

FIG. 1. (A) Alignment of amino acid sequences of African green monkey (AGM, CV1-TRIM5 α -type1), cynomolgus monkey (CM, HSC-F-TRIM5 α), and human TRIM5 α (Hu, MT4-TRIM5 α) predicted from the sequences of the cDNAs used in the present study with key domains indicated. (B) Alignment of amino acid sequences of the highly variable region within the SPRY (B30.2) domain of TRIM5 α . The rhesus monkey TRIM5 α (Rh) sequence published by Stremlau et al. (33) was added. A box indicates the 20-amino-acid duplication within African green monkey TRIM5 α . An open arrowhead denotes the recombination point in chimeric TRIM5 α s, HSC-delete-tag, HSC-insert-tag, and CV1-delete-tag (see Fig. 4A). (C) Phylogenetic tree of various TRIM5 α sequences produced by the UPGMA (unweighted pair-group method with arithmetic averages) method.

pressing MT4-, CV1-, and HSC-F-TRIM5 α were selected according to the method described in Materials and Methods. The levels of expression of TRIM5 α were determined by using a real-time PCR, and cells expressing comparable amounts of TRIM5 α (4×10^6 to 6×10^6 copies/ μ g of total RNA) were used for subsequent study. Restriction can be quantified by comparing the percentage of GFP-positive cells with or without TRIM5 α .

As can be seen in Fig. 2A, MT4-TRIM5 α had a very weak anti-HIV-1 effect (~ 1.2 -fold), a finding consistent with a previous study (33). In contrast, restriction of HIV-1 was clearly

evident over a wide range of initial MOIs in cells expressing HSC-F-TRIM5 α , CV1-TRIM5 α -type1, and CV1-TRIM5 α -type2 (ca. 5- to 10-fold). There was no significant difference between CV1-TRIM5 α -type1 and type2; therefore, we chose CV1-TRIM5 α -type1 to be representative of CV1-TRIM5 α in the subsequent experiments. We obtained the same results as described above when we used an HA-tagged version of TRIM5 α (data not shown).

African green monkey and cynomolgus monkey TRIM5 α inhibit CD4-dependent HIV-1 infection in human cells. To test the restriction properties of TRIM5 α in CD4-dependent

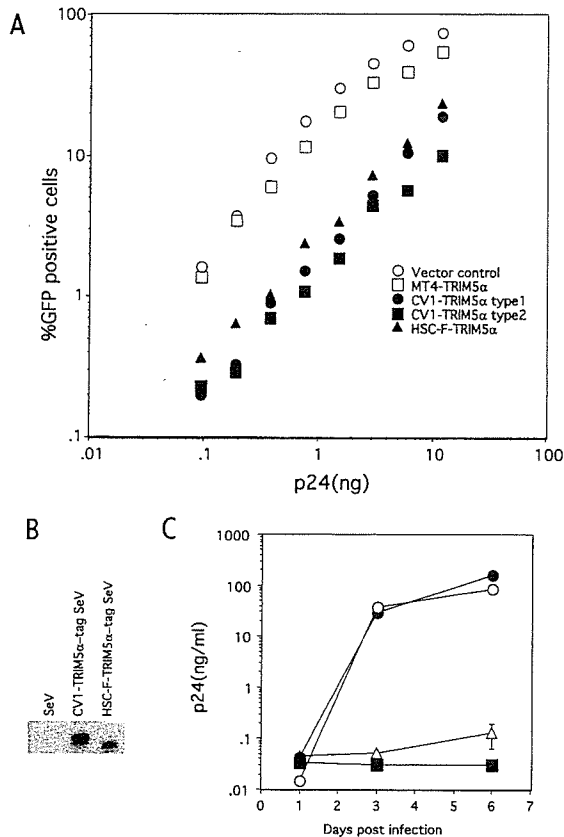


FIG. 2. (A) TK-ts13 cell clones expressing MT4-TRIM5 α (\square), CV1-TRIM5 α -type1 (\bullet), CV1-TRIM5 α -type2 (\blacksquare), HSC-F-TRIM5 α (\blacktriangle), or empty vector (\circ) were exposed to the indicated GFP-expressing HIV-1 vector. GFP-positive cells were counted with a flow cytometer. Data typical of at least three independent clones for each TRIM5 α are shown. (B) Lysates of MT4 cells infected with recombinant SeV expressing CV1-TRIM5 α -tag, HSC-F-TRIM5 α -tag, or the parental Z strain were immunoprecipitated by anti-HA antibody. Resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of four independent experiments is shown. (C) MT4 cells were mock infected (\circ), or infected with SeV expressing CV1-TRIM5 α -tag (\blacksquare), HSC-F-TRIM5 α -tag (\triangle), or the parental Z strain (\bullet). At 9 h after infection, cells were inoculated with an HIV-1 strain, NL43, and culture supernatants were periodically assayed for levels of p24. The datum points are means for triplicate samples with the SD.

HIV-1 infection, we constructed a recombinant SeV expressing TRIM5 α fused with the HA tag in the C-terminal of HSC-F-TRIM5 α or CV1-TRIM5 α (HSC-F-TRIM5 α -tag SeV or CV1-TRIM5 α -tag SeV). Human T-cell line MT4 cells were first infected with the SeV expressing TRIM5 α -tag (Fig. 2B), incubated at 37°C for 9 h, and then infected with an X4-tropic HIV-1 strain NL43. As can be seen in Fig. 2C, both HSC-F-TRIM5 α -tag and CV1-TRIM5 α -tag completely inhibited HIV-1 replication, whereas MT4 cells infected with SeV empty vector fully supported HIV-1 replication.

Distinct patterns of restriction for SIVmac among nonhuman primate TRIM5 α . In African green monkey CV1 cells, both HIV-1 and SIVmac239 were restricted, whereas only HIV-1 was restricted in cynomolgus monkey HSC-F cells (Fig. 3A). Therefore, we examined whether or not CV1-TRIM5 α also

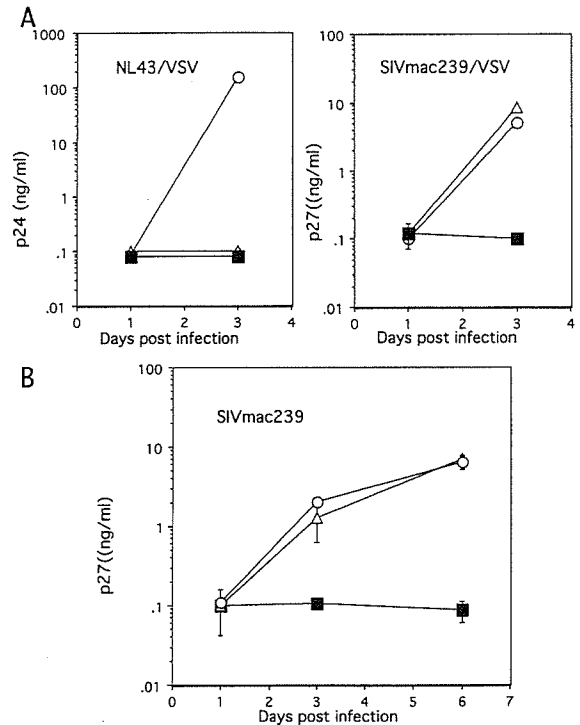


FIG. 3. (A) MT4 (\circ), HSC-F (\triangle), or CV1 (\blacksquare) cells were infected with VSV-pseudotyped NL43 or VSV-pseudotyped SIVmac239, and culture supernatants were periodically assayed for levels of p24 or p27. The datum points are means for triplicate samples with the SD. (B) MT4 cells were mock infected (\circ) or infected with SeV expressing CV1-TRIM5 α -tag (\blacksquare) or HSC-F-TRIM5 α -tag (\triangle). At 9 h after infection, cells were inoculated with SIVmac239, and culture supernatants were periodically assayed for levels of p27. The datum points are means for triplicate samples with the SD.

could inhibit the replication of SIVmac. In MT4 cells infected with recombinant SeV expressing HSC-F-TRIM5 α -tag, the replication of SIVmac239 was not suppressed at all (Fig. 3B), indicating that the HSC-F-TRIM5 α showed a similar specificity to rhesus monkey TRIM5 α (33). In MT4 cells infected with recombinant SeV expressing CV1-TRIM5 α -tag, in contrast, the replication of SIVmac239 was completely suppressed. These results suggested that the distinct sensitivity of African green monkey and cynomolgus monkey cells to HIV-1 and SIVmac infection was, at least partly, determined by TRIM5 α .

A small region of 37 amino acid residues in the SPRY domain of CV1-TRIM5 α determines SIVmac restriction. A comparison of the human and nonhuman primate TRIM5 α sequences showed the presence of a highly variable region in the N-terminal portion of the SPRY domain (Fig. 1A and B). In this region, CV1 and Vero TRIM5 α had a 20-amino-acid repetition that was totally absent in HSC-F-TRIM5 α . These findings prompted us to test whether this highly variable region of TRIM5 α determined the species-specific inhibition of SIVmac infection. We constructed chimeric TRIM5 α s from HSC-F-TRIM5 α -tag and CV1-TRIM5 α -tag by using SphI and BamHI restriction enzyme digestion (Fig. 4A). HSC-F+60tag contained the 242-bp fragment of CV1-TRIM5 α in the background of HSC-F-TRIM5 α -tag. The reciprocal chimera, CV1-60tag, contained a 182-bp fragment of HSC-F-TRIM5 α in the

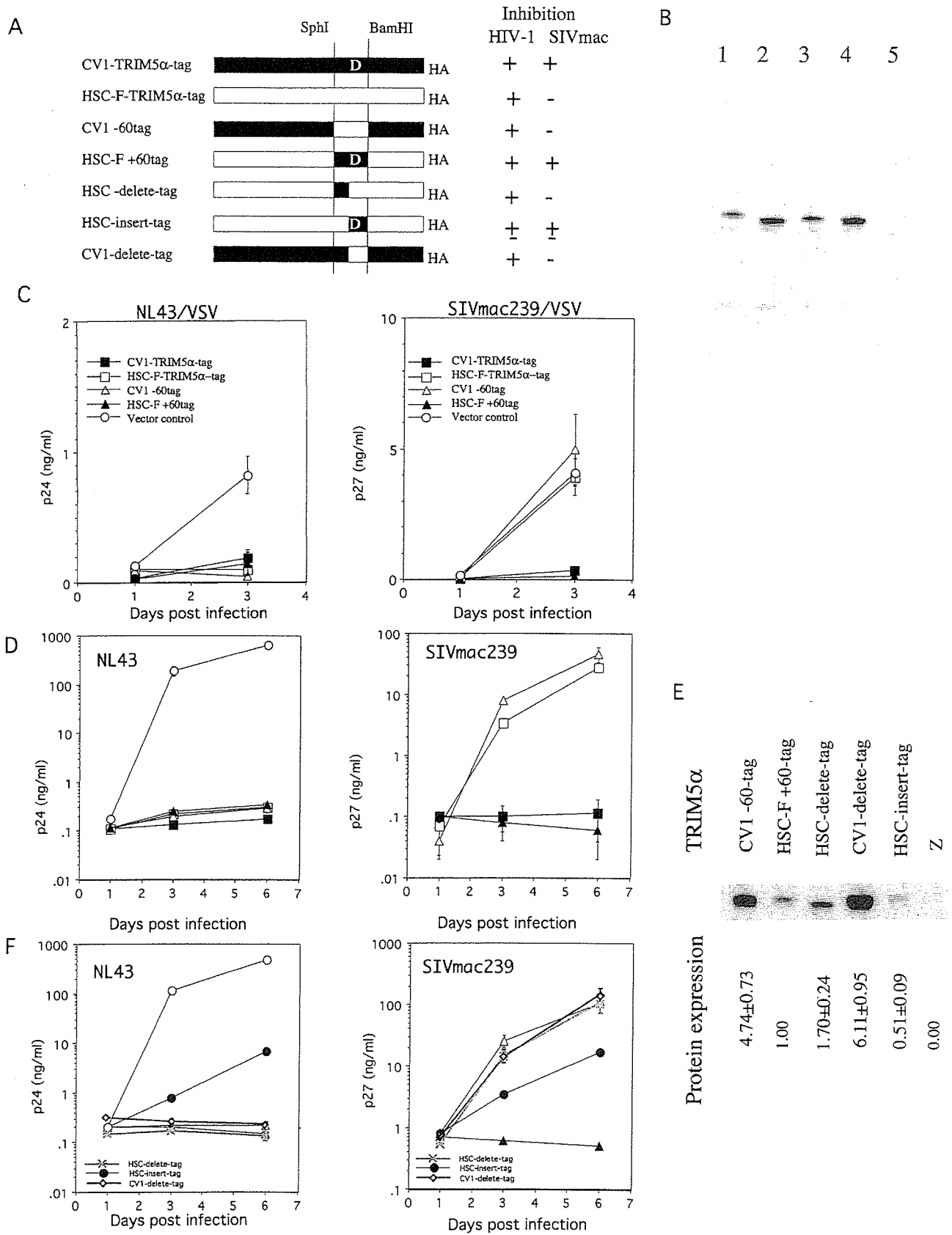


FIG. 4. (A) Schematic representation of chimeric TRIM5 α and summary of the results. Filled and open bars denote CV1 and HSC-F sequences, respectively. D, the CV-1-TRIM5 α -specific 20-amino-acid duplication. The +, \pm , and - symbols denote full, partial, and no

background of the CV1-TRIM5 α -tag. In this fragment, the differences between CV1 and HSC-F TRIM5 α , including the 20-amino-acid repetition, were located in a small region of 37 amino acid residues (Fig. 1B). Human osteosarcoma C143 cells stably expressing various TRIM5 α s (Fig. 4B) were infected with VSV-G-pseudotyped HIV-1 NL43, and levels of P24 in the culture supernatants were assayed periodically. As expected, both chimeric TRIM5 α s and parental TRIM5 α s clearly inhibited the replication of HIV-1 NL43 (Fig. 4C). When these cells were infected with VSV-G-pseudotyped SIVmac239, the parental CV1-TRIM5 α -tag could also inhibit the replication of SIVmac239, whereas CV1-60tag, which contained the 182-bp fragment of HSC-F-TRIM5 α in the background of CV1-TRIM5 α -tag, could not. Conversely, the parental HSC-F-TRIM5 α -tag did not inhibit the replication of SIVmac239 at all, whereas HSC-F+60tag, which contained the 242-bp fragment of CV1-TRIM5 α in the background of HSC-F-TRIM5 α -tag, clearly inhibited SIVmac239 (Fig. 4C). We obtained the same results as described above when we used SeVs to express parental TRIM5 α -tag or chimeras (Fig. 4D). These results indicated that the determinant of the species-specific inhibition of SIVmac239 replication is located in 37 amino acid residues in the SPRY domain of CV1-TRIM5 α .

To narrow the SIVmac restriction determinant more precisely, we generated two more chimeric TRIM5 α s (Fig. 4A). HSC-delete-tag contained a CV1-derived 17-amino-acid fragment without the 20-amino-acid duplication in the background of the HSC-TRIM5 α -tag. HSC-insert-tag contained the HSC-F-derived 17-amino-acid fragment with the CV-1 derived 20-amino-acid duplication in the background of HSC-TRIM5 α -tag. Recombinant SeVs expressing these chimeric TRIM5 α s were generated and used in the subsequent experiments. Although the expression levels of mRNA of each chimeric TRIM5 α was virtually identical to each other (data not shown), we observed considerable variations in the levels of TRIM5 α protein expression among chimeras (Fig. 4E). The HSC-delete-tag showed slightly higher levels of protein expression than those of HSC-F+60tag. However, HSC-delete-tag failed to inhibit SIVmac replication, whereas it restricted HIV-1 replication as completely as HSC-F+60tag did (Fig. 4F). These results clearly indicated that the CV1-derived 17-amino-acid region alone was not sufficient for SIVmac restriction. On the other hand, HSC-insert-tag partially restricted both HIV-1 and SIVmac, although this chimera showed lower levels of protein expression than other chimeras did (Fig. 4E

and F). These results indicated that the CV1-specific 20-amino-acid duplication was important in SIVmac restriction.

To determine whether the CV1-specific 20-amino-acid duplication was indispensable for SIVmac restriction, we generated CV1-delete-tag, which lacked the 20-amino-acid duplication in the CV1-TRIM5 α -tag (Fig. 4A). The protein expression level of CV1-delete-tag was comparable to that of CV1-60tag (Fig. 4E), and CV1-delete-tag inhibited HIV-1 replication as completely as CV1-60tag did. However, CV1-delete-tag was shown to lose the ability to inhibit SIVmac infection (Fig. 4F). Taken together, our data clearly indicated that the 20-amino-acid duplication of CV1-TRIM5 α was necessary for SIVmac restriction and suggested that the adjacent 17-amino-acid region of CV1-TRIM5 α was also necessary to fully restrict SIVmac infection.

HIV-2 GH123 is sensitive to cynomolgus monkey TRIM5 α , as well as African green monkey TRIM5 α . HIV-2 is closely related to SIVmac (9). We tested whether or not the sensitivity of HIV-2 to various TRIM5 α s was similar to that of SIVmac239. C143 cells expressing CV1, HSC-F, and their chimeric TRIM5 α s were infected with the VSV-G-pseudotyped HIV-2 strain GH123. Surprisingly, HSC-F-TRIM5 α -tag inhibited HIV-2 replication as CV1-TRIM5 α -tag had done (Fig. 5). Both chimeric TRIM5 α s, CV1-60tag and HSC-F+60tag, also inhibited HIV-2 replication to a similar extent (Fig. 5). These results indicated that HIV-2 strain GH123 was sensitive to cynomolgus monkey TRIM5 α despite its high level of sequence homology to SIVmac239.

DISCUSSION

In the present study, we showed that both cynomolgus and African green monkey TRIM5 α s could inhibit HIV-1 infection. African green monkey TRIM5 α could also inhibit SIVmac infection, whereas cynomolgus monkey TRIM5 α could not. Experiments on chimeras of the cynomolgus and African green monkey TRIM5 α s unequivocally demonstrated that a small region composed of 37 amino acid residues in the SPRY domain of African green monkey TRIM5 α was responsible for restricting the SIVmac infection.

A previous study showed that rhesus monkey TRIM5 γ , a splicing variant lacking the SPRY domain, did not suppress HIV-1 infection (33). In the case of TRIM7, the SPRY domain alone was sufficient for binding to its ligand glycogenin (38). Deletion of the entire SPRY domain from TRIM11 also abol-

suppression, respectively. (B) Lysates of C143 cells expressing CV1-TRIM5 α -tag (lane 1), HSC-F-TRIM5 α -tag (lane 2), HSC-F+60tag (lane 3), CV1-60tag (lane 4), or empty vector (lane 5) were immunoprecipitated by using anti-HA antibody. The resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of three independent experiments is shown. (C) C143 cells expressing CV1-TRIM5 α -tag (■), HSC-F-TRIM5 α -tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with VSV-pseudotyped NL43 or SIVmac239, and culture supernatants were periodically assayed for levels of p24 or p27. The datum points are means for triplicate samples with the SD. (D) MT4 cells infected with SeV expressing CV1-TRIM5 α -tag (■), HSC-F-TRIM5 α -tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with NL43 or SIVmac239, and culture supernatants were assayed for levels of p24 and p27. The datum points are means for triplicate samples with the SD. (E) Lysates of MT4 cells infected with recombinant SeVs expressing CV1-60tag, HSC-F+60tag, HSC-delete-tag, CV1-delete-tag, HSC-insert-tag, or parental Z strain were immunoprecipitated by using anti-HA antibody. Resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of three independent experiments is shown. The relative amounts of TRIM5 α protein to that of HSC-F+60tag were calculated, and means and SD values of three independent experiments are shown. (F) MT4 cells infected with SeV expressing CV1-60tag (△), HSC-F+60tag (▲), HSC-delete-tag (asterisks with blue lines), HSC-insert-tag (●), CV1-delete-tag (diamonds with red lines), or empty vector (○) were infected with NL43 or SIVmac239, and culture supernatants were assayed for levels of p24 and p27. The datum points are means for triplicate samples with the SD.

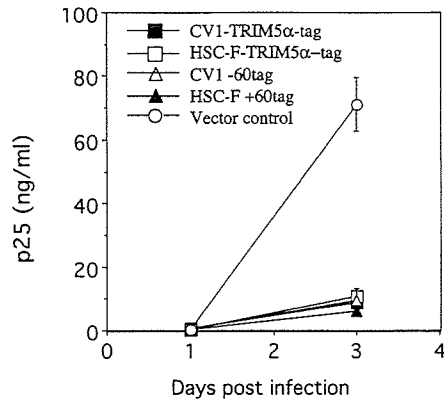


FIG. 5. C143 cells expressing CV1-TRIM5 α -tag (■), HSC-F-TRIM5 α -tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with VSV-pseudotyped GH123, and culture supernatants were periodically assayed for levels of p25. The datum points are means for triplicate samples with the SD.

ished its ability to bind Humanin (21). Therefore, it is reasonable to assume that the variable N-terminal region of the SPRY domain of TRIM5 α binds to HIV-1 or SIVmac CA protein. This assumption is consistent with the recent findings that in owl monkey cells, HIV-1 infection was restricted by a TRIM5-cyclophilin A fusion protein in which the SPRY domain was replaced with cyclophilin A, since cyclophilin A is a well-known ligand of HIV-1 CA protein (22, 28).

In the attempt to further narrow the SIVmac restriction determinant more precisely, we were able to demonstrate that the African green monkey-specific 20-amino-acid duplication was indispensable for SIVmac restriction and that the adjacent 17-amino-acid region of African green monkey alone was not sufficient. However, HSC-insert-tag carrying the cynomolgus monkey-derived 17-amino acid region with African green monkey-specific 20-amino-acid duplication showed low levels of protein expression and only partial suppression of HIV-1 and SIVmac replication. It is possible that an artificial combination of African green monkey-specific 20-amino-acid duplication with the cynomolgus monkey-derived 17-amino-acid region made TRIM5 α molecules unstable. Further studies, including mutational analysis of the African green monkey-specific 17-amino-acid region, are necessary to determine the precise role of this region in SIVmac restriction.

Despite its close similarity to SIVmac, HIV-2 strain GH123 was restricted by cynomolgus monkey TRIM5 α , as well as by African green monkey TRIM5 α . Although both HIV-2 and SIVmac were considered to come from SIVsm (9), it is possible that HIV-2 has been replicating in the human population in the absence of TRIM5 α restriction for a certain period and has lost its ability to escape from cynomolgus monkey TRIM5 α . However, it has also been reported that there was a considerable degree of variation in the ability to grow in monkey cells among HIV-2 strains (6, 8, 26). Therefore, it is necessary to examine various HIV-2 strains for their sensitivity to human and monkey TRIM5 α s before we can draw a definite conclusion. It would also be interesting to identify specific amino acid changes determining the sensitivity to cynomolgus monkey TRIM5 α in viral CA proteins, since nearly 90% of the amino

acid residues in SIVmac239 CA protein are conserved in HIV-2GH123.

In CV1 cells, the level of TRIM5 gene expression was ca. 3×10^6 copies/ μ g of total RNA, a level similar to that observed in other human cell lines examined (data not shown). Although HIV-1 infection was suppressed in hamster TK-ts13 or human C143 cells expressing CV1-TRIM5 α , relatively high levels of TRIM5 α (nearly 5×10^7 copies/ μ g of total RNA) appeared to be required for a level of suppression similar to that observed in CV1 cells. One possible explanation for this discrepancy is that certain molecules cooperating with TRIM5 α also showed species specificity, and CV1-TRIM5 α was not fully supported in hamster and human cells. Because TRIM5 gene products are suspected to be an E3 ubiquitin ligase (35), it is important to identify the E2 ubiquitin-conjugating enzyme interacting with TRIM5 α . Alternatively, restriction factors other than TRIM5 α may exist in CV1 cells, or certain molecules required for efficient lentivirus infection may be absent in CV1 cells.

After we submitted these findings for publication, small amino acid differences in the SPRY domain between human and rhesus monkey TRIM5 α s were reported to determine HIV-1 restriction (27, 34, 37). Our findings are in good agreement with the results of these studies.

ACKNOWLEDGMENTS

We thank Setsuko Bando for skillful technical assistance. HSC-F cells were kindly supplied by Hirofumi Akari. pGH123 was a gift from Akio Adachi.

This study was supported by grants from the Human Science Foundation; the Ministry of Education, Culture, Sports, Science, and Technology; and the Ministry of Health, Labour, and Welfare of Japan.

REFERENCES

- Adachi, A., H. E. Gendelman, S. Koeing, T. Folks, R. Willey, A. Rabson, and M. A. Martin. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* 59:284–291.
- 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.
- Akari, H., K. H. Nam, K. Mori, I. Otani, H. Shibata, A. Adachi, K. Terao, and Y. Yoshikawa. 1999. Effects of SIVmac infection on peripheral blood CD4⁺ CD8⁺ T lymphocytes in cynomolgus macaques. *Clin. Immunol.* 91:321–329.
- Alder, H., C. D. Chang, S. T. Chen, I. Beck, C. Y. Chang, and R. Baserga. 1989. Temporary complementation of temperature-sensitive mutants of the cell cycle by transfection with a wild-type or a mutant cDNA of ADP/ATP translocase. *J. Cell Physiol.* 141:90–96.
- Besnier, C., Y. Takeuchi, and G. Towers. 2002. Restriction of lentivirus in monkeys. *Proc. Natl. Acad. Sci. USA* 99:11920–11925.
- Castro, B. A., M. Nepomuceno, N. W. Lerche, J. W. Eichberg, and J. A. Levy. 1991. Persistent infection of baboons and rhesus monkeys with different strains of HIV-2. *Virology* 184:219–226.
- Chackerian, B., E. M. Long, P. A. Luciw, and J. Overbaugh. 1997. Human immunodeficiency virus type 1 coreceptors participate in postentry stages in the virus replication cycle and function in simian immunodeficiency virus infection. *J. Virol.* 71:3932–3939.
- Fujita, M., A. Yoshida, A. Sakurai, J. Tatsuki, F. Ueno, H. Akari, and A. Adachi. 2003. Susceptibility of HVS-immortalized lymphocytic HSC-F cells to various strains and mutants of HIV/SIV. *Int. J. Mol. Med.* 11:641–644.
- Gao, F., E. Bailes, D. L. Robertson, Y. Chen, C. M. Rodenburg, S. F. Michael, L. B. Cummins, L. O. Arthur, M. Peeters, G. M. Shaw, P. M. Sharp, and B. H. Hahn. 1999. Origin of HIV-1 in the chimpanzee *Pan troglodytes* troglodytes. *Nature* 397:436–441.
- Hatzioannou, T., S. Cowan, S. P. Goff, P. D. Bieniasz, and G. J. Towers. 2003. Restriction of multiple divergent retroviruses by Lv1 and Ref1. *EMBO J.* 22:385–394.
- Hatzioannou, T., S. Cowan, U. K. Von Schwedler, W. I. Sundquist, and P. D. Bieniasz. 2004. Species-specific tropism determinants in the human immunodeficiency virus type 1 capsid. *J. Virol.* 78:6005–6012.

12. Hatzioannou, T., D. Perez-Caballero, A. Yang, S. Cowan, and P. D. Bieniasz. 2004. Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5 α . *Proc. Natl. Acad. Sci. USA* 101:10774–10779.
13. Himathongkham, S., and P. A. Luciw. 1996. Restriction of HIV-1 (subtype B) replication at the entry step in rhesus macaque cells. *Virology* 219:485–488.
14. Keckesova, Z., L. M. Ylinen, and G. J. Towers. 2004. The human and African green monkey TRIM5 α genes encode Ref1 and Lv1 retroviral restriction factor activities. *Proc. Natl. Acad. Sci. USA* 101:10780–10785.
15. 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.
16. Kootstra, N. A., C. Munk, N. Tonnu, N. R. Landau, and I. M. Verma. 2003. Abrogation of postentry restriction of HIV-1-based lentiviral vector transduction in simian cells. *Proc. Natl. Acad. Sci. USA* 100:1298–1303.
17. Miyoshi, H., U. Blomer, M. Takahashi, F. H. Gage, and I. M. Verma. 1998. Development of a self-inactivating lentivirus vector. *J. Virol.* 72:8150–8157.
18. Miyoshi, H., M. Takahashi, F. H. Gage, and I. M. Verma. 1997. Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc. Natl. Acad. Sci. USA* 94:10319–10323.
19. Munk, C., S. M. Brandt, G. Lucero, and N. R. Landau. 2002. A dominant block to HIV-1 replication at reverse transcription in simian cells. *Proc. Natl. Acad. Sci. USA* 99:13843–13848.
20. Nakayama, E. E., Y. Tanaka, Y. Nagai, A. Iwamoto, and T. Shioda. 2004. A CCR2-V64I polymorphism affects stability of CCR2A isoform. *AIDS* 18:729–738.
21. Niikura, T., Y. Hashimoto, H. Tajima, M. Ishizaka, Y. Yamagishi, M. Kawasumi, M. Nawa, K. Terashita, S. Aiso, and I. Nishimoto. 2003. A tripartite motif protein TRIM11 binds and destabilizes Humanin, a neuroprotective peptide against Alzheimer's disease-relevant insults. *Eur. J. Neurosci.* 17:1150–1158.
22. Nisole, S., C. Lynch, J. P. Stoye, and M. W. Yap. 2004. A Trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. *Proc. Natl. Acad. Sci. USA* 101:13324–13328.
23. Owens, C. M., B. Song, M. J. Perron, P. C. Yang, M. Stremlau, and J. Sodroski. 2004. Binding and susceptibility to postentry restriction factors in monkey cells are specified by distinct regions of the human immunodeficiency virus type 1 capsid. *J. Virol.* 78:5423–5437.
24. Owens, C. M., P. C. Yang, H. Gottlinger, and J. Sodroski. 2003. Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells. *J. Virol.* 77:726–731.
25. Perron, M. J., M. Stremlau, B. Song, W. Ulm, R. C. Mulligan, and J. Sodroski. 2004. TRIM5 α mediates the postentry block to N-tropic murine leukemia viruses in human cells. *Proc. Natl. Acad. Sci. USA* 101:11827–11832.
26. Putkonen, P., B. Bottiger, K. Warstedt, R. Thorstensson, J. Albert, and G. Biberfeld. 1989. Experimental infection of cynomolgus monkeys (*Macaca fascicularis*) with HIV-2. *J. Acquir. Immune. Defic. Syndr.* 2:366–373.
27. Sawyer, S. L., L. I. Wu, M. Emerman, and H. S. Malik. 2005. Positive selection of primate TRIM5 α identifies a critical species-specific retroviral restriction domain. *Proc. Natl. Acad. Sci. USA* 102:2832–2837.
28. Sayah, D. M., E. Sokolskaja, L. Berthou, and J. Luban. 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* 430:569–573.
29. Shibata, R., M. Kawamura, H. Sakai, M. Hayami, A. Ishimoto, and A. Adachi. 1991. Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. *J. Virol.* 65:3514–3520.
30. Shibata, R., T. Miura, M. Hayami, K. Ogawa, H. Sakai, T. Kiyomasu, A. Ishimoto, and A. Adachi. 1990. Mutational analysis of the human immunodeficiency virus type 2 (HIV-2) genome in relation to HIV-1 and simian immunodeficiency virus SIV (AGM). *J. Virol.* 64:742–747.
31. Shibata, R., H. Sakai, M. Kawamura, K. Tokunaga, and A. Adachi. 1995. Early replication block of human immunodeficiency virus type 1 in monkey cells. *J. Gen. Virol.* 76(Pt. 11):2723–2730.
32. Shioda, T., E. E. Nakayama, Y. Tanaka, X. Xin, H. Liu, A. Kawana-Tachikawa, A. Kato, Y. Sakai, Y. Nagai, and A. Iwamoto. 2001. Naturally occurring deletional mutation in the C-terminal cytoplasmic tail of CCR5 affects surface trafficking of CCR5. *J. Virol.* 75:3462–3468.
33. Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier, and J. Sodroski. 2004. The cytoplasmic body component TRIM5 α restricts HIV-1 infection in Old World monkeys. *Nature* 427:848–853.
34. Stremlau, M., M. Perron, S. Welikala, and J. Sodroski. 2005. Species-specific variation in the B30.2(SPRY) domain of TRIM5 α determines the potency of human immunodeficiency virus restriction. *J. Virol.* 79:3139–3145.
35. Xu, L., L. Yang, P. K. Moitra, K. Hashimoto, P. Rallabhandi, S. Kaul, G. Meroni, J. P. Jensen, A. M. Weissman, and P. D'Arpa. 2003. BTBD1 and BTBD2 colocalize to cytoplasmic bodies with the RBCC/tripartite motif protein, TRIM5delta. *Exp. Cell Res.* 288:84–93.
36. Yap, M. W., S. Nisole, C. Lynch, and J. P. Stoye. 2004. Trim5alpha protein restricts both HIV-1 and murine leukemia virus. *Proc. Natl. Acad. Sci. USA* 101:10786–10791.
37. Yap, M. W., S. Nisole, and J. P. Stoye. 2005. A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction. *Curr. Biol.* 15:73–78.
38. Zhai, L., A. Dietrich, A. V. Skurat, and P. J. Roach. 2004. Structure-function analysis of GNIP, the glycogenin-interacting protein. *Arch. Biochem. Biophys.* 421:236–242.

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 Nagai¹⁰

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 (Δ 5G). In Δ 5G-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 Δ 5G 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 Δ 5G 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 Δ 5G 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 (NAbs) 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 are 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 (Δ 5G), 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 Δ 5G; although Δ 5G was replicated as extensively as SIVmac239 during the primary infection, the subsequent Δ 5G 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 Δ 5G-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 Δ 5G 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 Δ 5G infection after primary infection, we hypothesized that the Env of Δ 5G, 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 Δ 5G (Δ 5G 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 SIVmac239 SU or Δ 5G SU, pJWSUmac239 and pJWSUmac Δ 5G, 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 Δ 5G SU DNA sequence was amplified with a pair of primers, SUmAcA (5'-TGTGCTAGCTATGTCACAGTCTTTTATGGTGTAC-3') and SUmAcB (5'-CCAGGATCCTATTACCTCTTCACATCTGTGGGGC-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 pJWSUmac Δ 5G. 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 Δ 5G, WRv_{wt}mac239 or WRv_{wt} Δ 5G, respectively, were constructed using a vaccinia virus WR strain (WRv) 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'-GAAAGAGAAGAAGGATCCCGAAAAAGG-3') consisted of nt 6796 to 9822 and the underlined mutations of the BamHI site; S-9537 (5'-TATGAATACTCCCGGGAGAAACCC-3') consisted of nt 9527 to 9550 and the underlined mutations of the SmaI site. DNA fragments containing the *env* gene of SIVmac239 or Δ 5G 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 WRv, 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 were propagated and titrated in CV-1 cells. 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, pJWSUmac Δ 5G, or the vector pJW4303. Secreted SU metabolically labeled with ³⁵S 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 WRv_{wt}mac239, WRv_{wt} Δ 5G, or WRv at a multiplicity of infection of 10, metabolically labeled with ³⁵S 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 Δ 5G 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

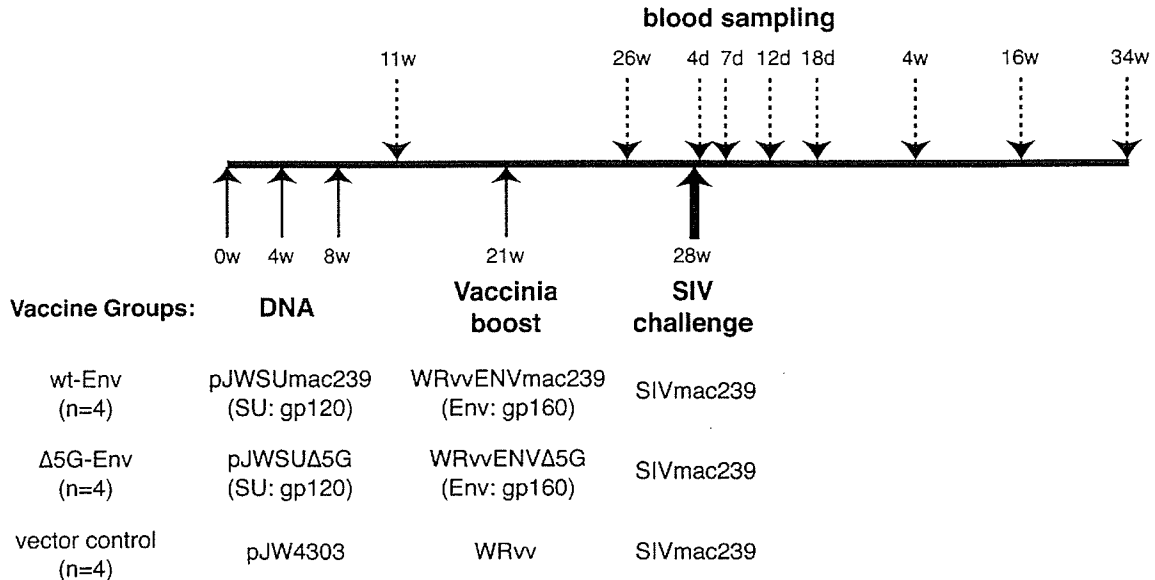


FIG. 1. Outline of immunization, challenge infection, and blood sampling. Twelve juvenile rhesus macaques were divided into three immunization groups of four animals each: the wt-Env immunization group (Mm0005, Mm0007, Mm0010, and Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, and Mm0009), 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 TCID₅₀ of SIVmac239 intravenously at 28 weeks p.p. w, weeks; d, day.

challenged with 10 50% tissue culture infective doses (TCID₅₀) 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'-GGACCAAGGCCTAAAAACCC-3'), and TaqMan probe 1272T (6-carboxyfluorescein-5'-ACCATGTTATGGCC AAATGCCAGAC-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 (OD₂₆₀) measurement and branched DNA assay for SIV viral RNA (Bayer Diagnostics, Tarrytown, N.Y.). RNA equivalent to 10 to 10⁷ 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 × 10⁵ 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 OD₄₉₂ 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

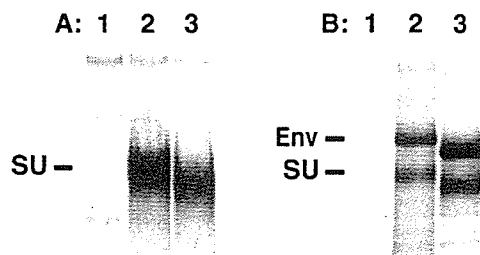


FIG. 2. Expression of SU and Env by SU-expressing DNA vaccines and Env-expressing vaccinia viruses. A: SU secreted in supernatant from CV-1 cells transfected with SU-expressing plasmids. Lane 1, pJW4303 vector; lane 2, pJWSUmac239; lane 3, pJWSUmac Δ 5G. B: Env in cell lysates of CV-1 cells infected with recombinant vaccinia viruses. Lane 1, WRv; lane 2, WRvmac239; lane 3, WRv Δ 5G.

(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, Δ 5G, 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 Δ 5G 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, pJWSU Δ 5G expressing SU of Δ 5G, 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 Δ 5G, or vaccinia virus (Fig. 1).

Expression of SU DNA plasmids and Env vaccinia viruses in vitro and in animals. Although Δ 5G 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 (pJWSUmac Δ 5G) were at similar levels (Fig. 2A). The expression and processing of Env in the wild type (WRvENVmac239) and in the deglycosylated Env mutant vaccinia virus (WRvENV Δ 5G) 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 WRvEnv, 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 TCID₅₀ 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 Δ 5G SU-specific T-cell response by using autologous B-LCLs infected with recombinant Sendai viruses expressing either wt SU and/or Δ 5G 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 Δ 5G 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 Δ 5G 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 Δ 5G 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 Δ 5G 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, Δ 5G, 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

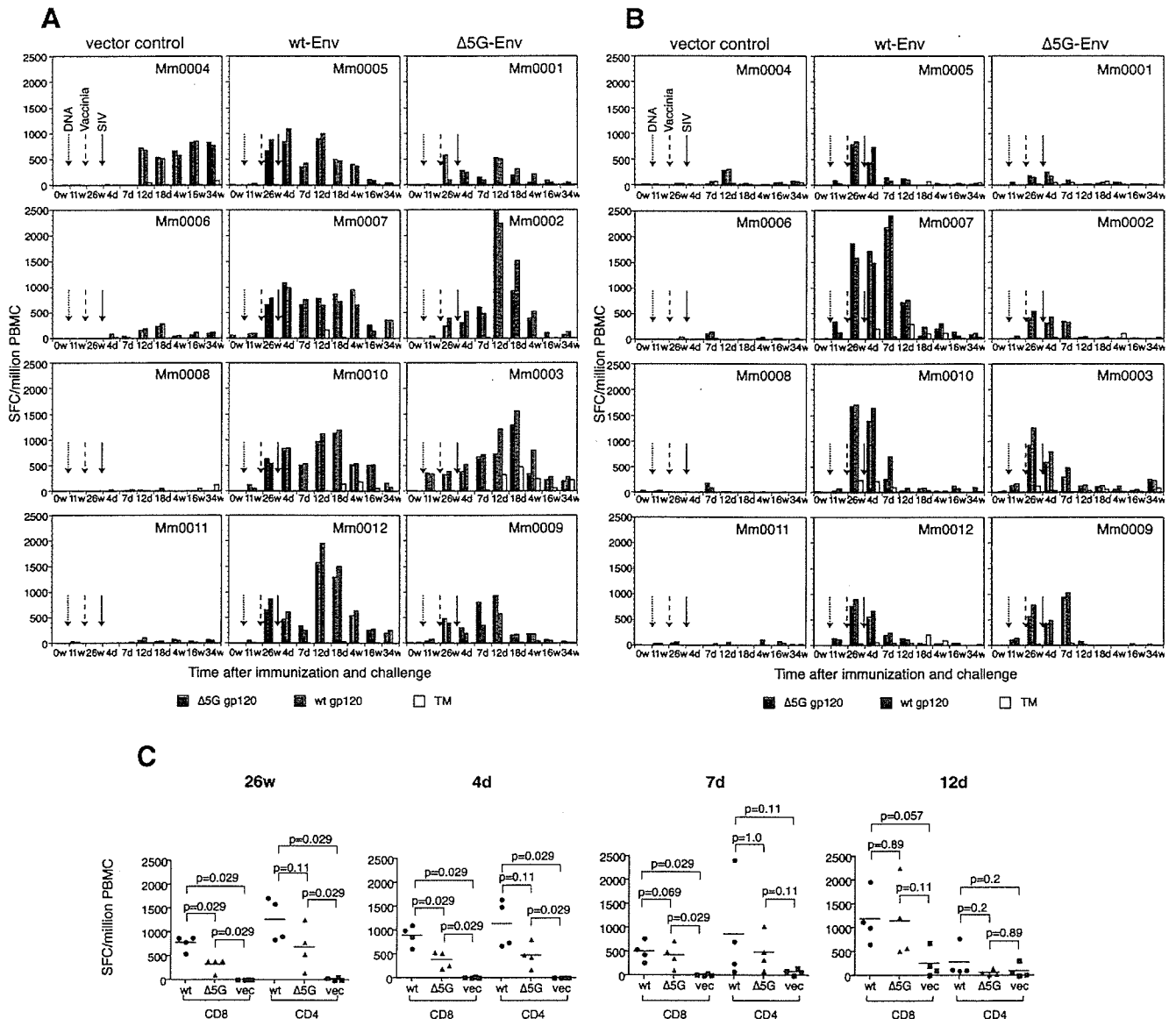


FIG. 3. Env-specific CD4⁺ T-cell and CD8⁺ T-cell responses in 12 macaques. A: Env-specific CD8⁺ T cells in PBMCs were measured by ELISPOT assay for IFN- γ in three groups. B: Env-specific CD4⁺ T cells in PBMCs were measured by ELISPOT assay for IFN- γ in three groups. ELISPOT results are colored as follows: Δ 5G 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 Δ 5G 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, Δ 5G (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 Δ 5G 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 Δ 5G 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

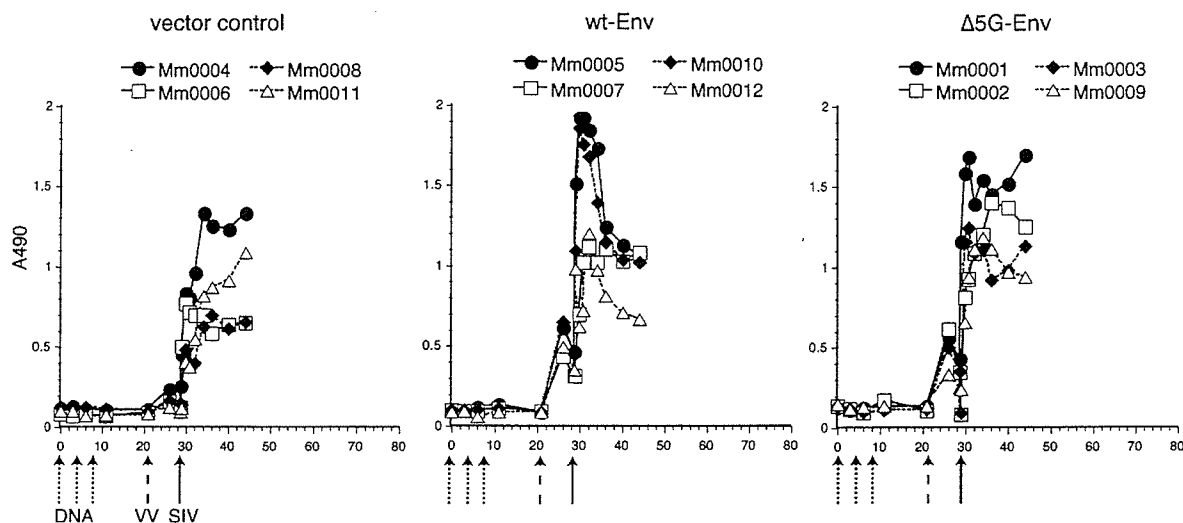


FIG. 4. Humoral immune response during immunization and after challenge infection. The OD_{492} was used as a relative measurement of anti-SIV ELISA antibody titer.

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 $\Delta 5G$ 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 $\Delta 5G$ 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 $\Delta 5G$ 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 $\Delta 5G$ Env vaccine group (Fig. 5E). Collectively, wt-Env vaccination induced significantly effective immunity to control SIVmac239 infection, whereas $\Delta 5G$ Env vaccination induced a marginal effect seen only in primary and not in chronic infection.

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 $\Delta 5G$ 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 $\Delta 5G$ 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

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

Vaccine	Animal	Neutralizing-antibody titer ^a			Mean ^b
		SIVmac239	$\Delta 5G$	239/envMERT	
wt-Env	Mm0005	<20	<20	800	400
	Mm0007	<20	<20	400	
	Mm0010	<20	<20	400	
	Mm0012	<20	<20	200	
$\Delta 5G$ -Env	Mm0001	<20	<20	100	50
	Mm0002	<20	<20	20	
	Mm0003	<20	<20	100	
	Mm0009	<20	<20	50	
		<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 ($P = 0.0029$).

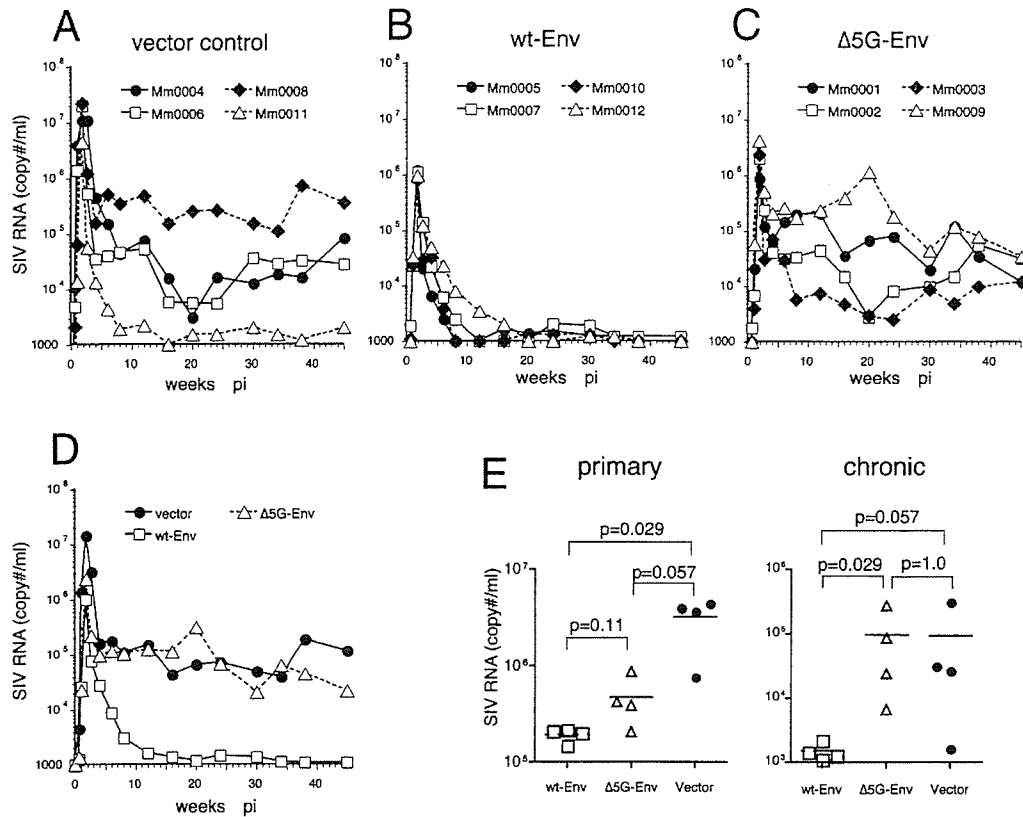


FIG. 5. Plasma viral loads after SIVmac239 challenge infection. Plasma viral load was measured by real-time PCR with a detection limit of 1,000 copies/ml. A: wt-Env vaccine group; B: Δ 5G Env vaccine group; C: vector controls; D: comparison of viral loads among three groups; E: comparison of viral loads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

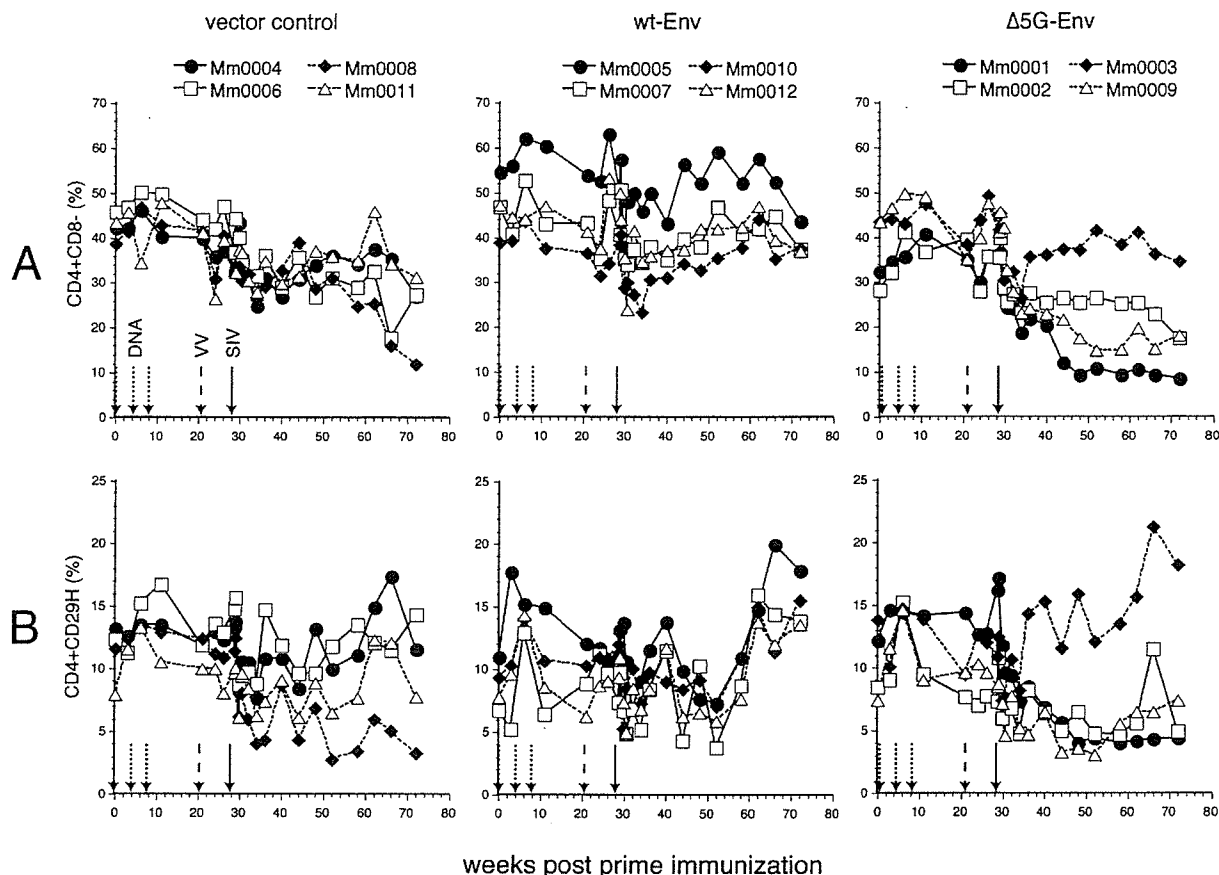


FIG. 6. CD4⁺ T cells in PBMCs from rhesus macaques during immunization and after the challenge infection. A: Percentage of CD4⁺ T cells in PBMCs; B: percentage of CD4⁺ CD29^{high} T cells in PBMCs.

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 $\Delta 5G$ infection in rhesus macaques, because the host response elicited by $\Delta 5G$ infection not only contained $\Delta 5G$ infection but also protected the animals from SIVmac239 challenge infection (36). This study therefore suggested that an almost sterilizing immunity against SIVmac239 induced in $\Delta 5G$ -infected animals could not be explained by the immunogenicity of $\Delta 5G$ Env; instead, it is likely associated with the property of $\Delta 5G$ as an attenuated virus. In fact, $\Delta 5G$ was more neutralization-

sensitive than SIVmac239 (36). Alternatively, the immunogenic property of Env in $\Delta 5G$ could not successfully be duplicated by immunization with a $\Delta 5G$ Env DNA prime-vaccinia virus boost regimen. Therefore, another immunization regimen might be able to elicit the protective immune response induced by $\Delta 5G$ infection.

The Env vaccine is superior to other vaccines containing other viral proteins with respect to the induction of NAb; however, both the $\Delta 5G$ Env vaccine and the wt-Env vaccine could not induce detectable NAb against either SIVmac239 or $\Delta 5G$. Instead, the wt-Env vaccine induced higher NAb against macrophage-tropic SIV than the $\Delta 5G$ 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 SIVmac239 (34), it might recognize unknown epitopes conserved between SIVmac239 and 239/envMERT. On the other hand, $\Delta 5G$ 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

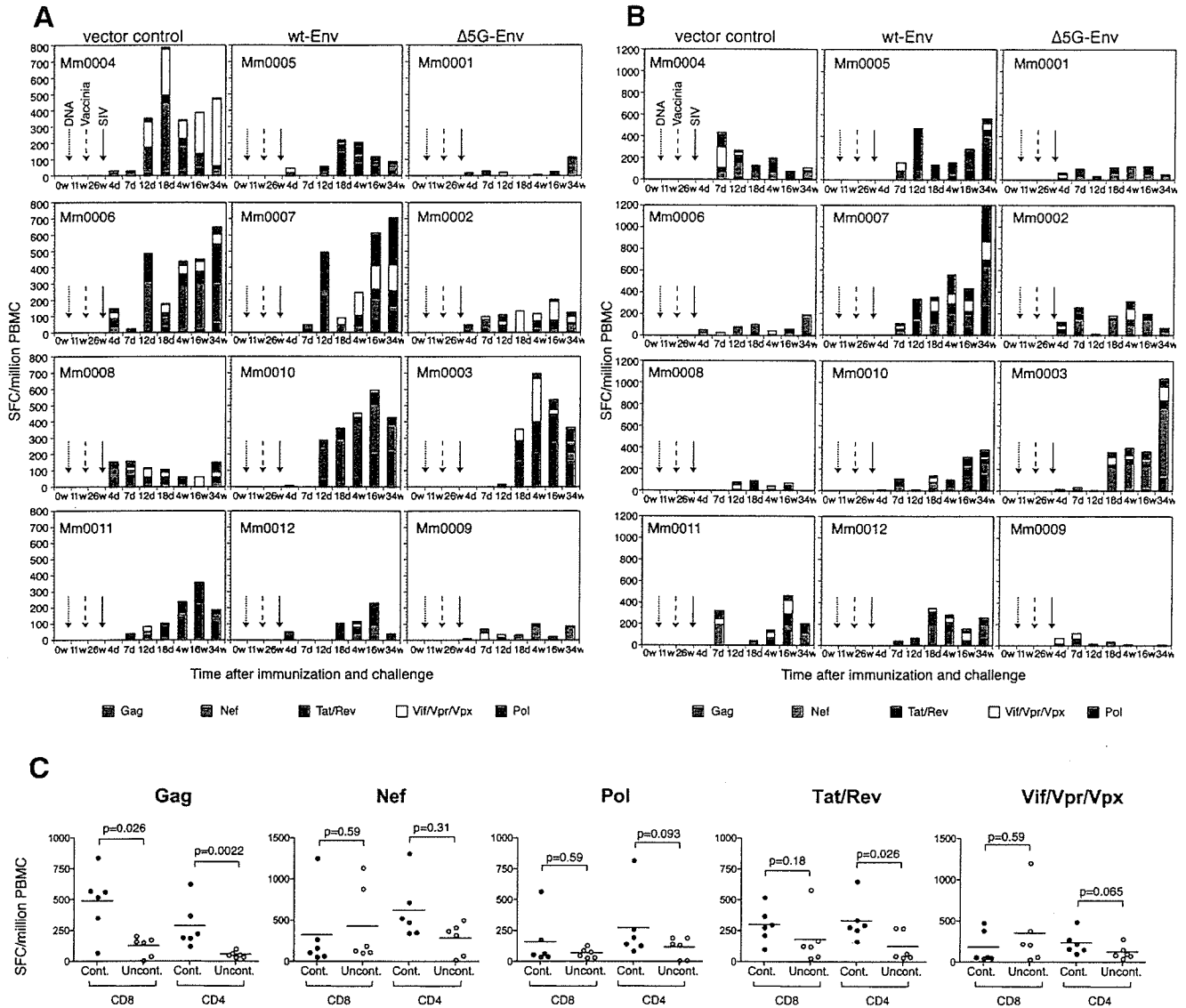


FIG. 7. SIV-specific CD8⁺ T-cell and CD4⁺ T-cell responses in 12 animals. A: SIV viral-protein-specific CD8⁺ T cells in PBMCs were measured by ELISPOT assay for IFN- γ in three groups: vector controls, wt-Env vaccine group, and Δ 5G Env vaccines. B: SIV viral-protein-specific CD4⁺ T cells in PBMCs were 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 Δ 5G 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 Δ 5G 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 Δ 5G Env vaccine were indistinguishable in cultured cells (Fig. 2), wt-Env might persist longer than Δ 5G 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 Δ 5G 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 cynomolgous 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. Hulse, 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. Triglona, 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. Suzuki, 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. Rubsamem-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 Haaf, 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.

Protective Effects of *IL4-589T* and *RANTES-28G* on HIV-1 disease progression in infected Thai females

Nuanjun Wichukchinda^{a,b}, Emi E. Nakayama^b, Archawin Rojanawiwat^a, Panita Pathipvanich^c, Wattana Auwanit^a, Suthon Vongsheree^a, Koya Ariyoshi^d, Pathom Sawanpanyalert^a and Tatsuo Shioda^b

Objective: To evaluate the effect of polymorphisms in interleukin-4 (*IL4*) and *RANTES* promoters on disease progression in HIV-1 infected Thais.

Design: Antiretroviral (ARV) drug-free HIV-1 infected females from the prospective cohort.

Methods: A total of 246 DNA samples were genotyped for *IL4* and *RANTES* promoter polymorphisms by PCR-RFLP. Associations of genotype with HIV-1 disease progression were assessed with respect to baseline clinical data including plasma HIV-1 load, CD4 cell counts, and proportion of symptomatic/AIDS, and survival status during 3 years of follow-up.

Results: Patients with homozygous *IL4-589T* allele showed a significantly lower HIV-1 viral load ($P = 0.005$) and a higher CD4 cell count ($P = 0.003$) than the other patients with heterozygous *IL4-589C/T* or homozygous *IL4-589C* allele. Kaplan-Meier analysis demonstrated an apparent but insignificant trend towards better survival in homozygous *IL4-589T* patients. On the other hand, patients with *RANTES-28G* allele showed a significantly better survival while those with *RANTES In1.1C* allele without *RANTES-28G* showed a significantly poorer survival compared with those who did not possess either *RANTES In1.1C* or *RANTES-28G* ($P = 0.02$), although those polymorphisms only weakly associated with baseline viral load and CD4 cell counts.

Conclusions: Our results implicate the significant protective effect of *IL4-589T* and *RANTES-28G* on HIV disease progression in Thais. In contrast, *RANTES In1.1C* without *RANTES-28G* had an accelerating effect on HIV disease progression.

© 2006 Lippincott Williams & Wilkins

AIDS 2006, 20:189–196

Keywords: *IL4-589T*, *RANTES* promoter polymorphisms, HIV disease progression, survival, Thailand, viral load, CD4 cells

Introduction

HIV-1 infected individuals have widely different rates of disease progression. Some infected individuals become symptomatic within 2–3 years while others remain asymptomatic for more than 10–15 years [1]. It is

important to investigate factors modulating rates of disease progression for designing novel therapies and vaccines.

RANTES is a natural CCR5 ligand and potently inhibits cell entry of HIV-1 that uses CCR5 as a coreceptor

From the ^aNational Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand, the ^bResearch Institute for Microbial Diseases, Osaka University, Osaka, Japan, the ^cDay Care Center, Lampang Hospital, Lampang, Thailand, and the ^dAIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan.

Correspondence to T. Shioda, Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita-shi, Osaka, 565-0871 Japan.

Tel: +81 6 6879 8346; fax: +81 6 6879 8347; e-mail: shioda@biken.osaka-u.ac.jp

Received: 26 May 2005; revised: 9 September 2005; accepted: 27 October 2005.

ISSN 0269-9370 © 2006 Lippincott Williams & Wilkins

189

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

(R5 virus). Two single nucleotide polymorphisms (SNP) in *RANTES* promoter: $-403G/A$ and $-28C/G$, were identified [2]. The *RANTES-28G* variant was shown to elevate promoter activity *in vitro* [2,3] and is associated with a slower rate of CD4 cell depletion in HIV-1 infected Japanese patients [2]. Although this result was not confirmed in subsequent studies in other ethnic groups in which the *RANTES-28G* frequency was lower than in the Japanese [3–5], McDermott *et al.* reported that patients with *RANTES-28G* showed a clear trend towards slower rate of CD4 cell depletion in HIV-1 infected European Americans [6].

With respect to *RANTES-403A* variants, HIV-1 infected European Americans with *RANTES-403A* were initially reported to progress more slowly to AIDS [6]. However, subsequent studies with a larger sample size showed that *RANTES-403A* or *RANTES In1.1C*, a *RANTES* intron polymorphism which is in a strong linkage disequilibrium with *RANTES-403A* and which negatively affects *RANTES* transcription *in vitro*, was reported to be associated with an increased rate of disease progression [3,4].

A variant of the interleukin-4 (*IL4*) promoter, $-589T$ was found to be associated with elevated levels of serum IgE and to accelerate phenotypic switches of HIV-1 from non-syncytium inducing (NSI) to syncytium-inducing (SI) type in HIV-infected Japanese patients probably due to the suppressive effect of *IL4* on CCR5 expression together with its enhancing effect on CXCR4 expression [7]. This allele was subsequently found to be associated with a lower viral load and a slower rate of disease progression in HIV-infected French patients [8,9]. However, studies in other ethnic groups did not show consistent results [10,11].

There is a considerable variation in allele frequencies at these loci among different ethnic groups. Furthermore, diverse strains of HIV-1 circulate in different geographical areas and effects of host genetic polymorphisms may differ in individuals infected with different HIV-1 subtypes. Therefore, it is important to study influences of host genetic polymorphisms in different populations; however, such data in Asian populations remain sparse. In this paper, we report that polymorphisms in *IL4* and *RANTES* influence HIV load, CD4 cell count and survival time in Thai patients infected with HIV-1 subtype E.

Materials and methods

Patients and samples

We conducted a prospective cohort study at the HIV clinic in the Day Care Center of Lampang Hospital in northern Thailand. The Lampang Hospital is a government referral hospital with approximately 800 beds. It is located in the center of Lampang province, a province

600 km to the north of Bangkok. We approached all HIV-1 infected individuals who attended the clinic from 6 July 2000 to 12 July 2001 and asked them to participate in this cohort study. After giving written informed consent, all participants were interviewed by trained study coordinators, and clinically examined by two designated doctors. EDTA-treated blood was taken from each individual. Plasma and buffy coat fractions were separated and stored at -80°C until use. For a control group we collected blood samples from 119 female blood donors at the blood bank of the Lampang Hospital.

Plasma HIV-1 RNA copy number was measured using a commercial kit (Amplicor HIV-1 Monitor Test, Roche Molecular System, Inc. Branchburg, New Jersey, USA), which has a lower limit of detection of 400 copies/ml. CD4 cell count was measured by FACScan (BD Biosciences, California, USA) using fresh EDTA-treated whole blood samples at the time of recruitment. The survival status of participants until 1 October 2003 was ascertained from the cohort database, mailing letters, and death certificates at the Lampang Provincial Health Office. Data were double entered and validated using the access program. This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand in January 2000.

Polymorphism genotyping

Genomic DNA was extracted and purified from the buffy coat fraction using QIAamp mini blood kit (QIAGEN GmbH, Hilden, Germany). Each specimen was analyzed for *IL4-589C/T*, *RANTES-403G/A*, *RANTES-28C/G*, *RANTES In1.1T/C*, and *RANTES 3'222T/C* by PCR-RFLP as described elsewhere [2,3,7].

Sample analysis

We conducted this study strictly in blinded manner so that field investigators were masked from any host genetic polymorphism data and laboratory investigators were masked from any clinical data. The data set was analyzed only after deleting sample and patient's identification number. Continuous variables of two groups with different genetic background were compared by a non-parametric Kruskal–Wallis test. Qualitative variables of two groups were compared by the Chi-square test. Significance in Kaplan–Meier analysis was determined by the log-rank test. Statistical analyses were carried out using Epi Info version 3.01 (US-CDC). *RANTES* haplotypes were constructed and calculated for their frequencies by an Expectation–Maximization algorithm using software (Arlequin version 2.01, Genetica and Biometry Laboratory, Geneva, Switzerland). D' measure for linkage disequilibrium was also calculated by using Arlequin. Incidence rate ratios were calculated by STATA (Stata Corp., Texas, USA). Crude and adjusted relative risks (RR) and their 95% confidence intervals (CI) were calculated by Cox proportional hazard models using StatView (SAS Institute Inc. North Carolina, USA).

Results

Baseline characteristics of study subjects

During the study period, 487 HIV-infected individuals participated in this study. There were 187 males and 300 females; baseline CD4 cell counts and antiretroviral (ARV) drug use information was available in 185 males and 297 females. We found that HIV-disease was significantly more advanced in men than in women. Median [interquartile range (IQR)] CD4 cell count at the time of recruitment in men [66 (15–258) cells/ μ l] was significantly lower ($P < 0.0001$) than that in women [262 (90–418)]. The proportion of patients diagnosed as AIDS was also higher ($P < 0.0001$) in men (77/185; 41.6%) than in women (44/297; 14.8%). Because the aim of this study was to investigate the influence of host genetic polymorphisms on disease progression and the majority of male individuals were already in the advanced stage of HIV disease, we analyzed the data of men and women separately and in this paper we present our analysis on the 297 females.

Among these females, 186 were ARV drug naive and 111 were ARV drug experienced: 51 were receiving ARV drugs and 60 reported prior ARV treatment but were not on ARV at the time of recruitment. The latter group included 18 females who were given zidovudine (ZDV) as prophylaxis for HIV mother-to-child transmission. The remaining 42 subjects stopped ARV drugs because of financial constraints.

All females reported that they had contracted HIV-1 through heterosexual contact. Baseline characteristics of ARV drug naive and ARV drug interrupted groups were similar (Table 1). In this analysis, we excluded the 51 individuals who were on ARV drug treatment, because on-going ARV drug treatment considerably affects CD4 cell count and viral load so that an association between these parameters and genetic polymorphisms may be obscured.

Allele frequencies of *IL4-589T* and *RANTES-403A*, *RANTES-28G*, *RANTES In1.1C*, and *RANTES 3'222C*

We genotyped 246 ARV drug-free females and 119 blood donors for *IL4-589C/T*, *RANTES-403G/A*, *RANTES-*

28C/G, *RANTES In1.1T/C*, and *RANTES 3'222T/C*. Allele frequency of *IL4-589T* was 0.774 in 246 ARV drug-free females and 0.798 in 119 blood donors. Frequencies of four *RANTES* SNP in ARV drug-free females and blood donors are shown at the bottom of Table 2. There was no difference in frequency of those five SNP among subgroups, including the ARV-treated group (data not shown). All of the observed genotypes were in Hardy–Weinberg equilibrium (data not shown).

Table 2 shows estimated *RANTES* haplotype distribution in 246 ARV drug-free females and 119 blood donors. As reported in European- and African-Americans, the haplotype R1 was the most common. In European- and African-Americans, approximately 75% and 55% of *RANTES-403A* was associated with *RANTES In1.1C*, respectively. *RANTES-403A* was more closely associated with *RANTES In1.1C* in Thai patients than in European- and African-Americans, since 93.4% of *RANTES-403A* were associated with *RANTES In1.1C* in 246 ARV drug-free female patients ($D' = 1.0$, Table 2). Therefore, we focused on *RANTES-28G* and *RANTES In1.1C* in the subsequent analysis among four *RANTES* polymorphisms, as *RANTES-28G* and *RANTES In1.1C* were shown to be functional and actually affected *RANTES* promoter activity [2,3], while roles of *RANTES-403A* and *RANTES 3'222T/C* as functional alleles were not yet confirmed.

Genotype variation and HIV disease status (clinical, CD4 cell count, viral load)

We analyzed viral load, CD4 cell count, and clinical status among ARV drug-free females at recruitment by stratifying individuals according to their host genetic background (Table 3). We found that patients with the homozygous *IL4-589T* allele showed a significantly lower HIV-1 viral load ($P = 0.005$) and higher CD4 cell count ($P = 0.003$) than patients with heterozygous *IL4-589C/T* or homozygous *IL4-589C* alleles. Furthermore the proportion of symptomatic individuals was lower among females with homozygous *IL4-589T* allele than among others, although this trend did not reach statistical significance ($P = 0.183$).

Patients with *RANTES-28G* allele (*C/G* or *G/G*) showed a weak trend toward a higher CD4 cell count

Table 1. Baseline data of ARV drug-free HIV-1 infected females.

Characteristics	ARV drug naive (n = 186)	ARV treatment interrupted (n = 60)	Combined (ARV drug free) (n = 246)
Age at recruitment: median years (IQR)	31 (28–36)	31 (27.5–38)	31 (28–36)
CD4 cell count [median cells/ μ l (IQR)]	269 (71–422)	259.5 (63–400.5)	262 (67–421)
HIV-1 load [median log ₁₀ HIV RNA copies/ml (IQR)]	5.084 (4.424–5.549)	5.0194 (3.897–5.592)	5.078 (4.331–5.571)
AIDS-related symptoms [% (n)]	37.1(69)	45(27)	39 (96)
95% CI	30.1–44.5	32.1–58.4	32.9–45.4
Diagnose of AIDS [% (n)]	15.6 (29)	20 (12)	16.7 (41)
95% CI	10.7–21.6	10.8–32.3	12.3–21.9

ARV, Antiretroviral; IQR, interquartile range; CI, confidence interval.