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	8/10	V145A, L216S, D244E, P376S
	Viral RNA	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-

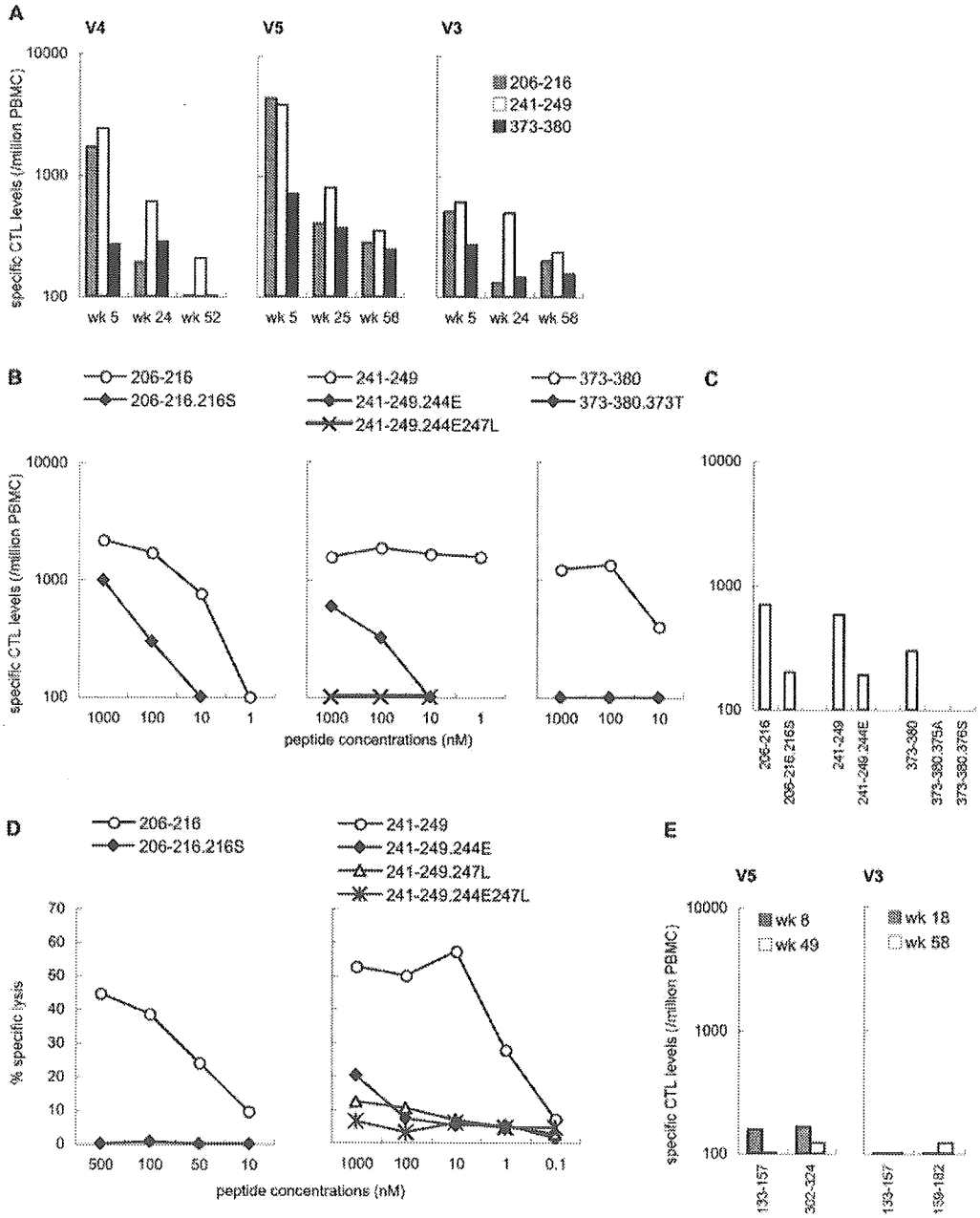

FIG. 2. CTL responses in the controllers (V4, V5, and V3) possessing MHC-I haplotype *90-120-Ia*. (A) $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{373-380}$-specific CTL levels in the macaques V4, V5, and V3. (B) IFN-γ induction in macaque V5 after stimulation with the wild-type or the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

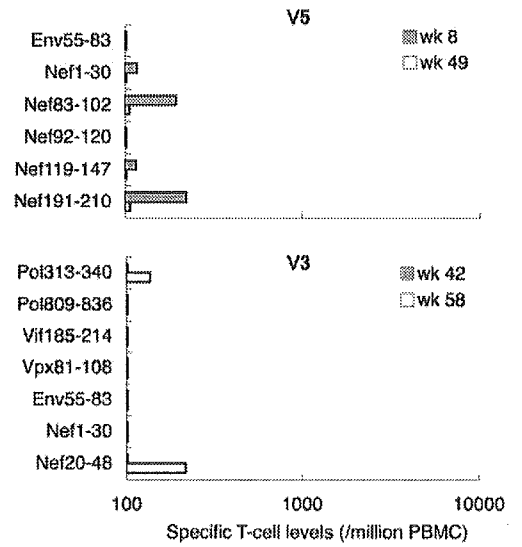

FIG. 3. Mutations in viral genomes encoding SIV proteins other than Gag. (A) Viral mutations in macaques V5 and V3. Dominant mutations leading to amino acid changes are shown. (B) CTL responses to the peptides corresponding to the region around the mutation sites. PBMCs derived from macaque V5 at week 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.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology; grants from the Japan Health Sciences Foundation; and grants from the Ministry of Health, Labor, and Welfare in Japan.

We thank T. Kodama and R. C. Desrosiers for providing an SIVmac239 molecular clone; Y. Ami, F. Ono, K. Komatsuzaki, A. Hiyaoka, A. Oyama, H. Ogawa, K. Hanari, K. Oto, H. Oto, H. Akari, and K. Terao for assistance in the animal experiments; and DNAVEC Corp., A. Kato, M. Kano, M. Miyazawa, M. Yasunami, A. Kimura, K. Mori, N. Yamamoto, T. Takemori, T. Sata, T. Kurata, A. Nomoto, and Y. Nagai for their help.

REFERENCES

1. **Akari, H., K. Mori, K. Terao, I. Otani, M. Fukasawa, R. Mukai, and Y. Yoshikawa.** 1996. In vitro immortalization of old world monkey T lymphocytes with herpesvirus saimiri: its susceptibility to infection with simian immunodeficiency viruses. Virology **218**:382–388.
2. **Alexander, L., L. Denekamp, S. Czajak, and R. C. Desrosiers.** 2001. Suboptimal nucleotides in the infectious, pathogenic simian immunodeficiency virus clone SIVmac239. J. Virol. **75**:4019–4022.
3. **Amara, R. R., F. Villinger, J. D. Altman, S. L. Lydy, S. P. O'Neil, S. I. Staprans, D. C. Montefiori, Y. Xu, J. G. Herndon, L. S. Wyatt, M. A. Candido, N. L. Kozyr, P. L. Earl, J. M. Smith, H. L. Ma, B. D. Grimm, M. L. Hulsey, J. Miller, H. M. McClure, J. M. McNicholl, B. Moss, and H. L. Robinson.** 2001. Control of a mucosal challenge and prevention of AIDS in rhesus macaques by a multiprotein DNA/MVA vaccine. Science **292**:69–74.
4. **Arguello, J. R., A. M. Little, A. L. Pay, D. Gallardo, I. Rojas, S. G. Marsh, J. M. Goldman, and J. A. Madrigal.** 1998. Mutation detection and typing of polymorphic loci through double-strand conformation analysis. Nat. Genet. **18**:192–194.
5. **Barouch, D. H., J. Kunstman, J. Glowczwskie, K. J. Kunstman, M. A. Egan, F. W. Peyerl, S. Santra, M. J. Kuroda, J. E. Schmitz, K. Beaudry, G. R. Krivulka, M. A. Lifton, D. A. Gorgone, S. M. Wolinsky, and N. L. Letvin.** 2003. Viral escape from dominant simian immunodeficiency virus epitope-specific cytotoxic T lymphocytes in DNA-vaccinated rhesus monkeys. J. Virol. **77**:7367–7375.
6. **Barouch, D. H., J. Kunstman, M. J. Kuroda, J. E. Schmitz, S. Santra, F. W. Peyerl, G. R. Krivulka, K. Beaudry, M. A. Lifton, D. A. Gorgone, D. C. Montefiori, M. G. Lewis, S. M. Wolinsky, and N. L. Letvin.** 2002. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature **415**:335–339.
7. **Barouch, D. H., J. Powers, D. M. Truitt, M. G. Kishko, J. C. Arthur, F. W. Peyerl, M. J. Kuroda, D. A. Gorgone, M. A. Lifton, C. I. Lord, V. M. Hirsch, D. C. Montefiori, A. Carville, K. G. Mansfield, K. J. Kunstman, S. M. Wolinsky, and N. L. Letvin.** 2005. Dynamic immune responses maintain cytotoxic T lymphocyte epitope mutations in transmitted simian immunodeficiency virus variants. Nat. Immunol. **6**:247–252.
8. **Barouch, D. H., S. Santra, J. E. Schmitz, M. J. Kuroda, T. M. Fu, W. Wagner, M. Bilska, A. Craiu, X. X. Zheng, G. R. Krivulka, K. Beaudry, M. A. Lifton, C. E. Nickerson, W. L. Trigona, K. Punt, D. C. Freed, L. Guan, S. Dubey, D. Casimiro, A. Simon, M. E. Davies, M. Chastain, T. B. Strom, R. S. Gelman, D. C. Montefiori, M. G. Lewis, E. A. Emini, J. W. Shiver, and N. L. Letvin.** 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science **290**:486–492.
9. **Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone.** 1994. Virus-specific $CD8^+$ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. **68**:6103–6110.
10. **Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw.** 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTL) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. **3**:205–211.
11. **Brander, C., and B. D. Walker.** 2003. Gradual adaptation of HIV to human host populations: good or bad news? Nat. Med. **9**:1359–1362.
12. **Feinberg, M. B., and J. P. Moore.** 2002. AIDS vaccine models: challenging challenge viruses. Nat. Med. **8**:207–210.
13. **Friedrich, T. C., C. A. Frye, L. J. Yant, D. H. O'Connor, N. A. Kriewaldt, M. Benson, L. Vojnov, E. J. Dodds, C. Cullen, R. Rudersdorf, A. L. Hughes, N. Wilson, and D. I. Watkins.** 2004. Extra-epitopic compensatory substitutions partially restore fitness to simian immunodeficiency virus variants that escape from an immunodominant cytotoxic T-lymphocyte response. J. Virol. **78**:2581–2585.
14. **Friedrich, T. C., E. J. Dodds, L. J. Yant, L. Vojnov, R. Rudersdorf, C. Cullen, D. T. Evans, R. C. Desrosiers, B. R. Mothe, J. Sidney, A. Sette, K. Kunstman, S. Wolinsky, M. Piatak, J. Lifson, A. L. Hughes, N. Wilson, D. H. O'Connor, and D. I. Watkins.** 2004. Reversion of CTL escape-variant immunodeficiency viruses in vivo. Nat. Med. **10**:275–281.
15. **Goulder, P. J., and D. I. Watkins.** 2004. HIV and SIV CTL escape: implications for vaccine design. Nat. Rev. Immunol. **4**:630–640.
16. **Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgana, A. Edwards, A. J. McMichael, and S. Rowland-Jones.** 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat. Med. **3**:212–217.
17. **Horton, H., T. U. Vogel, D. K. Carter, K. Vielhuber, D. H. Fuller, T. Shipley, J. T. Fuller, K. J. Kunstman, G. Sutter, D. C. Montefiori, V. Erfle, R. C. Desrosiers, N. Wilson, L. J. Picker, S. M. Wolinsky, C. Wang, D. B. Allison, and D. I. Watkins.** 2002. Immunization of rhesus macaques with a DNA prime/modified vaccinia virus Ankara boost regimen induces broad simian immunodeficiency virus (SIV)-specific T-cell responses and reduces initial viral replication but does not prevent disease progression following challenge with pathogenic SIVmac239. J. Virol. **76**:7187–7202.
18. **Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho.** 1999. Dramatic rise in plasma viremia after $CD8^+$ T cell depletion in simian immunodeficiency virus-infected macaques. J. Exp. Med. **189**:991–998.
19. **Kano, M., T. Matano, A. Kato, H. Nakamura, A. Takeda, Y. Suzaki, Y. Ami, K. Terao, and Y. Nagai.** 2002. Primary replication of a recombinant Sendai viral vector in macaques. J. Gen. Virol. **83**:1377–1386.
20. **Kato, A., Y. Sakai, T. Shioda, T. Kondo, M. Nakanishi, and Y. Nagai.** 1996. Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. Genes Cells **1**:569–579.
21. **Kelleher, A. D., C. Long, E. C. Holmes, R. L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, C. Brander, G. Ogg, J. S. Sullivan, W. Dyer, I. Jones, A. J. McMichael, and S. Rowland-Jones.** 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. J. Exp. Med. **193**:375–386.
22. **Kestler, H. W., III, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **65**:651–662.
23. **Kobayashi, M., H. Igarashi, A. Takeda, M. Kato, and T. Matano.** 2005. Reversion in vivo after inoculation of a molecular proviral DNA clone of simian immunodeficiency virus with a cytotoxic-T-lymphocyte escape mutation. J. Virol. **79**:11529–11532.
24. **Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. **68**:4650–4655.
25. **Leslie, A. J., K. J. Pfafferott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St. John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V. Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker, and P. J. Goulder.** 2004. HIV evolution: CTL escape mutation and reversion after transmission. Nat. Med. **10**:282–289.
26. **Li, H. O., Y. F. Zhu, M. Asakawa, H. Kuma, T. Hirata, Y. Ueda, Y. S. Lee, M. Fukumura, A. Iida, A. Kato, Y. Nagai, and M. Hasegawa.** 2000. A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. J. Virol. **74**:6564–6569.
27. **Matano, T., M. Kano, H. Nakamura, A. Takeda, and Y. Nagai.** 2001. Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen. J. Virol. **75**:11891–11896.
28. **Matano, T., M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D. H. O'Connor, D. I. Watkins, and Y. Nagai.** 2004. Cytotoxic T lymphocyte-based control of sim-

ian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J. Exp. Med. **199**:1709–1718.
29. **Matano, T., R. Shibata, C. Siemon, M. Connors, H. C. Lane, and M. A. Martin.** 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J. Virol. **72**:164–169.
30. **McMichael, A. J., and T. Hanke.** 2003. HIV vaccines 1983–2003. Nat. Med. **9**:874–880.
31. **O'Connor, D. H., A. B. McDermott, K. C. Krebs, E. J. Dodds, J. E. Miller, E. J. Gonzalez, T. J. Jacoby, L. Yant, H. Piontkivska, R. Pantophlet, D. R. Burton, W. M. Rehrauer, N. Wilson, A. L. Hughes, and D. I. Watkins.** 2004. A dominant role for $CD8^+$-T-lymphocyte selection in simian immunodeficiency virus sequence variation. J. Virol. **78**:14012–14022.
32. **O'Connor, D. H., T. M. Allen, T. U. Vogel, P. Jing, I. P. DeSouza, E. Dodds, E. J. Dunphy, C. Melsaether, B. Mothe, H. Yamamoto, H. Horton, N. Wilson, A. L. Hughes, and D. I. Watkins.** 2002. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. Nat. Med. **8**:493–499.
33. **Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D. D. Ho, D. F. Nixon, and A. J. McMichael.** 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science **279**:2103–2106.
34. **Peyerl, F. W., D. H. Barouch, W. W. Yeh, H. S. Bazick, J. Kunstman, K. J. Kunstman, S. M. Wolinsky, and N. L. Letvin.** 2003. Simian-human immunodeficiency virus escape from cytotoxic T-lymphocyte recognition at a structurally constrained epitope. J. Virol. **77**:12572–12578.
35. **Phillips, R. E., S. Rowland-Jones, D. F. Nixon, F. M. Gotch, J. P. Edwards, A. O. Ogunlesi, J. G. Elvin, J. A. Rothbard, C. R. Bangham, C. R. Rizza, and A. J. McMichael.** 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. Nature **354**:453–459.
36. **Price, D. A., P. J. Goulder, P. Klenerman, A. K. Sewell, P. J. Easterbrook, M. Troop, C. R. Bangham, and R. E. Phillips.** 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. Proc. Natl. Acad. Sci. USA **94**:1890–1895.
37. **Rose, N. F., P. A. Marx, A. Luckay, D. F. Nixon, W. J. Moretto, S. M. Donahoe, D. Montefiori, A. Roberts, L. Buonocore, and J. K. Rose.** 2001. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. Cell **106**:539–549.
38. **Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann.** 1999. Control of viremia in simian immunodeficiency virus infection by $CD8^+$ lymphocytes. Science **283**:857–860.
39. **Shibata, R., F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, T. Fredrickson, and M. A. Martin.** 1997. Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. J. Infect. Dis. **176**:362–373.
40. **Shiver, J. W., T. M. Fu, L. Chen, D. R. Casimiro, M. E. Davies, R. K. Evans, Z. Q. Zhang, A. J. Simon, W. L. Trigona, S. A. Dubey, L. Huang, V. A. Harris, R. S. Long, X. Liang, L. Handt, W. A. Schleif, L. Zhu, D. C. Freed, N. V. Persaud, L. Guan, K. S. Punt, A. Tang, M. Chen, K. A. Wilson, K. B. Collins, G. J. Heidecker, V. R. Fernandez, H. C. Perry, J. G. Joyce, K. M. Grimm, J. C. Cook, P. M. Keller, D. S. Kresock, H. Mach, R. D. Troutman, L. A. Isopi, D. M. Williams, Z. Xu, K. E. Bohannon, D. B. Volkin, D. C. Montefiori, A. Miura, G. R. Krivulka, M. A. Lifton, M. J. Kuroda, J. E. Schmitz, N. L. Letvin, M. J. Caulfield, A. J. Bett, R. Youil, D. C. Kaslow, and E. A. Emini.** 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature **415**:331–335.
41. **Takeda, A., H. Igarashi, H. Nakamura, M. Kano, A. Iida, T. Hirata, M. Hasegawa, Y. Nagai, and T. Matano.** 2003. Protective efficacy of an AIDS vaccine, a single DNA-prime followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model. J. Virol. **77**:9710–9715.
42. **Voss, G., S. Nick, C. Stahl-Hennig, K. Ritter, and G. Hunsmann.** 1992. Generation of macaque B lymphoblastoid cell lines with simian Epstein-Barr-like viruses: transformation procedure, characterization of the cell lines and occurrence of simian foamy virus. J. Virol. Methods **39**:185–195.
43. **Yang, O. O., P. T. Sarkis, A. Ali, J. D. Harlow, C. Brander, S. A. Kalams, and B. D. Walker.** 2003. Determinant of HIV-1 mutational escape from cytotoxic T lymphocytes. J. Exp. Med. **197**:1365–1375.

JOURNAL OF VIROLOGY, Aug. 2005, p. 10386–10396
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.16.10386–10396.2005

Vol. 79, No. 16

Influence of Glycosylation on the Efficacy of an Env-Based Vaccine against Simian Immunodeficiency Virus SIVmac239 in a Macaque AIDS Model

Kazuyasu Mori,[1,2,3]* Chie Sugimoto,[1,2,3] Shinji Ohgimoto,[4] Emi E. Nakayama,[5] Tatsuo Shioda,[5] Shigeru Kusagawa,[1] Yutaka Takebe,[1] Munehide Kano,[1] Tetsuro Matano,[6] Takae Yuasa,[7] Daisuke Kitaguchi,[7] Masaaki Miyazawa,[7] Yumiko Takahashi,[8] Michio Yasunami,[8] Akinori Kimura,[8] Naoki Yamamoto,[1] Yasuo Suzuki,[3,9] and Yoshiyuki Nagai[10]

AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640,[1] Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki 305-0843,[2] CREST, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012,[3] Microbiology and Genomics, Department of Genome Sciences, Kobe University School of Medicine, Kobe, Hyogo 650-0017,[4] Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871,[5] Department of Microbiology, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033,[6] Department of Immunology, Kinki University School of Medicine, Osaka-Sayama, Osaka 589-8511,[7] Department of Molecular Pathogenesis, Division of Medical Science, Medical Research Institute, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062,[8] Department of Biochemistry, University of Shizuoka School of Pharmaceutical Sciences and COE Program in the 21st Century, Shizuoka, Shizuoka 422-8526,[9] and Toyama Institute of Health, Kosugi, Toyama 939-0363,[10] Japan

Received 8 December 2004/Accepted 2 May 2005

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

* Corresponding author. Mailing address: Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan. Phone: 81-29-837-2121. Fax: 81-29-837-0218. E-mail: mori@nibio.go.jp.

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**GCTAGC**TATGTCACAGTCTTTTATGGTGTAC-3′) and SUmacB (5′-CC**AGGATCC**TATTACCTCTTCACATCTGTGGGGGC-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

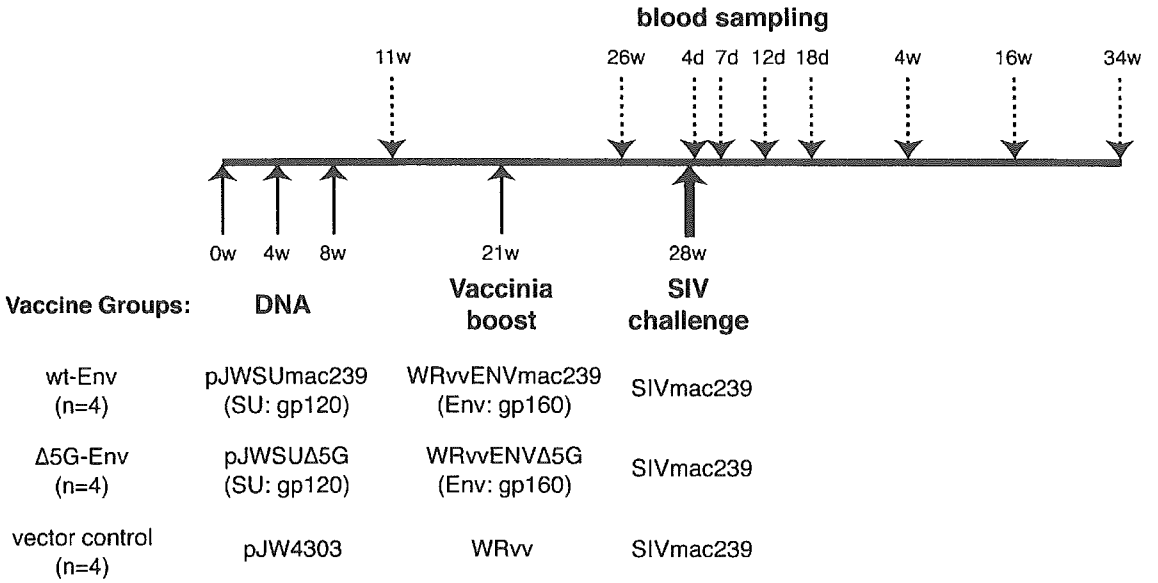

FIG. 1. Outline of immunization, challenge infection, and blood sampling. Twelve juvenile rhesus macaques were divided into three immunization groups of four animals each: the wt-Env immunization group (Mm0005, Mm0007, Mm0010, and Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, and Mm0009), 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

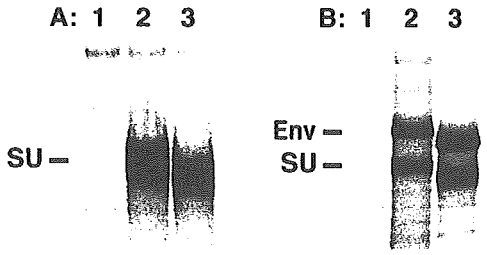

FIG. 2. Expression of SU and Env by SU-expressing DNA vaccines and Env-expressing vaccinia viruses. A: SU secreted in supernatant from CV-1 cells transfected with SU-expressing plasmids. Lane 1, pJW4303 vector; lane 2, pJWSUmac239; lane 3, pJWSUmacΔ5G. B: Env in cell lysates of CV-1 cells infected with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

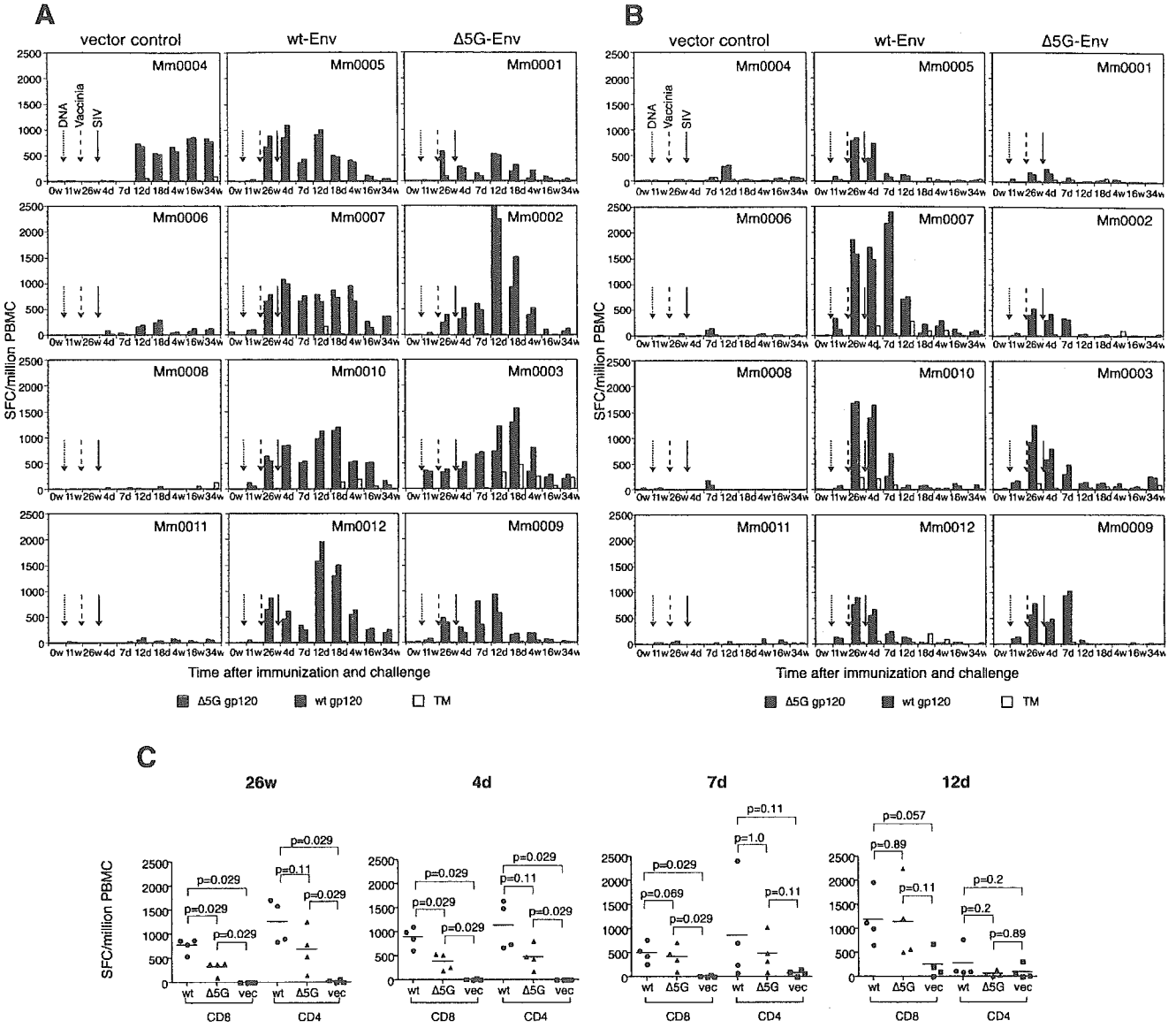

FIG. 3. Env-specific CD4$^+$ T-cell and CD8$^+$ T-cell responses in 12 macaques. A: Env-specific CD8$^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. B: Env-specific CD4$^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results are colored 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

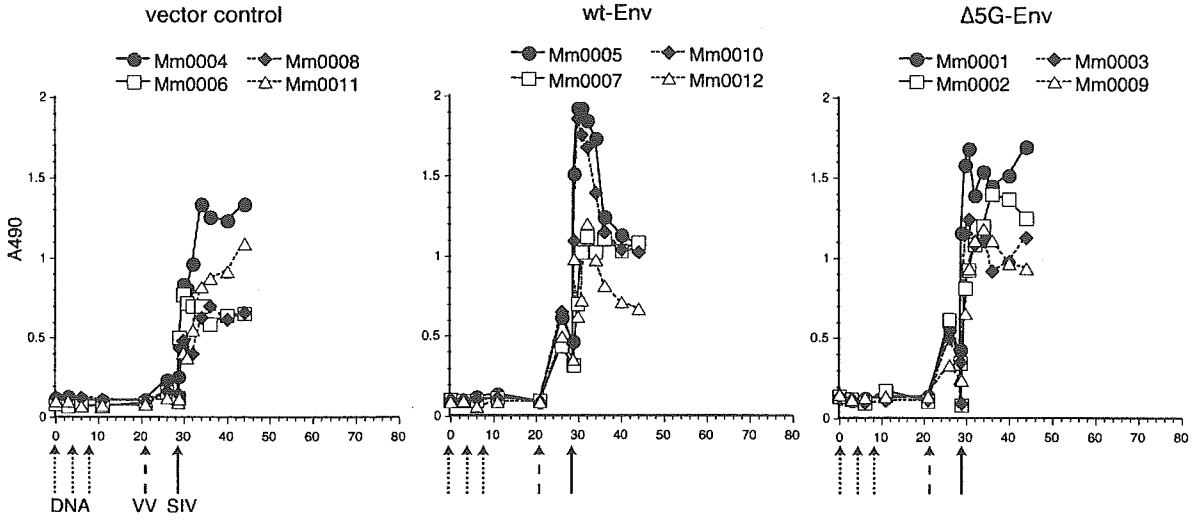

FIG. 4. Humoral immune response during immunization and after challenge infection. The OD_{492} was used as a relative measurement of anti-SIV ELISA antibody titer.

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

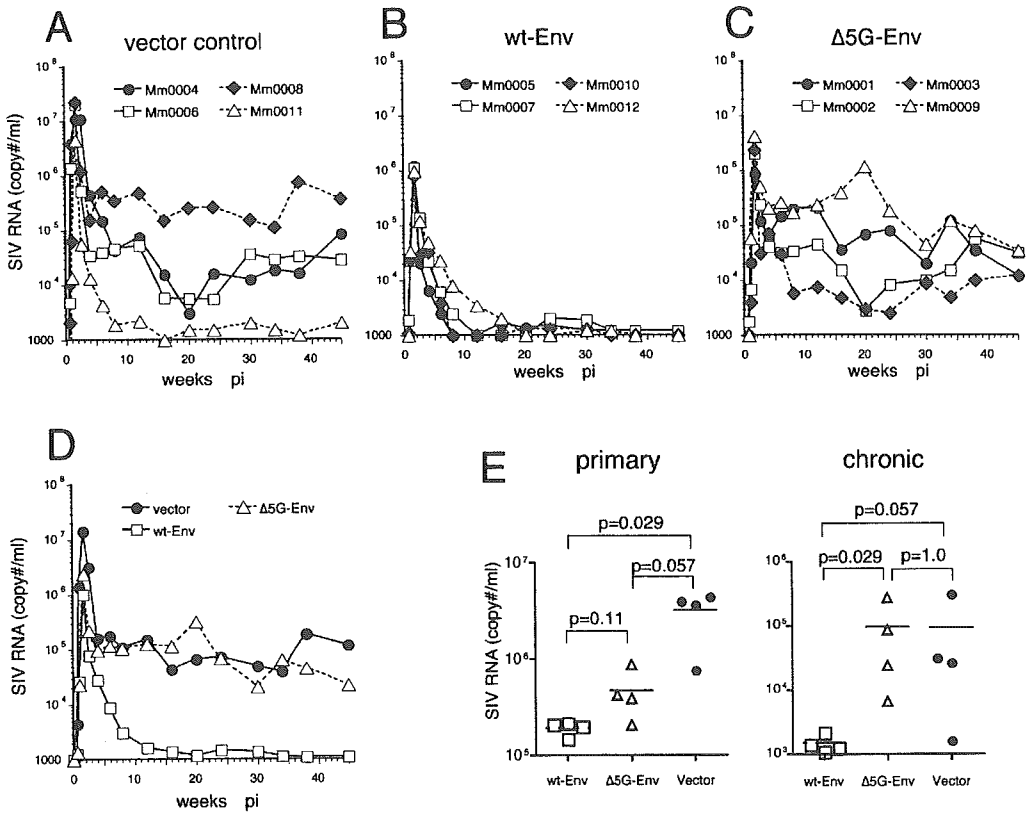

FIG. 5. Plasma viral loads after SIVmac239 challenge infection. Plasma viral load was measured by real-time PCR with a detection limit of 1,000 copies/ml. A: wt-Env vaccine group; B: Δ5G Env vaccine group; C: vector controls; D: comparison of viral loads among three groups; E: comparison of viral loads 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

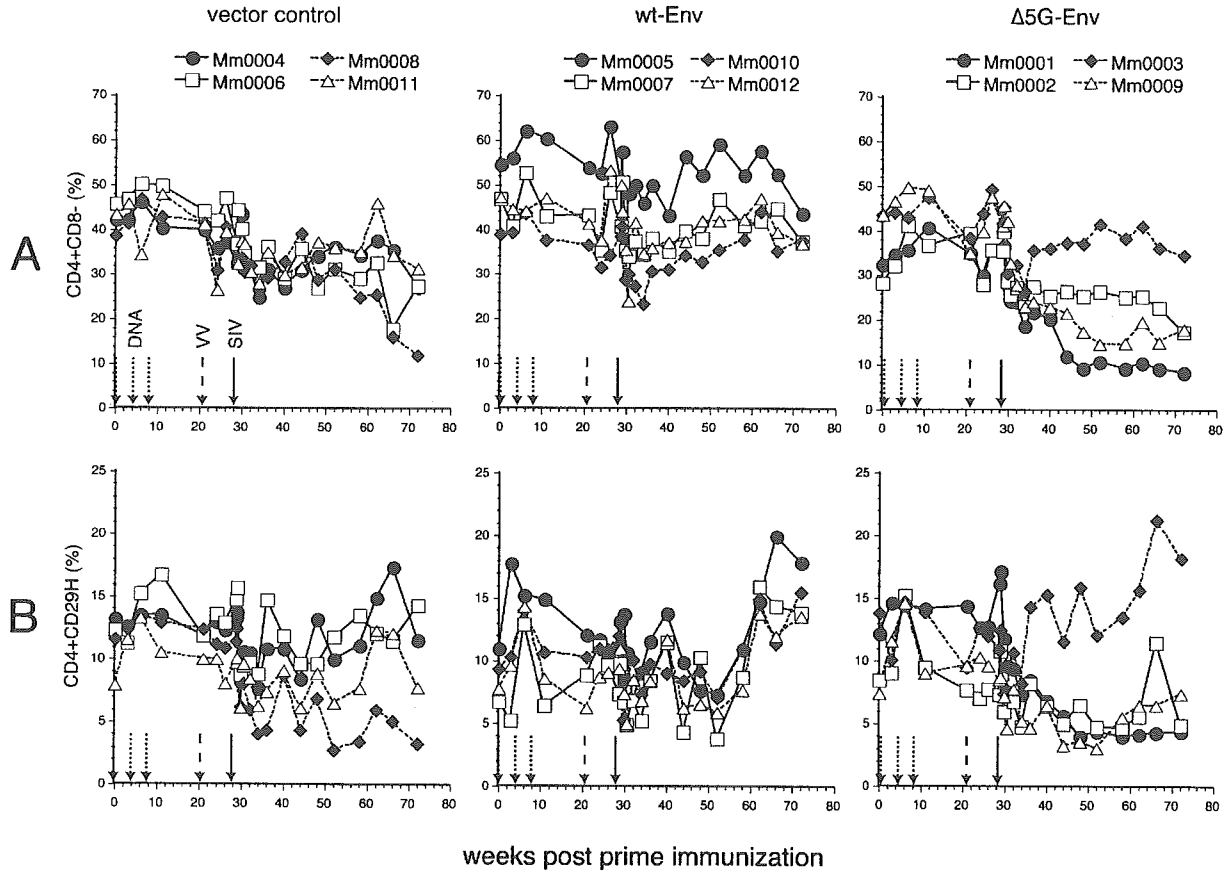

FIG. 6. $CD4^+$ T cells in PBMCs from rhesus macaques during immunization and after the challenge infection. A: Percentage of $CD4^+$ T cells in PBMCs; B: percentage of $CD4^+$ CD29high T cells in PBMCs.

were induced, with statistical significance ($P < 0.05$), in the control animals.

DISCUSSION

The heavily glycosylated structure of Env has been considered a main cause of chronically persistent viral replication and the pathogenicity of HIV/SIV, primarily because it potentially interferes with the development of the host immune response associated with protective immune functions, such as NAb and CTL (10, 36, 44). This characteristic constitutes the primary reason for the difficulty of developing effective vaccines. We therefore examined the efficacy of a deglycosylated-Env vaccine and compared it with the wt-Env vaccine. This study showed that quintuple deglycosylation neither improved the immunogenicity of the wt-Env vaccine nor elicited NAb against SIVmac239. This was in contrast to what occurred with Δ5G infection in rhesus macaques, because the host response elicited by Δ5G infection not only contained Δ5G infection but also protected the animals from SIVmac239 challenge infection (36). This study therefore suggested that an almost sterilizing immunity against SIVmac239 induced in Δ5G-infected animals could not be explained by the immunogenicity of Δ5G Env; instead, it is likely associated with the property of Δ5G as an attenuated virus. In fact, Δ5G was more neutralization-sensitive than SIVmac239 (36). Alternatively, the immunogenic property of Env in Δ5G could not successfully be duplicated by immunization with a Δ5G Env DNA prime-vaccinia virus boost regimen. Therefore, another immunization regimen might be able to elicit the protective immune response induced by Δ5G infection.

The Env vaccine is superior to other vaccines containing other viral proteins with respect to the induction of NAb; however, both the Δ5G Env vaccine and the wt-Env vaccine could not induce detectable NAb against either SIVmac239 or Δ5G. Instead, the wt-Env vaccine induced higher NAb against macrophage-tropic SIV than the Δ5G Env vaccine. Notably, this parameter most significantly correlated with the efficacies of the two Env vaccines. As Ab neutralized the macrophage-tropic variant 239/envMERT, which has only four separate amino acid substitutions distributed in *env* of SVmac239 (34), it might recognize unknown epitopes conserved between SIVmac239 and 239/envMERT. On the other hand, Δ5G Env may not sufficiently present this epitope due to mutations. Regarding the role of nonneutralizing Ab for the control of SIVmac239 infection, it is assumed that, as the neutralization assay did not necessarily reflect in vivo conditions, such nonneutralizing Ab with potential virus-binding ability may interfere with SIVmac239 infection in animals. Alternatively, Ab

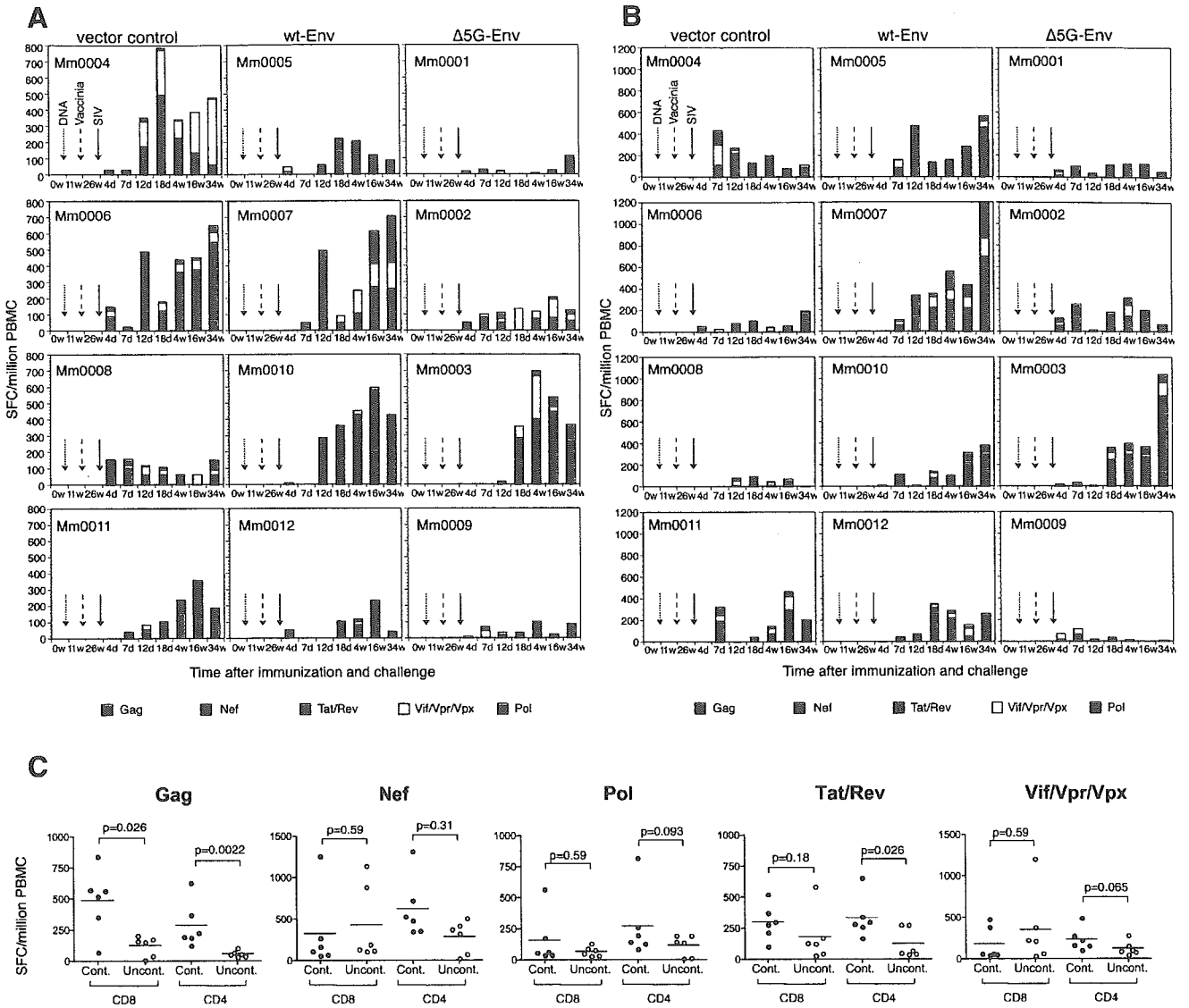

FIG. 7. SIV-specific $CD8^+$ T-cell and $CD4^+$ T-cell responses in 12 animals. A: SIV viral-protein-specific $CD8^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups: vector controls, wt-Env vaccine group, and Δ5G Env vaccines. B: SIV viral-protein-specific $CD4^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results of individual SIV proteins are colored as follows: Gag (red), Nef (green), Tat/Rev (blue), Vif/Vpr/Vpx (yellow), and Pol (pink). C: Comparison of cumulated $CD8^+$ T cells or $CD4^+$ T cells specific to the viral proteins Gag, Pol, Nef, Tat/Rev, and Vif/Vpr/VpX between SIV infection-controlled and uncontrolled animals. w, weeks; d, days.

might play a role in other effector functions, such as antibody-dependent cell-mediated cytotoxicity to eliminate the infected cells. The antibody-mediated enhancement of viral antigen processing and cross presentation is also a mechanism potentially related to the control of SIV infection in vivo (49).

Reduced immunogenicity in the Δ5G Env vaccine was also noted in cellular immunity. The levels of stimulation of antigen-specific $CD8^+$ T cells and $CD4^+$ T cells are MHC I and MHC II dependent, respectively. As the macaques in this study have different MHC haplotypes (data not shown), the magnitude and breadth of SIV-specific T cells should vary among the animals. Nevertheless, the magnitude of SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs was greater in the wt-Env vaccine group than in the Δ5G Env vaccine group. Although the expression of SU by expressing plasmids and that of Env by the vaccinia virus vector elicited by either the wt-Env vaccine or Δ5G Env vaccine were indistinguishable in cultured cells (Fig. 2), wt-Env might persist longer than Δ5G Env in vaccinated animals. T-cell epitopes in the wt-Env vaccine might therefore be more efficiently presented on MHC molecules in antigen-presenting cells than in the Δ5G Env vaccine. Differences in glycosylation levels might also affect some processes in antigen-presenting cells associated with the presentation of T-cell epitopes in Env.

Taking all results together, Env glycosylation might affect the presentation of B-cell epitopes and T-cell epitopes required for Ab-mediated and T-cell-mediated immunities related to the control of SIV infection.

As seen in viral loads and SU-specific T cell levels after challenge infection (Fig. 3 and 5), the effect of vaccination was limited. That seemed related to the development of escape mutants. Therefore, distinctive cellular immune responses after the challenge infection were also implicated in the control of SIVmac239 replication. The magnitude of virus-specific $CD8^+$ T cells did not always correlate with the suppression of viral replication as reported previously (1, 6), particularly in vector controls (Fig. 5 and 7A); however, selected epitope-specific CTL responses might be associated with infection control. Gag-specific CTLs are such candidates, because a high magnitude of Gag-specific $CD8^+$ T cells was significantly elicited in five control animals (Fig. 7C). The magnitude of Gag- or Tat/Rev-specific $CD4^+$ T cells was statistically correlated with infection control (Fig. 7C). This may simply indicate a lower depletion of virus-specific $CD4^+$ T cells in animals with lower viral loads as reported previously (11). Alternatively, these virus-specific $CD4^+$ T cells may play an important role in protective immunity (39). Taken together, these results implicated the dominant role of selected epitope-specific $CD4^+$ T cells and $CD8^+$ T cells for the control of SIVmac239 infection.

The challenge virus that should be used has been an important issue in AIDS vaccine studies (8, 10, 12). Many studies have reported impressive efficacy in a pathogenic-SHIV macaque model (3, 4, 45, 46); however, pathogenic SHIVs use CXCR4 as a coreceptor, whereas the majority of clinical isolates of HIV-1 use CCR5 (13, 27). Therefore, the challenge virus for an AIDS vaccine study should be an R5 virus, such as SIV (10). Consistent with this concern, a DNA prime–modified-vaccinia virus Ankara boost regimen, inducing broad SIV-specific T-cell responses, reduced the initial viral replication but did not prevent disease progression against SIVmac239 challenge (18). Thus, vaccine studies using pathogenic SHIV should be reevaluated by using an R5 virus (10).

Matano et al. reported that a DNA prime-Sendai virus boost regimen induced the CTL-based control of SIVmac239 in rhesus macaques (27). This study demonstrated that a DNA prime-vaccinia virus WR boost regimen expressing only Env controlled the chronic infection of SIVmac239 in rhesus macaques. The relatively lower viral loads in macaques from Myanmar or Laos than in those of Indian origin might contribute to the control of SIVmac239 infection. Nevertheless, it is important that these two studies demonstrated the efficacies of the two vaccine regimens against highly pathogenic SIVmac239. In earlier studies, other R5 SIVs were used as a challenge virus for an efficacy study of vaccine candidates. An Env-based vaccine in vaccinia virus vector priming and subunit protein boosting protected cynomologous macaques against homologous SIVmne clone E11S (42). In recombinant modified vaccinia virus, Ankara viruses expressing Gag-Pol and/or Env exhibited vaccine efficacy because of reduced viremia and the increased survival of rhesus macaques infected with uncloned SIVsmE660 (41). Accordingly, the efficacy of vaccine candidates might be influenced by the experimental conditions. Thus, well-defined animal models with detailed virological, immunological, and genetic information and suitable challenge viruses are required for the evaluation of vaccine candidates and the development of an AIDS vaccine.

This study demonstrated the importance of Env as a component of the AIDS vaccine, and Env-specific $CD8^+$ and $CD4^+$ T cells and nonneutralizing Env-specific Ab were suggested as protective immunity components. Quintuple deglycosylation in Env reduced vaccine efficacy and Env-specific immune responses. Env may therefore be comprised of appropriate antigenic properties to elicit humoral and cellular immune responses required for protective immunity against homologous or allele-specific target SIV/HIV. These properties could be modified by the alteration of glycosylation.

In conclusion, although Env is an important immunogen for the AIDS vaccine, Env properties, including glycosylation, should be carefully considered to design vaccines specific to the targeted viruses.

ACKNOWLEDGMENTS

We thank Kayoko Ueda for excellent technical assistance.

This work was supported by AIDS research grants from the Health Sciences Research Grants, from the Ministry of Health, Labor, and Welfare in Japan, and from the Ministry of Education, Culture, Sports, Science and Technology in Japan.

REFERENCES

1. **Addo, M. M., X. G. Yu, A. Rathod, D. Cohen, R. L. Eldridge, D. Strick, M. N. Johnston, C. Corcoran, A. G. Wurcel, C. A. Fitzpatrick, M. E. Feeney, W. R. Rodriguez, N. Basgoz, R. Draenert, D. R. Stone, C. Brander, P. J. Goulder, E. S. Rosenberg, M. Altfeld, and B. D. Walker.** 2003. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J. Virol. **77:**2081–2092.
2. **Allen, T. M., and D. I. Watkins.** 2001. New insights into evaluating effective T-cell responses to HIV. AIDS **15**(Suppl. 5)**:**S117–S126.
3. **Amara, R. R., F. Villinger, J. D. Altman, S. L. Lydy, S. P. O'Neil, S. I. Staprans, D. C. Montefiori, Y. Xu, J. G. Herndon, L. S. Wyatt, M. A. Candido, N. L. Kozyr, P. L. Earl, J. M. Smith, H. L. Ma, B. D. Grimm, M. L. Hulsey, J. Miller, H. M. McClure, J. M. McNicholl, B. Moss, and H. L. Robinson.** 2001. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science **292:**69–74.
4. **Barouch, D. H., S. Santra, J. E. Schmitz, M. J. Kuroda, T. M. Fu, W. Wagner, M. Bilska, A. Craiu, X. X. Zheng, G. R. Krivulka, K. Beaudry, M. A. Lifton, C. E. Nickerson, W. L. Trigona, K. Punt, D. C. Freed, L. Guan, S. Dubey, D. Casimiro, A. Simon, M. E. Davies, M. Chastain, T. B. Strom, R. S. Gelman, D. C. Montefiori, M. G. Lewis, E. A. Emini, J. W. Shiver, and N. L. Letvin.** 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science **290:**486–492.
5. **Berger, E. A., P. M. Murphy, and J. M. Farber.** 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. **17:**657–700.
6. **Betts, M. R., D. R. Ambrozak, D. C. Douek, S. Bonhoeffer, J. M. Brenchley, J. P. Casazza, R. A. Koup, and L. J. Picker.** 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. J. Virol. **75:**11983–11991.
7. **Burton, D. R.** 2002. Antibodies, viruses and vaccines. Nat. Rev. Immunol. **2:**706–713.
8. **Burton, D. R., R. C. Desrosiers, R. W. Doms, W. C. Koff, P. D. Kwong, J. P. Moore, G. J. Nabel, J. Sodroski, I. A. Wilson, and R. T. Wyatt.** 2004. HIV vaccine design and the neutralizing antibody problem. Nat. Immunol. **5:**233–236.
9. **Chapman, B. S., R. M. Thayer, K. A. Vincent, and N. L. Haigwood.** 1991. Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. Nucleic Acids Res. **19:**3979–3986.
10. **Desrosiers, R. C.** 2004. Prospects for an AIDS vaccine. Nat. Med. **10:**221–223.
11. **Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors, and R. A. Koup.** 2002. HIV preferentially infects HIV-specific CD4+ T cells. Nature **417:**95–98.
12. **Emini, E. A., and W. C. Koff.** 2004. AIDS/HIV. Developing an AIDS vaccine: need, uncertainty, hope. Science **304:**1913–1914.
13. **Feinberg, M. B., and J. P. Moore.** 2002. AIDS vaccine models: challenging challenge viruses. Nat Med. **8:**207–210.
14. **Gardner, M. B.** 2003. Simian AIDS: an historical perspective. J. Med. Primatol. **32:**180–186.
15. **Gotoh, H., T. Shioda, Y. Sakai, K. Mizumoto, and H. Shibuta.** 1989. Rescue

of Sendai virus from viral ribonucleoprotein-transfected cells by infection with recombinant vaccinia viruses carrying Sendai virus L and P/C genes. Virology **171**:434–443.
16. **Haigwood, N. L., and L. Stamatatos.** 2003. Role of neutralizing antibodies in HIV infection. AIDS **17**(Suppl. 4):S67–S71.
17. **Hirsch, V. M.** 2004. What can natural infection of African monkeys with simian immunodeficiency virus tell us about the pathogenesis of AIDS? AIDS Rev. **6**:40–53.
18. **Horton, H., T. U. Vogel, D. K. Carter, K. Vielhuber, D. H. Fuller, T. Shipley, J. T. Fuller, K. J. Kunstman, G. Sutter, D. C. Montefiori, V. Erfle, R. C. Desrosiers, N. Wilson, L. J. Picker, S. M. Wolinsky, C. Wang, D. B. Allison, and D. I. Watkins.** 2002. Immunization of rhesus macaques with a DNA prime/modified vaccinia virus Ankara boost regimen induces broad simian immunodeficiency virus (SIV)-specific T-cell responses and reduces initial viral replication but does not prevent disease progression following challenge with pathogenic SIVmac239. J. Virol. **76**:7187–7202.
19. **Johnson, R. P., R. L. Glickman, J. Q. Yang, A. Kaur, J. T. Dion, M. J. Mulligan, and R. C. Desrosiers.** 1997. Induction of vigorous cytotoxic T-lymphocyte responses by live attenuated simian immunodeficiency virus. J. Virol. **71**:7711–7718.
20. **Johnson, W. E., J. D. Lifson, S. M. Lang, R. P. Johnson, and R. C. Desrosiers.** 2003. Importance of B-cell responses for immunological control of variant strains of simian immunodeficiency virus. J. Virol. **77**:375–381.
21. **Johnson, W. E., H. Sanford, L. Schwall, D. R. Burton, P. W. Parren, J. E. Robinson, and R. C. Desrosiers.** 2003. Assorted mutations in the envelope gene of simian immunodeficiency virus lead to loss of neutralization resistance against antibodies representing a broad spectrum of specificities. J. Virol. **77**:9993–10003.
22. **Kano, M., T. Matano, A. Kato, H. Nakamura, A. Takeda, Y. Suzaki, Y. Ami, K. Terao, and Y. Nagai.** 2002. Primary replication of a recombinant Sendai virus vector in macaques. J. Gen. Virol. **83**:1377–1386.
23. **Kano, M., T. Matano, H. Nakamura, A. Takeda, A. Kato, K. Ariyoshi, K. Mori, T. Sata, and Y. Nagai.** 2000. Elicitation of protective immunity against simian immunodeficiency virus infection by a recombinant Sendai virus expressing the Gag protein. AIDS **14**:1281–1282.
24. **Letvin, N. L., J. E. Schmitz, H. L. Jordan, A. Seth, V. M. Hirsch, K. A. Reimann, and M. J. Kuroda.** 1999. Cytotoxic T lymphocytes specific for the simian immunodeficiency virus. Immunol. Rev. **170**:127–134.
25. **Lifson, J. D., M. A. Nowak, S. Goldstein, J. L. Rossio, A. Kinter, G. Vasquez, T. A. Wiltrout, C. Brown, D. Schneider, L. Wahl, A. L. Lloyd, J. Williams, W. R. Elkins, A. S. Fauci, and V. M. Hirsch.** 1997. The extent of early viral replication is a critical determinant of the natural history of simian immunodeficiency virus infection. J. Virol. **71**:9508–9514.
26. **Matano, T., M. Kano, T. Odawara, H. Nakamura, A. Takeda, K. Mori, T. Sato, and Y. Nagai.** 2000. Induction of protective immunity against pathogenic simian immunodeficiency virus by a foreign receptor-dependent replication of an engineered avirulent virus. Vaccine **18**:3310–3318.
27. **Matano, T., M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D. H. O'Connor, D. I. Watkins, and Y. Nagai.** 2004. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J Exp. Med. **199**:1709–1718.
28. **McMichael, A. J., and S. L. Rowland-Jones.** 2001. Cellular immune responses to HIV. Nature **410**:980–987.
29. **Means, R. E., T. Greenough, and R. C. Desrosiers.** 1997. Neutralization sensitivity of cell culture-passaged simian immunodeficiency virus. J. Virol. **71**:7895–7902.
30. **Means, R. E., T. Matthews, J. A. Hoxie, M. H. Malim, T. Kodama, and R. C. Desrosiers.** 2001. Ability of the V3 loop of simian immunodeficiency virus to serve as a target for antibody-mediated neutralization: correlation of neutralization sensitivity, growth in macrophages, and decreased dependence on CD4. J. Virol. **75**:3903–3915.
31. **Mellors, J. W., L. A. Kingsley, C. R. Rinaldo, Jr., J. A. Todd, B. S. Hoo, R. P. Kokka, and P. Gupta.** 1995. Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. Ann. Intern. Med. **122**:573–579.
32. **Moore, J. P., S. G. Kitchen, P. Pugach, and J. A. Zack.** 2004. The CCR5 and CXCR4 coreceptors—central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res. Hum. Retrovir. **20**:111–126.
33. **Mori, K., D. J. Ringler, and R. C. Desrosiers.** 1993. Restricted replication of simian immunodeficiency virus strain 239 in macrophages is determined by Env but is not due to restricted entry. J. Virol. **67**:2807–2814.
34. **Mori, K., D. J. Ringler, T. Kodama, and R. C. Desrosiers.** 1992. Complex determinants of macrophage tropism in Env of simian immunodeficiency virus. J. Virol. **66**:2067–2075.
35. **Mori, K., M. Rosenzweig, and R. C. Desrosiers.** 2000. Mechanisms for adaptation of simian immunodeficiency virus to replication in alveolar macrophages. J. Virol. **74**:10852–10859.
36. **Mori, K., Y. Yasutomi, S. Ohgimoto, T. Nakasone, S. Takamura, T. Shioda, and Y. Nagai.** 2001. Quintuple deglycosylation mutant of simian immunodeficiency virus SIVmac239 in rhesus macaques: robust primary replication, tightly contained chronic infection, and elicitation of potent immunity against the parental wild-type strain. J. Virol. **75**:4023–4028.
37. **Mori, K., Y. Yasutomi, S. Sawada, F. Villinger, K. Sugama, B. Rosenwith, J. L. Heeney, K. Uberla, S. Yamazaki, A. A. Ansari, and H. Rubsamen-Waigmann.** 2000. Suppression of acute viremia by short-term postexposure prophylaxis of simian/human immunodeficiency virus SHIV-RT-infected monkeys with a novel reverse transcriptase inhibitor (GW420867) allows for development of potent antiviral immune responses resulting in efficient containment of infection. J. Virol. **74**:5747–5753.
38. **Munch, J., N. Adam, N. Finze, N. Stolte, C. Stahl-Hennig, D. Fuchs, P. Ten Haaft, J. L. Heeney, and F. Kirchhoff.** 2001. Simian immunodeficiency virus in which *nef* and U3 sequences do not overlap replicates efficiently in vitro and in vivo in rhesus macaques. J. Virol. **75**:8137–8146.
39. **Norris, P. J., and E. S. Rosenberg.** 2001. Cellular immune response to human immunodeficiency virus. AIDS **15**(Suppl. 2):S16–S21.
40. **Ohgimoto, S., T. Shioda, K. Mori, E. E. Nakayama, H. Hu, and Y. Nagai.** 1998. Location-specific, unequal contribution of the N glycans in simian immunodeficiency virus gp120 to viral infectivity and removal of multiple glycans without disturbing infectivity. J. Virol. **72**:8365–8370.
41. **Ourmanov, I., C. R. Brown, B. Moss, M. Carroll, L. Wyatt, L. Pletneva, S. Goldstein, D. Venzon, and V. M. Hirsch.** 2000. Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV. J. Virol. **74**:2740–2751.
42. **Polacino, P., V. Stallard, J. E. Klaniecki, D. C. Montefiori, A. J. Langlois, B. A. Richardson, J. Overbaugh, W. R. Morton, R. E. Benveniste, and S. L. Hu.** 1999. Limited breadth of the protective immunity elicited by simian immunodeficiency virus SIVmne gp160 vaccines in a combination immunization regimen. J. Virol. **73**:618–630.
43. **Reeves, J. D., and R. W. Doms.** 2002. Human immunodeficiency virus type 2. J. Gen. Virol. **83**:1253–1265.
44. **Reitter, J. N., R. E. Means, and R. C. Desrosiers.** 1998. A role for carbohydrates in immune evasion in AIDS. Nat. Med. **4**:679–684.
45. **Robinson, H. L., D. C. Montefiori, R. P. Johnson, K. H. Manson, M. L. Kalish, J. D. Lifson, T. A. Rizvi, S. Lu, S. L. Hu, G. P. Mazzara, D. L. Panicali, J. G. Herndon, R. Glickman, M. A. Candido, S. L. Lydy, M. S. Wyand, and H. M. McClure.** 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. Nat. Med. **5**:526–534.
46. **Rose, N. F., P. A. Marx, A. Luckay, D. F. Nixon, W. J. Moretto, S. M. Donahoe, D. Montefiori, A. Roberts, L. Buonocore, and J. K. Rose.** 2001. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. Cell **106**:539–549.
47. **Stebbing, J., B. Gazzard, and D. C. Douek.** 2004. Where does HIV live? N. Engl. J. Med. **350**:1872–1880.
48. **Sugimoto, C., K. Tadakuma, I. Otani, T. Moritoyo, H. Akari, F. Ono, Y. Yoshikawa, T. Sata, S. Izumo, and K. Mori.** 2003. *nef* gene is required for robust productive infection by simian immunodeficiency virus of T-cell-rich paracortex in lymph nodes. J. Virol. **77**:4169–4180.
49. **Villinger, F., A. E. Mayne, P. Bostik, K. Mori, P. E. Jensen, R. Ahmed, and A. A. Ansari.** 2003. Evidence for antibody-mediated enhancement of simian immunodeficiency virus (SIV) Gag antigen processing and cross presentation in SIV-infected rhesus macaques. J. Virol. **77**:10–24.
50. **Watanabe, M. E.** 2003. Skeptical scientists skewer VaxGen statistics. Nat. Med. **9**:376.
51. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422**:307–312.
52. **Yu, D., T. Shioda, A. Kato, M. K. Hasan, Y. Sakai, and Y. Nagai.** 1997. Sendai virus-based expression of HIV-1 gp120: reinforcement by the V(−) version. Genes Cells **2**:457–466.

D. Shichi
E.F. Kikkawa
M. Ota
Y. Katsuyama
A. Kimura
A. Matsumori
J.K. Kulski
T.K. Naruse
H. Inoko

The haplotype block, *NFKBIL1-ATP6V1G2-BAT1-MICB-MICA*, within the class III – class I boundary region of the human major histocompatibility complex may control susceptibility to hepatitis C virus-associated dilated cardiomyopathy

Key words:
dilated cardiomyopathy (DCM); hepatitis C virus (HCV); human leukocyte antigen region; hypertrophic cardiomyopathy (HCM); microsatellite; single nucleotide polymorphism (SNP)

Acknowledgments
We are grateful to Drs A. Hasegawa, R. Nagai, T. Izumi, N. Aoyama, K. Yamauchi-Takihara, Y. Sato, Y. Takatsu, S. Maruyama, E. Matsuyama, K. Nakamura, T. Ohe, Y. Sakai, H. Kotoura, M. Matsuzaki, and T. Yamamura for their contributions in clinical evaluation and blood sampling from patients with HCV-DCM or HCV-HCM. This study was supported by grants as follows: Japanese Ministry of Health and Welfare, Mitsui Life Social Welfare Foundation, and Tokai University, School of Medicine Research Aid 2002.

Abstract: Cardiomyopathy is a heart muscle disease with impaired stretch response that can result in severe heart failure and sudden death. A small proportion of hepatitis C virus (HCV)-infected patients may be predisposed to develop dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM). The molecular mechanisms involved in the predisposition remain unknown due in part to the lack of information on their genetic background. Because the human leukocyte antigen (HLA) region has a pivotal role in controlling the susceptibility to HCV-induced liver disease, we hypothesized that particular *HLA* alleles and/or non-*HLA* gene alleles within the human major histocompatibility complex (MHC) genomic region might control the predisposition to HCV-associated DCM (HCV-DCM) and/or HCV-associated HCM (HCV-HCM). Here, we present mapping results of the MHC-related susceptibility gene locus for HCV-associated cardiomyopathy by analyzing microsatellite and single nucleotide polymorphism markers. To delineate the susceptibility locus, we genotyped 44 polymorphic markers scattered across the entire MHC region in a total of 59 patients (21 HCV-DCM and 38 HCV-HCM) and 120 controls. We mapped HCV-DCM susceptibility to a non-*HLA* gene locus spanning from *NFKBIL1* to *MICA* gene loci within the MHC class III – class I boundary region. Our results showed that HCV-DCM was more strongly associated with alleles of the non-*HLA* genes rather than the *HLA* genes themselves. In addition, no significant association was found between the MHC markers and HCV-HCM. This marked difference in the MHC-related disease susceptibility for HCV-associated cardiomyopathy strongly suggests that the development of HCV-DCM and HCV-HCM is under the control of different pathogenic mechanisms.

Authors' affiliation:

D. Shichi[1,2],
E.F. Kikkawa[1],
M. Ota[3],
Y. Katsuyama[3],
A. Kimura[2,4],
A. Matsumori[5],
J.K. Kulski[1,6],
T.K. Naruse[1],
H. Inoko[1]

[1]Department of Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, Isehara, Kanagawa, Japan

[2]Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo, Japan

[3]Department of Legal Medicine, Shinshu University School of Medicine, Matsumoto, Nagano, Japan

[4]Laboratory of Genome Diversity, School of Biomedical Science, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo, Japan

[5]Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto, Japan

[6]Center for Bioinformatics and Biological Computing, Murdoch University School of Information Technology, Murdoch, Western Australia, Australia

Correspondence to:
Hidetoshi Inoko, PhD, MD
Department of Basic Medical Science and Molecular Medicine
Tokai University School of Medicine
Bohseidai, Isehara
Kanagawa 259-1193
Japan
Tel.:
+81 463 93 1121x2312
Fax: +81 463 94 8884
e-mail: hinoko@is.icc.u-tokai.ac.jp

Hepatitis C virus (HCV) is a major pathogen of liver disease (1). It also infects various extrahepatic tissues (2) and causes clinical manifestations that do not originate from hepatopathy (3). The extrahepatic manifestation includes cardiomyopathy that is defined as a heart muscle disease with impaired stretch response that can result in heart failure and sudden death (4–7). Dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) are two major clinical phenotypes of the HCV-associated cardiomyopathy. DCM is characterized by chamber dilation with contractile dysfunction. In contrast, HCM exhibits cardiac hypertrophy with diastolic ventricular failure. Not all of the patients infected with HCV develop cardiomyopathy, and there is a report showing DCM and HCM can be found in 5.7 and 6.6%, respectively, of random patients with positive HCV antibody (8).

Received 3 March 2005, revised 24 May 2005, accepted for publication 30 May 2005

doi: 10.1111/j.1399-0039.2005.00457.x

Tissue Antigens 2005: **66:** 200–208
Printed in Singapore. All rights reserved

This epidemiological study suggests that there may be predisposing factors in the virus and/or infected host, which affect the clinical outcome of viral pathogenesis. To date, it is not clear whether or not there are cardiotropic HCV strains, and how they can escape cellular immune responses. It also remains unknown which genetic factors would predispose the infected patients to develop HCV-associated DCM (HCV-DCM) or HCV-associated HCM (HCV-HCM).

Human major histocompatibility complex (MHC), also called human leukocyte antigen (HLA) complex, on chromosome 6p21.3 plays a pivotal role in antiviral defense as a genetic factor controlling the immune response (9). The MHC genomic region consists of densely packed gene clusters including the *HLA* genes (10), the most polymorphic genes in the human genome (11–13). In addition to the classical and non-classical *HLA* class I and class II genes that regulate the cell-mediated immune response, there are at least 126 non-*HLA* coding genes within the MHC genomic region, which might affect acute and/or chronic viral infection and susceptibility to disease. Of particular interest are the 60 non-*HLA* coding genes within the MHC class III region, many of which have a role in regulating inflammation or have strong associations with susceptibility to diseases, such as rheumatoid arthritis (14), myocardial infarction (15), malaria (16), septic shock (17), and Ehlers – Danlos syndrome (18). The statistical analysis of extensive linkage disequilibrium (LD) across the entire 3.6-Mb MHC region has resulted in the detection and categorization of population-specific HLA haplotypes based on the allelic distribution and allelic combination of different MHC genes (19–21).

Although HCV appears to infect humans regardless of their *HLA* genotypes, the classical *HLA* alleles may determine the natural history of the viral infection in the post-infectious stage, such as viral resolution, the progression/suppression of persistent infection, and liver disease. For example, *HLA-B*44-DRB1*1302-DQB1*0604* haplotype carriers remain asymptomatic, whereas *B*54-DRB1*0405-DQB1*0401* carriers tend to develop chronic liver disease (22). Therefore, we hypothesized that either particular *HLA* or non-*HLA* gene alleles might regulate the development of DCM and/or HCM after HCV infection. In this study, we undertook genotyping to map the HLA-linked susceptibility loci for HCV-DCM and HCV-HCM in order to better understand the immuno-genetic factors controlling the clinical outcome of chronic HCV infection. This is the first genetic study to demonstrate that a non-*HLA* locus within the MHC class III – class I boundary region may confer the susceptibility to HCV-DCM in Japanese.

Materials and methods

Subjects

Diagnosis of DCM and HCM was based on the criteria of the Japan Research Committee on Idiopathic Cardiomyopathy developed with guidance from the report by the 1980 World Health Organization/International Society and Federation of Cardiology task force on the definition and classification of cardiomyopathies (23, 24). Among the Japanese patients who conformed with the diagnostic criteria, a total of 59 patients positive for the anti-HCV antibody (21 patients of HCV-DCM and 38 patients of HCV-HCM) were enrolled in this study along with 120 healthy controls after obtaining informed consent. All of them were genetically unrelated. Genomic DNAs were extracted by the guanidine hydrochloride method from each peripheral blood sample. The study protocol was approved by the Ethics Reviewing Committee of Tokai University School of Medicine.

Genotyping for microsatellite polymorphisms

Genotyping was performed with 19 microsatellite markers located within the MHC genomic region to identify the susceptibility locus for HCV-DCM and/or HCV-HCM. The marker order, genomic distances, and primer sequence were described previously (25, 26). Each forward primer was synthesized by labeling at the 5′ end with a fluorochrome 6-FAM, HEX, or TET (Applied Biosystems, Foster City, CA, USA). The fragment samples were mixed with formamide-containing stop buffer and GeneScan-500 ROX size standard (Applied Biosystems) and separated by GeneScan system on a 377 DNA sequencer (Applied Biosystems). Repeat fragment sizes were assigned by GENOTYPER software (Applied Biosystems).

Genotyping for two classical *HLA* genes, *HLA-B*, and *HLA-Cw*

The high resolution typing for *HLA-B* and *HLA-Cw* was performed by the sequence-based typing (SBT) method according to standard procedures (Forensic Analytical, Hayward, CA, USA). The MATCHMAKER allele Identification program (Applied Biosystems) was used to assign the *HLA* alleles.

Genotyping for five non-*HLA* genes, *TNF*, *LTA*, *NFKBIL1*, *ATP6V1G2*, and *BAT1*

A total of 23 single nucleotide polymorphisms (SNPs) as shown in Fig. 2 were examined in this study. The tumor necrosis factor (*TNF*), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1 also known as *IκBL* (*NFKBIL1*), and HLA-B associated transcript 1 (*BAT1*) genes were genotyped by the direct sequencing method using the primer sets as previously reported

Primers used for genotyping the candidate genes, *LTA* and *ATP6V1G2*

Gene	Target	Name	Primer sequence (5′–3′)	Length (mer)	Product (bp)	Annealing (°C)
LTA						
	+80C/A	LTA1F2	CTC CAC ACA GCA GGT GAG G	19	172	58
		LTA1R2	CCA AAA CCA AAC CCA CCA AG	20		
	+252A/G	LTA1F	GCT TCG TGC TTT GGA CTA CC	20	714	60
		LTA3R	GGG AGG TCA GGT GGA TGT TTA C	22		
ATP6V1G2 (ATP6G)						
	Exon1	ATPex1F	AGG AGG ACC AGT CAT CAA TAG GAG	24	306	66
		ATPex1R	CTA AGG GAG GAA AGA GGA GAC TCA	24		
	Exon2 and *+760A/T* (rs2523502)	ATPex2F	GGG ACT GAC TCC TGC TAT TAC ATT G	25	247	64
		ATPex2R	CAC CCT TAC ACA CCT CAC TAG ATG C	25		
	Exon3 and *+1222C/T* (rs2239705)	ATPex3F	CTT TCT TCT AGG CTT TGT TTC AGG A	25	527	62
		ATPex3R	CAA ATT TCA CAG AGG GTT TAG GTG A	25		

Table 1

(27–29). The *ATP6V1G2* gene was analyzed for all exons in addition to two intronic SNPs, *ATP6V1G2*+760* (dbSNP accession number rs2523502) and *ATP6V1G2*+1222* (rs2239705), through the direct sequencing method by primer sets as listed in Table 1. Cycle sequencing was performed using the Big-Dye Terminator system (Applied Biosystems). The sequencing analysis was conducted with an ABI Prism 377 DNA sequencer (Applied Biosystems). The genotypes were determined by manual comparison of patients' sequences with a public reference sequence (GeneBank accession number AB063177). In the *LTA* (lymphotoxin-alpha) gene, alleles carried at +80 and +252 (30) were screened by the PCR-single strand conformation polymorphism analysis and the PCR-restriction fragment length polymorphism method at the *Nco* I site, respectively.

Statistical analysis

The Hardy – Weinberg equilibrium distribution for the SNPs and their haplotypes was assessed by Fisher's exact test with Bonferroni's inequality method. The statistical significance was assigned at P and corrected P (Pc) values of less than 0.05. The strength of the association with HCV-DCM and/or HCV-HCM was evaluated by odds ratio (OR) with 95% confidence interval. The R package 'haplo.stats' (http://www.r-project.org/) was used to evaluate haplotype structure carrying the disease-associated markers around the candidate region. On the basis of the haplotype structure, the LD around the candidate region was measured in both patients and controls with two LD coefficients, Lewontin's D' and Hill's r^2, obtained from the R package 'genetics' (http://www.r-project.org/).

Results

Susceptibility gene mapping for HCV-associated cardiomyopathy with microsatellite markers encompassing the MHC genomic region

In an initial association analysis, we carried out a region-wide scan using 19 microsatellite markers dispersed throughout the MHC region. The multipoint analysis displayed different association profiles between HCV-DCM and HCV-HCM as illustrated in Fig. 1. In the analysis of HCV-DCM, significant associations were observed with two markers positioned around the boundary region between class III and class I: *STR-MICA*183* (OR = 4.59, $P = 0.005$, $Pc = 0.026$) and *C1-4-1*225* (OR = 3.57, $P = 0.006$, $Pc = 0.042$). Because LD of microsatellite markers usually extend some hundred kb, we considered a approximately 300-kb interval as a potential susceptibility locus for HCV-DCM, which contains 9 genes as follows; *TNF*, *LTA*, *NFKBIL1*, *ATP6V1G2* (vacuolar ATP synthase subunit G 2; also known as *ATP6G*), *BAT1*, *MICB* (MHC class I′ chain-related protein B), *MICA* (MHC class I chain-related gene A protein), *HLA-B*, and *HLA-Cw* (Fig. 2). In clear contrast, there were no significant associations between the HCV-HCM and the microsatellite markers in the MHC region.