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Induction of Gag-specific T-cell responses by therapeutic immunization with a Gag-expressing Sendai virus vector in macaques chronically infected with simian-human immunodeficiency virus

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Abstract

Recent prophylactic vaccine trials inducing virus-specific $CD8^+$ T-cell responses have shown control of primary infections of a pathogenic simian-human immunodeficiency virus (SHIV) in macaques. In the chronic phase, therapeutic immunization replenishing virus-specific $CD8^+$ T-cells is likely to contribute to sustained control of virus replication. In this study, we have administered a recombinant Sendai virus (SeV) vector into five rhesus macaques that had received prophylactic vaccinations and had controlled SHIV replication for more than 1 year after challenge. Our results indicate that virus-specific $CD8^+$ T-cell responses can be expanded and broadened by therapeutic immunization with SeV vectors in the chronic phase after prophylactic vaccine-based control of primary immunodeficiency virus infections.

Keywords: AIDS; Sendai virus; Therapeutic vaccine

1. Introduction

Virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses play a central role in the control of immunodeficiency virus infections. The importance of CTL in the control has been indicated not only in the acute phase but also in the chronic phase of infections by several clinical correlations in human immunodeficiency virus type 1 (HIV-1)-infected humans [1–3] and $CD8^+$ T-cell-depletion experiments in macaque AIDS models [4–6]. Therefore, AIDS vaccine studies have been making efforts to develop methods efficiently inducing virus-specific $CD8^+$ T-cell responses.

Recombinant viral vectors can be a promising tool for AIDS vaccines because of their potential for inducing virus-specific $CD8^+$ T-cell responses. Recently, preclinical trials of prophylactic vaccines using recombinant viral vectors have shown control of primary infections of a pathogenic simian-human immunodeficiency virus (SHIV) that induces acute $CD4^+$ T-cell depletion in macaques [7–10]. These vaccinated macaques have contained the challenge virus leading to reduction in plasma viral loads to be undetectable at the setpoint and maintained peripheral $CD4^+$ T-cell counts, although they have failed to eliminate the virus and shown detectable levels of proviral DNA in lymphocytes in the chronic phase

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[11]. $CD8^+$ T-cell depletion by anti-CD8 monoclonal antibody treatment in these macaques in the chronic phase after prophylactic vaccine-based control of primary SHIV infection has shown a rise in plasma viral loads [12]. Additionally, loss of the control due to appearance of a CTL escape mutant has been observed in the chronic phase in a macaque that had controlled primary SHIV infection [13]. Thus, virus-specific $CD8^+$ T-cell responses have been indicated to play a central role in maintaining the control of virus replication in the chronic phase and therapeutic immunization replenishing virus-specific $CD8^+$ T-cell responses are likely to contribute to the sustained control.

We previously developed a prophylactic DNA vaccine system that uses FMSIV [14], which is a chimeric SHIV with ecotropic Friend murine leukemia virus (FMLV) *env* in place of SHIV *env*, in combination with the FMLV receptor, mCAT1 [15], which is not normally expressed in primate cells. Vaccination of macaques with both of the FMSIV proviral DNA and an mCAT1-expression plasmid DNA induced mCAT1-dependent FMSIV replication leading to efficient elicitation of virus-specific $CD8^+$ T-cell responses. We also established a prophylactic Sendai virus (SeV) vector-based vaccine system [16–19]. Not only the replication-competent (transmissible) but also the replication-defective (non-transmissible) SeV vector showed the potential for efficiently inducing virus-specific $CD8^+$ T-cell responses [20,21]. Additionally, combination of the DNA vaccine and the SeV vector vaccine, DNA-prime/SeV-boost, elicited extremely high levels of virus-specific $CD8^+$ T-cell responses [8]. Preclinical trials of these prophylactic vaccine systems showed control of replication of a pathogenic SHIV89.6PD and prevented macaques from acute AIDS progression [8,21].

In this study, we have examined if the SeV vector can be used for therapeutic immunization to induce virus-specific $CD8^+$ T-cell responses in the chronic phase. We considered Gag as a promising vaccine-antigen candidate to avoid CTL escape because it has been indicated that Gag CTL escape variants mostly diminish viral fitness and require multiple additional compensatory mutations to restore their replicative competence [22]. We administered a Gag-expressing SeV (SeV-Gag) vector into those macaques that had controlled SHIV replication for more than 1 year after challenge and analyzed Gag-specific T-cell responses.

2. Materials and methods

2.1. Animals

Male rhesus macaques (*Macaca mulatta*) were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases. These macaques were tested negative for SeV, simian immunodeficiency virus (SIV), and simian retrovirus type D before use. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia.

Macaques used in this study were previously subjected to prophylactic vaccination and challenge experiments (Table 1) [8,23]. In brief, macaques R011 and R012 received four times FMSIV plus mCAT1 DNA vaccinations and single intranasal SeV-Gag booster, whereas macaques R003 and R006 were boosted intranasally with a recombinant SeV expressing HIV-1 Tat (SeV-Tat) after the DNA vaccinations. Macaque R022 received the DNA vaccinations only. An infectious FMSIV clone DNA obtained by replacing the gene fragment encoding Env surface protein of $SHIV_{MD14YE}$ [24] with an FMLV *env* fragment [25] has simian immunodeficiency virus-derived long terminal repeat, *gag*, *pol*, *vif*, *vpx*, and partial *vpr* sequences, HIV-1-derived partial *vpr*, *tat*, *rev*, and partial env (containing the second exon of *tat*, the second exon of *rev*, and RRE) sequences, and FMLV-derived *env* sequences [14]. At each DNA vaccination, animals received 800 μg of individual DNA intramuscularly and 10 μg of individual DNA by gene gun. At the booster, animals received 1×10^8 cell infectious units (CIU) of replication-competent F(+)SeV-Tat or F(+)SeV-Gag. Animals were challenged intravenously with 10 TCID50 (50% tissue culture infective doses) of SHIV89.6PD [26].

2.2. Therapeutic immunization

We used two kinds of SeV vectors expressing SIVmac239 gag, a replication-competent one (F[+]SeV-gag) and a replication-defective F-deleted one (F[−]SeV-gag), for therapeutic immunization. Recombinant F(+)SeV-Gag and F(−)SeV-Gag were prepared as described previously [16,19,20]. Animals received 1×10^8 CIU of F(+)SeV-Gag (macaques R003 and R006) or 6×10^9 CIU of F(−)SeV-

Table 1
Vaccination and challenge protocol in macaques

Macaques	Prophylactic vaccination	Challenge	Therapeutic vaccination
R003	DNA and F(+)SeV-Tat	SHIV89.6PD	F(+)SeV-Gag at week 56
R006	DNA and F(+)SeV-Tat	SHIV89.6PD	F(+)SeV-Gag at week 56
R011	DNA and F(+)SeV-Gag	SHIV89.6PD	F(−)SeV-Gag at week 176
R012	DNA and F(+)SeV-Gag	SHIV89.6PD	F(−)SeV-Gag at week 176
R022	DNA	SHIV89.6PD	F(−)SeV-Gag at weeks 139 and 146

DNA vaccinations were performed four times at weeks 0, 0.5, 1, and 6 after the initial vaccination. SeV-Tat or SeV-Gag vaccination for booster was performed once at week 12 after the initial vaccination. Macaques R003, R006, R011, and R012 were challenged with SHIV89.6PD at week 26 after the initial vaccination, whereas macaque R022 at week 14. Therapeutic SeV-Gag vaccination was performed at indicated time points after challenge. F(+)SeV-Tat and F(+)SeV-Gag are replication-competent and F(−)SeV-Gag is replication-defective.

Gag (macaques R011, R012, and R022) intranasally for the immunization.

2.3. Detection of SeV RNA in lymph nodes (LN)

Lymphocytes were prepared from minced lymph nodes (LN) by using Ficoll-Paque Plus (Amersham Biosciences). RNA was isolated from 1×10^6 lymphocytes by using RNeasy Mini kit (Qiagen K.K.) and eluted by 50 μl of water. Ten microliters of RNA was subjected to reverse transcription and nested PCR (RT-PCR) using SeV NP-specific primers (ATGGCCGGGTTGTTGAG and GGGCTCTTGTTGACCATAGG for the first RT-PCR, and AGTCGGGAAGAGGTGCTG and CGTCTTCACAATGAATCCGTC for the second DNA PCR) for detection of SeV RNA.

2.4. Quantitation of plasma viral loads

Plasma RNA was extracted using high pure viral RNA kit (Roche Diagnostics). Serial 5-fold dilutions of RNA samples were amplified in quadruplicate by nested RT-PCR using SIV *gag*-specific primers (AGAAACTCCGTCTTGTCAGG and TGATAATCTGCATAGCCGC for the first RT-PCR, and GATTAGCAGAAAGCCTGTTGG and TGCAACCTTCTGACAGTGC for the second DNA PCR) to determine the end-point. Plasma SIV RNA levels were calculated according to the Reed–Muench method as described [24,27]. The lower limit of detection in this assay is about 4×10^2 copies/ml.

2.5. Quantitation of proviral DNA levels in peripheral blood mononuclear cells (PBMC)

Genomic DNA was extracted from PBMC by using DNeasy kit (Qiagen K.K.). For quantitation of proviral SIV DNA copy numbers in cell lysates, serial 5-fold dilutions of cell lysates were amplified in quadruplicate by nested DNA-PCR using SIV *gag*-specific primers to determine the end-point as described [24]. The lower limit of detection in this assay is about 5 copies/μg DNA.

2.6. Measurement of antigen-specific T-cell frequencies

We measured antigen-specific T-cell frequencies by flow-cytometric analysis of interferon-γ (IFN-γ) induction after specific stimulation as described previously [8]. In brief, PBMC were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCL) [28] infected with a vaccinia virus (Vv) vector [29] for non-specific Vv-control-stimulation, B-LCL infected with a Vv vector expressing SIVmac239 Gag for Gag-specific Vv-Gag-stimulation, and B-LCL infected with SeV for SeV-specific stimulation, respectively. Intracellular IFN-γ staining was performed by using Cytofix-Cytoperm kit (BD Biosciences). Fluorescein isothiocyanate (FITC)-conjugated anti-human CD4, peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8, allophycocyanin (APC)-conjugated anti-human CD3, and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (BD Biosciences) were used. Gag-specific T-cell frequencies and SeV-specific T-cell frequencies were calculated by subtracting the IFN-γ^+ T-cell frequencies after non-specific Vv-control-stimulation from those after Gag-specific Vv-Gag-stimulation and those after SeV-specific stimulation, respectively. The background IFN-γ^+ T-cell frequencies after non-specific Vv-control-stimulation were less than 200 cells per million PBMC. Gag-specific T-cell frequencies (and SeV-specific T-cell frequencies) less than 100 cells per million PBMC were considered negative, those between 100 and 200 borderline, and those greater than 200 positive.

In case of examining peptide-specific T-cell frequencies, B-LCL were pulsed with peptide mixture (final concentration of each peptide, 1–10 μM) for peptide-specific stimulation or incubated without peptide for non-specific stimulation. A panel of 117 overlapping peptides (15–17 amino acid (aa) in length and overlapping by 10–12 aa) spanning the entire SIVmac239 Gag aa sequence were purchased from Sigma Genosys, Japan, and divided into 10 pools, each consisting of 11 or 12 peptides. The background IFN-γ^+ T-cell frequencies were less than 100 cells per million PBMC. Peptide-specific T-cell levels less than 100 cells per million PBMC were considered negative, and those greater than 100 positive.

3. Results

3.1. SeV-Gag immunization into macaques chronically infected with SHIV

In this study, we used five rhesus macaques that had received prophylactic vaccines and had controlled SHIV replication for more than 1 year after challenge (Table 1). All five macaques (R003, R006, R011, R012, and R022) had received DNA vaccinations; additionally, macaques R003 and R006 had been boosted with SeV-Tat whereas macaques R011 and R012 boosted with SeV-Gag as described previously [8,23]. They had been challenged with SHIV89.6PD 8 weeks (in R022) or 14 weeks (in R003, R006, R011, and R012) after the last vaccination. Plasma viral loads had been below the detectable levels after the setpoint and peripheral CD4$^+$ T-cell counts had been maintained until therapeutic immunization in all five macaques (data not shown). Macaques R003 and R006 received therapeutic immunization with replication-competent F(+)SeV-Gag at week 56 post-challenge, whereas macaques R011 and R012 with replication-defective F(−)SeV-Gag at week 176. Macaque R022 was immunized with F(−)SeV-Gag twice at weeks 139 and 146.

No macaques displayed apparent clinical symptoms after the therapeutic SeV-Gag immunization. No apparent pathological signs were observed by histological analysis of tissues obtained at autopsy from macaque R003 euthanized 1 week post-immunization (p.i.) (at week 57 after challenge),

Table 2
Detection of SeV RNA by nested RT-PCR

Macaques	Therapeutic vaccination	Autopsy	SeV RNA[a]		
			SM-LN	MC-LN	IG-LN
R003	At week 56	At week 57	Positive	Negative	Negative
R006	At week 56	At week 58	Negative	Negative	Negative
R011	At week 176	At week 181	Negative	Negative	Negative
R012	At week 176	At week 181	Positive	Negative	Negative
R022	At weeks 139 and 146	At week 147	Positive	Negative	Negative

[a] RNA was extracted from LN-derived lymphocytes and nested RT-PCR was performed for detection of SeV RNA. SM-LN, submandibular LN; MC-LN, mesenchymal LN; IG-LN, inguinal LN.

macaque R006 euthanized 2 weeks p.i. (at week 58), R011 or R012 euthanized 5 weeks p.i. (at week 181) or R022 euthanized 1 week after the second SeV-Gag immunization (at week 147). SeV RNA was detected by nested RT-PCR in the submandibular lymph node in three macaques (R003, R012, and R022) but undetectable in other two (R006 and R011) (Table 2). In the mesenchymal LN and the inguinal LN, however, SeV RNA was undetectable in all five macaques.

3.2. Gag-specific T-cell responses after therapeutic SeV-Gag immunization

To see the effect of therapeutic SeV-Gag immunization on Gag-specific T-cell responses, we measured Gag-specific T-cell frequencies in PBMC before and after the immunization by detection of Gag-specific IFN-γ induction. In all five macaques, Gag-specific $CD8^+$ T-cell levels were increased after SeV-Gag immunization (Fig. 1). The second SeV-Gag immunization at week 146, 7 weeks after the first immunization, also increased the levels in macaque R022.

In macaques R012 and R022, Gag-specific $CD8^+$ T-cell responses were not clearly detected before SeV-Gag immunization but appeared after that, indicating that new epitope-specific $CD8^+$ T-cell responses were induced by the immunization. In contrast, Gag-specific $CD8^+$ T-cells were detectable even before immunization and their levels were largely increased after that in macaques R003, R006, and R011. We then examined whether increases in their levels were only due to expansion of epitope-specific $CD8^+$ T-cells that had been detectable before immunization or new-epitope specific $CD8^+$ T-cells were induced by the immunization. Using a panel of overlapping peptides spanning the entire SIV Gag aa sequence, IFN-γ induction was assessed after stimulation with pools of peptides (Fig. 2). In macaque R003, analysis of PBMC at week 56 (just before immunization) detected $CD8^+$ T-cells specific for three pools of peptides, #4 (corresponding to the 155th–213th aa in SIV Gag), #5 (202nd–265th aa), and #10 (453rd–510th aa). At week 57, 1 week p.i., no significant changes were observed in #4-specific $CD8^+$ T-cell or #10-specific $CD8^+$ T-cell levels, but #5-specific $CD8^+$ T-cells expanded efficiently. Additionally, the immunization induced #7 (306th–364th aa)-specific $CD8^+$ T-cells that had been undetectable before immunization. In

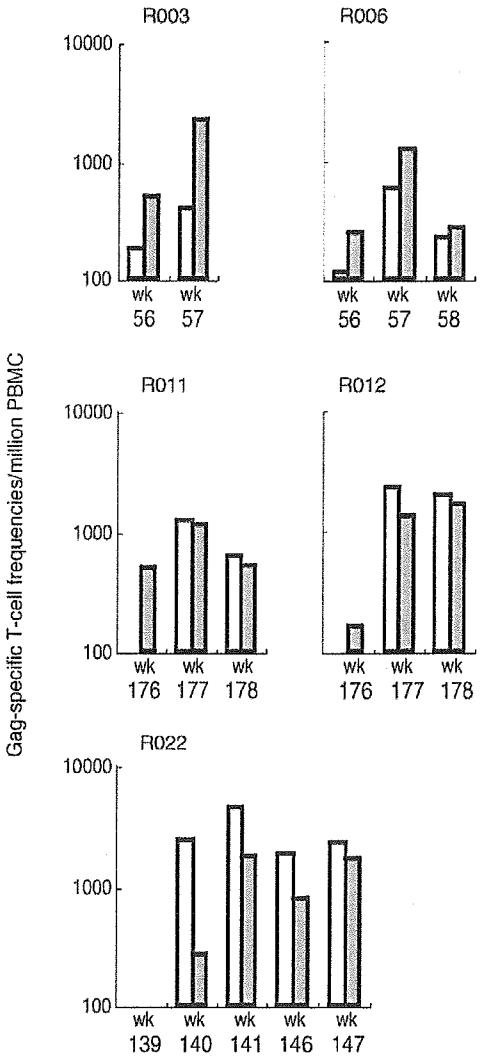

Fig. 1. Frequencies of Gag-specific $CD4^+$ T-cells (open bar) and $CD8^+$ T-cells (shaded bar) in PBMC before and after therapeutic SeV-Gag immunization. Macaques R003 and R006 were immunized at week 56 post-challenge, macaques R011 and R012 at week 176, and macaque R022 at weeks 139 and 146.

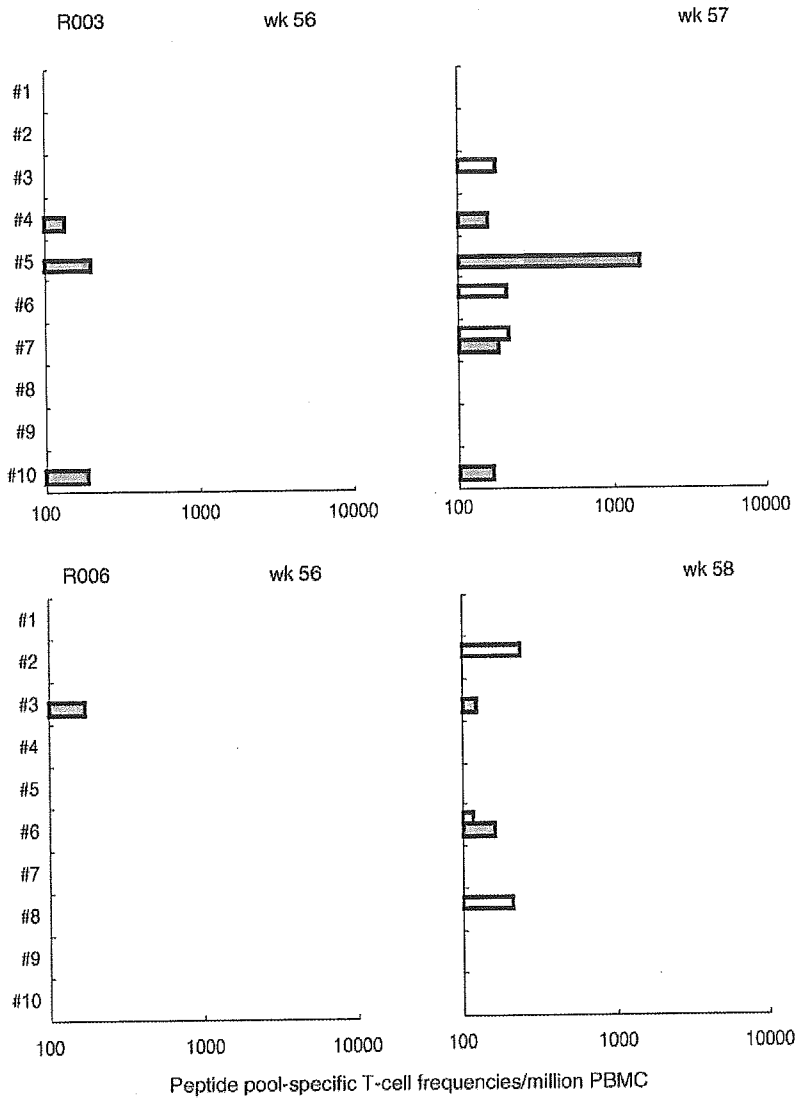

Fig. 2. Frequencies of $CD4^+$ T-cells (open bar) and $CD8^+$ T-cells (shaded bar) specific for pools of SIV Gag peptides in PBMC. A panel of overlapping peptides spanning the entire SIV Gag aa sequence were divided into 10 pools (each consisting of 11 or 12 peptides), #1 (corresponding to the 1st–65th aa in SIV Gag), #2 (55th–114th aa), #3 (104th–165th aa), #4 (155th–213th aa), #5 (202nd–265th aa), #6 (255th–316th aa), #7 (306th–364th aa), #8 (354th–416th aa), #9 (406th–464th aa), and #10 (453rd–510th aa), and used for the stimulation to detect peptide pool-specific T-cells.

macaque R006, #3 (104th–165th aa)-specific $CD8^+$ T-cell levels remained unchanged at week 58, 2 weeks p.i. The immunization, however, induced #6 (255th–316th aa)-specific $CD8^+$ T-cells that had been undetectable before immunization. Thus, the immunization induced new epitope-specific $CD8^+$ T-cells that had been undetectable before immunization in macaques R003 and R006 as well as in macaques R012 and R022, indicating that therapeutic SeV-Gag immunization not only expanded but also broadened Gag-specific $CD8^+$ T-cell responses. We failed to obtain enough PBMC samples for analysis of peptide-specific responses in macaque R011.

Therapeutic SeV-Gag immunization efficiently induced Gag-specific $CD4^+$ T-cell responses also although the responses were undetectable before immunization in all five macaques (Fig. 1). In macaques R003 and R006, several epitope-specific $CD4^+$ T-cells became detectable after immunization (Fig. 2).

3.3. *SeV-specific T-cell responses after therapeutic SeV-Gag immunization*

We also examined SeV-specific T-cell responses in macaques (Fig. 3). SeV-specific $CD8^+$ T-cells and $CD4^+$ T-cells both were undetectable just before immunization not only in macaque R022 that had been naive to SeV but also in other four macaques that had received a prophylactic vaccination with SeV vectors before challenge. In the latter, efficient induction of SeV-specific $CD8^+$ T-cell and $CD4^+$ T-

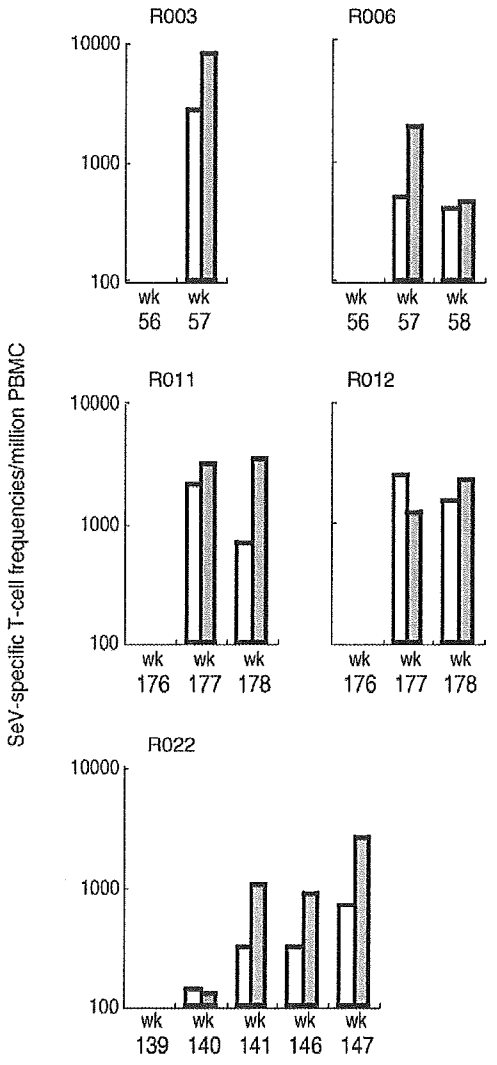

Fig. 3. Frequencies of SeV-specific $CD4^+$ T-cells (open bar) and $CD8^+$ T-cells (shaded bar) in PBMC before and after therapeutic SeV-Gag immunization.

cell responses both was observed 1 week p.i. In the former (R022), SeV-specific T-cell responses appeared with some delay and became apparent 2 weeks after the first immunization, whereas the second SeV-Gag immunization resulted in efficient expansion of SeV-specific T-cells in a week.

3.4. Viral loads after therapeutic SeV-Gag immunization

In all five macaques, plasma viremia remained undetectable after therapeutic SeV-Gag immunization. We then examined proviral DNA levels in PBMC in macaques R011, R012, and R022 (Fig. 4). These macaques kept proviral loads at low levels and we found no significant changes in their levels after immunization.

4. Discussion

The purpose of current therapeutic AIDS vaccines is to maintain HIV-1-specific CTL in the HIV-1-infected individuals who control viral replication and show little CTL responses, because CTL is crucial for sustained control in the chronic phase. The first object is HIV-1-infected individuals who control HIV-1 replication due to antiretroviral therapy, and therapeutic vaccines with recombinant viral vectors for replenishing CTL responses have been studied in HIV-1-infected individuals and SIV-infected macaques during antiretroviral treatment [30–32]. Further, HIV-1-infected individuals who show prophylactic vaccine-based control of HIV-1 replication can be an object of therapeutic vaccines, if an effective prophylactic AIDS vaccine is developed. Indeed, advances in recombinant viral vector technologies have contributed to progress in the development of CTL-based prophylactic AIDS vaccines, and recent studies in macaques have shown the importance of CTL maintenance for keeping prophylactic vaccine-based control of SHIV replication in the chronic phase [12]. Our study presents the first trial of therapeutic immunization into macaques that

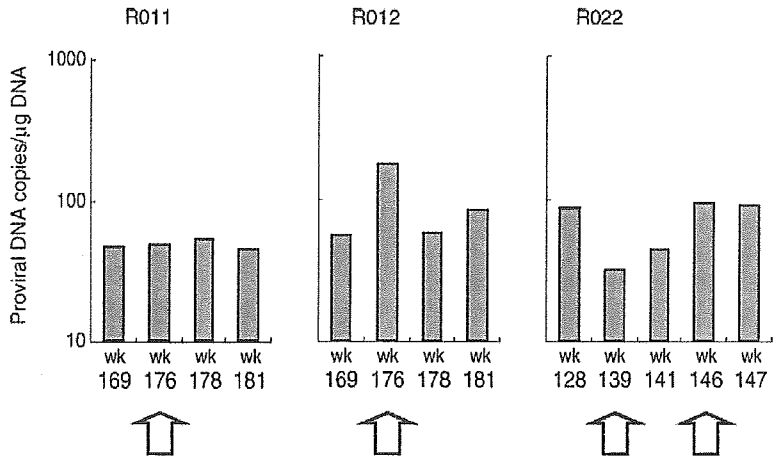

Fig. 4. SHIV proviral DNA copy numbers in PBMC before and after therapeutic SeV-Gag immunization. Arrows indicate the time-points of immunization.

have maintained prophylactic vaccine-based control of viral replication without antiretroviral help and suggests that the therapeutic SeV vector immunization can contribute to the maintenance of virus-specific CTL responses in the chronic phase.

The potential of the SeV vector as a prophylactic AIDS vaccine for inducing virus-specific T-cell responses have been studied, but its potential as a therapeutic vaccine has not yet been examined. The present study is the first report on its therapeutic administration in the chronic phase of immunodeficiency virus infections. Histological analysis revealed no pathological signs after immunization. The SeV vector distribution after therapeutic immunization was consistent with the previous analysis of prophylactic vaccination indicating that the vector was not disseminated but localized in the nasal mucosa and its primary LN [19]. These results support the notion that this vector can be safely used as a therapeutic vaccine.

Four of five macaques in this study had received a prophylactic SeV vaccination before SHIV challenge. SeV-specific T-cell responses that can interfere with the vector expression were undetectable before therapeutic SeV-Gag immunization but appeared rapidly after that. However, Gag-specific T-cell responses were efficiently induced in the presence of SeV-specific T-cell responses. Notably, SeV-Gag re-immunization only 7 weeks after the first immunization showed rapid expansion of SeV-specific T-cell responses but was able to augment Gag-specific T-cell responses although not so efficiently in macaque R022. These results suggest feasibility of SeV vector re-administration for induction of virus-specific T-cell responses.

In this study, Gag-specific $CD8^+$ T-cell responses were diminished in the chronic phase but augmented by therapeutic SeV-Gag immunization. Importantly, the immunization not only expanded but also broadened CTL responses. Broader responses may be advantageous for avoiding appearance of CTL escape mutants. A long-term follow-up study would be required to see if such CTL expansion and broadening by therapeutic immunization can contribute to sustained control of immunodeficiency virus replication.

Therapeutic SeV-Gag immunization elicited Gag-specific $CD4^+$ T-cell as well as Gag-specific $CD8^+$ T-cell responses in this study. It has been indicated that virus-specific $CD4^+$ T-cell as well as $CD8^+$ T-cell responses play an important role in the control of immunodeficiency virus infections [33–35]. Recent studies, however, have reported that HIV-1-infected patients with viremia frequently keep HIV-1-specific $CD4^+$ T-cells able to produce IFN-γ but do not have those able to proliferate and produce interleukin-2 in response to HIV-1 antigens, suggesting that the HIV-1-specific $CD4^+$ T-cell subpopulation able to produce IFN-γ may not contribute to the proliferative responses for the $CD4^+$ T-cell helper function [36,37]. Therefore, it has remained unclear if Gag-specific $CD4^+$ T-cell responses induced by SeV-Gag immunization can contribute to sustained control of virus replication. On the other hand, virus-specific $CD4^+$ T-cell induction may result in augmentation of virus replication because HIV-1 has been reported to preferentially infect HIV-1-specific specific $CD4^+$ T-cells [38]. However, no significant changes were observed in proviral loads after therapeutic SeV-Gag immunization in the present study.

In conclusion, we administered SeV-Gag as a therapeutic immunization into macaques that had maintained prophylactic vaccine-based control of SHIV replication for more than 1 year. Our results indicate that the therapeutic immunization can induce higher and broader virus-specific T-cell responses that may contribute to sustained control of immunodeficiency virus replication.

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Involvement of Multiple Epitope-Specific Cytotoxic T-Lymphocyte Responses in Vaccine-Based Control of Simian Immunodeficiency Virus Replication in Rhesus Macaques

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Cytotoxic T-lymphocyte (CTL) responses are crucial for the control of immunodeficiency virus replication. Possible involvement of a dominant single epitope-specific CTL in control of viral replication has recently been indicated in preclinical AIDS vaccine trials, but it has remained unclear if multiple epitope-specific CTLs can be involved in the vaccine-based control. Here, by following up five rhesus macaques that showed vaccine-based control of primary replication of a simian immunodeficiency virus, SIVmac239, we present evidence indicating involvement of multiple epitope-specific CTL responses in this control. Three macaques maintained control for more than 2 years without additional mutations in the provirus. However, in the other two that shared a major histocompatibility complex haplotype, viral mutations were accumulated in a similar order, leading to viral evasion from three epitope-specific CTL responses with viral fitness costs. Accumulation of these multiple escape mutations resulted in the reappearance of plasma viremia around week 60 after challenge. Our results implicate multiple epitope-specific CTL responses in control of immunodeficiency virus replication and furthermore suggest that sequential accumulation of multiple CTL escape mutations, if allowed, can result in viral evasion from this control.

Virus-specific cytotoxic T-lymphocyte (CTL) responses are crucial for the control of immunodeficiency virus infections. The importance of CTLs for control has been indicated by temporal association of CTL appearance with the resolution of primary viremia in human immunodeficiency virus type 1 (HIV-1)-infected humans (9, 24, 33) and by monoclonal anti-CD8 antibody-mediated CD8-depletion experiments in macaque AIDS models (18, 29, 38). Therefore, AIDS vaccine researchers have been making efforts to develop methods efficiently eliciting CTL responses (15, 30), and most of them have used multiple antigens for CTL induction (3, 8). However, it has remained unclear if multiple epitope-specific CTLs can really take part in vaccine-based control of viral replication.

Several preclinical trials of CTL-based AIDS vaccines in macaques have succeeded in the control of replication of a simian-human immunodeficiency virus, SHIV89.6P, that induces acute $CD4^+$ T-cell depletion (3, 8, 27, 37, 40). Unfortunately, most of these vaccine regimens have failed to contain the more realistic challenge of pathogenic simian immunodeficiency viruses (SIVs) that induce chronic disease progression (12, 17). Recently, however, CTL-based control of replication of a pathogenic SIV clone, SIVmac239, has been shown in a preclinical vaccine trial using Burmese rhesus macaques (28). In that study, macaques immunized with a DNA prime/Gag-expressing Sendai virus (SeV-Gag) vector-boost vaccine were challenged intravenously with SIVmac239. Five of eight vaccinees controlled viral replication and had undetectable levels of plasma viremia after 5 weeks of infection. All of the five macaques showed rapid selection of CTL escape mutations in *gag*, indicating that vaccine-induced CTLs were crucial for the containment of the wild-type, challenge virus. Of the five, three vaccinees that share a major histocompatibility complex class I (MHC-I) haplotype, *90-120-Ia*, showed high levels of $Gag_{206–216}$ (IINEEAADWDL) epitope-specific CTL and rapid selection of a mutant escaping from this CTL. The virus with the CTL escape mutation, GagL216S, leading to an alteration from leucine (L) to serine (S) at the 216th amino acid (aa) in Gag showed diminished replicative ability compared to the wild type. Inoculation of naive macaques with this mutant resulted in persistent viral replication and reversion in the absence of the $Gag_{206–216}$-specific CTL responses (23). These results have suggested that additional adaptive immune responses as well as $Gag_{206–216}$-specific CTLs are important for containment of this CTL escape mutant virus with lower viral fitness.

Viral escape from CTL recognition has been frequently observed in HIV-1 and SIV infections, and it may be critical for viral evasion from immune control (5, 6, 10, 15, 16, 32, 35, 36). Indeed, viral evasion from immune control with a single escape mutation from a dominant CTL has been reported in preclinical AIDS vaccine trials, indicating involvement of the single epitope-specific CTL in this control (5, 6). However, these reports have not made it clear whether multiple epitope-spe-

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cific CTLs can be involved in the vaccine-based control of immunodeficiency virus replication.

In the present study, we have followed, for more than 2 years, the five macaques that showed vaccine-based control of SIVmac239 replication. We have found that three of them maintained control of viral replication for more than 2 years while the other two lost control at approximately week 60 after challenge. Analysis of the latter two has revealed viral evasion from the vaccine-based control by accumulation of multiple CTL escape mutations, indicating involvement of multiple epitope-specific CTLs in this control.

MATERIALS AND METHODS

Animal experiments. Twelve male Burmese rhesus macaques (*Macaca mulatta*) used in our previous SIVmac239 challenge experiment (28) were followed up in the present study. These macaques were maintained in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia. Four of the macaques were naive whereas the other eight macaques received a DNA vaccine followed by a single boost with SeV-Gag before an intravenous SIVmac239 challenge. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA (39) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx; SIVmac239-$HIV\text{-}1_{DH12}$ chimeric Vpr; and $HIV\text{-}1_{DH12}$ Tat and Rev as described previously (28). At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals intranasally received a single boost with 1×10^8 cell infectious units of replication-competent SeV-Gag (V1, V2, V3, and V4) or 6×10^9 cell infectious units of F-deleted replication-defective F(−)SeV-Gag (19, 20, 26, 41). Thirteen weeks after the boost, animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239 (22).

Quantitation of plasma viral loads. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). Serial fivefold dilutions of RNA samples were amplified in quadruplicate by reverse transcription (RT) and nested PCR using SIV *gag*-specific primers (AGAAACTCCGTCTTGT CAGG and TGATAATCTGCATAGCCGC for the first RT-PCR and GATTA GCAGAAAGCCTGTTGG and TGCAACCTTCTGACAGTGC for the second DNA PCR) to determine the endpoint. Plasma SIV RNA levels were calculated according to the Reed-Muench method as described previously (28, 39). The lower limit of detection in this standard assay is about 4×10^2 copies/ml. For fivefold concentration of plasma, after centrifugation of 1 ml of plasma at 25,000 $\times g$ for 2 h, 0.8 ml of its supernatant was discarded and the remaining 0.2 ml was subjected to RNA extraction.

Sequencing. Fragments corresponding to nucleotides (nt) 1231 to 2958 (containing the entire *gag* region), nt 2827 to 3960, nt 3811 to 4970, nt 4829 to 5986, nt 5852 to 7000, nt 6843 to 7901, nt 7684 to 8831, nt 8677 to 9723, and nt 9499 to 10196 in the SIVmac239 genome (GenBank accession number M33262) were amplified by nested RT-PCR. Alternatively, genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) by using the DNeasy kit (QIAGEN K.K., Tokyo, Japan), and the *gag* fragment was amplified by nested PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan). Alternatively, the PCR products were subcloned into plasmids by using the TOPO cloning system (Invitrogen, Tokyo, Japan) and sequenced.

Peptide-specific CTL responses. We measured virus-specific T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously (28). In brief, PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCL) (42) pulsed with 1 μM or indicated concentrations of peptides (Sigma Genosys, Ishikari, Japan) for peptide-specific stimulation or unpulsed B-LCL for nonspecific stimulation. Intracellular IFN-γ staining was performed by using the Cytofix-Cytoperm kit (Becton Dickinson, San Jose, California). Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting the IFN-γ^+ T-cell frequencies after nonspecific stimulation from those after peptide-specific stimulation. Specific T-cell levels less than 100 cells per million PBMCs were considered negative.

Generation of CTL clones and CTL assay. $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL clones were obtained from macaque V5 PBMCs cocultured with irradiated, V5-derived B-LCL pulsed with the corresponding peptides. Cytotoxicity was measured in a standard ^{51}Cr release assay. In brief, target cells (5×10^5) were incubated with 150 μCi $Na_2{}^{51}CrO_4$ for 1 h, pulsed with the corresponding peptides for 1 h, and cocultured with effector cells for 4 h. The culture supernatants were analyzed with a gamma counter. The spontaneous ^{51}Cr release (cpm spn) was determined by measuring the ^{51}Cr release from the culture containing only target cells. The maximum release (cpm max) was determined by measuring the ^{51}Cr release from target cells in the presence of 2.5% Triton X-100. Percent specific lysis was calculated as follows: percent specific lysis = 100 × (cpm exp − cpm spn)/(cpm max − cpm spn), where cpm exp is the ^{51}Cr release from the culture containing both target and effector cells.

Viral competition assay. SIV molecular clone DNAs with mutations in *gag* were constructed by site-directed mutagenesis from the wild-type SIV molecular clone DNA pBRmac239, provided by T. Kodama and R. C. Desrosiers. COS1 cells were transfected with mutant SIV molecular DNAs to obtain mutant SIV stocks. Two million cells of a herpesvirus saimiri-immortalized macaque T-cell (MTC) line (1) were infected with one of the mutant SIVs at the dose of 2 ng of SIV CA (p27), and 1 day later, half of them were cocultured with those infected with another mutant SIV. Two million MTCs were added into the culture on days 8, 12, 16, and 20 after infection. RNA was extracted from the culture supernatant on day 24. The fragment (nt 1231 to nt 3016 in SIVmac239) containing the entire *gag* region was amplified from the RNA by RT-PCR and was subcloned into plasmids for sequencing to determine dominant sequences.

RESULTS

Reappearance of viremia after 1 year of control in two of the five controllers. Twelve Burmese rhesus macaques used in our previous SIVmac239 challenge experiment (28) were followed up in the present study (Table 1). Of the 12, eight macaques descended from a male breeder, R-90-120, and four of them shared an MHC-I haplotype, *90-120-Ia*. Four macaques were naive whereas eight macaques received a DNA vaccine followed by a single boost with SeV-Gag before an intravenous SIVmac239 challenge. All four naive animals and three of the vaccinees failed to control SIV replication, but five of eight vaccinees controlled SIV replication with undetectable levels of plasma viremia (less than 400 RNA copies/ml) after 5 weeks of infection. We have termed the former seven animals noncontrollers and the latter five controllers in the present study.

During 2 years of follow-up, all the seven noncontrollers maintained high levels of plasma viremia (Fig. 1A). Four of them developed AIDS and had to be euthanized. By contrast, plasma viremia was undetectable and peripheral $CD4^+$ T-cell counts were maintained even after 2 years of infection in three (V4, V6, and V8) of five controllers (Fig. 1A and B). In the other two controllers (V5 and V3), however, plasma viremia reappeared and was detectable (more than 400 RNA copies/ml) at week 58 after challenge (Fig. 1A). Thus, three of five controllers maintained control of SIV replication for more than 2 years, whereas the other two controllers lost control after 1 year of infection. We have termed the former three animals sustained controllers and the latter two transient controllers in the present study.

Of four macaques possessing the MHC-I haplotype *90-120-Ia*, all of the three vaccinees, V5, V3, and V4, successfully controlled SIV replication, although one naive macaque, N2, failed. Remarkably, two of the three controllers possessing *90-120-Ia* lost control around week 60.

We examined viral loads in the controllers by detection of viral genomes in concentrated plasma (Fig. 1C). The cutoff line of this assay is about 80 RNA copies/ml whereas that of our standard assay for quantitation of plasma viral RNA is approximately 400 RNA copies/ml. In both of the transient control-

TABLE 1. SIVmac239 challenge experiments

Macaque	MHC-I haplotype[a]	Naive or vaccinee[b]	Set point VL[c] around wk 12	CTL escape[d] at wk 5	VL around wk 60
R-90-120 descendants					
N2	*90-120-Ia*	Naive	10^4–10^6		10^4–10^6
V5	*90-120-Ia*	Vaccinee	<400	GagL216S	$>10^3$
V3	*90-120-Ia*	Vaccinee	<400	GagL216S	$>10^3$
V4	*90-120-Ia*	Vaccinee	<400	GagL216S	<400
V2	*90-120-Ib*	Vaccinee	10^4–10^6		Dead[e]
N3	*90-122-Ie*	Naive	10^4–10^6		10^4–10^6
V7	*90-122-Ie*	Vaccinee	10^4–10^6		10^4–10^6
V6	*90-122-Ie*	Vaccinee	<400	GagI377T	<400
R-90-088 descendants					
N1	*90-088-Ij*	Naive	10^4–10^6		10^4–10^6
V1	*90-088-Ij*	Vaccinee	10^4–10^6		10^4–10^6
R-90-010 descendants					
N4	*90-010-Id*	Naive	10^4–10^6		10^4–10^6
V8	*90-010-Id*	Vaccinee	<400	GagQ58K	<400

[a] MHC-I haplotype was determined by reference strand-mediated conformation analysis (4) as described previously (28). Macaques N2, V3, and V2 are sons of male breeder R-90-120; V5, V4, N3, V7, and V6 are sons of R-94-027; N1 and V1 are sons of R-90-088; N4 and V8 are sons of R-90-010. Breeder R-94-027 is the son of male R-90-120 and female R-90-122 and possesses *90-120-Ia* and *90-122-Ie* haplotypes. MHC-I haplotypes *90-120-Ia* and *90-120-Ib* are derived from breeder R-90-120, *90-122-Ie* is from R-90-122, *90-088-Ij* is from R-90-088, and *90-010-Id* is from R-90-010.

[b] All the animals were challenged intravenously with SIVmac239. Vaccinees received a prophylactic DNA prime/SeV-Gag boost vaccine before challenge.

[c] Plasma viral load (RNA copies/ml plasma). VL, viral load.

[d] Rapidly selected CTL escape mutations in Gag as described previously (28).

[e] Macaques N3, V1, V2, and V7 developed AIDS and were euthanized at weeks 104, 105, 42, and 77, respectively.

lers, viral RNA was detected in the concentrated plasma during the period of control although it was undetectable by our standard assay. In contrast, viral RNA was undetectable even in the concentrated plasma in all of the sustained controllers. These results indicate that SIV replication was contained to much lower levels in the sustained controllers compared to the rather high levels in the transient controllers.

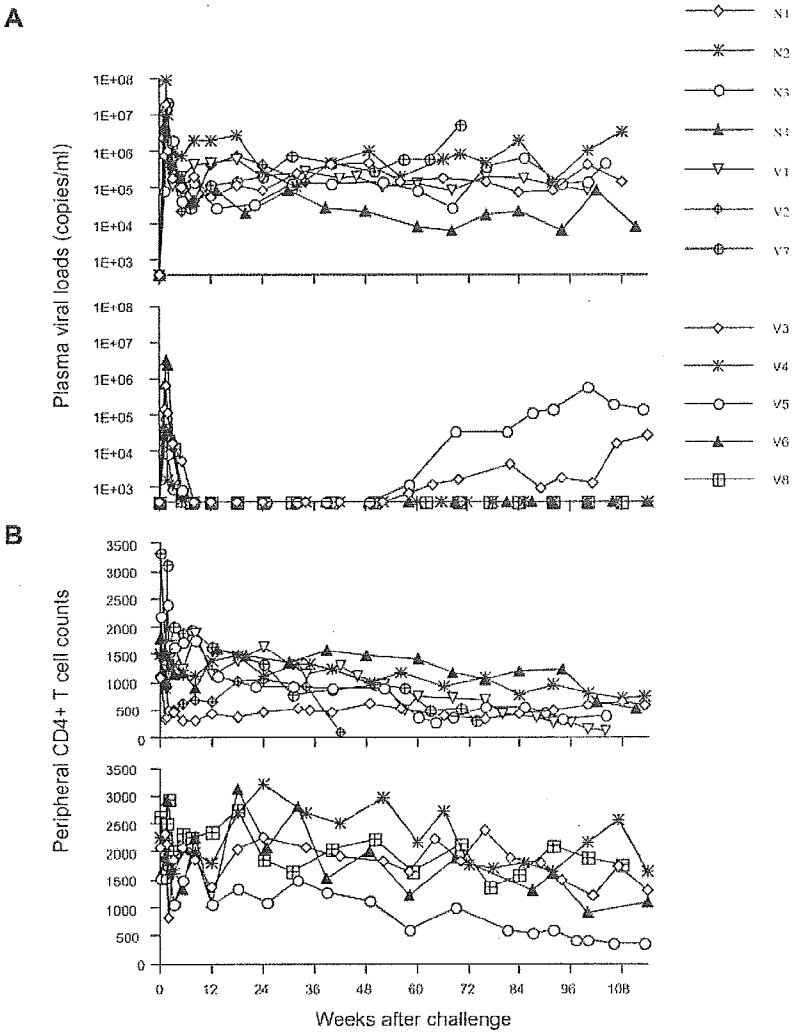
Viral mutations in the transient controllers. The previous study (28) showed rapid selection of CTL escape mutations in *gag* in all of the controllers (Table 1), indicating the importance of the CTL responses in the control of SIV replication. We then examined *gag* sequences to see if additional viral mutations were involved in the loss of control in the transient-controllers (Table 2). In a sustained controller (V4) possessing the MHC-I haplotype *90-120-Ia*, we observed rapid selection of the GagL216S mutation leading to escape from $Gag_{206–216}$-specific CTL responses (referred to as $Gag_{206–216}$-CTL-escape mutation) both in plasma viral RNA and in proviral DNA of PBMCs. This mutation was maintained, but no other mutation became dominant even at week 85. In the other two sustained controllers (V6 and V8), the rapidly selected CTL escape mutations were observed in viral RNA but not in proviral DNA. This may reflect the possibility that accumulated mutant copies were too small for their detection in provirus compared to the wild type in these two macaques.

In both of the transient controllers (V5 and V3) possessing the MHC-I haplotype *90-120-Ia*, the $Gag_{206–216}$-CTL-escape mutation was rapidly selected and still maintained at approximately week 60. In contrast to the sustained controllers, we found multiple additional mutations in the reemerged viruses in both of these macaques. In macaque V5, viral genomes with GagL216S, GagD244E (aspartic acid [D]-to-glutamic acid [E] alteration at the 244th aa in Gag), GagI247L (isoleucine [I] to L at the 247th aa), GagA312V (alanine [A] to valine [V] at the 312th aa), and GagA373T (A to threonine [T] at the 373rd aa) mutations were dominant at week 58. In macaque V3, viral genomes with GagV145A (V to A at the 145th aa), GagL216S, GagD244E, and GagP376S (proline [P] to serine[S] at the 376th aa) mutations were dominant, but those with GagP172S (P to S at the 172nd aa), GagL216S, GagD244E, and GagV375A (V to A at the 375th aa) mutations were also detected at week 64.

We then examined *gag* sequences during control in both of the transient controllers (Tables 3 and 4). This analysis showed that, in addition to the GagL216S mutation, the GagD244E mutation was initially selected, followed by selection of the mutations leading to alterations around the 375th aa in Gag in both of these macaques. In this regard, the two transient controllers showed similar patterns of sequential accumulation of mutations.

Accumulation of CTL escape mutations in the transient controllers. To see if the mutations observed in the transient controllers were CTL escape mutations, we examined IFN-γ induction after stimulation with peptides corresponding to the regions around the mutation sites. In addition to the $Gag_{206–216}$ epitope, we mapped two CTL epitopes, $Gag_{241–249}$ (SSVDEQ IQW) and $Gag_{373–380}$ (APVPIPFA). High levels of these three epitope-specific ($Gag_{206–216}$-specific, $Gag_{241–249}$-specific, and $Gag_{373–380}$-specific) CTL responses were observed in all the three controllers possessing MHC-I haplotype *90-120-Ia* in the early phase of infection (Fig. 2A). The $Gag_{206–216}$-specific and $Gag_{241–249}$-specific CTL responses were especially dominant. These CTL levels were considerably reduced in the chronic phase, probably reflecting diminished SIV replication during the control. Reduction in $Gag_{206–216}$-specific CTL responses was faster, consistent with the fastest selection of the $Gag_{206–216}$-CTL-escape mutation.

Both of the transient controllers (V5 and V3) showed di-



C

SIV *gag* RNA in concentrated plasma

V5	wk 12 positive	wk 25 positive		
V3	wk 12 positive	wk 24 positive		
V4	wk 12 negative	wk 24 negative	wk 60 negative	wk 100 negative
V6	wk 12 negative	wk 25 negative	wk 58 negative	wk 100 negative
V8	wk 12 negative	wk 24 negative	wk 50 negative	wk 100 negative

FIG. 1. Follow-up of 12 macaques after SIVmac239 challenge. (A) Plasma viral loads. Top, noncontrollers; bottom, controllers. (B) Peripheral $CD4^+$ T-cell counts (per μl). Top, noncontrollers; bottom, controllers. (C) Detection of viral genomes in concentrated plasma obtained from the controllers. Positive, detected ($>8 \times 10^1$ copies/ml); negative, undetectable.

minished recognition of the peptide with the GagD244E mutation, $Gag_{241-249}$.244E (SSVEEQIQW), by $Gag_{241-249}$-specific CTL responses (Fig. 2B and 2C). The peptide with the GagI247L mutation in addition to the GagD244E (SSVEEQLQW) showed further-reduced sensitivity to CTL recognition. This indicates that the GagD244E and GagI247L mutations were selected for by $Gag_{241-249}$-specific CTLs (referred to as $Gag_{241-249}$-CTL-escape mutations). Furthermore, the GagA373T, GagV375A, and GagP376S mutations in the $Gag_{373-380}$ peptide (APVPIPFA) resulted in diminished recognition by $Gag_{373-380}$-specific CTL responses (Fig. 2B and 2C), indicating that the GagA373T, GagV375A, and GagP376S mutations were selected for by $Gag_{373-380}$-specific CTLs (referred to as $Gag_{373-380}$-CTL-escape mutations). Thus, viruses in both of the transient controllers accumulated the $Gag_{241-249}$-CTL-escape mutation and the $Gag_{373-380}$-CTL-es-

TABLE 2. Dominant sequences in Gag in the five controllers

Macaque	Wk	Sample	Amino acid change(s) in Gag[a]
V5	5	Plasma viral RNA	L216S
	58	Plasma viral RNA	L216S, D244E, I247L, A312V, A373T
V3	5	Plasma viral RNA	L216S
	64	Plasma viral RNA	(V145A), (P172S), L216S, D244E, (V375A), (P376S)
V4	5	Plasma viral RNA	L216S
	12	PBMC proviral DNA	L216S
	85	PBMC proviral DNA	L216S
V6	5	Plasma viral RNA	I377T
	12	PBMC proviral DNA	No mutation
	100	PBMC proviral DNA	No mutation
V8	5	Plasma viral RNA	Q58K
	12	PBMC proviral DNA	No mutation
	100	PBMC proviral DNA	No mutation

[a] Fragments containing the SIV *gag* region were amplified by nested RT-PCR and subjected to sequencing. Dominant mutations leading to amino acid changes are shown. The parentheses indicate that both the wild-type and the mutant sequences were detected clearly at the position.

cape mutation in addition to the $Gag_{206-216}$-CTL-escape mutation. Additionally, we obtained a $Gag_{206-216}$-specific CTL clone and a $Gag_{241-249}$-specific CTL clone and confirmed these escapes (Fig. 2D).

To determine if the remaining mutations, GagV145A, GagP172S, and GagA312V, that were observed in the reemerged viruses were within CTL epitope regions, we further examined IFN-γ induction after stimulation with peptide mixtures corresponding to the 133rd to 157th aa, the 159th to 182nd aa, and the 302nd to 324th aa, respectively. The responses were at marginal levels (Fig. 2E), and we were unable to determine whether these mutations were selected for by CTLs.

Loss of viral fitness by the accumulated mutations. Next, we examined the effect of the mutations observed in viruses from the transient controllers on viral fitness. We constructed three groups of mutant SIV clones from an SIVmac239 molecular clone by site-directed mutagenesis as shown in Table 5. The

TABLE 3. Accumulation of mutations in macaque V5

Wk	Sample	Frequency[a]	Amino acid change(s) in Gag[b]
5	Plasma Viral RNA	10/10	L216S
18	PBMC	7/10	L216S, D244E
	Proviral DNA	3/10	L216S, D244E, A373T
32	PBMC	6/11	L216S, D244E, A373T
	Proviral DNA	5/11	L216S
58	Plasma	8/10	L216S, D244E, I247L, A312V, A373T
	Viral RNA	2/10	V145A, L216S, D244E, I247L, A312V, A373T

[a] Number of clones with change(s)/total number of clones.
[b] Amplified *gag* fragments were subcloned into plasmids for sequencing. In general, mutations detected more than once are shown.

TABLE 4. Accumulation of mutations in macaque V3

Wk	Sample	Frequency[a]	Amino acid change(s) in Gag[b]
5	Plasma Viral RNA	10/10	L216S
24	Concentrated plasma Viral RNA[c]	2/9	L216S
		1/9	L216S, D244E
		3/9	L216S, D244E, V375A
		2/9	L216S, D244E, V375M
		1/9	L216S, D244E, V375I
64	Plasma Viral RNA	8/10	V145A, L216S, D244E, P376S
		2/10	P172S, L216S, D244E, V375A

[a] Number of clones with change(s)/total number of clones.
[b] Amplified *gag* fragments were subcloned into plasmids for sequencing. In general, mutations detected more than once are shown.
[c] We successfully obtained the *gag* fragments for sequencing from concentrated plasma in macaque V3 although we failed to amplify them in macaque V5 during the period of viral control.

group P virus (P1), SIVmac239Gag216S, contains a single CTL escape mutation selected in 5 weeks in both macaques V5 and V3 and has diminished replicative ability compared to the wild-type SIVmac239 as described previously (28). The group Q viruses have the $Gag_{206-216}$-CTL-escape, $Gag_{241-249}$-CTL-escape, and $Gag_{373-380}$-CTL-escape mutations. The group R viruses contain the four or five mutations dominant in the reemerged viruses.

We then compared viral fitness of the mutant viruses by determination of dominant viruses in the coculture of mutant virus-infected cells with cells infected by another mutant (Table 6). The competitions between groups P and Q revealed that the group Q viruses with $Gag_{206-216}$-CTL-escape, $Gag_{241-249}$-CTL-escape, and $Gag_{373-380}$-CTL-escape mutations showed lower viral fitness than did group P with a single $Gag_{206-216}$-CTL-escape mutation, indicating that additions of $Gag_{241-249}$-CTL-escape and $Gag_{373-380}$-CTL-escape mutations reduced viral fitness. The competitions between groups Q and R did not show recovery of viral fitness by the GagI247L, GagA312V, GagP172S, or GagV145A mutation. Consistent with these results, the group R viruses showed lower viral fitness than did the group P virus. Thus, CTLs from both of the transient controllers (V5 and V3) selected for $Gag_{241-249}$-CTL-escape and $Gag_{373-380}$-CTL-escape mutations in addition to the $Gag_{206-216}$-CTL-escape mutation with viral fitness costs. Viruses with the Gag mutations observed at viremia reappearance showed lower viral fitness than did the SIVmac239Gag216S selected in 5 weeks of infection.

DISCUSSION

In the present study, we have followed five rhesus macaques that showed vaccine-based control of SIVmac239 replication in a preclinical trial of a CTL-based AIDS vaccine (28). Two of them showed increases in plasma viral loads after 1 year of control, but the other three maintained the control without detectable plasma viremia for more than 2 years. This result suggests that vaccine induction of CTLs can result in sustained control of immunodeficiency virus replication.

Among the five macaques we followed, three (V5, V3, and V4) shared an MHC-I haplotype, *90-120-Ia*, and rapidly se-

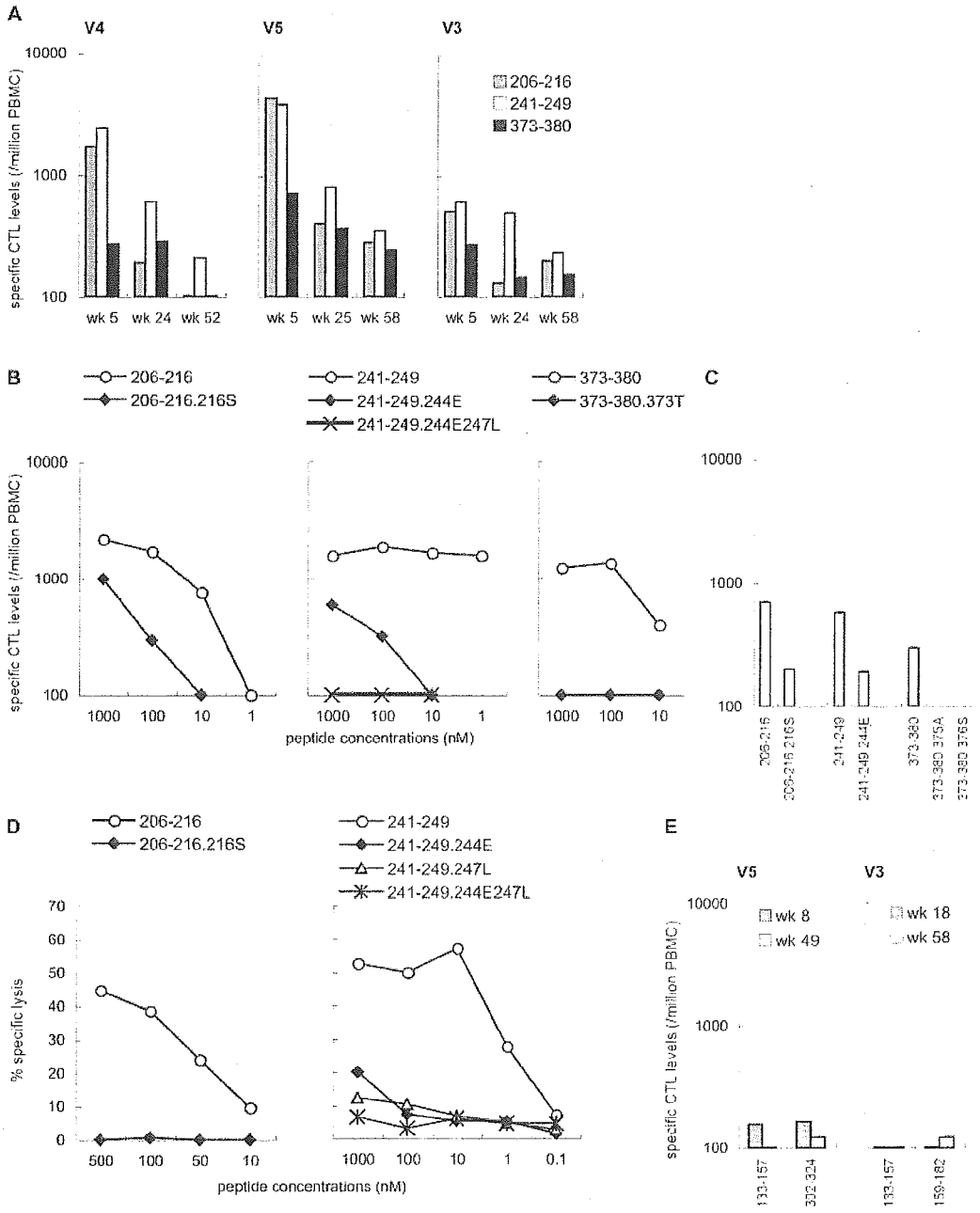


FIG. 2. CTL responses in the controllers (V4, V5, and V3) possessing MHC-I haplotype *90-120-Ia*. (A) $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{373-380}$-specific CTL levels in the macaques V4, V5, and V3. (B) IFN-γ induction in macaque V5 after stimulation with the wild-type or the mutant peptides. In the left panel, PBMCs obtained at 2 weeks after SeV-Gag boost were stimulated by coculture with B-LCL pulsed with indicated concentrations of the wild-type $Gag_{206-216}$-epitope peptide (206–216, IINEEAADWDL) or the mutant peptide with an L216S alteration (206–216.216S, IINEEAADWDS) corresponding to the 206th to 216th aa in Gag. In the middle panel, PBMCs at 2 weeks after SeV-Gag boost were stimulated by coculture with B-LCL pulsed with the wild-type $Gag_{241-249}$-epitope peptide (241–249, SSVDEQIQW), the mutant peptide with a D244E alteration (241–249.244E, SSVEEQIQW), or the mutant peptide with D244E and I247L alterations (241–249.244E247L, SSVEEQLQW) corresponding to the 241st to 249th aa in Gag. In the right panel, PBMCs at 1 week after SeV-Gag boost were stimulated by coculture with B-LCL pulsed with the wild-type $Gag_{373-380}$ epitope peptide (373–380, APVPIPFA) or the mutant peptide with an A373T alteration (373–380.373T, TPVPIPFA) corresponding to the 373rd to 380th aa in Gag. (C) IFN-γ induction in macaque V3 after stimulation with the wild-type or the mutant peptides. PBMCs at week 5 (206–216, 206–216.216S, 373–380, 373–380.375A, and 373–380.376S) or week 8 (241–249 and 241–249.244E) after challenge were used. (D) Recognition of wild-type and mutant epitope peptides by $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL clones. In the left panel, the cytotoxic activities of a $Gag_{206-216}$-specific CTL clone for target cells pulsed with the wild-type $Gag_{206-216}$ epitope (206–216) or the L216S mutant epitope (206–216.216S) peptide were measured at an effector-to-target ratio (E:T) of 2:1. In the right panel, the cytotoxic activities of a $Gag_{241-249}$-specific CTL clone for target cells pulsed with the wild-type $Gag_{241-249}$ epitope (241–249) or mutant epitope peptides with D244E (241–249.244E), I247L (241–249.247L), or D244E-I247L (241–249.244E247L) alterations were measured at an E:T of 2:1. (E) CTL responses to the peptides corresponding to the region around the sites of GagV145A, GagP172S, and GagA312V mutations. PBMCs were cocultured with B-LCL pulsed with a mixture of peptides corresponding to the 133rd to 147th, 137th to 153rd, and 143rd to 157th aa in Gag; those corresponding to the 159th to 174th, 164th to 178th, and 168th to 182nd aa in Gag; or those corresponding to the 302nd to 316th, 306th to 320th, and 310th to 324th aa in Gag for $Gag_{133-157}$-specific (133–157), $Gag_{159-182}$-specific (159–182), or $Gag_{302-324}$-specific (302–324) stimulation.

TABLE 5. List of SIV mutants

Group and abbreviation[a]	Name	Amino acid change(s) in Gag	Macaque(s) in which selected
P			
P1	SIVmac239Gag216S	L216S	V5 and V3
Q			
Q1	SIVmac239Gag216S244E373T	L216S, D244E, A373T	V5
Q2	SIVmac239Gag216S244E375A	L216S, D244E, V375A	V3
Q3	SIVmac239Gag216S244E376S	L216S, D244E, P376S	V3
R			
R1	SIVmac239Gag216S244E247L312V373T	L216S, D244E, I247L, A312V, A373T	V5
R2	SIVmac239Gag172S216S244E375A	L216S, D244E, V375A, P172S	V3
R3	SIVmac239Gag145A216S244E376S	L216S, D244E, P376S, V145A	V3

[a] Group P, Gag206–216-CTL-escape mutant rapidly selected in 5 weeks; group Q, Gag206–216-, Gag241–249-, and Gag373–380-CTL escape mutants; group R, mutants selected in the reemerged viruses.

lected for a $Gag_{206-216}$-specific CTL-escape mutant by 5 weeks after challenge. Among these three, one macaque (V4) maintained this control without additional mutations in the provirus, while the other two (V5 and V3) accumulated viral mutations and lost control with reappearance of plasma viremia (more than 400 RNA copies/ml). Because the rapidly selected $Gag_{206-216}$-CTL-escape mutant virus with the GagL216S mutation showed diminished replicative ability, it was expected that the additional mutations accumulated in macaques V5 and V3 might contribute to recovery of viral fitness. Indeed, some CTL escape mutant viruses with lower viral fitness are known to require additional compensatory mutations to restore their replicative competence (13, 21, 34, 43). However, our results have revealed that mutations accumulated in macaques V5 and V3 did not result in recovery of viral fitness. Viruses accumulated the $Gag_{241-249}$-CTL-escape mutation (GagD244E) and the $Gag_{373-380}$-CTL-escape mutation (GagA373T, GagV375A, or GagP376S) with viral fitness costs. Therefore, escape from $Gag_{241-249}$-specific and $Gag_{373-380}$-specific CTLs as well as $Gag_{206-216}$-specific CTLs was essential in the process of viral evasion from the control. This suggests that these three epitope-specific ($Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{373-380}$-specific) CTL responses were crucial for the control in these macaques. This is the first evidence indicating multiple epitope-specific CTL-based control of SIV replication.

It remains unclear what determines the time and the order of appearance of CTL escape mutations. These may be influenced by CTL levels and selective pressure, viral fitness costs by mutations, and mutation rates (T-to-C change in L216S mutation, T-to-G in D244E, G-to-A in A373T, T-to-C in V375A, and C-to-T in P376S). In macaques V5 and V3, $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were detected dominantly in the early phase of SIV infection, and the $Gag_{206-216}$-CTL-escape and $Gag_{241-249}$-CTL-escape mutations were selected for first. These results might suggest that $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses played a central role in the control of SIV replication in both of these macaques. Interestingly, the SIV $Gag_{241-249}$ epitope (SSVDEQIQW) is homologous to the HLA-B57/5801-restricted CTL epitope, TW10 (TSTLQEQIAW), in HIV-1 Gag ($Gag_{240-249}$). Like the D244E mutation within the SIV $Gag_{241-249}$ epitope, an escape mutation within the HIV-1 Gag TW10 epitope has been reported to be selected for with viral fitness costs by this TW10-specific CTL (25). Thus, this region in Gag CA could be a promising epitope candidate for CTL-based AIDS vaccines.

The viruses that reemerged around week 60 in macaques V5 and V3 had other Gag mutations (GagA312V in V5 and GagV145A or GagP172S in V3) in addition to the $Gag_{206-216}$-CTL-escape, the $Gag_{241-249}$-CTL-escape, and the $Gag_{373-380}$-CTL-escape mutations. Our results did not show recovery of viral fitness by these mutations, either, although we failed to determine whether these mutations might result in evasion from another epitope-specific CTL response. Importantly, viruses with the Gag mutations observed at viremia reappearance showed lower replicative ability than did the SIVmac239Gag216S selected around week 5. Therefore, it is inferred that the viruses with lower viral fitness can replicate to detectable levels in plasma because of their evasion from multiple epitope-specific CTL responses essential for this control. Whereas Barouch et al. (5, 6) reported a single CTL escape mutation followed by viral breakthrough (viremia recrudescence) in SHIV89.6P and SIVsmE660 infection, our results indicate that accumulation of multiple CTL escape mutations can result in viral breakthrough from the vaccine-based control of SIVmac239 replication.

In a sustained controller (V4) sharing the MHC-I haplotype *90-120-Ia* with macaques V5 and V3, $Gag_{206-216}$-specific CTL responses are considered to be involved in the sustained control even at week 85, because the GagL216S mutation was maintained without reversion (7, 11, 14, 23, 25). In addition, $Gag_{241-249}$-specific and $Gag_{373-380}$-specific CTLs are expected to play an important role in this control, and failure in accumulating $Gag_{241-249}$-CTL-escape and $Gag_{373-380}$-CTL-escape mutations may be associated with the sustained control. In contrast, it is inferred that, in macaques V5 and V3, viruses were allowed to accumulate CTL escape mutations leading to reappearance of plasma viremia. The magnitude of $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, $Gag_{373-380}$-specific, or total Gag-specific CTL responses did not appear to correlate with the level of control (Fig. 2) (25). It may be that, in macaque V4, additional effective CTLs that were not induced in V3 or V5 contributed to sustained control of SIV replication together

TABLE 6. Competition between SIV mutants[a]

Competition no.	SIV mutant used	Amino acid mutation(s)	Frequency[b]
1	P1	L216S	13/17
	Q1	L216S, D244E, A373T	2/17
		L216S, D244E,	1/17
		L216S, A373T	1/17
2	P1	L216S	15/15
	R1	L216S, D244E, I247L, A312V, A373T	0/15
3	Q1	L216S, D244E, A373T	12/14
	R1	L216S, D244E, I247L, A312V, A373T	1/14
		L216S, D244E, A312V, A373T	1/14
4	P1	L216S	11/12
	Q2	L216S, D244E, V375A	0/12
		L216S, V375A	1/12
5	P1	L216S	11/15
	R2	P172S, L216S, D244E, V375A	0/15
		L216S, V375A	3/15
		P172S, L216S, V375A	1/15
6	Q2	L216S, D244E, V375A	8/12
	R2	P172S, L216S, D244E, V375A	4/12
7	P1	L216S	12/12
	Q3	L216S, D244E, P376S	0/12
8	P1	L216S	7/12
	R3	V145A, L216S, D244E, P376S	0/12
		V145A, L216S	1/12
		L216S, D244E	1/12
		L216S, P376S	1/12
		V145A, L216S, D244E	1/12
		L216S, D244E, P376S	1/12
9	Q3	L216S, D244E, P376S	7/12
	R3	V145A, L216S, D244E, P376S	5/12

[a] MTCs infected with one SIV mutant were cocultured with those infected with another SIV mutant. RNA was extracted from the culture supernatant on day 24 after infection, and the *gag* fragment amplified from the RNA was subcloned into plasmids for sequencing.

[b] Number of clones with mutation(s)/total number of clones.

A

macaque	week	mutations (the positions of aa substitution)				
V5	wk 5	751st	in Env	&	40th	in Rev
	wk 58	67th	in Env			
		751st	in Env	&	40th	in Rev
		12th	in Nef			
		90th	in Nef			
		105th	in Nef			
		136th	in Nef			
		201st	in Nef			
V3	wk 5	751st	in Env	&	40th	in Rev
		12th	in Nef			
	wk 64	326th	in Pol			
		821st	in Pol			
		196th	in Vif			
		92nd	in Vpx			
		67th	in Env			
		751st	in Env	&	40th	in Rev
		12th	in Nef			
		34th	in Nef			

B

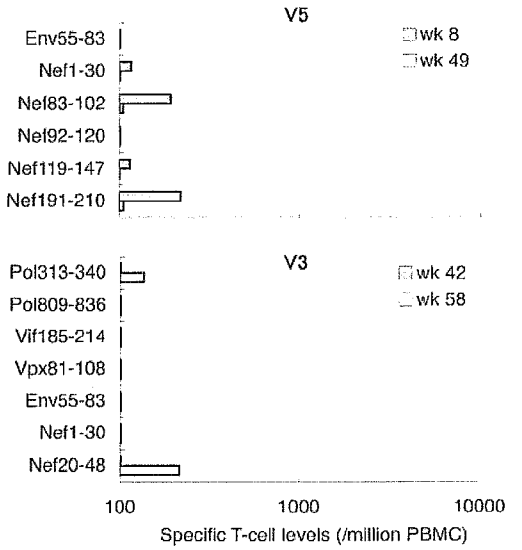


FIG. 3. Mutations in viral genomes encoding SIV proteins other than Gag. (A) Viral mutations in macaques V5 and V3. Dominant mutations leading to amino acid changes are shown. (B) CTL responses to the peptides corresponding to the region around the mutation sites. PBMCs derived from macaque V5 at week 8 or 49 were stimulated by coculture with B-LCL pulsed with a mixture of peptides corresponding to the 55th to 83rd aa in Env (Env55-83), the 1st to 30th aa in Nef (Nef1-30), the 83rd to 102nd aa in Nef (Nef83-102), the 92nd to 120th aa in Nef (Nef92-120), the 119th to 147th aa in Nef (Nef119-147), or the 191st to 210th aa in Nef (Nef191-210). PBMCs from V3 at week 42 or 58 were stimulated by coculture with B-LCL pulsed with a mixture of peptides corresponding to the 313th to 340th aa in Pol (Pol313-340), the 809th to 836th aa in Pol (Pol809-836), the 185th to 214th aa in Vif (Vif185-214), the 81st to 108th aa in Vpx (Vpx81-108), Env55-83, Nef1-30, or the 20th to 48th aa in Nef (Nef20-48).

with $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{373-380}$-specific CTLs.

We focused on SIV *gag* sequences because we used a Gag-expressing vector for the boost in our vaccine system and because vaccine-induced CTL responses were detectable only to Gag (28). In macaques V5 and V3, however, we examined sequences of all of the viral protein coding regions in the SIV genomes at week 5 and around week 60 (Fig. 3). We found that a mutation leading to an arginine (R)-to-glycine (G) alteration at the 751st aa in Env and a lysine (K)-to-R alteration at the 40th aa in Rev was dominant at week 5 in both of them. The wild-type sequence at this position in the SIVmac239 molecular clone is considered to be a suboptimal nucleotide that frequently reverts to an alternative sequence in vivo (2, 31). Indeed, we found this mutation also in the noncontrollers, indicating no association of this mutation with viral control or evasion in the present study. At week 5, no other nonsynonymous mutation became dominant in macaque V5, while one additional mutation in *nef* was found in macaque V3. Around week 60, several additional mutations were dominant in both macaques. Positions of some of the mutations were within or around epitopes for CTLs, but those CTL responses were only at marginal levels. Even considering the possible contribution of some of these mutations in the viral genome outside *gag* to the loss of control, it is reasonable to conclude that escape

from $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{373-380}$-specific CTL responses was crucial for the viral evasion in macaques V5 and V3.

In summary, our follow-up study of macaques that showed vaccine-based control of primary SIV replication has revealed that sequential accumulation of multiple CTL escape mutations, if allowed, can result in viral evasion from this control. This finding indicates, for the first time, that multiple epitope-specific CTLs can be involved in control of immunodeficiency virus replication. This has an important implication for vaccine design, suggesting the rationale for eliciting multiple epitope-specific CTL responses to contain HIV replication.

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