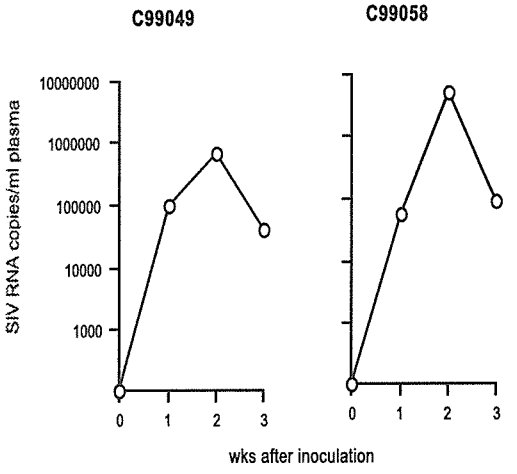


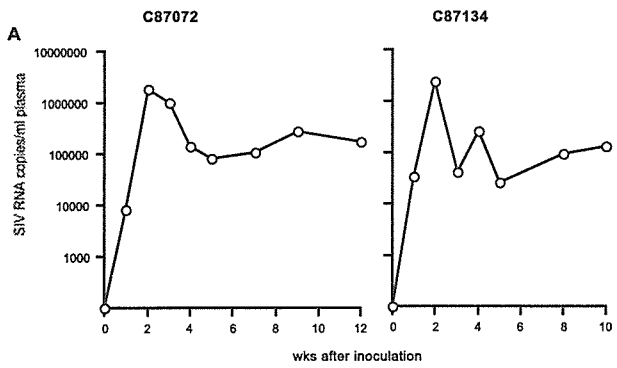
B

	wk 1	wk 2	wk 3	wk 1	wk 2	wk 3
wt clones	4	13	9	4	6	11
mt clones	6	3	1	6	2	1

C

	SM	Mes	Ing	SM	Mes	Ing
wt clones	11	9	10	11	11	6
mt clones	0	1	2	1	3	2

FIG. 1. Comparison of replication efficiencies in vivo of the wild-type SIVmac239 and the CTL escape mutant SIVmac239Gag216S. Macaques C99049 (left panels) and C99058 (right panels) were coinoculated with both wild-type and mutant SIV molecular clone DNAs. (A) Plasma viral loads after the inoculation. These indicate the sums of the wild-type and the mutant SIV RNA levels. The lower limit of detection in this assay is about 4×10^2 copies/ml. (B) Frequencies of the wild-type and mutant viral genomes in plasma. At each time point, a *gag* gene fragment was amplified by nested reverse transcription-PCR from plasma RNA and subcloned into plasmids for sequencing. The numbers of clones carrying the wild-type sequence (wt clones) and the mutant sequence (mt clones) at the region encoding the 216th aa in Gag are shown. (C) Frequencies of the wild-type and the mutant proviral genomes in the submandibular (SM) LN, the mesenchymal (Mes) LN, and the inguinal (Ing) LN. Genomic DNAs were extracted from LNs at euthanasia, and *gag* gene fragments amplified by nested PCR from the DNAs were subcloned into plasmids for sequencing.



B

	wk 4	wk 5	wk 7	wk 9	wk 4	wk 5	wk 8	wk 10
wt clones	0	1	9	11	0	1	3	12
mt clones	26	9	1	1	22	13	8	9

C

	SM	Mes	Ing	SM	Mes	Ing
wt clones	12	9	10	3	6	3
mt clones	0	2	0	10	4	8

FIG. 2. Reversion in vivo from the CTL escape mutant molecular clone. Macaques C87072 (left panels) and C87134 (right panels) were inoculated with the mutant SIV molecular clone DNA. (A) Plasma viral loads after the inoculation. (B) Frequencies of the wild-type and mutant viral genomes in plasma. See the legend for Fig. 1B. (C) Frequencies of the wild-type and the mutant proviral genomes in LNs. See the legend for Fig. 1C.

that of the wild type, confirming the previous results obtained with rhesus macaques (17). After euthanasia of macaques at week 3, we extracted genomic DNA, by using the DNeasy kit (QIAGEN K.K., Tokyo, Japan), from the submandibular lymph node (LN), the mesenchymal LN, and the inguinal LN and subcloned proviral *gag* gene fragments amplified by nested PCR from the DNA into plasmids for sequencing. The mutant Gag216S was detected in most of the LNs, but the wild-type sequence was dominant in the proviral genomes in all the LNs (Fig. 1C).

Second, two cynomolgus macaques, C87072 and C87134, were inoculated intramuscularly with 5 mg of the mutant SIVmac239Gag216S molecular clone DNA alone. Plasma viremia was maintained until euthanasia of macaques at week 12 (C87072) or week 10 (C87134) after the inoculation (Fig. 2A). We subcloned viral *gag* gene fragments amplified from plasma RNA and found the wild-type Gag216L sequence at week 5, although it was undetectable at week 4 in both of the animals (Fig. 2B). In macaque C87072, 9 of 10 viral *gag* clones showed the wild-type Gag216L sequence at week 7 (Fig. 2B), and most of the proviral *gag* clones were the wild type in the submandibular LN, the mesenchymal LN, and the inguinal LN at week 12 (Fig. 2C). In macaque C87134, the ratio of wild-type to total viral *gag* clones was 3/11 at week 8 but 12/21 at week 10 (Fig. 2B). The wild-type Gag216L sequence was detected but was not predominant in the proviral *gag* clones from the LNs at week 10 (Fig. 2C). These results indicate that the mutant SIVmac239Gag216S proliferated in all the LNs but was outgrown by the wild-type virus.

We further examined virus-specific $CD8^+$ T-cell responses in macaques by flow cytometric analysis of antigen-specific interferon-γ (IFN-γ) induction as described previously (16, 17). In brief, peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cells infected with a vesicular stomatitis virus G-pseudotyped SIV for SIV-specific stimulation. Alternatively, PBMCs were cocultured with B lymphoblastoid cells pulsed with the $Gag_{206\text{-}216}$-epitope peptide for $Gag_{206\text{-}216}$-spe-

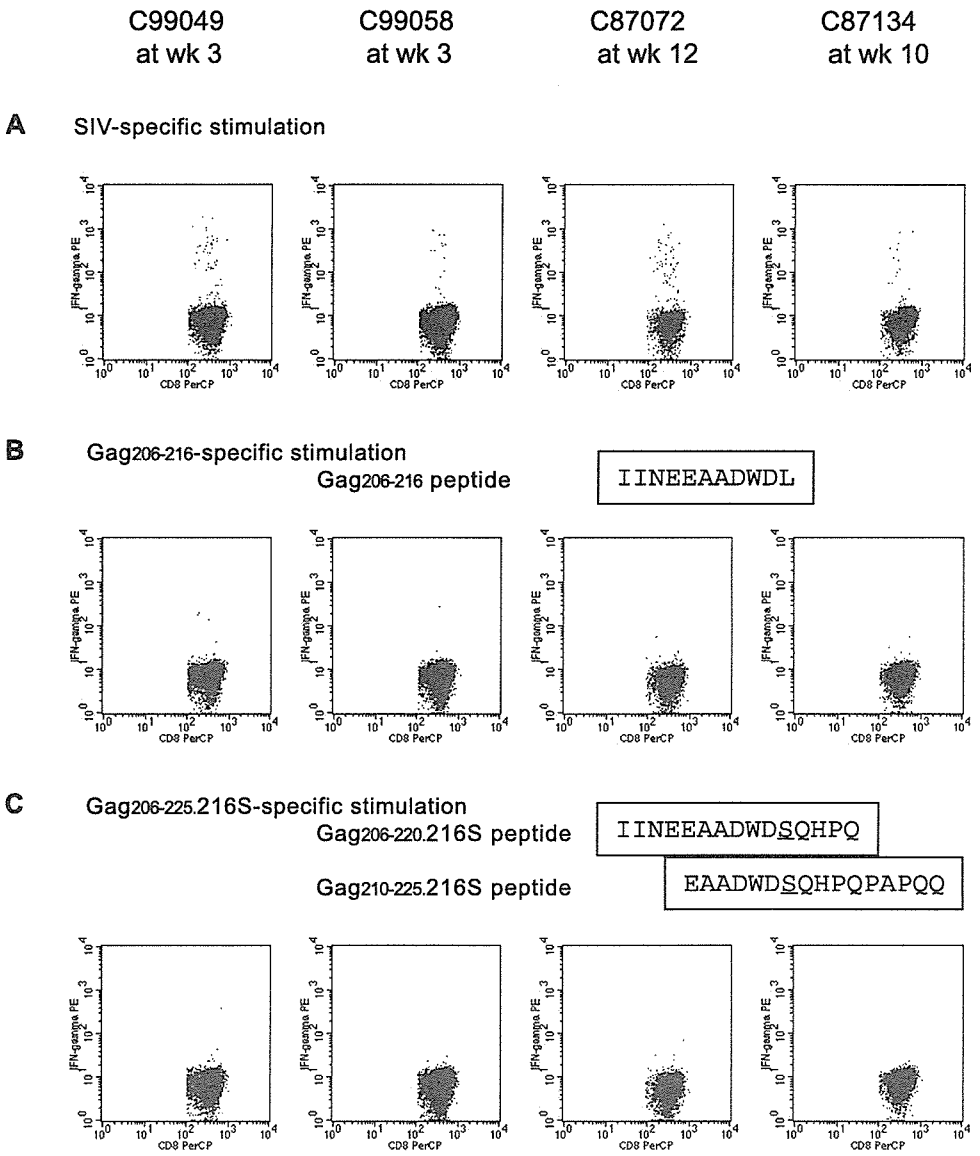


FIG. 3. SIV-specific $CD8^+$ T-cell responses in macaques. We examined IFN-γ induction after SIV-specific stimulation using vesicular stomatitis virus G-pseudotyped SIV (A), $Gag_{206-216}$-specific stimulation using $Gag_{206-216}$ peptide (B), or $Gag_{206-225}$.216S-specific stimulation using a mixture of $Gag_{206-220}$.216S and $Gag_{210-225}$.216S peptides (C). The aa sequences of $Gag_{206-216}$, $Gag_{206-220}$.216S, and $Gag_{210-225}$.216S peptides are indicated. Dot plots gated on $CD3^+CD8^+$ lymphocytes are shown. PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

cific stimulation or a mixture of the peptides with a 216S mutation corresponding to the 206th through 220th aa and the 210th through 225th aa in Gag ($Gag_{206-220}$.216S and $Gag_{210-225}$.216S peptides, respectively) for $Gag_{206-225}$.216S-specific stimulation. PBMCs derived from macaques C99049 at week 3, C99058 at week 3, C87072 at week 12, and C87134 at week 10 were subjected to this assay (Fig. 3). SIV-specific $CD8^+$ T-cell responses were detected but $Gag_{206-216}$-specific IFN-γ induction was undetectable, confirming no $Gag_{206-216}$-specific CTL responses in any of four animals. $Gag_{206-225}$.216S-specific $CD8^+$ T-cell responses were also undetectable, indicating that the predominance of the wild-type virus in these four macaques was not due to immune pressure exerted by the mutant-specific CTL recognizing an epitope with the mutant Gag216S sequence.

In the previous study (17), the Gag216S mutant virus escaping from $Gag_{206-216}$-specific CTL was rapidly selected in the vaccinees possessing the MHC-I haplotype *90-120-Ia* after SIVmac239 challenge. However, the CTL escape mutant with lower viral fitness was rapidly contained and became undetectable in plasma after week 5 postchallenge. The present study shows that this mutant SIV, which was rapidly contained in the vaccinees in the previous study, can replicate and is unable to be rapidly contained in naive macaques, leading to the appearance of the wild-type virus in the absence of $Gag_{206-216}$-specific CTL responses. This suggests the requirement of additional

adaptive immune responses as well as $Gag_{206-216}$-specific CTLs for containment of this CTL escape mutant virus with lower viral fitness.

Viral adaptation by escape mutations under CTL pressure and reversion after transmission to MHC-I-mismatched hosts have been indicated in immunodeficiency virus infection (2, 3, 7, 14). It has recently been shown that reversion by de novo mutation can really occur after challenge of macaques with a cloned SIV with CTL escape mutations (7). In that study, preparation of the challenge virus stock from a molecular clone DNA of the mutant SIV required viral replication in vitro for more than a week. In the present study, to see the reversion by de novo mutation only in vivo by deleting the in vitro replication process for virus stock preparation, we directly inoculated macaques with a molecular clone DNA of the mutant SIV. Our results show that the reversion by de novo mutation can really occur and be detected in 5 weeks after inoculation of the mutant molecular clone DNA. Thus, this is the first report describing the reversion in vivo from an inoculated, molecular proviral DNA clone of immunodeficiency virus with a CTL escape mutation.

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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-1_{DH12} chimeric Vpr; and HIV-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.

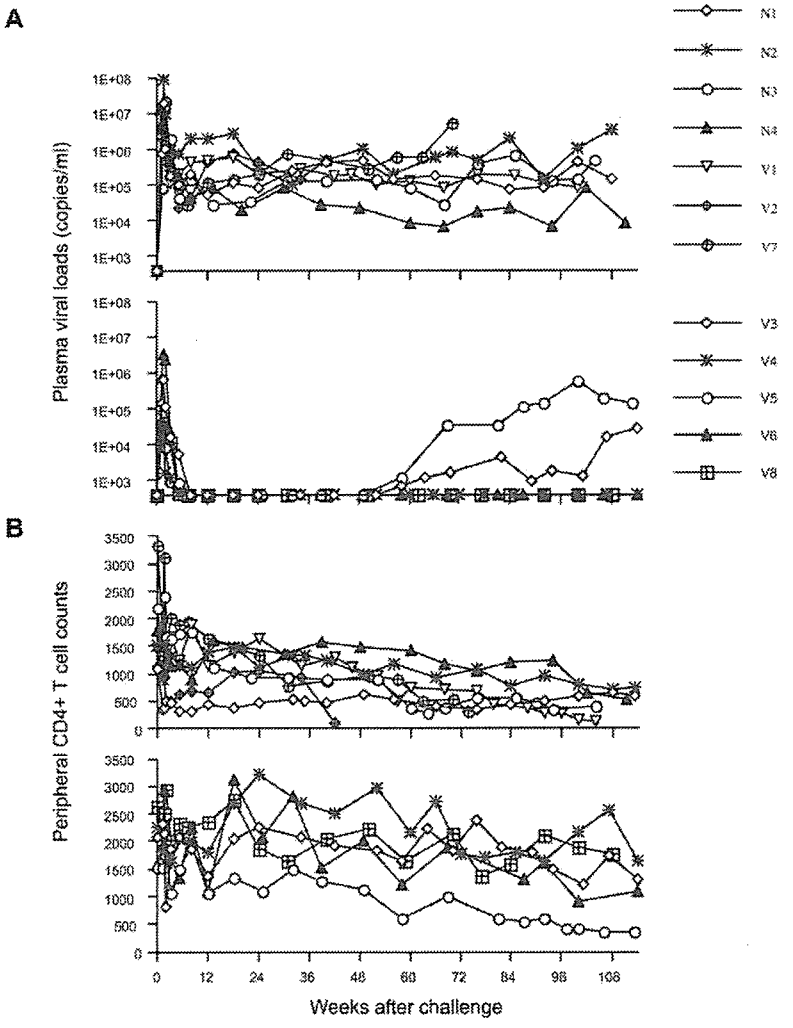
Viral mutations in the transient controllers. The previous study (28) showed rapid selection of CTL escape mutations in *gag* in all of the controllers (Table 1), indicating the importance of the CTL responses in the control of SIV replication. We then examined *gag* sequences to see if additional viral 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 Proviral DNA	7/10	L216S, D244E
		3/10	L216S, D244E, A373T
32	PBMC Proviral DNA	6/11	L216S, D244E, A373T
		5/11	L216S
58	Plasma Viral RNA	8/10	L216S, D244E, I247L, A312V, A373T
		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-

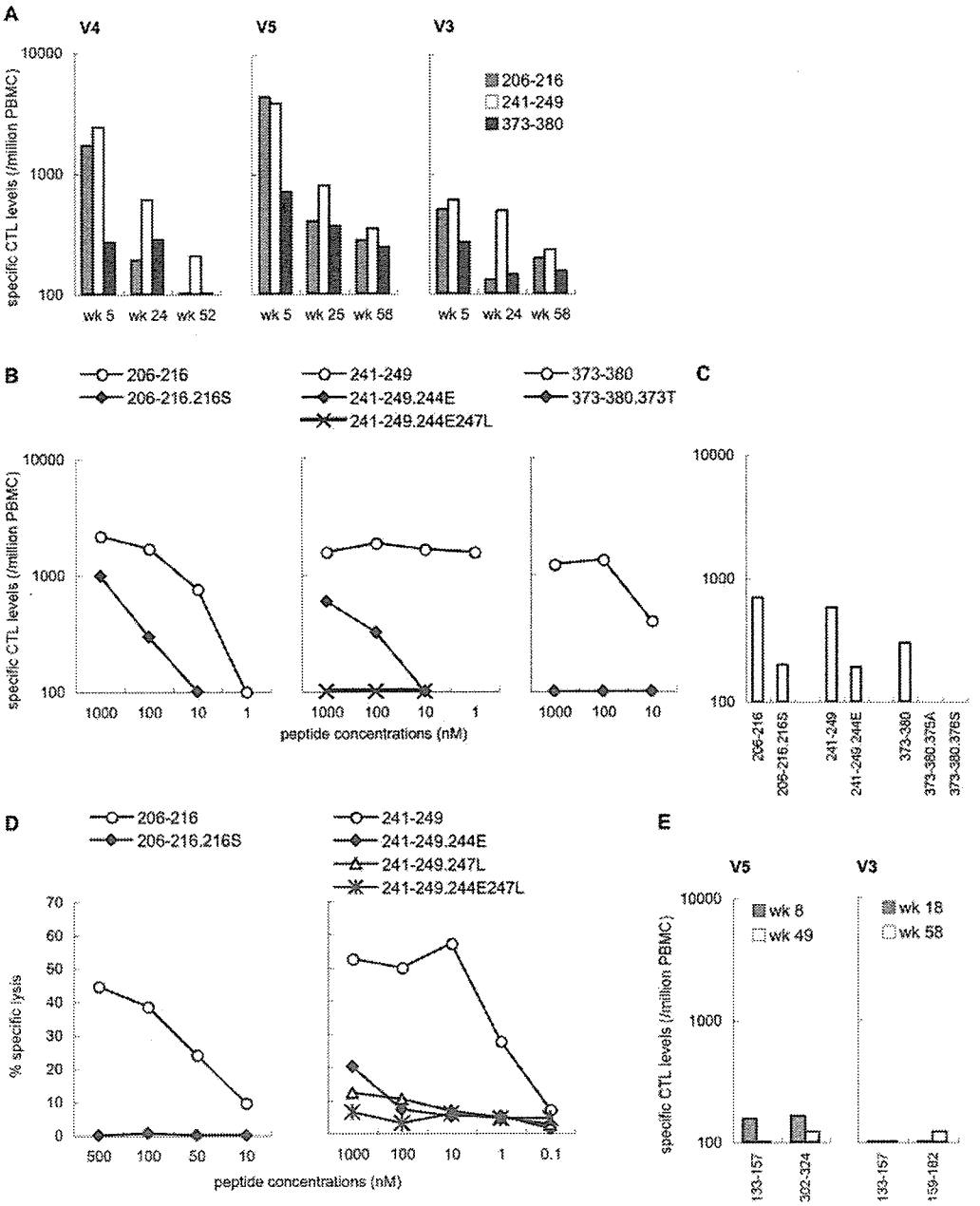


FIG. 2. CTL responses in the controllers (V4, V5, and V3) possessing MHC-I haplotype *90-120-Ia*. (A) $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{373-380}$-specific CTL levels in the macaques V4, V5, and V3. (B) IFN-γ induction in macaque V5 after stimulation with the wild-type or the 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

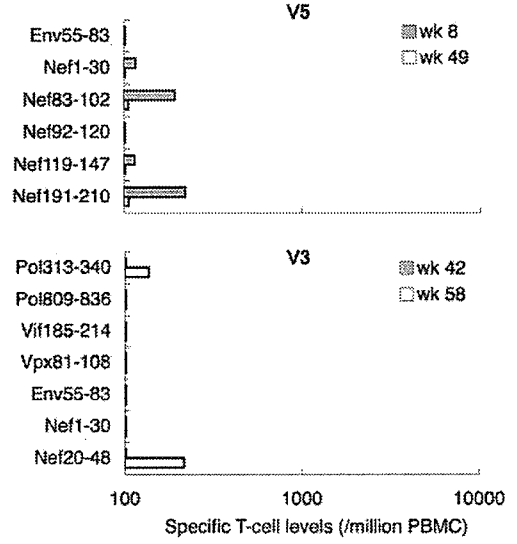


FIG. 3. Mutations in viral genomes encoding SIV proteins other than Gag. (A) Viral mutations in macaques V5 and V3. Dominant mutations leading to amino acid changes are shown. (B) CTL responses to the peptides corresponding to the region around the mutation sites. PBMCs derived from macaque V5 at week 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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Influence of Glycosylation on the Efficacy of an Env-Based Vaccine against Simian Immunodeficiency Virus SIVmac239 in a Macaque AIDS Model

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The envelope glycoprotein (Env) of human immunodeficiency viruses (HIVs) and simian immunodeficiency viruses (SIVs) is heavily glycosylated, and this feature has been speculated to be a reason for the insufficient immune control of these viruses by their hosts. In a macaque AIDS model, we demonstrated that quintuple deglycosylation in Env altered a pathogenic virus, SIVmac239, into a novel attenuated mutant virus (Δ5G). In Δ5G-infected animals, strong protective immunity against SIVmac239 was elicited. These HIV and SIV studies suggested that an understanding of the role of glycosylation is critical in defining not only the virological properties but also the immunogenicity of Env, suggesting that glycosylation in Env could be modified for the development of effective vaccines. To examine the effect of deglycosylation, we constructed prime-boost vaccines consisting of Env from SIVmac239 and Δ5G and compared their immunogenicities and vaccine efficacies by challenge infection with SIVmac239. Vaccination-induced immune responses differed between the two vaccine groups. Both Env-specific cellular and humoral responses were higher in wild-type (wt)-Env-immunized animals than in Δ5G Env-immunized animals. Following the challenge, viral loads in SIVmac239 Env (wt-Env)-immunized animals were significantly lower than in vector controls, with controlled viral replication in the chronic phase. Unexpectedly, viral loads in Δ5G Env-immunized animals were indistinguishable from those in vector controls. This study demonstrated that the prime-boost Env vaccine was effective against homologous SIVmac239 challenge. Changes in glycosylation affected both cell-mediated and humoral immune responses and vaccine efficacy.

Primate lentiviruses, human immunodeficiency viruses (HIVs), and simian immunodeficiency viruses (SIVs) share common genetic and biological properties. As SIVmac, originally isolated from macaques in primate research centers in the United States, causes AIDS in macaques with remarkable similarities to HIV type 1 (HIV-1) infection in humans, this AIDS monkey model has been utilized to study vaccine development and the pathogenesis of HIV infection (for reviews, see references 10, 14, 17, 43, and 47).

HIV/SIV infection in the host consists of two phases, the primary infection and chronic infection. During the primary infection, extensive viral replication and dissemination of the infection occur. In chronic infection, viral replication continues for a long period, eventually leading to AIDS. Due to the host immune response against the infection, these two phases are separated by a set point at which the viral load reaches its lowest level. The viral loads of the set point and chronic infection are inversely correlated with the control of SIV/HIV infection and predict disease progression (25, 31); however, it remains unclear which host responses determine the viral loads of the set point and chronic infection. Nevertheless, virus-specific immune responses have been implicated in the host's control of the infection. Cellular immunity, such as that shown by cytotoxic T lymphocytes (CTL) and helper T cells, has been reported to correlate with the control of HIV/SIV infection (for reviews, see references 2, 24, 28, and 39). The role of the neutralizing antibody (NAb) in the control of infection and the

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emergence of escape mutants has also been reported previously (7, 16, 51).

Despite these immune responses against HIV/SIV infection, humans and macaques fail to contain the infection due to the virus properties. HIV/SIV infects major target cells, such as $CD4^+$ T cells and macrophages, by binding viral envelope glycoproteins (Env) to cellular surface proteins and CD4 and chemokine receptors (CCR5, CXCR4, or others) on target cells (5, 32). Since viral entry consists of multiple steps (virion binding to these viral receptors, conformational change of Env, and fusion between the virion and the cellular membrane) and the critical parts of Env used in these steps are exposed only during each step, naturally generated antibodies are only partly effective in preventing HIV/SIV infection in their hosts (7, 8). Primary isolates can be neutralized to various degrees by HIV-infected patient serum but not by contemporaneous autologous samples. Consequently, escape mutants against preexisting NAb are selectively replicated (51). Thus, effective NAb is rarely induced in HIV/SIV infection (8, 10). This could partly explain the failure of Env-based vaccine trials against HIV-1 (8, 50).

The heavy glycosylation of Env is a unique feature of HIV/SIV that is distinctive from features of other enveloped viruses and is significantly related to their neutralization-resistant property (8, 29, 44). We therefore assumed that the insufficient immune containment of HIV/SIV might be due to heavy glycosylation in Env and that the removal of some glycans might allow the host to mount a protective immune response against the infection. Thus, we studied the influence of deglycosylation on the replication of SIVmac239 in a T-cell line and created a quintuple deglycosylation mutant of SIVmac239 (Δ5G), which has maximal removal of N-glycans at amino acid residues 79, 146, 171, 460, and 479 in Env and retains a replication capability similar to that of SIVmac239 in phytohemagglutinin-stimulated rhesus peripheral blood mononuclear cells (PBMCs) (36, 40). We then examined the infection of rhesus macaques with Δ5G; although Δ5G was replicated as extensively as SIVmac239 during the primary infection, the subsequent Δ5G infection was restricted to a level less than the detection sensitivity of a plasma viral load assay by 8 weeks postinfection (p.i.), in contrast to high chronic viral replication in SIVmac239 infection. Furthermore, an almost sterilizing immunity against SIVmac239 was induced in Δ5G-infected animals (36). Interestingly, another quintuple-deglycosylation-mutation strain with mutations at amino acid residues 146, 156, 184, 244, and 247 in Env was created (44) and was demonstrated to share common features with Δ5G in viral replication in animals and in functions as an attenuated vaccine (20). Since these two viruses share only one deglycosylation mutation and other mutations distributed differently in surface envelope protein gp120 (SU), these two studies suggest that heavily glycosylated Env determines the pathogenicity of HIV/SIV.

To dissect the mechanism for notable containment of Δ5G infection after primary infection, we hypothesized that the Env of Δ5G, a viral protein that differs from that in SIVmac239, might elicit protective immunity against SIVmac239, because deglycosylation in Env might alter antigenic properties such as B-cell and T-cell epitopes and enhance the protective immunity against SIVmac239. For this purpose, we immunized animals with Env of Δ5G (Δ5G Env) or Env of SIVmac239 (the wild type; wt Env), and examined the effect of these vaccinations against SIVmac239 infection.

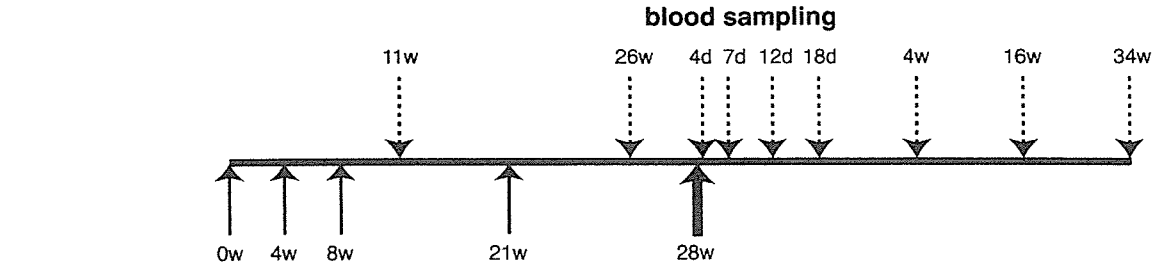
MATERIALS AND METHODS

Generation of SU DNA vaccines. DNA vaccine plasmids expressing SIV mac239 SU or Δ5G SU, pJWSUmac239 and pJWSUmacΔ5G, were constructed using the expression vector pJW4303 (45). To produce secreted SU efficiently, the native signal sequence in the SIVmac239 SU gene was replaced with the human tissue plasminogen activator signal in plasmid pJW4303, and a termination codon was created at the cleavage site for SU transmembrane (TM) protein (9). An SIVmac239 SU or Δ5G SU DNA sequence was amplified with a pair of primers, SUmacA (5′-TGT**GCTAG**CTATGTCACAGTCTTTTATGGTGTAC-3′) and SUmacB (5′-CC**AGGATCC**TATTACCTCTTCACATCTGTGGGGG C-3′). The SUmacA primer consisted of nucleotides (nt) 6923 to 6955 of the SIVmac239 sequence (GenBank accession number M33262) and the boldface nucleotides, which were changed to create a NheI site; primer SUmacB consisted of nt 8412 to 8381 and the boldface nucleotides, which were changed to create a BamHI site, and the underlined nucleotides, which generated tandem termination codons. The PCR-amplified fragments were digested with NheI and BamHI and cloned into the NheI- and BamHI-digested eukaryotic expression vector pJW4303 to yield pJWSUmac239 and pJWSUmacΔ5G. These plasmids were prepared using a Plasmid Mega kit (QIAGEN, Tokyo, Japan).

Generation of Env vaccinia vaccines. Recombinant vaccinia viruses expressing Env of SIVmac239 or Δ5G, WRvvmac239 or WRvvΔ5G, respectively, were constructed using a vaccinia virus WR strain (WRvv) as described previously (15). To excise the entire coding region of the *env* gene from the cloned SIV plasmid, BamHI and SmaI sites were introduced by in vitro mutagenesis at 5′- and 3′-end-flanking sites of the *env* gene, respectively. Primer B-6808 (5′-GAA AGAGAAGAAGGATCCCGAAAAAGG-3′) consisted of nt 6796 to 9822 and the underlined mutations of the BamHI site; S-9537 (5′-TATGAATACTCCC GGGAGAAACCC-3′) consisted of nt 9527 to 9550 and the underlined mutations of the SmaI site. DNA fragments containing the *env* gene of SIVmac239 or Δ5G were isolated by digesting the mutated plasmids with BamHI and SmaI and were cloned into the SmaI- and BamHI-digested vaccinia virus vector plasmid pNZ68K2. To transfer the *env* gene from a recombinant plasmid to WRvv, the standard homologous recombination method using CV-1 cells was performed. Env expression in the recombinant vaccinia virus was confirmed by immunoprecipitation. The function of Env was confirmed by CD4- and CCR5-dependent fusion activity. The recombinant Env-expressing vaccinia viruses obtained were propagated and titrated in CV-1cells. The two recombinant viruses were propagated with similar kinetics in CV-1 cells.

Expression of SU-expressing plasmids and Env-expressing vaccinia virus in vitro. CV-1 cells were transfected with equal amounts of the following SU-expressing plasmids: pJWSUmac239, pJWSUmacΔ5G, or the vector pJW4303. Secreted SU metabolically labeled with ^{35}S protein labeling mix (PerkinElmer, Boston, MA) in culture supernatant was concentrated, immunoprecipitated with plasma from SIVmac239-infected monkeys, and then analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) as described previously (40). To examine Env-expressing vaccinia viruses, CV-1 cells were infected with WRvvmac239, WRvvΔ5G, or WRvv at a multiplicity of infection of 10, metabolically labeled with ^{35}S protein labeling mix overnight, lysed, immunoprecipitated with plasma from SIVmac239-infected monkeys, and then analyzed by SDS-PAGE as described for the expression of SU-expressing plasmids.

Animals, immunization, and challenge. Twelve juvenile rhesus macaques from Myanmar or Laos that were seronegative for SIV, simian T-cell lymphotropic virus, B virus, and type D retroviruses were used. As the polymorphism of major histocompatibility complex (MHC) genes influenced cellular immune responses against SIV/HIV infection, MHC II haplotypes and alleles of the macaques were determined (data not shown). All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare stated by the National Institute of Infectious Diseases. As shown in Fig. 1, the 12 animals were divided into three immunization groups of four animals each: the SIVmac239 (wt)-Env immunization group (Mm0005, Mm0007, Mm0010, Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, Mm0009), and the vector control immunization group (Mm0004, Mm0006, Mm0008, Mm0011). All animals were inoculated with 1 mg of plasmid DNA in 1 ml of saline, one into each quadriceps femoris at 0, 4, and 8 weeks after the initial prime immunization (weeks p.p.). The boost consisted of 5×10^7 PFU of vaccinia virus in 1 ml of phosphate-buffered saline (PBS), administered in two 0.1-ml intradermal inoculations, one into the skin of each femur, and two 0.4-ml inoculations, one into each quadriceps femoris at 21 weeks p.p. All animals were



Vaccine Groups:	DNA	Vaccinia boost	SIV challenge
wt-Env (n=4)	pJWSUmac239 (SU: gp120)	WRvvENVmac239 (Env: gp160)	SIVmac239
Δ5G-Env (n=4)	pJWSUΔ5G (SU: gp120)	WRvvENVΔ5G (Env: gp160)	SIVmac239
vector control (n=4)	pJW4303	WRvv	SIVmac239

FIG. 1. Outline of immunization, challenge infection, and blood sampling. Twelve juvenile rhesus macaques were divided into three immunization groups of four animals each: the wt-Env immunization group (Mm0005, Mm0007, Mm0010, and Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, and Mm0009), and the vector control immunization group (Mm0004, Mm0006, Mm0008, and Mm0011). Animals were inoculated with a DNA vaccine (pJWSUmac239 for the wt-Env vaccine group, pJWSUΔ5G for the Δ5G Env vaccine group, and pJW4303 for the vector control group) at 0, 4, and 8 weeks p.p. The boost vaccine consisted of vaccinia virus (WRvvENVmac239 for the wt-Env vaccine group, WRvvENVΔ5G for the Δ5G Env vaccine group, and the WR strain for the vector control group) administered at 21 weeks p.p. All animals were challenged with 10 $TCID_{50}$ of SIVmac239 intravenously at 28 weeks p.p. w, weeks; d, day.

challenged with 10 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 intravenously at 28 weeks p.p.

Viral load measurement. To monitor SIV infection, the plasma viral load was measured by the real-time-PCR method described previously (36). Viral RNA was isolated from plasma from the infected animals using a commercial viral-RNA isolation kit (PE Applied Biosystems, Urayasu, Japan). SIV *gag* RNA was amplified and quantified using a commercial RNA reverse transcription (RT)-PCR kit (TaqMan EZ RT-PCR; PE Applied Biosystems) with the two *gag* primers, namely, the forward primer 1224F (5′-AATGCAGAGCCCCAAGAA GAC-3′), the reverse primer 1326R (5′-GGACCAAGGCCTAAAAAACCC-3′), and TaqMan probe 1272T (6-carboxyfluorescein-5′-ACCATGTTATGGCC AAATGCCCAGAC-3′-6-carboxymethylrhodamine). Purified viral RNA (10 μl) was reverse transcribed and amplified in a MicroAmp optical 96-well reaction plate (PE Applied Biosystems) according to the manufacturer's instructions and with the following thermal cycle conditions: 1 cycle of three sequential incubations (50°C for 2 min, 60°C for 30 min, and 95°C for 5 min) and then 50 cycles of amplification (95°C for 5 s, 62°C for 30 s) in a 7000 Prism sequence detection system (PE Applied Biosystems). In vitro RNA transcripts were quantified by optical density at 260 nm (OD_{260}) measurement and branched DNA assay for SIV viral RNA (Bayer Diagnostics, Tarrytown, N.Y.). RNA equivalent to 10 to 10^7 copies per reaction was used as the standard for each assay. The detection sensitivity of plasma viral RNA using this method was 1,000 copies/ml.

Flow cytometry. CD4 depletion was monitored by measuring the percentage of $CD4^+$ T cells, memory cells (CD29 high $CD4^+$) T cells (48) in PBMCs. PBMC samples were purified from a citrate anticoagulant containing blood using standard Ficoll-Hypaque gradient centrifugation. For flow cytometry, 2×10^5 PBMCs were reacted with fluorescein isothiocyanate or phycoerythrin-labeled antibodies (anti-human CD4, Nu-Th/I [Nichirei, Tokyo, Japan]; anti-human CD8, Leu2a [Becton Dickinson, San Jose, CA]; anti-human CD29, 4B4 [Coulter, Miami, FL]; anti-monkey CD3, FN-18 [Biosource, Camarillo, CA]; and anti-human CD20, Leu16 [Becton Dickinson, San Jose, CA]) as previously described (36, 37, 48).

Peptides. Overlapping peptides were synthesized by Emory University, Microchemical Facility, Winship Cancer Center (Atlanta, GA.). All SIVmac239 viral proteins except Env, Gag, Pol, Vif, Vpr, Vpx, Tat, Rev, and Nef were covered by consecutive 20-mer peptides overlapped by 12 amino acids. Env of SIVmac239 was covered by 72 consecutive 25-mer peptides overlapped by 13 amino acids. Peptides were dissolved in PBS with 10% dimethyl sulfoxide (Sigma Chemical, St. Louis, Mo.).

rSeV. Recombinant Sendai viruses (rSeV) expressing SIVmac239 Gag, SU, or Δ5G SU were used to infect herpesvirus papio-transformed B-lymphoblastoid cell lines (B-LCLs) to prepare autologous B-LCLs presenting these viral antigens. rSeV Gag expressing unprocessed SIVmac239 Gag and p55 (22, 23) and rSeV SU and rSeV/Δ5G SU expressing wt SU and Δ5G SU were constructed as described previously (52) and were also used to infect autologous B-LCLs.

Anti-SIV ELISA. A 1:100 dilution of each plasma sample in PBS (pH 7.4) containing a blocking reagent (Dainippon Seiyaku, Osaka, Japan) was assayed for SIV-specific antibody by using a standard enzyme-linked immunosorbent assay (ELISA) technique with 96-well plates precoated with SIVmac239 virion lysate. The OD_{492} was measured using a microplate reader (range of absorbance with linearity, 0 to 3.0; Tecan Japan, Tokyo, Japan) and utilized as a relative measurement of the antibody titer.

ELISPOT assay. Virus-specific $CD4^+$ T cells and $CD8^+$ T cells in PBMCs were measured using a monkey γ-IFN ELISPOT assay kit (U-CyTech, Utrecht, The Netherlands).

Cryopreserved PBMCs were thawed and cultured overnight in R-10 medium (RPMI 1640 [Sigma] supplemented with 10% heat-inactivated, defined fetal bovine serum [HyClone, Logan, Utah], 55 μM 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin). PBMCs were subjected to the depletion of $CD4^+$ cells with magnet beads coated with anti-human CD4 Ab (Dynal ASA, Oslo, Norway) or subjected to the depletion of $CD8^+$ cells with magnet beads coated with anti-human CD8 Ab (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion of $CD4^+$ or $CD8^+$ cells from PBMCs was confirmed by flow cytometry. Using this depletion method, more than 95% of $CD4^+$ or CD8 cells were removed from PBMCs. These PBMCs were used for ELISPOT assay for virus-specific $CD8^+$ T cells and virus-specific $CD4^+$ T cells. Virus-specific stimulation of T cells was performed with autologous B-LCLs pulsed with pooled peptides for Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef or B-LCLs infected with an rSeV for Gag, wt Env, and Δ5G Env. B-LCLs were incubated with pooled peptides corresponding to each viral protein at a final concentration of 2 μg/ml or infected with rSeV at a multiplicity of infection of 10 at 37°C overnight. Peptide-pulsed or infected B-LCLs were inactivated with long-wave UV irradiation (19) in the presence of 10 μg/ml psoralen (Sigma) for 10 min at a distance of 3.5 cm from a UV light, washed three times with R-10, and then used as stimulators in an ELISPOT assay. $CD4^+$ or $CD8^+$ cell-depleted PBMCs were cultured with these stimulators in an anti-γ-IFN Ab-coated ELISPOT plate (U-CyTech) overnight according to the protocol for the kit. Spots on the ELISPOT plate were imaged using an Olympus model SZX12 microscope

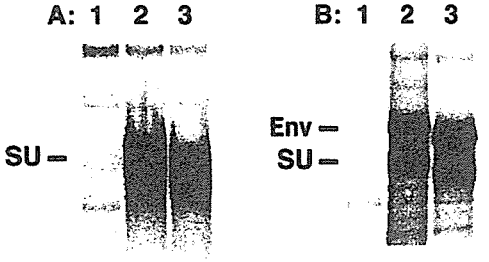


FIG. 2. Expression of SU and Env by SU-expressing DNA vaccines and Env-expressing vaccinia viruses. A: SU secreted in supernatant from CV-1 cells transfected with SU-expressing plasmids. Lane 1, pJW4303 vector; lane 2, pJWSUmac239; lane 3, pJWSUmacΔ5G. B: Env in cell lysates of CV-1 cells infected with recombinant vaccinia viruses. Lane 1, WRvv; lane 2, WRvvmac239; lane 3, WRvvΔ5G.

(Olympus, Tokyo, Japan) equipped with a digital camera, PDMCIe/OL (Polaroid, Cambridge, MA), and analyzed using a personal computer with MAC SCOPE version 2.61 (Mitani Corporation, Toyama, Japan). The results were calculated as numbers of spot-forming cells (SFC) per million PBMCs after subtraction of the background.

Neutralization assay. The original protocol of this neutralization assay was reported by Means et al. (29). Plasma that was heat inactivated at 56°C for 30 min was serially diluted and incubated with a fixed concentration of SIVmac239, Δ5G, or a macrophage-tropic SIV, 239/envMERT, at room temperature for 1 h. CEMx174/SIVLTR-SEAP cells were added to the mixture and then incubated at 37°C for 3 days. Secreted alkaline phosphatase activity in the culture supernatant was measured using a Phospha-Light System (Applied Biosystems). Chemiluminescence was detected with a Wallac Microbeta plate reader.

Statistical analysis. Statistical analysis was based on the Mann-Whitney test and performed using GraphPad Prism 4.0 software.

RESULTS

Experimental design. We adopted a DNA prime-vaccinia virus boost regimen to immunize rhesus macaques with wt Env or Δ5G Env as shown in Fig. 1. Twelve macaques were immunized at 0, 4, and 8 weeks after the initial prime immunization (weeks p.p.) with one of three different DNA expression plasmids (n = 4): pJWSUmac239 expressing SU of SIVmac239, pJWSUΔ5G expressing SU of Δ5G, or the vector pJW4303. At 21 weeks p.p., all animals were boosted with recombinant WR vaccinia viruses expressing the respective Env proteins: vaccinia virus expressing Env of SIVmac239, vaccinia virus expressing Env of Δ5G, or vaccinia virus (Fig. 1).

Expression of SU DNA plasmids and Env vaccinia viruses in vitro and in animals. Although Δ5G replicated similarly to wild-type SIVmac239 in animals (36), quintuple deglycosylation might affect the expression of SU in a plasmid vector and the expression of Env in the vaccinia virus vector. Thus, we examined the expression of these vaccines in CV-1 cells. SU expressions in the wild-type plasmid (pJWSUmac239) and in the deglycosylated SU plasmid (pJWSUmacΔ5G) were at similar levels (Fig. 2A). The expression and processing of Env in the wild type (WRvvENVmac239) and in the deglycosylated Env mutant vaccinia virus (WRvvENVΔ5G) were also at similar levels (Fig. 2B). The reduced molecular size of the proteins due to deglycosylation was confirmed by PAGE (Fig. 2). As the amount of secreted SU in the supernatant by DNA transfection was comparable to that of Env in the cell lysate from CV-1 cells infected with WRvvEnv, a high expression of SU was achieved in a *rev*-independent manner by the pJW403 expression plasmid as described previously (9).

The expression of Env vaccines in the immunized animals was indirectly estimated by Env-specific antibody responses measured by a peptide ELISA using overlapping Env peptides. Env peptide-specific Ab was detected from 11 weeks p.p. after immunization with DNA vaccines, whereas there was no significant difference in the titers and the specificity of the responses between the two vaccine groups (data not shown), suggesting similar amounts of Env expressed in animals immunized with either Env vaccine. To examine the protective effect of the Env vaccines, all animals were challenged with 10 $TCID_{50}$ of SIVmac239 intravenously at 28 weeks p.p.

Cellular immune responses elicited by Env vaccines. The DNA prime-vaccinia virus boost regimen has been used in many studies, has successfully induced a high frequency of virus-specific $CD8^+$ T cells in macaques, and has conferred protective immunity against chimeric simian/human immunodeficiency virus (SHIV) (3, 27, 45). We therefore examined the vaccine-induced Env-specific T-cell responses by IFN-γ ELISPOT assay. Since deglycosylation in Env might change T-cell epitopes in SIVmac239, we measured the wt-SU and Δ5G SU-specific T-cell response by using autologous B-LCLs infected with recombinant Sendai viruses expressing either wt SU and/or Δ5G SU, respectively.

Although there was a tendency for more ELISPOT-positive cells to be observed by homologous SU than heterologous SU, comparable results were obtained by both assays (Fig. 3A and B). As vaccinated animals were challenged with SIVmac239, the results from the wt-SU assay were subsequently used to assess the SU-specific immune response. Immunization with the DNA vaccine induced only marginal SU-specific $CD8^+$ T cells or $CD4^+$ T cells at 11 weeks p.p.; however, boost immunization with recombinant WR vaccinia virus significantly increased SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs at 26 weeks p.p. (Fig. 3A, B, and C). Notably, SIVmac239 Env (wt Env) induced twofold more SU-specific CD8 T cells (mean, 770 SFC per million PBMCs; range, 540 to 880) responding to wt SU than Δ5G Env (mean, 320; range, 110 to 400) (P = 0.029) (Fig. 3A and C). Similarly, twofold more SU-specific $CD4^+$ T cells were observed in wt-Env vaccinees (mean, 1,260; range, 840 to 1,710) than in Δ5G Env vaccinees (mean, 680; range, 150 to 1,260) at 26 weeks p.p. (P = 0.11) (Fig. 3B and C). Thus, a twofold-greater number of both SU-specific $CD4^+$ T cells and $CD8^+$ T cells were induced in SIVmac239 Env vaccinees than in Δ5G Env vaccinees at 26 weeks p.p. In vector controls, only negligible SU-specific $CD4^+$ T cells and $CD8^+$ T cells were detected in PBMCs at 26 weeks p.p. (Fig. 3A and B).

Humoral immune response elicited with Env vaccines. The anti-Env Ab titer was examined by SIVmac239 virion lysate ELISA. Anti-SIV Ab was detected in both wt-Env vaccinees and Δ5G Env vaccinees after an rVV boost (Fig. 4) (26 weeks p.p.). Anti-SIV Ab titers were comparable between the two vaccine groups.

Next, we examined the NAb against either SIVmac239, Δ5G, or a macrophage-tropic mutant, 239env/MERT (33, 35), in the two vaccine groups. Macrophage-tropic SIVs were highly susceptible to neutralization by plasma from most SIV-infected macaques (29), whereas SIVmac239 was highly resistant to neutralization as were most clinical isolates of HIV-1

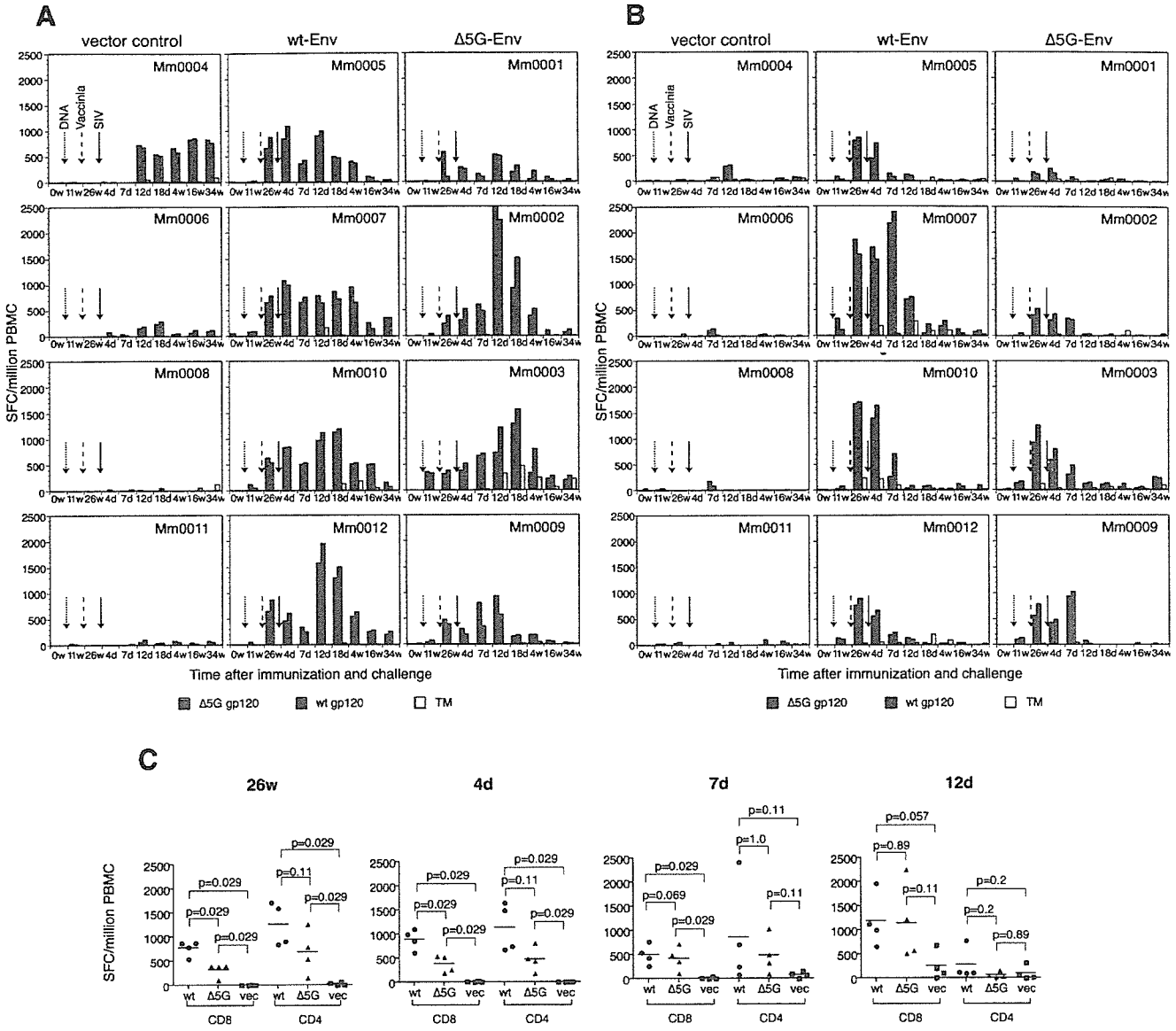


FIG. 3. Env-specific $CD4^+$ T-cell and $CD8^+$ T-cell responses in 12 macaques. A: Env-specific $CD8^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. B: Env-specific $CD4^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results are colored as follows: Δ5G SU-specific T cells (red), wt-SU-specific T cells (green), and TM-specific T cells (yellow). Arrows with a dotted line, arrows with broken line, and arrows with a solid line indicate the time of the third DNA vaccination at 8 weeks p.p., the time of the vaccine boost at 21 weeks p.p., and the time of SIVmac239 challenge at 28 weeks p.p., respectively. C: Comparison of SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs among the wt-Env vaccine group, the Δ5G Env vaccine group, and the vector control group at 26 weeks p.p. and 4, 7, and 12 days p.i. The numbers of SFC responding to SIVmac239 SU were used to compare the effects of the two vaccines. w, weeks; d, days.

(21, 29, 30). Plasma at 26 weeks p.p. from all immunized animals failed to neutralize not only SIVmac239 but also a multiple-deglycosylation-mutation strain, Δ5G (Table 1); in contrast, these plasma specimens did neutralize 239env/MERT. Furthermore, a marked difference was observed between the two vaccine groups. The NAb titer in the wt-Env vaccine group was eightfold higher than in the Δ5G Env vaccine group (Table 1). The difference of this immune response between the two vaccine groups was significant ($P = 0.029$).

SIV replication in Env-immunized animals. As described above, wt-Env vaccine and Δ5G Env vaccine induced different magnitudes of virus-specific cellular and humoral immunity in macaques. To examine the effect of the two vaccines, we challenged the vaccinated animals with SIVmac239. Viral loads in vector controls were mostly consistent with our previous results with SIVmac239-infected rhesus macaques (36, 48). The mean peak viral load at 2 weeks p.i. was 1.4×10^7 copies/ml, with a range of 0.5×10^7 to 2.2×10^7 copies/ml. Viral loads in chronic infection diverged into two patterns (Fig. 5A). Subsequent to the set point at 20 weeks p.i., the viral loads in three animals increased more than 10^4 copies/ml. In contrast, viral loads in one animal (Mm0011) remained as low as 1,000 copies/ml up to 45 weeks p.i.

Compared with the vector controls, viral loads in wt-Env

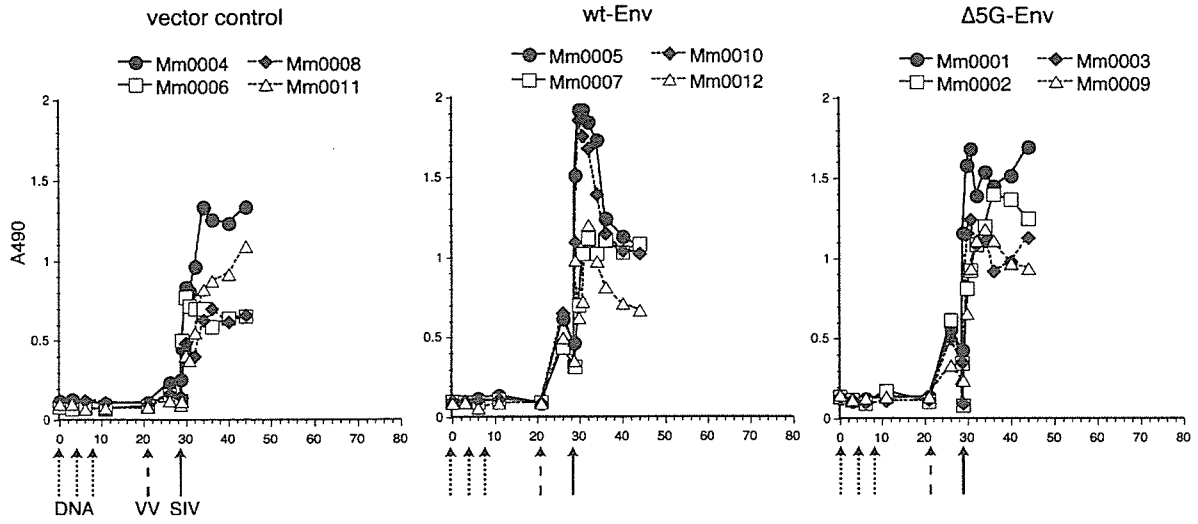


FIG. 4. Humoral immune response during immunization and after challenge infection. The OD_{492} was used as a relative measurement of anti-SIV ELISA antibody titer.

vaccinees were markedly reduced (Fig. 5B). Peak viral loads at 2 weeks p.i. (mean, 1×10^6 copies/ml; range, 0.8×10^6 to 1.2×10^6 copies/ml) were 1-log lower than those in the vector controls. Furthermore, viral loads decreased to as low as 1,000 copies/ml by 8 to 20 weeks p.i., remaining low until autopsy at 45 weeks p.i.

Unexpectedly, viral loads in the Δ5G Env vaccine group resembled those in vector controls (Fig. 5C). Peak viral loads (mean, 2.4×10^6 copies/ml; range, 0.9×10^6 to 4.2×10^6 copies/ml) were slightly lower than those in vector controls. Set points and viral loads in the chronic phase were similar to those of vector controls.

In summary, as shown by the mean viral loads in primary and chronic infection (Fig. 5D) and statistical analysis (Fig. 5E), the effects of vaccination differed between the wt-Env vaccine and Δ5G Env vaccine. In the effect on primary infection (up to 6 weeks p.i.), wt-Env vaccination decreased viral loads more extensively and significantly than Δ5G Env vaccination (P = 0.029 versus P = 0.057); however, in chronic infection (viral loads after 8 weeks p.i.), significant reductions in viral loads compared with those in vector controls were seen only in the wt-Env vaccine group and not the Δ5G Env vaccine group (Fig. 5E). Collectively, wt-Env vaccination induced significantly effective immunity to control SIVmac239 infection, whereas Δ5G Env vaccination induced a marginal effect seen only in primary and not in chronic infection.

TABLE 1. Neutralizing-antibody titers in the vaccinated macaques at 26 weeks p.p.

Vaccine	Animal	Neutralizing-antibody titer[a]			Mean[b]
		SIVmac239	Δ5G	239/envMERT	
wt-Env	Mm0005	<20	<20	800	
	Mm0007	<20	<20	400	
					400
	Mm0010	<20	<20	400	
	Mm0012	<20	<20	200	
Δ5G-Env	Mm0001	<20	<20	100	
	Mm0002	<20	<20	20	
					50
	Mm0003	<20	<20	100	
	Mm0009	<20	<20	50	

[a] Reciprocal of the dilution of plasma giving 50% inhibition of SIV replication.
[b] The difference in NAb levels between the two vaccine groups was significant (P = 0.0029).

$CD4^+$ T-cell subsets in PBMCs. CD4 cell depletion is a primary manifestation indicating immune disorder in HIV/SIV infection. As CD4 depletion results from HIV/SIV infection in lymphatic tissue, it correlates with the extent of viral replication. Accordingly, viral loads were correlated mostly with CD4 depletion (Fig. 5 and 6A). Despite fluctuations due to immunizations and the challenge infection, the percentage of $CD4^+$ T cells in wt-Env-immunized animals in the chronic phase recovered to the levels at the initiation of the experiment. By contrast, in vector controls and Δ5G Env vaccinees, the percentage of $CD4^+$ T cells decreased in the chronic phase. Among them, an extensive decrease in $CD4^+$ T cells occurred in animals with high viral loads in the chronic phase (Mm0001, Mm0008, and Mm0009) (Fig. 5 and 6A). However, in the other animals, the levels of $CD4^+$ T cells remained as before the challenge (Mm0003, Mm0011).

A subset of $CD4^+$ CD29 high cells, approximately corresponding to memory $CD4^+$ T cells, is useful for diagnosing a deterioration in the immune function in animals with AIDS (26, 38, 48). Although this parameter usually correlates with the percentage of $CD4^+$ T cells, remarkable differences were noted between two Env vaccine groups after the challenge infection. First, all animals in the wt-Env vaccine group showed an increased percentage of this subset in the chronic phase (Fig. 6B). Second, three of the Δ5G Env vaccinees had a marked decrease after the challenge infection (Mm0001, Mm0002 and Mm0009), whereas the remaining animal (Mm0003) showed an increased percentage of this subset. In

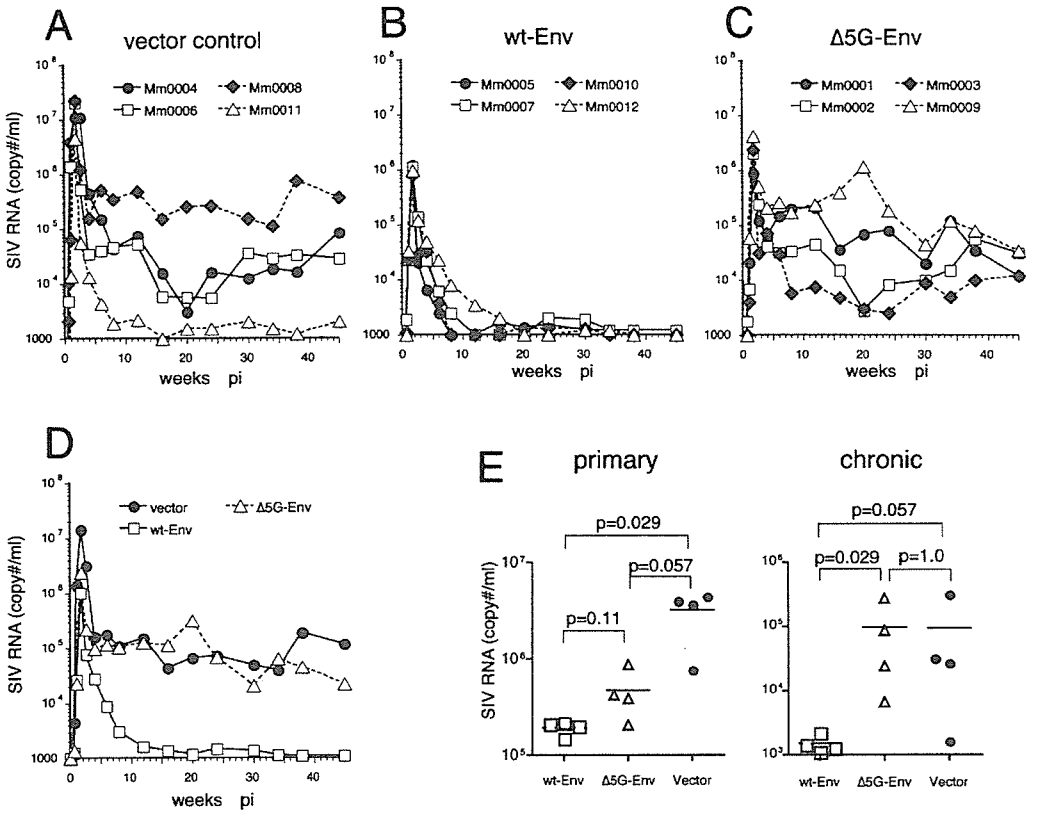


FIG. 5. Plasma viral loads after SIVmac239 challenge infection. Plasma viral load was measured by real-time PCR with a detection limit of 1,000 copies/ml. A: wt-Env vaccine group; B: Δ5G Env vaccine group; C: vector controls; D: comparison of viral loads among three groups; E: comparison of viral loads during the primary infection (5 days to 6 weeks p.i.) and chronic infection (8 weeks to 45 weeks p.i.) among three groups. Viral load was determined by averaging over a period of time.

vector controls, this subset remained in the range before the challenge infection in all animals but one (Fig. 6B).

Env-specific-T-cell immunity after the challenge infection. The magnitude of Env-specific T cells after the challenge infection is assumed to be influenced not only by vaccination but also by viral replication. Namely, SU-specific T cells at 4 days p.i. and those at 12 days p.i. were likely influenced by the former and the latter respectively. The magnitudes of SU-specific $CD4^+$ T cells and $CD8^+$ T cells at 4 days p.i. were comparable to those before challenge at 26 weeks p.p. (Fig. 3A and B); therefore, twofold-more SU-specific $CD8^+$ T cells and $CD4^+$ T cells were present in wt-Env vaccinees than in Δ5G Env vaccinees up to 4 days p.i. (Fig. 3C). However, this difference in the magnitudes of SU-specific $CD8^+$ T and $CD4^+$ T cells was not sustained at 7 and 12 days p.i. (Fig. 3C). Present with robust viral replication in primary infection, SU-specific $CD4^+$ T cells immediately decreased to an undetectable level at 12 days p.i. In contrast, SU-specific $CD8^+$ T cells increased (Fig. 3A and B). Subsequently, SU-specific $CD8^+$ T cells gradually decreased to very low or undetectable levels by 34 weeks p.i. (Fig. 3A). Thus, vaccine-induced SU-specific $CD8^+$ T and $CD4^+$ T cells were sustained only for a short period of time after challenge infection in both Env vaccine groups.

SIV-specific T-cell immunity after challenge infection. Despite an Env vaccination, robust SIV infection occurred shortly after the challenge infection (Fig. 5B and C). Consequently, SIV-specific $CD8^+$ T cells and $CD4^+$ T cells were elicited not only in vector controls but also in Env vaccine groups (Fig. 7A and B). To examine the effect of these SIV-specific T cells on the control of SIV infection, all animals were divided into SIV infection-controlled (controlled) and SIV infection-uncontrolled (uncontrolled) animals. Viral loads in chronic infection and the percentage of $CD4^+$ cells in PBMCs were used to classify the animals as controlled or uncontrolled (Fig. 6A). All animals in the wt-Env vaccine group, Mm00011 in vector controls, and Mm0003 in the Δ5G Env vaccine group were grouped as control animals. The remaining animals, Mm0004, Mm0006, and Mm0008 in vector controls and Mm0001, Mm0002, and Mm0009 in the Δ5G Env vaccine group were grouped as uncontrolled animals. Notably, SIV-specific $CD4^+$ T cells as well as the percentage of $CD4^+$ CD29H cells remained high in the chronic phase in controlled animals (Fig. 7B and 6B, respectively).

Although overall SIV-specific $CD8^+$ T cells were high in Env-vaccinated controlled animals, such correlation was not seen in vector controls grouped as uncontrolled animals (Fig. 7A). Therefore, to examine the relevance of virus-specific T cells to the control of SIV infection, the magnitudes of every viral-protein-specific T cell in controlled and uncontrolled animals were compared. As shown in Fig. 7C, Gag-specific $CD8^+$ T cells and $CD4^+$ T cells, and Tat/Rev-specific $CD4^+$ T cells