative approaches to the immune control of viral replication has been less than satisfactory, especially in patients with long-standing HIV-1 infection.

Here we investigated the basis of unabated virological failure in rhesus macaques that expressed the MHC class I molecule Mamu-A*01 through a course of STI during acute and long-standing infection with SIVmac251. We measured the frequency of SIV-specific CD8\(^+\) T cells staining with the Mamu-A*01-restricted SIV Gag\(_{181-189}\) CM9 tetramer (Allen et al., 1998; Kuroda et al., 1998) and tracked changes in the targeted immunodominant Gag\(_{181-189}\) CM9 epitope over time. Certain rhesus macaques received concurrent low-dose IL-2 as a means to overcome defects in IL-2 production demonstrated previously for people infected with HIV-1 (Allen et al., 1998; Allouche et al., 1999; Nicastri et al., 1999; Pal et al., 2001; Sidney et al., 2000; Westby et al., 1998). We found that five of six macaques with acute infection with SIVmac251 had had transient increases in the level of SIV RNA in plasma (up to \(10^7\) copies per ml) and an expansion of the frequency of SIV-specific CTL after the interruption of antiretroviral drug therapy. One macaque (683) had had a significant increase in the level of SIV RNA in plasma (>\(10^7\) copies per ml) with a rapid progression to SIV disease and death from AIDS-related complications. It is uncertain whether the mutation in the Gag\(_{181-189}\) CM9 epitope resulted in high viremia or whether the high viremia resulted in selection of this mutant virus. However, it seems reasonable to hypothesize that escape to this dominant response may be an underlying cause of uncontrolled viral replication, as demonstrated also by others (Barouch et al., 2002).

All four macaques with long-standing infection with SIVmac251 had had substantial increases in plasma viral RNA (>\(10^7\) copies per ml) after the first interruption of antiretroviral drug therapy that persisted despite a second cycle of STI. Measurable SIV-specific CD8\(^+\) T cells staining with the Mamu-A*01-restricted SIV Gag\(_{181-189}\) CM9 tetramer were found in the two macaques that received IL-2 as well. All four of these macaques had preexisting or developed nonsynonymous substitutions in the second position of the Mamu-A*01-restricted SIV Gag\(_{181-189}\) CM9 epitope that affect binding to the cognate MHC class I molecule (Sidney et al., 2000). One monkey had plasma SIV RNA with a mutation that conferred PMPA resistance.

In the rhesus macaque model for AIDS, antiretroviral therapy alone given during the early stage of infection has long-term clinical benefit in most cases (Franchini, 2002; Hel et al., 2000; Lifson et al., 2000; Lori et al., 2000; Mori et al., 2000), suggesting that early treatment gives the immune system the opportunity to establish some measure of long-term control of viral replication, perhaps by avoiding viral escape from epitopes with high avidity (O'Connor et al., 2002). In rhesus macaques with long-standing infection with SIVmac251, however, this approach did not appear to provide the same advantage, likely because viral escape to high-avidity CTL epitopes has already occurred in most cases, as demonstrated by others (O'Connor et al., 2002) and our work here, since we already observed changes in the Gag\(_{181-189}\) CM9 epitope in two of the four chronically infected macaques at the beginning of the study. Thus, the clinical benefit of STI may be restricted to those patients infected with HIV-1 who present early in the course of their disease. The failure of STI to contain viral replication in macaques with primary and long-standing infection with SIVmac251 was associated with evolution and emergence of viral escape from immune recognition. All the amino acid changes that we found in the second position of the immunodominant Gag\(_{181-189}\) CM9 epitope in the four macaques decreased the binding capacity of the corresponding peptides to the MHC class I Mamu-A*01 molecule (Sidney et al., 2000) and in the case of the T \(\rightarrow\) A also escape from CTL has been clearly demonstrated (Chen et al., 2000). Thus, escape from this dominant response may contribute to disease progression as also observed previously in one animal infected with SHIV89.6P (Barouch et al., 2002). Unlike virus escape from antiretroviral drug treatment brought about by constant selection pressure on the virus population, virus escape from immune control changes the selection pressure applied by the immune system. In the setting of long-term infection with SIVmac, STI may drive this process further and therefore precipitate the changes that allow the virus to escape from CTL.

Taken together, these data suggest that controlled interruptions of antiretroviral therapy may contribute to the selection of viral immune escape in both primary and long-standing infection. The extent of virus diversity and evolution (Wolinsky et al., 1996), particularly during chronic infection, functional impairment of virus-specific CD8\(^+\) T cells (Appay et al., 2000; Goepfert et al., 2000; Hel et al., 2001; Kostenae et al., 2001; Shankar et al., 2000; Vogel et al., 2001; Westby et al., 1998), and lack of adequate CD4\(^+\) T cell help (Rosenberg et al., 1997; Villinger et al., 2002), are all potential additional underlying mechanisms. Caution is therefore warranted when considering STI as a therapeutic approach in HIV-1-infected individuals. Perhaps strengthening the host immune response by vaccination before STI may result in containment of viral replication and a decreased viral diversification.

**MATERIALS AND METHODS**

**Animals and treatments**

All macaques were colony-bred rhesus macaques (*Macaca mulatta*) housed and handled in accordance with the standards of the American Association for the Accreditation of Laboratory Animal Care. No macaque
had confirmed STLV-1 or herpesvirus B infection before entering the study. MHC class I haplotype restriction was determined by a high-resolution molecular typing assay as described previously (Knapp et al., 1997). Macaques were inoculated with the same stock of pathogenic SIVmac251 (561) (Pal et al., 2001). All macaques received a triple-drug antiviral regimen that included the intravenous administration of DDI (10 mg/kg) once a day, oral administration of d4T (1.2 mg/kg/dose) twice a day, and subcutaneous administration of PMPA (20 mg/kg) once a day as previously described (Hel et al., 2000).

Six treatment-naive rhesus macaques were infected with SIVmac251 (day 0) and then treated with the triple-drug antiviral regimen 15 days later. Four of these macaques were also vaccinated with NYVAC-SIV-gpe (Benson et al., 1998) (Fig. 1, legend). Antiviral therapy continued unabated until day 154 (see legend, Fig. 1A). Daily administration of IL-2 (120,000 IU) by the subcutaneous route was chosen and based on the pharmacokinetics of IL-2 in macaques (our unpublished results) and began on day 70 and ended on day 196. Four treatment-naive rhesus macaques with long-standing SIVmac251 infection (11–15 months) received the same antiviral therapy and IL-2 at the same dose. Table 1 shows the mean levels of SIV RNA in plasma, average CD4 T cell numbers, and previous immunization schedule for these four macaques.

### Quantification of plasma SIV RNA

We quantified the level of SIVmac251 RNA in plasma by nucleic acid sequence-based isothermal amplification assay using SIVmac251-specific oligonucleotide primers as described previously (Romano et al., 2000). The limit of sensitivity of the assay was 2 × 10^3 copies per 100 μl of plasma.

### Immunological assays

Mononuclear cells isolated from peripheral blood were stained with anti-human CD3 antibody (FITC-labeled, clone SP34, Becton–Dickinson, San Jose, CA), anti-human CD8* antibody (PerCP-labeled, Becton–Dickinson), and analyzed by flow cytometry assays as described previously. To assess the frequency of SIV-specific CTL, we measured the number of CD8* T cells stained with the Mamu-A*01-restricted SIV Gag<sub>181–189</sub> CM9 tetramer-PE by flow cytometry (FACSCalibur, Becton–Dickinson).

### DNA sequence analysis

We used end-point dilution DNA sequencing of plasma viral RNA to screen for the presence of mutations in the Mamu-A*01-restricted Gag<sub>181–189</sub> CM9 epitope. Viral particles were isolated from plasma by centrifugation at 25,000 g for 1 h. The particles were lysed in a solution containing 48% guanidine thiocyanate, 1.4% dithiothreitol, 1% N-lauroylsarcosine, and 1% sodium citrate. Viral RNA was precipitated with 100% isopropanol and 70% ethanol and then resuspended in 50 μl of RNA diluent. The viral RNA was amplified by PCR with SIV gag-F outer primer (nucleotides 319 to 342: 5’ACCTAGTTGGTGGAACACAG-GAACAG3’) and SIV gag-R outer primer (nucleotides 930 to 903; 5’TGTGTGCTCTGCTTAAGCTTTTGTAG3’); SIV gag-F inner primer (nucleotides 374 to 395; 5’AGCAC-CATCTAGTGCCAGAGA3’) and SIV gag-R inner primer (nucleotides 883 to 863; 5’GAAATGGCCTCTTTTGGC-CCTT3’). The positions of the oligonucleotide primers are numbered according to the gag gene of the SIVmac251 isolate. After extraction and amplification, the PCR-product DNA inserted into vector pCR II by the principles of T-A cloning (Invitrogen TOPO TA Cloning Kit) and 8 to 13 inserts were sequenced and analyzed with a sequencing system (Prism 377, Applied Biosystems, Foster City, CA) as described previously.

We used direct sequencing of plasma viral RNA to assess the frequency of mutations coding for drug resistance in the reverse transcriptase region of the SIVmac251 pol gene. Plasma viral RNA was amplified by PCR with SIV pol-F outer primer (nucleotides 658 to 676; 5’TAAAGCCAGAAAGATGG3’) and SIV pol-R outer primer (nucleotides 1365 to 1346; 5’TGGCAACTC-TATTTTGGCA3’); SIV pol-F inner primer (nucleotides 681 to 698; 5’AAATTGAAGCAGTGGCCA3’) and SIV pol-R inner primer (nucleotides 1333 to 1316; 5’TGGCCACAATTTGGTACC3’). The positions of the oligonucleotide primers were numbered according to the pol gene of the SIVmac251 isolate. The PCR-product DNA was sequenced directly and analyzed for mutations coding for resistance to DDI (K45R, L74V, and M184V), d4T (M41L, D67N, K70R, L210W), or PMPA (K65R) in the reverse transcriptase region of the pol gene of SIVmac251 with a sequencing system as described above.

The positions of the deduced amino acid substitutions are numbered according to the reverse transcriptase region in the pol gene of the SIVmac251 isolate. In reconstruction experiments, this assay will discriminate a genetic variant when represented in the virus population at a frequency of more than 30%.

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immunodominant CTL epitope from simian immunodeficiency virus. 


