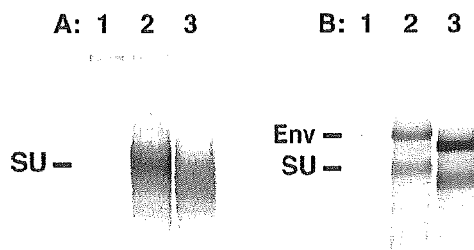

FIG. 2. Expression of SU and Env by SU-expressing DNA vaccines and Env-expressing vaccinia viruses. A: SU secreted in supernatant from CV-1 cells transfected with SU-expressing plasmids. Lane 1, pJW4303 vector; lane 2, pJWSUmac239; lane 3, pJWSUmacΔ5G. B: Env in cell lysates of CV-1 cells infected with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

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

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

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

RESULTS

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

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

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

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

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

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

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

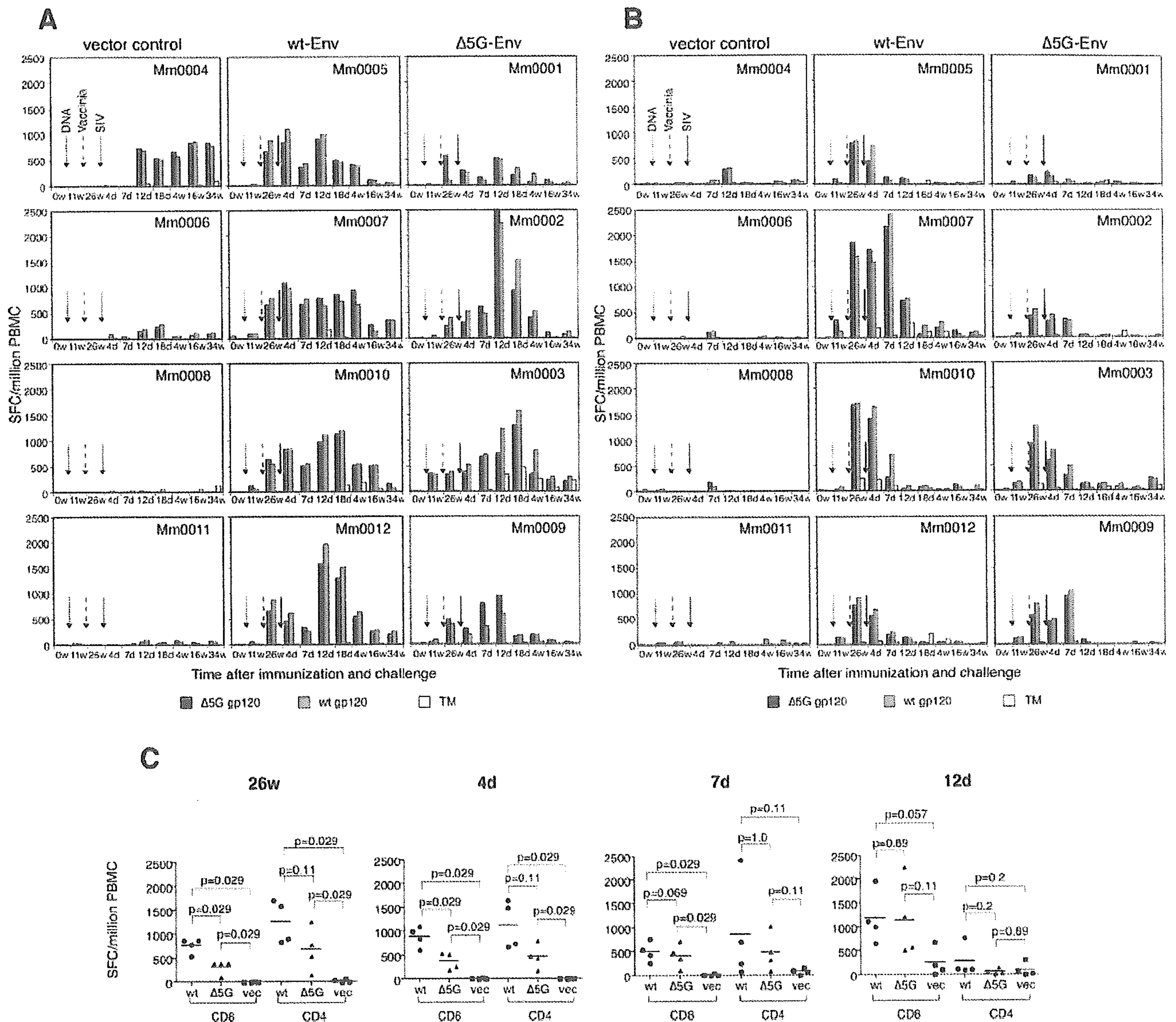

FIG. 3. Env-specific $CD4^+$ T-cell and $CD8^+$ T-cell responses in 12 macaques. A: Env-specific $CD8^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. B: Env-specific $CD4^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results are colored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

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

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

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

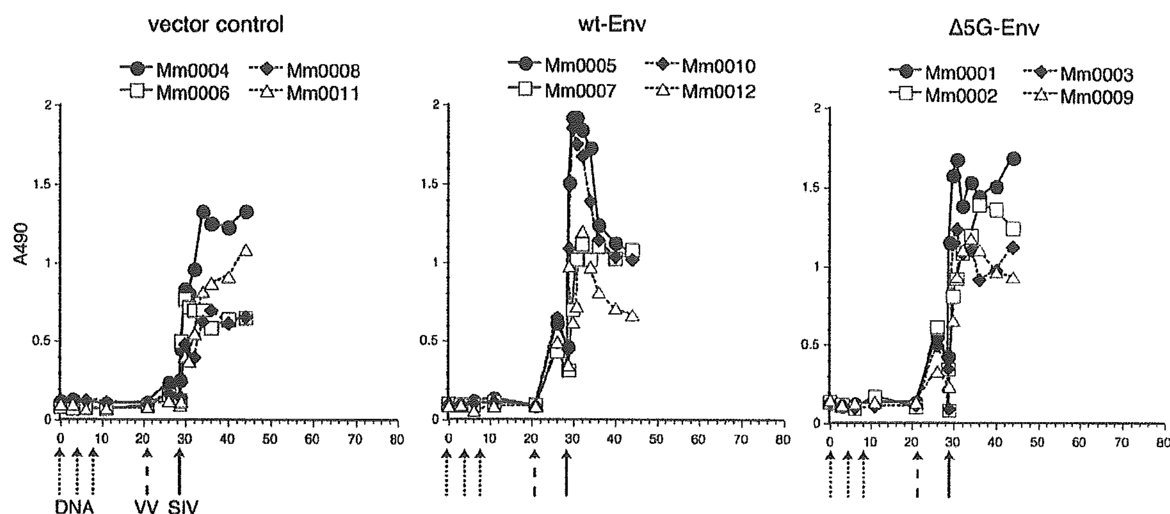

FIG. 4. Humoral immune response during immunization and after challenge infection. The OD_{492} was used as a relative measurement of anti-SIV ELISA antibody titer.

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

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

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

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

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

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

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

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

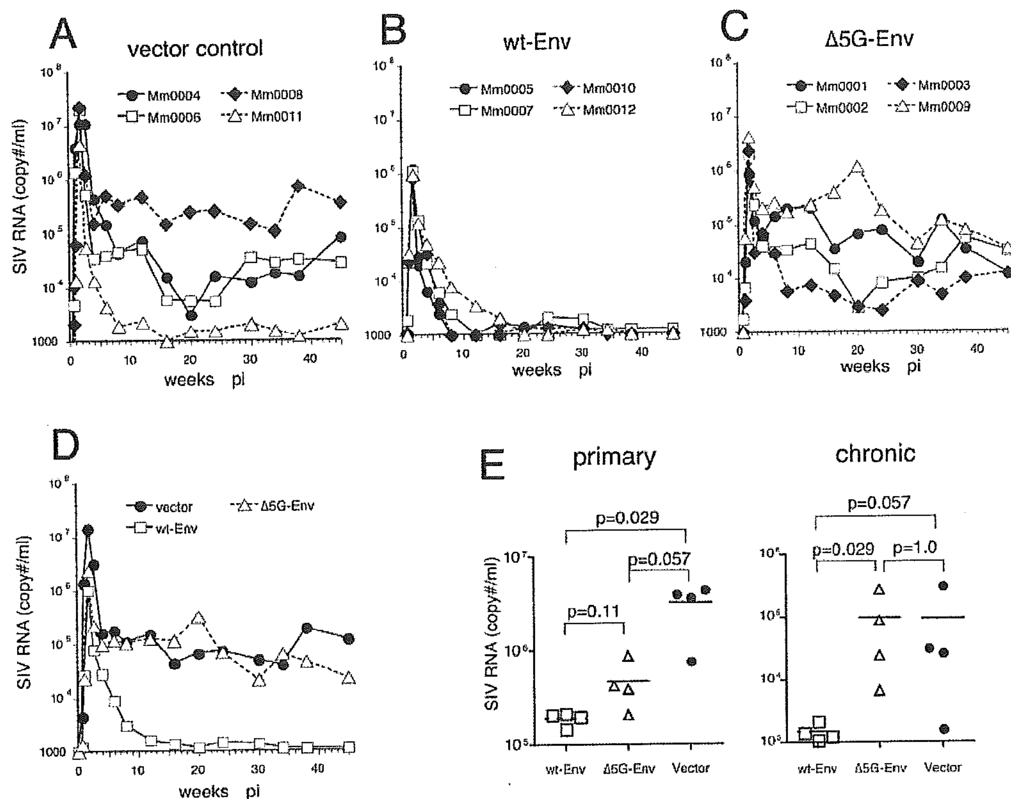

FIG. 5. Plasma viral loads after SIVmac239 challenge infection. Plasma viral load was measured by real-time PCR with a detection limit of 1,000 copies/ml. A: wt-Env vaccine group; B: Δ5G Env vaccine group; C: vector controls; D: comparison of viral loads among three groups; E: comparison of viral loads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

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

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

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

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

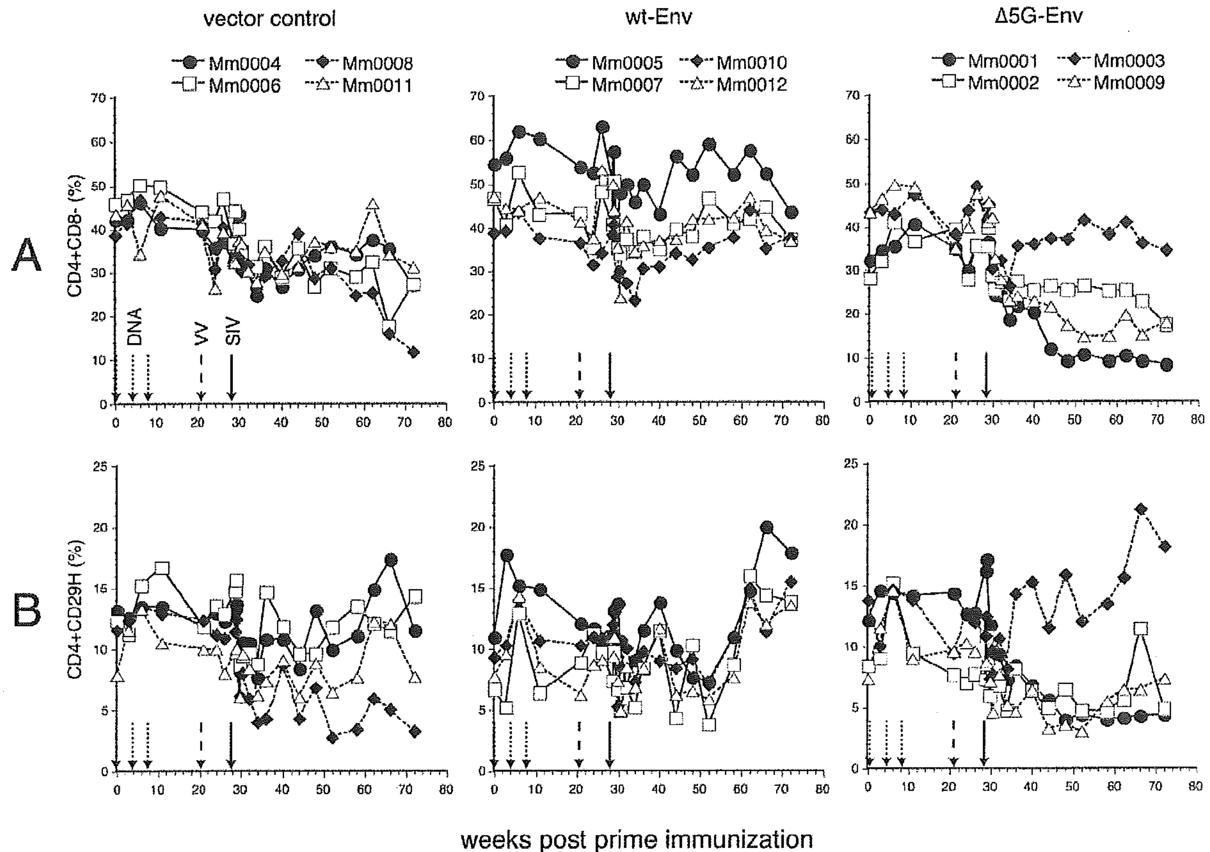

FIG. 6. $CD4^+$ T cells in PBMCs from rhesus macaques during immunization and after the challenge infection. A: Percentage of $CD4^+$ T cells in PBMCs; B: percentage of $CD4^+$ CD29high T cells in PBMCs.

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

DISCUSSION

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

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

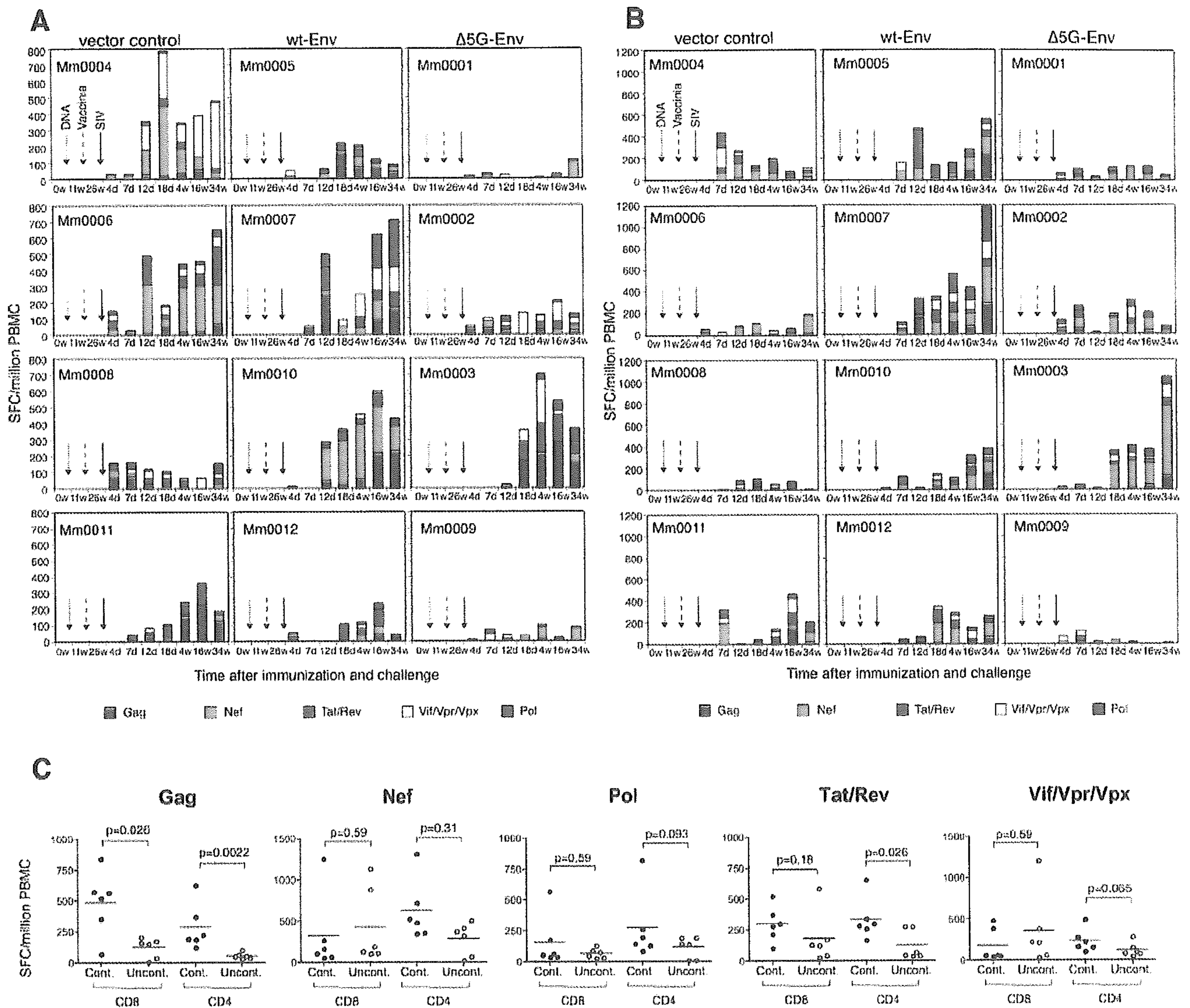

FIG. 7. SIV-specific CD8⁺ T-cell and CD4⁺ T-cell responses in 12 animals. A: SIV viral-protein-specific CD8⁺ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups: vector controls, wt-Env vaccine group, and Δ5G Env vaccines. B: SIV viral-protein-specific CD4⁺ T cells in PBMCs were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

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

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

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

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

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

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

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

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

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Ag85B of Mycobacteria Elicits Effective CTL Responses through Activation of Robust Th1 Immunity as a Novel Adjuvant in DNA Vaccine[1]

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CD4$^+$ T cells play a crucial role in CTL generation in a DNA vaccination strategy. Several studies have demonstrated the requirement of CD4$^+$ T cells for the induction of a sufficient immune response by coadministrating DNAs. In the present study we investigated the effectiveness of Ag85B of mycobacteria, which is known to be one of the immunogenic proteins for Th1 development, as an adjuvant of a DNA vaccine. HIV gp120 DNA vaccine mixed with Ag85B DNA as an adjuvant induced HIV gp120-specific Th1 responses, as shown by delayed-type hypersensitivity, cytokine secretion, and increasing HIV-specific CTL responses. Moreover, these responses were enhanced in mice primed with *Mycobacterium bovis* bacillus Calmette-Guérin before immunization of HIV DNA vaccine mixed with Ag85B DNA. Furthermore, these immunized mice showed substantial reduction of HIV gp120-expressing recombinant vaccinia virus titers compared with the titers in other experimental mice after recombinant vaccinia virus challenge. Because most humans have been sensitized by spontaneous infection or by vaccination with mycobacteria, these findings indicate that Ag85B is a promising adjuvant for enhancing CTL responses in a DNA vaccination strategy. ***The Journal of Immunology,* 2005, 175: 2541–2547.**

The use of adjuvant in vaccination is thought to be useful for enhancing the immune responses to various pathogens and tumors. One of the major advantages of plasmid DNA vaccination is the induction of MHC class I-restricted CTL responses through endogenous production of an Ag similar to viral infection (1). However, plasmid DNA immunization does not fully elicit cellular immune responses against infectious pathogens in some cases. Unlike viral infection, generation of CTLs after DNA vaccination appears to be critically dependent on functions of CD4$^+$ T cells, such as secretion of Th1 cytokines, which facilitate CTL expansion and activity (2), and activation of professional APCs through CD40-CD40L interaction to increase the expression of costimulatory molecules (3–5). Accordingly, simultaneous activation of CD4$^+$ T cells, especially Th1 cells, during priming is a promising strategy for the generation of substantial CTL responses when using a noninflammatory Ag expression system. In many cases, some helper epitopes are already present in a DNA vaccine, and epitope-specific CD4$^+$ Th cell responses are induced after vaccination. However, because CD4$^+$ T cell help for CTL generation does not require a pathogen including a CTL epitope, pathogen-specific CD4$^+$ T cell responses are not necessary for eliciting pathogen-specific CTL immunity (6, 7). This prompted us to use an appropriate molecule as an adjuvant for the induction of an effective CTL response by the activation of CD4$^+$ T cells.

Mycobacterium bovis bacillus Calmette-Guérin (BCG),[3] a currently available vaccine to prevent tuberculosis, is thought to have powerful immunogenic adjuvant activity that augments cell-mediated immune responses by induction of several Th1 cytokines (8). It is also well known that CFA, which contains heat-killed mycobacteria, augments immune responses by activating Th cells. However, the specific proteins that elicit Th1 immunity of BCG are not clear. One immunogenic protein that can induce a strong Th1-type immune response in hosts sensitized by BCG is Ag85B (also known as α Ag or MPT59) (9). Ag85B belongs to the Ag85 family, which participates in cell wall mycolic acid synthesis (10). Moreover, Ag85B is one of most dominant protein Ags secreted from all mycobacterial species, shows extensive cross-reactivity between different species, and has been shown to induce substantial Th cell proliferation and vigorous Th1 cytokine production in humans and mice infected with mycobacterial species, including individuals vaccinated with BCG (9). We previously showed that inoculation of Ag85B-transfected tumor cells enhances the immunogenicity of tumor-associated Ags and elicits a strong tumor-specific CTL response (11). In the present study we evaluated the effectiveness of Ag85B from *Mycobacterium kansassi* as an adjuvant for enhancing cellular immune responses induced by DNA vaccine.

Materials and Methods

Mice

In mice, unlike humans, studies using inbred and congenic strains have demonstrated different fastness against BCG infection among each strain (12). To give resistance to BCG infection, BALB/c (BCG-susceptible strain) × C3H/HeN (BCG-resistant strain; CC3HF1, H-$2^{d/k}$) female mice were used in this study. The mice were housed at the Laboratory Animal Center of Mie University School of Medicine.

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[3] Abbreviations used in this paper: BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; DC, dendritic cell; DTH, delayed-type hypersensitivity; FN, fibronectin; MMC, mitomycin C; rVV, recombinant vaccinia virus.

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Plasmid

A highly efficient mammalian expression vector, pJW4303, was used for efficient expression of HIV env gp120 of the NL432 strain (pJWNL432) (13). The Ag85B expression vector pcDNA-Ag85B has been constructed by cloning a PCR product that possesses an Ag85B of *M. kansasii* open reading frame lacking a signal sequence into *KpnI-ApaI* sites of pcDNA 3.1 (11).

Peptide synthesis

The peptides used in this study were an HIV-1 env helper epitope (315–329; RIQRGPGRAFVTIGK; p18) and CTL epitope (318–327; RGPGRAFVTI; p18-I10) in association with the class II MHC molecule I-A^d and the class I MHC molecule H-$2D^d$, respectively (14).

Priming to BCG

Six- to 8-wk-old female mice were primed to BCG by i.p. inoculation of 0.01 mg (dry weight) of BCG (Japan BCG Laboratory).

Immunization

Four weeks after BCG priming, groups of mice were i.m. injected four times with 100 μg of pJWNL432 mixed with or without 100 μg of pcDNA-Ag85B, and then the site of inoculation was immediately given an electric pulse by an Electric Square Porator (T820; BTX) to express both Ags of Ag85B and HIV gp120 in the same tissue, as previously described (15). Pulses were delivered to the muscle using a pair of electrode needles. Eight electric pulses of 50 V were delivered at a rate of one pulse per second. Each electric pulse was 99 ms in duration. Resistance was monitored with a graphic pulse analyzer (Optimizer 500; BTX). To test the dose dependency of Ag85B as an adjuvant, mice primed with BCG were coadministered various doses of pcDNA-Ag85B. Insufficiency of the amount of DNA by reduction of pcDNA-Ag85B was compensated for by mock DNA pcDNA3.1, the original expression vector of pcDNA-Ag85B, to equalize the total volume of administered DNA.

Immunohistochemical analysis

Immunized leg muscles were examined immunohistochemically for in vivo expression of HIV gp120 and Ag85B. Three days after injection, the tibialis anterior muscle was removed, fixed with 4% paraformaldehyde in PBS, and embedded in paraffin wax. Serial sections were prepared and deparaffinized and then incubated with proteinase K for 5 min at room temperature (gp120) or heated by microwave oven three times for 5 min each time (Ag85B) to reactivate the Ag. After incubation with 3% H_2O_2/methanol for 30 min to quench endogenous peroxidase activity, the sections were blocked with normal serum and incubated with anti-HIV gp120 Ab (OEM Concepts) diluted 1/100 or rabbit anti-Ag85B antiserum (16) diluted 1/250 for 30 min at room temperature. Subsequently, the sections were additionally incubated with a biotinylated secondary Ab and HRP-labeled avidin-biotin complex (ABC-peroxidase staining kit Elite; Vector Laboratories). They were then reacted with 0.5% 3.3′-diaminobenzidine tetrachloride and 0.01% H_2O_2 to visualize the bound Abs. Sections incubated with an irrelevant Ab instead of the primary Ab were used as negative controls. Sections were slightly counterstained with hematoxylin.

Delayed-type hypersensitivity (DTH) responses

DTH responses to HIV were elicited by injecting 5 μg of p18 peptide into the footpad of each immunized mouse. The degree of footpad swelling 24 h after the injection was measured using a micrometer and was expressed as the mean increment $\pm$ SE of three mice per group (11).

Determination of cytokine production

Spleen cells from immunized mice (5×10^6) were cultured with 2.5×10^6 mitomycin C (MMC)-treated autologous spleen cells labeled with p18 peptide in 24-well culture plates at a volume of 2 ml. After incubation at 37°C in a humidified incubator (5% CO_2) for 48 h, culture supernatants were collected and analyzed for IFN-γ (BioSource International) or IL-4 (Quantikine; R&D Systems) production by an ELISA according to the manufacturer's protocol.

RT-PCR

Total RNA was isolated from leg muscles of the site of immunization using TRIzol (Invitrogen Life Technologies), then reverse transcribed to cDNAs using a SuperScript system (Invitrogen Life Technologies). The resulting cDNA was amplified using TLR sequence-specific primers for 30 cycles of PCR (94°C for 1 min, 58°C for 1 min, and 72°C for 1 min). The following primers corresponding to each TLR were used: 5′-ATGGCAGAAGATGTGTCCG-3′ and 5′-GTCACCATGGCCAATGTAGG-3′ for TLR2, 5′-TGGATTCTTCTGGTGTCTTCC-3′ and 5′-AGTTCTTCACTTCGCAACGC-3′ for TLR3, 5′-CTGGCATCATCTTCATTGTCC-3′ and 5′-GCTTAGCAGCCATGTGTTCC-3′ for TLR4, 5′-CAGAACCTTCCTGGCTATTGC-3′ and 5′-AGAGGTTGACCAGACCTTGG-3′ for TLR9, and 5′-AGAAGAGCTATGAGCTGCCTGACG-3′ and 5′-CTTCTGCATCCTGTCAGCAATGCC-3′ for β-actin.

Generation of CTL effector cells

Effector cells were derived from spleen cells as precursor CTLs. $CD8^+$ T cells were purified with anti-CD8 magnetic beads (Miltenyi Biotec) by positive selection according to the recommended protocol (MACS system). Aliquots of 1×10^6 $CD8^+$ T cells were cocultured with 5×10^6 MMC-treated autologous spleen cells labeled with p18-I10 peptide at 37°C in a 5% CO_2 atmosphere. Two days after stimulation, human rIL-2 (Shionogi) was added to all wells at a final concentration of 5 ng/ml. The effector cells generated were harvested after 5 days of culture.

Cytotoxicity assay

MHC-matched (A20.2j) and unmatched (FBL-3) target cells (2×10^6) were incubated at 37°C in a 5% CO_2 atmosphere with or without 10 μg/ml p18-I10 peptide for 16 h. Then the target cells were washed and labeled with ^{51}Cr. The ^{51}Cr-labeled target cells were incubated for 5 h with effector cells. Spontaneous release varied from 5 to 10%. Percent lysis was calculated as [(experimental release − spontaneous release)/(100% release − spontaneous release)] × 100. All experiments were performed more than three times, and each group consisted of three mice.

Blocking of cytolysis

Blocking of cytolysis was performed by a method previously described (13). ^{51}Cr-labeled target cells were preincubated at 37°C for 20 min with anti-H-2 K^d, D^d, or L^d mAb (Meiji Institute of Health Science), and effector cells were then added. In a separate experiment, effector cells were preincubated with anti-CD4 mAb (GK1.5) or anti-CD8 mAb (Lyt2.2) at a 1/50 dilution with complement (Sigma-Aldrich) for 20 min at 37°C, and then labeled target cells were added. Blocking of cytolytic activities by these mAbs was assessed by a 5-h ^{51}Cr release assay.

Evaluation of HIV gp120-specific $CD8^+$ T cells by ELISPOT assay

The number of gp120-specific, IFN-γ-secreting cells was determined by ELISPOT assay. Briefly, 96-well nitrocellulose plates (Millipore) were each coated with 8 μg/ml anti-mouse IFN-γ mAb R4-6A2 (BD Pharmingen) in 100 μl of PBS. After overnight incubation at 4°C, the wells were washed three times with PBS. Then 100 μl of complete medium supplemented with 10% FCS was added to each well, and the plates were incubated at 37°C for 1 h. Triplicate samples of $CD8^+$ T cells separated from the spleen were plated in 2-fold dilutions from 5×10^5 to 6.25×10^4 cells/well. The p18-I10-labeled MMC-treated P815 cells were used as APCs. APCs (1×10^5) were added to each well, and the plates were incubated for 24 h in a 37°C incubator with a 5% CO^2 atmosphere. After stimulation, plates were washed intensively with PBS containing 0.05% Tween 20 and incubated overnight at 4°C with a solution of 2 μg/ml biotinylated anti-mouse IFN-γ mAb XMG1.2 (BD Pharmingen). Afterward, plates were washed with PBS containing 0.05% Tween 20 and 100 μl of streptavidin-alkaline phosphatase (Mabtech) at a 1/1000 dilution was added to each well. Spots were visualized using alkaline phosphatase color development buffer (Bio-Rad) and counted using KS ELISPOT (Zeiss).

Study of protection from vaccinia virus expressing HIV env gp120

The protective ability in immunized mice against systemic infection of recombinant vaccinia virus (rVV) was analyzed by real-time detection PCR as described previously (16). Twelve weeks after the first immunization, mice were challenged i.p. with 5×10^7 PFU of rVV carrying the HIV IIIB gp120 gene (rVV-HIV gp120). Five days after the challenge, the ovaries were harvested and homogenized, and DNA was isolated using a Genomic DNA Isolation kit (Promega). Primers (forward, 5′-GTTCCTTCGCCAACAGGTTAA-3′; reverse, 5′-ACTCGCGATCCTCAAAATGC-3′) and a TaqMan probe (5′-FAM-TTGGAAGCGCCACGGTTACATTCACT-3′) were selected from the core 4b gene of vaccinia virus. Amplification and detection were performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). After incubation at 50°C for 2 min, amplification was begun at 95°C for 10 min, followed by 45 two-step cycles of 95°C for 15 s and 60°C for 60 s.

Statistical analysis

Statistical analysis was performed using Mann-Whitney's U test and the Kruskal-Wallis test. Values are expressed as the mean ± SD. A 95% confidence limit was taken as significant ($p < 0.05$).

Results

In vivo expression of HIV gp120 and Ag85B

It has been reported that CD4$^+$ T cells play a critical role in the generation of CTLs at the site of Ag presentation (17). To induce the effect of Ag85B as an adjuvant for augmentation of cellular immune responses, two DNA vaccines, encoding HIV gp120 and Ag85B, were mixed and administered simultaneously using in vivo electroporation. Three days after immunization, transgene expression was assessed by immunohistochemical analysis in serial sections. Except for slight transient inflammation, no pathological changes were detected in muscles after DNA injection and in vivo electroporation (data not shown) (15). Both HIV gp120 (Fig. 1*A*) and Ag85B (Fig. 1*B*) were observed inside bundles of muscle cells and connective tissue among the muscle fascicles in the same area. Transgene expressions were only seen in the area between the electrode needles.

Coadministration of Ag85B DNA induces strong HIV gp120-specific Th1-type immune responses

As in cases of tuberculosis, one of the important markers of Th1-mediated acquired immunity (not synonymous with protection) is the DTH response. To confirm the ability of Ag85B to induce Th1 responses against coadministered Ag, immunized mice were injected with HIV env helper epitope p18 into footpads, and HIV gp120-specific DTH responses were assessed. As shown in Fig. 2, mice coadministered pcDNA-Ag85B showed greater footpad swelling than mice not administered pcDNA-Ag85B. The effectiveness of Ag85B for inducing Th1-type immune responses to vaccine Ag was augmented by BCG sensitization. In contrast, no significant responses were observed in nonimmunized mice and immunized mice injected with a control peptide (data not shown).

Next, to determine the effect of Ag85B on the patterns of Th1/Th2 cytokine production, we quantified the production of IFN-γ and IL-4 by ELISA in supernatants obtained from 48-h cocultures of peptide-pulsed syngeneic APCs with spleen cells of immunized mice. The production of IL-4 from spleen cells stimulated by p18 in mice immunized with both pcDNA-Ag85B and pJWNL432 was much less than that in mice immunized with pJWNL432 alone. Relatively high levels of HIV gp120-specific IFN-γ production were observed in mice coadministered pcDNA-Ag85B. Furthermore, these Th1-type immune responses were clearly observed when mice were sensitized by BCG inoculation before DNA immunization (Fig. 3). These results are in accordance with the results for DTH responses against HIV gp120 in in vivo experiments and indicate that predominant HIV gp120-specific Th1 responses were induced by coadministration of pcDNA-Ag85B.

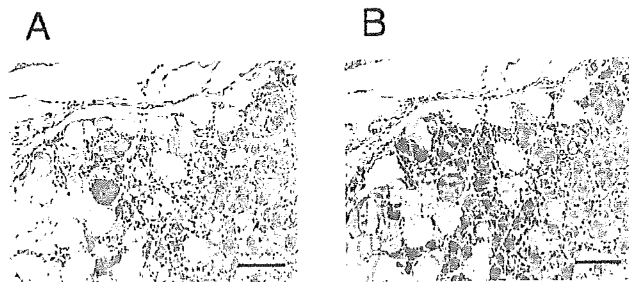

FIGURE 1. Immunostaining of serial sections of muscle tissue from a mouse 3 days after electric administration of pJWNL432 mixed with pcDNA-Ag85B. HIV gp120 (*A*) and Ag85B (*B*) were observed in muscle cells and connective tissue among the muscle fascicles in the same area. Bars represent 100 μm.

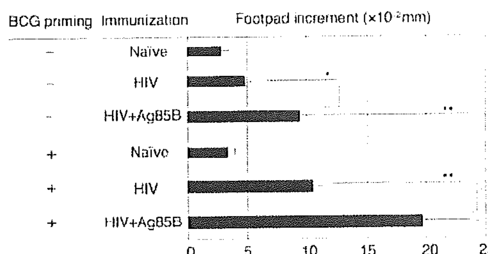

FIGURE 2. Anti-HIV gp120 DTH responses in immunized mice. BCG-primed or unprimed mice were immunized with pJWNL432 with or without pcDNA-Ag85B. The helper epitope peptide of HIV gp120 (p18) was injected into the footpads of immunized mice. The degree of footpad swelling was measured 24 h after the challenge. The results are expressed as the mean footpad increment ± SE of five mice per group. *, $p < 0.01$; **, $p < 0.001$.

Alteration of TLR mRNA expression after Ag85B DNA administration

Various proteins derived from pathogens promote Th1 responses through stimulation of TLRs and subsequently through secretion of cytokines (18). We therefore compared TLR mRNA expression profiles at the site of DNA injection with electroporation in pcDNA-Ag85B-immunized mice and mice immunized with pcDNA alone using RT-PCR. Ag85B stimulated the expression of TLR2, TLR3, and TLR4 mRNA, whereas mock immunization with electroporation resulted in only modest increases in the levels of these TLR mRNAs (Fig. 4). TLR9 mRNA was not detected in either group of mice (Fig. 4). Plasmid pcDNA3.1 has immunostimulatory sequence (six 5′-GACGTC-3′), whereas TLR9 mRNA was not detected in either group of mice under these conditions (Fig. 4). Positive reactions, however, were observed in both Ag85B-injected tissues and control tissues using high cycles (>60) of PCR (data not shown). This positive reaction was not thought to be the effect of Ag85B. These results suggested that Ag85B immunization plays a role in enhancement of the expression of these TLRs, although the possibility of indirect responses by cytokine production cannot be ruled out (18).

Ag85B enhances anti-HIV gp120-specific CTL responses

CD8$^+$ cells from BCG-primed mice and unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were stimulated in vitro with p18-I10-pulsed syngenic splenocytes, and the

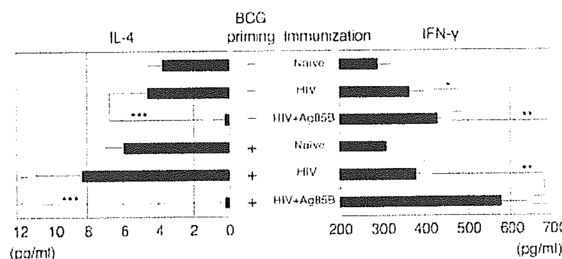

FIGURE 3. Induction of HIV gp120-specific Th1 immune responses by spleen cells obtained from immunized mice. Spleen cells obtained from BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were stimulated with p18-labeled spleen cells, and supernatants were assessed for cytokine concentrations. The results are expressed as the mean concentration ± SE of five mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

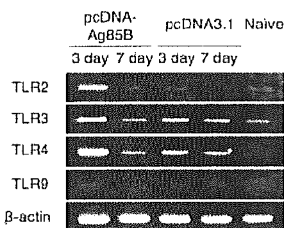

FIGURE 4. TLR mRNA expression profiles of the DNA injection site with electroporation in mice immunized with pcDNA-Ag85B or pcDNA3.1 alone. Total RNA was isolated 3 and 7 days after injection and was analyzed by RT-PCR for TLR2, TLR3, TLR4, and TLR9 mRNA expression. Equality of the RT reaction of isolated RNA between samples was confirmed by amplification of β-actin. Data are representative of three independent experiments.

lytic activities of the cells against p18-I10-pulsed target cells were assessed. Effector cells from mice immunized with pJWNL432 and pcDNA-Ag85B showed higher levels of p18-I10-specific lytic activity than effector cells from BCG-unprimed mice. Moreover, these cytolytic activities were clearly enhanced by BCG sensitization in mice immunized with pJWNL432 mixed with pcDNA-Ag85B (Fig. 5*A*). These effector cells from immunized mice were cultured in a medium containing anti-CD4 or anti-CD8 mAb during the ^{51}Cr release assay. Anti-CD8 mAb inhibited cytolysis against target cells pulsed with the peptide, whereas anti-CD4 mAb did not affect this effector cell function (Fig. 5*B*). Therefore, these results indicate that effector cells expressed CD8 and used this molecule to recognize the target cells. Furthermore, lytic activities of peptide-specific effector cells from immunized mice against MHC-matched or mismatched target cells labeled with the peptide were assessed. These p18-I10-specific effector cells lysed MHC-matched, H-2^d target cells, but not mismatched, H-2^b target cells pulsed with the peptide (Fig. 5*C*). Moreover, the functions of these p18-I10-specific effector cells were inhibited by anti-H-2D^d mAb, but not by anti-H-2K^d mAb or anti-H-2L^d mAb (Fig. 5*D*).

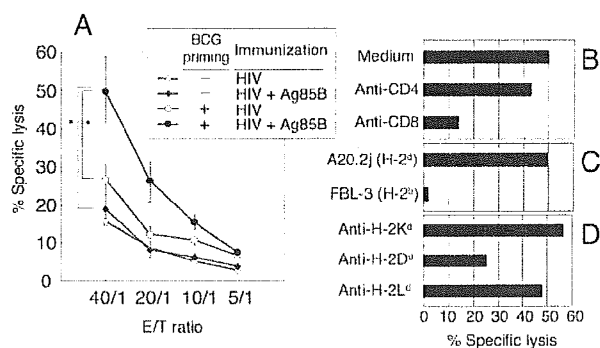

FIGURE 5. Spleen cells from BCG-primed mice coadministered pJWNL432 and pcDNA-Ag85B showed high levels of HIV gp120-specific MHC class I-restricted lytic activity. *A*, CD8$^+$ T cells were purified from spleens of BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B, and the cells were then stimulated with p18-I10-labeled spleen cells and assessed for lytic activities against p18-I10-pulsed target cells. *B*, Lytic activities of effector cells were assessed in the presence of anti-CD4 mAb, anti-CD8 mAb with complement, or medium. The E:T cell ratio was 40:1. *C*, Lytic activities of effector cells against p18-I10-pulsed H-2^d or H-2^b target cells were assessed. The E:T cell ratio was 40:1. *D*, Effector cells were examined for p18-I10-specific lytic activities in the presence of anti-H-2K^d, anti-H-2D^d, or anti-H-2L^d mAb. The E:T cell ratio was 40:1. Each value is the mean percentage of the specific lysis values obtained from five mice. *, $p < 0.01$.

These results indicated that effector cells elicited in immunized mice were CD8$^+$ and MHC class I-restricted CTLs and suggested that Ag85B has potent adjuvant activities for enhancement of CTL responses by being mixed with DNA vaccine Ag.

Ag85B increase the number of HIV gp120-specific, IFN-γ-secreting, CD8$^+$ T cells

ELISPOT assays were performed to compare the numbers of HIV gp120-specific, IFN-γ-secreting, CD8$^+$ cells in immunized mice. CD8$^+$ T cells purified from spleens of immunized mice were stimulated with peptide-pulsed P815 cells in ELISPOT filter plates coated with an IFN-γ capture mAb for 24 h. The numbers of spots were counted automatically using a KS ELISPOT system. In BCG-unprimed mice, the number of p18-I10-specific IFN-γ-secreting CD8$^+$ T cells was slightly increased in mice coadministered pcDNA-Ag85B (20.3 ± 10.0/10^6 cells) compared with that in mice immunized with pJWNL432 alone (14.0 ± 3.6/10^6 cells). In BCG-primed mice, however, the number of p18-I10-specific IFN-γ-secreting CD8$^+$ T cells was ~3.7-fold greater in mice coadministered pcDNA-Ag85B (96.7 ± 13.3/10^6 cells) than in mice immunized pJWNL432 alone (26.3 ± 5.1/10^6 cells; Fig. 6*A*). To confirm whether the improved CTL responses strictly depend on the presence of Ag85B, BCG-primed mice were coadministered various doses of pcDNA-Ag85B, and the frequency of anti-p18-I10-specific IFN-γ-secreting CD8$^+$ T cells was determined by ELISPOT assay. The number of anti-p18-I10-specific, IFN-γ-secreting, CD8$^+$ T cells was gradually increased by coadministration of Ag85B in a dose-dependent manner (Fig. 6*B*). In addition, dose dependency in improving the anti-p18-I10-specific response was not found in mice coadministered a control plasmid, which expresses an unrelated protein constructed by the same expression vector (data not shown). These results clearly indicate that the

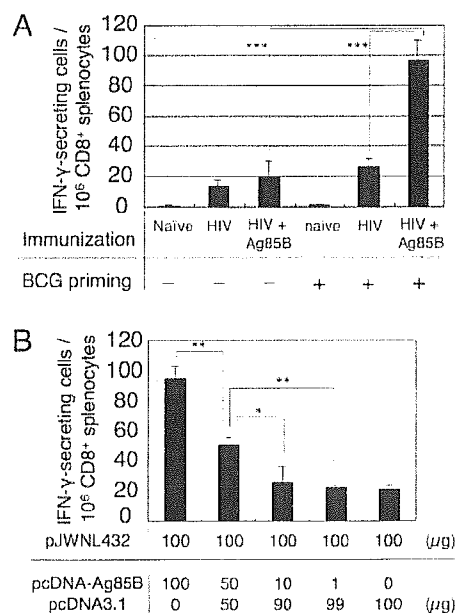

FIGURE 6. pcDNA-Ag85B coadministration in BCG-primed mice enhances HIV gp120-specific, IFN-γ-secreting cell frequency. CD8$^+$ T cells were purified from spleens of BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B (*A*) or with various doses of pcDNA-Ag85B (*B*) and were examined for IFN-γ production in an ELISPOT assay after stimulation with p18-I10-labeled P815 cells. Data are presented as the mean number of p18-I10-specific spots per 10^6 CD8$^+$ spleen cells ± SE of five mice per group. *, $p < 0.02$; **, $p < 0.01$; ***, $p < 0.001$.

anamnestic response to Ag85B could enhance the simultaneously induced CTL responses. These data also support the results for CTL responses and suggest that coadministration of pcDNA-Ag85B, especially in BCG-primed mice, induces high frequency, Ag-specific, responding $CD8^+$ T cells.

Ag85B enhances protective immunity against rVV-HIVenv infection

Finally, to determine the functional relevance of HIV gp120-specific CTLs generated by immunization with these DNA vaccines to eliminate the virus infection, immunized mice were challenged with 5×10^6 PFU of rVV-HIV gp120. Five days after the challenge, the mice were killed, and the ovaries were harvested and used for estimation of the vaccinia virus titer by RT-PCR, because the ovary is the organ in which the vaccinia virus preferentially replicates. The titers of rVV-HIV gp120 in mice coadministered pcDNA-Ag85B were much lower than those in mice immunized with pJWNL432 alone. Moreover, this inhibitory effect on replication of rVV gp120 was clearly demonstrated in mice primed with BCG before immunization (Fig. 7). These results indicated that immunization of mice with pJWNL432 mixed with pcDNA-Ag85B resulted in the generation of an effector T cell response capable of recognizing endogenously processed viral protein, and that DNA immunization inhibited the replication of rVV-expressing HIV gp120 in vivo.

Discussion

DNA vaccination is a practical and effective way to induce cellular immune responses, especially a CTL response, and has shown great promise for initiating cellular immune responses that are regulated for the prevention of various disease such as tumors, HIV, tuberculosis, hepatitis C virus, and malaria. In humans and large animal models, however, immune responses induced by DNA vaccination are not sufficient for prevention or control of infection. Thus, there is a need to increase the potency of DNA vaccines for use in humans. In the present study we investigated the effectiveness of a novel approach for enhancing the ability of a DNA vaccine to induce cellular immune responses by using previously experienced immunogenic proteins that induce a strong Th1-dominant immune response.

$CD4^+$ T cells play a critical role in the generation and maintenance of CTL responses in a noninflammable vaccination strategy including conventional plasmid DNA vaccination. Convincing evidence that dendritic cells (DCs) are the principal cells for priming

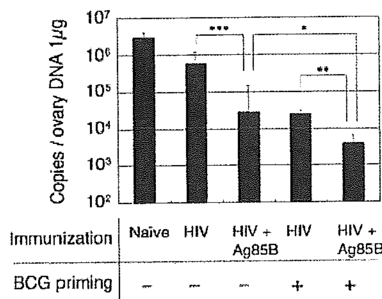

FIGURE 7. pcDNA-Ag85B coadministration in BCG-primed mice enhances HIV gp120-specific protective immunity. BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were challenged with 5×10^7 PFU of rVV-HIV-gp120. The bars show the virus as the log of the number of virus copies in ovaries of mice. The data represent the mean copies of virus obtained from five mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

$CD8^+$ T cells in DNA vaccination through direct transfection of DNA (19–21) or cross-presentation of a vaccine-derived Ag has been presented (22–24). Activation of $CD4^+$ T cells is required for maturation of DCs through CD40 signaling (3–5); however, this requirement is not sufficient in many cases of DNA vaccine immunization (25, 26). Various studies on compensation for the insufficiency of $CD4^+$ T cell help by coadministration of costimulatory molecules, such as B7-1 or B7-2 (27, 28), or by treatment with a CD40 agonist during immunization have therefore been conducted (26). Another $CD4^+$ T cell-mediated helper effect for induction of CTL by DNA vaccine is thought to be the production of cytokines for enhancement of activity and proliferation of CTLs (29). Cognate $CD4^+$ T cell help is important for inducing pathogen-specific CTLs (30), and cognate $CD4^+$ T cell help should be induced to elicit HIVgp120-specific CTLs by injection of DNA vaccine. The Ag85B in our system enhances this cognate manner and polarizes Th1-type immune responses as a vaccine adjuvant. Numerous studies have focused on the effectiveness of coadministration of Th1 cytokine-encoding plasmids, such as IL-2, IL-12, IL-15, IL-18, IL-23, and IFN-γ, for enhancing CTL responses (31, 32). However, adjuvant effects of cytokines on CTL generation are different (33, 34), suggesting that it is necessary to augment the immune responses by these strategies for administration not only of the combination of cytokines codelivered, but also for the timing of administration (35, 36). The expression of both Ag85B and HIV Ag in the same tissues provides this advantage, because Ag85B is thought to be a strong potentiator of Th1-type cytokines. In fact, our results showed production of IFN-γ from spleen cells after stimulation with HIV Ag (Fig. 3).

BCG is an important clinical tool because of its strong immunostimulatory properties. Humans as well as resistant mouse strains infected with BCG predominantly exhibit a Th1 cytokine secretion profile (37). Although specific Ags eliciting Th1 cell responses in mycobacteria are not yet known, a recent study suggested that one of the immunogenic proteins for Th1 development is Ag85B (9). Apparently, strong Th1 responses have been elicited in vitro from purified protein derivative-positive asymptomatic individuals using purified Ag85B (38–40). Furthermore, vaccination of mice with plasmid DNA encoding Ag85B induced strong Ag85B-specific CD4 T cell proliferation and vigorous IFN-γ secretion, resulting in the protection of further *Mycobacterium tuberculosis* infection (41). We have also shown that Ag85B-specific recall responses enhance tumor-specific cellular immune responses in Ag85B gene-transfected tumor cell immunization (11). One possible reason for Th1 domination by Ag85B is that the immunogenic Th1-inducing helper epitope, known as peptide-25, is included in Ag85B protein (42, 43). Peptide-25 was able to stimulate proliferation and a high amount of IFN-γ production in *M. tuberculosis*-primed cells (42). It remains unclear why peptide-25 can induce potent Th1 responses; however, several recent studies have suggested that the avidity of the peptide for its specific TCR may be strong enough to induce Th1 development (9, 44). It is now generally accepted that MHC class II-dependent activation of $CD4^+$ T cells, mainly Th1-polarized cells, potently enhances concomitantly existing unrelated CTL responses (7, 44). According to this line of reasoning, coadministration of Ag85B DNA is a promising tool for enhancement of CTL responses through Ag85B-specific Th cell proliferation and Th1 polarization in a DNA vaccination strategy.

The roles of some proteins and peptides in the polarized development of Th1 cells have been reported, and Ag85B is considered to be one such protein. In fact, we found therapeutic effects of Ag85B on Th2-type allergic disease, asthma, and atopic dermatitis (unpublished observations). The mechanisms, however, are still

not clear. Various products with adjuvant activities, such as LPS, CpG motif, or polyinosinic-polycytidylic acid, involve TLRs and show augmentation of Th1-type immune responses (18). Bacterial components, mycobacterial lipoprotein, bacterial peptidoglycan, and flagellin, also associate with TLRs (18). A correlation between the adjuvant activities of Ag85B and TLRs has not been found. Mycobacteria can bind some TLRs and show typical Th1-type immune responses (45). In a transfection model using Chinese hamster ovary cells (which are relatively deficient in TLRs), the expression of TLR2 or TLR4 conferred responsiveness to both virulent and attenuated *M. tuberculosis* (46). Lipoarabinomannan, a major mycobacterial cell wall component, appears to resemble the cell wall component of Gram-negative bacterial LPS. TLR2 was shown to be necessary for signaling of mycobacterial LPS lipoarabinomannan (47). An undefined, heat-labile, cell-associated, mycobacterial factor was found to be the ligand for TLR4 (47). Ag85B might be included in one of these factors, if it is involved in innate immunity through TLRs. In fact, our results showed enhancement of the expression of TLR2, TLR3, and TLR4 in Ag85B DNA-injected mice (Fig. 4). Because it has been reported that not only microbial components, but also several cytokines regulate the expression of TLRs, there is the possibility of secondary responses for the expression of TLRs by induction of cytokine (18).

Another important biological role of Ag85B is binding of fibronectins (FNs) (48–50). FNs are a family of high molecular weight glycoproteins found in plasma and tissues and are involved in cell motility and adhesion, regulation of cell morphology, phagocytic function, and wound healing (51). Many integrin-binding sites have been identified in amino acid sequences of FNs (52), and adhesion of FN-binding proteins to FNs helps the phagocytosis of proteins into integrin-expressing APCs, especially monocytes, macrophages, and DCs (53). Binding of FNs to human monocytes enhances the phagocytic function of monocytes for bacilli (51), and inhibition of FN-integrin receptor interaction can prevent *M. kansasii* phagocytosis (54). Moreover, Ag85B from *M. tuberculosis* and FNs synergistically stimulate TNF-α expression in human monocytes (55), suggesting that the binding ability of Ag85B with FNs influences not only the enhancement of incorporation of Ags into phagocytic cells, but also the construction of the Th1 milieu at the site of injection.

The results of the present study suggest that coadministration of Ag85B DNA has several potential advantages over other genetic adjuvants due to the existence of multiple mechanisms for elicitation of CTL responses by a DNA vaccine. The results also showed the effectiveness of mycobacterial sensitization for enhancing adjuvanticity of Ag85B. Because most humans have been sensitized by spontaneous infection or by vaccination with mycobacteria, this finding is valuable for the possible use of Ag85B as a genetic adjuvant of a DNA vaccine. The results of this study have provided evidence of the potential utility of Ag85B for the development of a DNA vaccination strategy for successful human use.

Disclosures

The authors have no financial conflict of interest.

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Sequence Note

Molecular Epidemiology of the Heterosexual HIV-1 Transmission in Kunming, Yunnan Province of China Suggests Origin from the Local IDU Epidemic

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ABSTRACT

Molecular epidemiological investigation was conducted among injecting drug users (IDUs) (n = 11) and heterosexuals (n = 15) in Kunming, Yunnan Province of China. HIV-1 genotypes were determined based on the nucleotide sequences of 2.6-kb *gag*-RT region. The distribution of genotypes among IDUs was as follows: CRF07_BC (5/11) and CRF08_BC (5/11); subtype B′ (1/11). Similarly, a majority of Kunming heterosexuals (14/15) were infected with CRF07_BC (4/15), CRF08_BC (6 /15), or subtype B′ (4/15), known to predominate among IDUs in China. This contrasts with trends in the coastal regions of China and surrounding southeastern Asian countries, where CRF01_AE predominates among heterosexuals. The heterosexual HIV-1 epidemic in Kunming thus appears to derive from the local IDU epidemic. Of note, subtype B′ was the most prevalent strain among heterosexuals before 1997, while CRF07_BC and CRF08_BC became predominant in 2002, indicating a transition of HIV-1 genotype distribution between the early and the more recent samples from Kunming heterosexuals.

THE HIV-1 EPIDEMIC IN CHINA was first detected among injecting drug users (IDUs) in the western part of Yunnan Province in 1989. HIV prevalence among IDUs in initial epidemic sites reached 50–80% by 1993.[1] Yunnan Province accounted for more than 80% of the HIV-1 infections reported in China through 1996[1] and is thought to be an epicenter of the HIV epidemic in China. According to recent HIV-1 sentinel surveys, the HIV-1 prevalence rate among newly tested IDUs in Yunnan has been stable (19.7–24.7% in 1997–1999).[2] However, HIV-1 prevalence rates among female commercial sex workers (CSWs) and wives of heroin users have increased steadily. For example, HIV prevalence among CSWs in Yunnan increased from 1.0% in 1997 to 3.4% in 2001.[2] Figure 1 shows the study site and the geographical distribution of the numbers of HIV reported cases in China as of June 2003 (http://www.aids.net.cn).

HIV-1 strains circulating in Yunnan showed extremely high genetic diversity. Various HIV-1 strains, including subtypes B, B′[3] (Thailand variant of subtype B, also referred to as Thai-B[4]) and C,[5,6] and CRF07_BC and CRF08_BC[7]), have been detected among IDUs. Moreover, in addition to these HIV-1 strains, diverse forms of unique recombinants between subtypes B′ and

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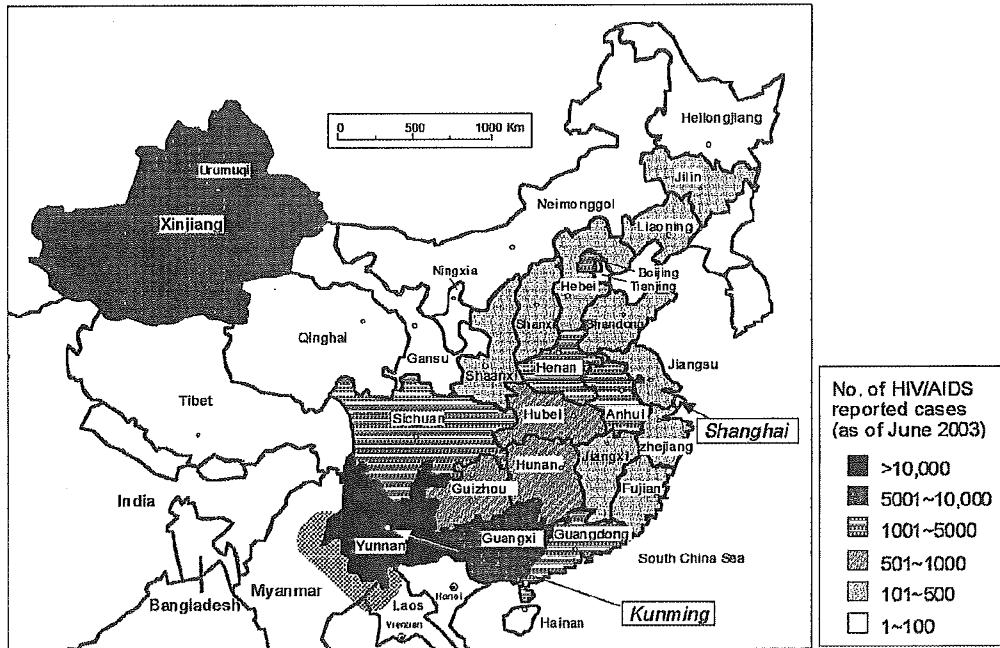

FIG. 1. Map of China. The study site (Kunming, Yunnan Province) and the geographical distribution of the numbers of HIV reported cases (as of June 2003) are shown (http://www.aids.net.cn). The so-called "Golden Triangle," a major heroin production, refining, and trading area, at the borders of Thailand, Myanmar, and Laos, near Yunnan Province, is marked.

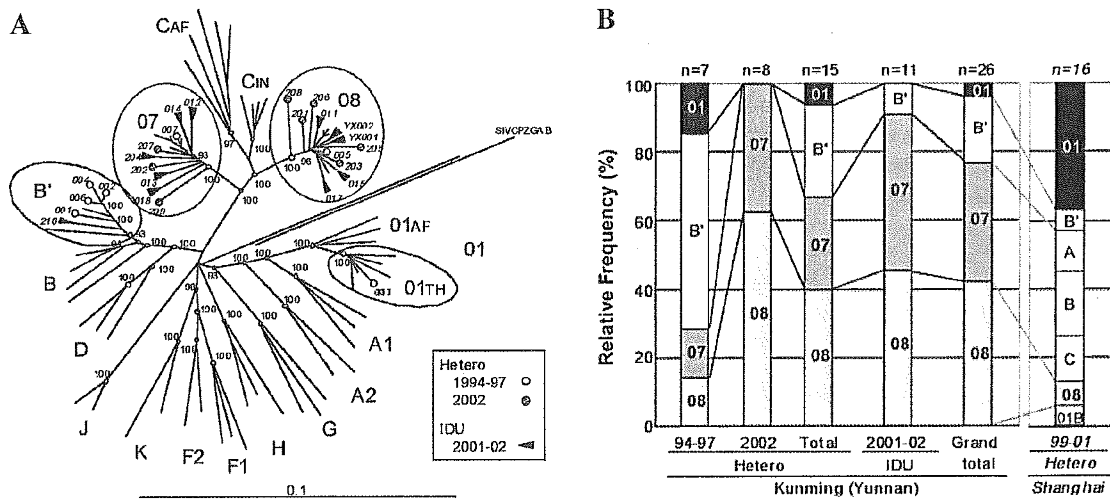

FIG. 2. Phylogenetic tree analysis and the distribution of HIV-1 strains circulating among different risk populations in Kunming, Yunnan. (**A**) Neighbor-joining tree based on the nucleotide sequences of 2.6-kb *gag*-RT regions with HIV-1 group M reference strains (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/Table1.html). $SIV_{CPZ}GAB$ was used as an outgroup. Bootstrap values (>90) are shown at the corresponding nodes. Subtype and CRF designations are shown outside the tree. HIV-1 specimens from IDUs were collected in 2001–2002 (closed arrowheads); HIV-1 specimens from heterosexuals sampled in 1994–1997 (open circles) and in 2002 (striped circles). Three-digit numbers indicate the specimen codes. (**B**) Distribution of HIV-1 genotypes in different risk populations. Bars indicate the relative frequency (%) of the indicated HIV-1 genotype in the respective sample category shown at the bottom. The data on HIV-1 genotype distribution among heterosexuals in Shanghai are adopted from Zhong *et al.*[15] *n* indicates the number of the specimens analyzed in each sample category shown below. B′, HIV-1 subtype B′ (Thailand variant of subtype B); C, subtype C; 01, CRF01_AE; 07, CRF07_BC; 08, CRF08_BC; C_{AF}, African subtype C; C_{IN}, Indian subtype C; 01_{AF}, African CRF01_AE; 01_{TH}, Thailand CRF01_AE.

C,[7] and even the second-generation recombinants comprised of CRF07_BC and CRF08_BC,[8] have been reported among IDUs in Yunnan. In contrast, however, information on the HIV-1 genotypes circulating among heterosexuals in Yunnan is very limited. Although an early study detected CRF01_AE in women who had returned from commercial sex work in Thailand[9] and HIV-1 subtype B/B′, C, CRF01_AE, and CRF08_BC have recently been reported in a small number of heterosexuals in Yunnan,[2] HIV-1 genotypes circulating among persons at heterosexual risk have not been well studied. Ongoing monitoring of the HIV-1 genotype distribution in Yunnan would be important for understanding the evolution of the epidemic as well as for future vaccine strategies in China.

To track the HIV-1 genotype distribution in Yunnan, we collected a total of 26 HIV-1-positive plasma samples from persons in the capital city of Kunming and environs during 1994–2002. Fifteen specimens were from persons who acquired HIV-1 infection through heterosexual contact (7 were sampled in 1994–1997 and 8 were collected in 2002). Eleven specimens were collected from IDUs in 2001–2002. The nucleotide sequences of HIV-1 *gag*-RT regions (2.6 kb) were determined on both strands using BigDye terminator reaction kits on an ABI 373 DNA sequencer as described previously.[7] A multiple alignment with HIV-1 group M references (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/Table1.html) was generated by the Se-Al program.[10] HIV-1 genotypes were screened and determined based on phylogenetic tree (Fig. 2A) and recombination breakpoint analyses of *gag*-RT regions. Phylogenetic trees were constructed by the neighbor-joining method[11] using PHYLIP package version 3.6a3[12] and the reliability of topologies of trees was tested by bootstrap analysis with 100 bootstrap replicates.[13] Bootscanning analyses were performed on neighbor-joining trees for a window of 200 bp moving along the alignment in 30-bp increments, using the Simplot program.[14]

The distribution of HIV-1 genotypes in a total of 26 samples is as follows (Fig. 2): HIV-1 subtype B′ (Thailand variant of subtype B) (5, 19%); CRF01_AE (1, 4%); CRF07_BC (9, 35%); and CRF08_BC (11, 42%). As shown in Fig. 2B, CRF07_BC (5 of 11, 45%) and CRF08_BC (5 of 11, 45%) are predominantly distributed among IDUs. In contrast, HIV-1 subtype B′ (4 of 7, 57%) was the most common strain among specimens from heterosexuals before 1997, while CRF01_AE, CRF07_BC, and CRF08_BC occurred only infrequently (1 of 7, 14% each). Interestingly, however, CRF07_BC (3 of 8, 38%) and CRF08_BC (5 of 8, 63%) were more common among specimens collected from heterosexuals in 2002, indicating a transition of HIV-1 genotype distribution between the early (before 1997) and the more recent samples (in 2002) from Kunming heterosexuals.

It is noted that the specimens, 208 (02CNKM208) and 209 (02CNKM209), are placed slightly outside the clusters of CRF08_BC and CRF07_BC, respectively (Fig. 2A). The raw direct sequencing data of theses specimens contained several ambiguous signals. The clonal sequence analysis by TA cloning revealed that they were coinfected with another lineage of the HIV-1 strain (X.-J. Li, in preparation).

The small proportion of CRF01_AE among heterosexuals in Kunming (Fig. 2) contrasts with the findings in surrounding Southeast Asian countries, where CRF01_AE shows a strong founder effect triggering the explosive epidemic among heterosexuals.[5] As shown in Fig. 2, it appears that CRF01_AE has not accounted for the majority of sexual transmission in Kunming. Although CRF01_AE was detected in the early 1990s among returnees from Thailand,[9] it has not gained the momentum of dissemination through the sexual route in Kunming, as it has in other Southeast Asian countries. In contrast, CRF01_AE constituted a significant proportion of HIV-1 strains among heterosexuals (6 of 16, 38%) in the city of Shanghai in 1999–2001[15] (Fig.2B. right). This suggests a difference in the structure and the genesis of heterosexual epidemics in Kunming and the coastal areas represented by Shanghai. Heterosexual transmission of HIV-1 in Kunming thus appears to be strongly influenced by the local IDU epidemic, while CRF01_AE shows a significant founder effect among heterosexuals in some coastal regions in China.

In conclusion, the apparent predominance of CRF07_BC and CRF08_BC among heterosexuals in Kunming suggests that a large proportion of these infections are related to IDU networks in China. These findings would contribute to our understanding of the HIV-1 epidemic in China.

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Potent Anti-R5 Human Immunodeficiency Virus Type 1 Effects of a CCR5 Antagonist, AK602/ONO4128/GW873140, in a Novel Human Peripheral Blood Mononuclear Cell Nonobese Diabetic-SCID, Interleukin-2 Receptor γ-Chain-Knocked-Out AIDS Mouse Model

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We established human peripheral blood mononuclear cell (PBMC)-transplanted R5 human immunodeficiency virus type 1 isolate JR-FL ($HIV\text{-}1_{JR\text{-}FL}$)-infected, nonobese diabetic-SCID, interleukin 2 receptor γ-chain-knocked-out (NOG) mice, in which massive and systemic HIV-1 infection occurred. The susceptibility of the implanted PBMC to the infectivity and cytopathic effect of R5 HIV-1 appeared to stem from hyperactivation of the PBMC, which rapidly proliferated and expressed high levels of CCR5. When a novel spirodiketopiperazine-containing CCR5 inhibitor, AK602/ONO4128/GW873140 (molecular weight, 614), was administered to the NOG mice 1 day after R5 HIV-1 inoculation, the replication and cytopathic effects of R5 HIV-1 were significantly suppressed. In saline-treated mice ($n = 7$), the mean human $CD4^+/CD8^+$ cell ratio was 0.1 on day 16 after inoculation, while levels in mice ($n = 8$) administered AK602 had a mean value of 0.92, comparable to levels in uninfected mice ($n = 7$). The mean number of HIV-RNA copies in plasma in saline-treated mice were $\sim10^6$/ml on day 16, while levels in AK602-treated mice were 1.27×10^3/ml ($P = 0.001$). AK602 also significantly suppressed the number of proviral DNA copies and serum p24 levels ($P = 0.001$). These data suggest that the present NOG mouse system should serve as a small-animal AIDS model and warrant that AK602 be further developed as a potential therapeutic for HIV-1 infection.

Highly active antiretroviral therapy has brought about a major impact on the AIDS epidemics in the industrially advanced nations (5, 22). However, eradication of human immunodeficiency virus type 1 (HIV-1) is thought to be currently impossible, due in part to the viral reservoirs remaining in blood and infected tissues (6). The limitation of antiviral therapy of AIDS is exacerbated by complicated regimens, the development of drug-resistant HIV-1 variants (11), and a number of inherent adverse effects (2, 31). Hence, the identification of new antiretroviral drugs that have unique mechanisms of action and produce no or minimal adverse effects remains an important therapeutic objective. In regard to development of potential anti-HIV therapies or vaccines, experimental animal models for AIDS which allow the determination of the possible efficacy of antiviral agents or vaccines have been sought since severe combined immunodeficiency (SCID) mice engrafted with human fetal thymus, liver, or peripheral blood mononuclear cells (PBMC) were first exploited to examine antiretroviral agents (19, 25). However, a number of mouse models have suffered from false-positive and false-negative results in detecting or quantifying HIV-1 infection and replication and have required a large number of samples and mice for testing (25, 29).

In the present work, we established human PBMC-transplanted R5 $HIV\text{-}1_{JR\text{-}FL}$-infected, nonobese diabetic (NOD)-SCID, interleukin 2 receptor γ (IL-2Rγ)-chain-knocked-out (NOG) mice, in which massive and systemic HIV-1 infection occurs, human $CD4^+/CD8^+$ cell ratios significantly decrease, and high levels of R5 HIV-1 viremia reaching as high as 10^6 copies/ml are achieved. Furthermore, we demonstrated that this unprecedented susceptibility of the implanted human PBMC to the infectivity and cytopathic effects of R5 HIV-1 infection stems from hyperactivation of the PBMC. Here, we also report a novel small nonpeptide CCR5 antagonist, AK602/ONO4128/GW873140, which exerts potent anti-HIV-1 activity in vitro against laboratory and clinical strains of HIV-1, including highly multidrug-resistant (MDR) variants.

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