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 *Kpn*I-*Apa*I 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 ± 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′-ATGGCAGAAGAT GTGTCCG-3′ and 5′-GTCACCATGGCCAATGTAGG-3′ for TLR2, 5′-TGGATTCTTCTGGTGTCTTCC-3′ and 5′-AGTTCTTCACTTCGCAA CGC-3′ for TLR3, 5′-CTGGCATCATCTTCATTGTCC-3′ and 5′-GCTTAGCAGCCATGTGTTCC-3′ for TLR4, 5′-CAGAACCTTCCTG GCTATTGC-3′ and 5′-AGAGGTTGACCAGACCTTGG-3′ for TLR9, and 5′-AGAAGAGCTATGAGCTGCCTGACG-3′ and 5′-CTTCTG CATCCTGTCAGCAATGCC-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′-GTTCCT TCGCCAACAGGTTAA-3′; reverse, 5′-ACTCGCGATCCTCAAAATG C-3′) and a TaqMan probe (5′-FAM-TTGGAAGCGCCACGGTTACAT TCACT-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.

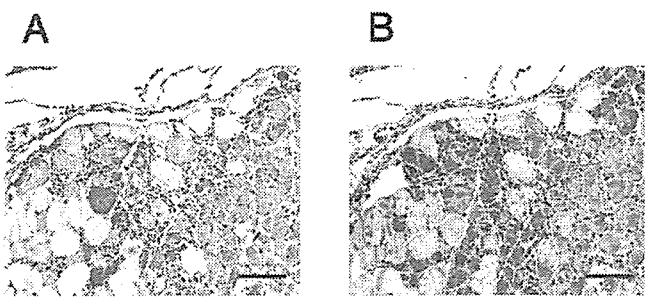

FIGURE 1. Immunostaining of serial sections of muscle tissue from a mouse 3 days after electric administration of pJWNL432 mixed with pcDNA-Ag85B. HIV gp120 (*A*) and Ag85B (*B*) were observed in muscle cells and connective tissue among the muscle fascicles in the same area. Bars represent 100 μm.

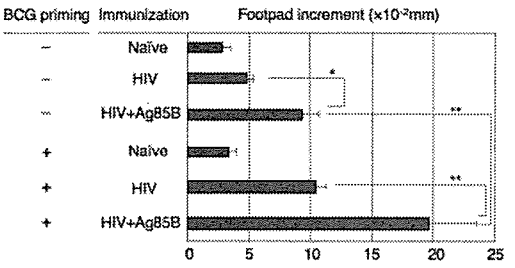

FIGURE 2. Anti-HIV gp120 DTH responses in immunized mice. BCG-primed or unprimed mice were immunized with pJWNL432 with or without pcDNA-Ag85B. The helper epitope peptide of HIV gp120 (p18) was injected into the footpads of immunized mice. The degree of footpad swelling was measured 24 h after the challenge. 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

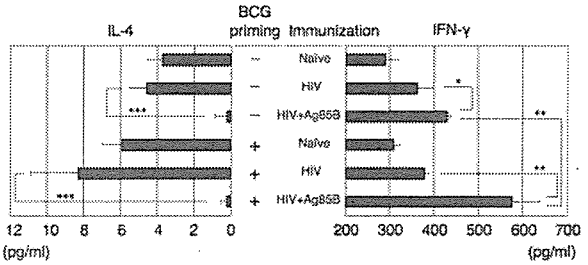

FIGURE 3. Induction of HIV gp120-specific Th1 immune responses by spleen cells obtained from immunized mice. Spleen cells obtained from BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were stimulated with p18-labeled spleen cells, and supernatants were assessed for cytokine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

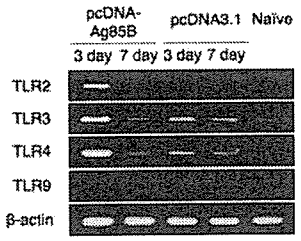

FIGURE 4. TLR mRNA expression profiles of the DNA injection site with electroporation in mice immunized with pcDNA-Ag85B or pcDNA3.1 alone. Total RNA was isolated 3 and 7 days after injection and was analyzed by RT-PCR for TLR2, TLR3, TLR4, and TLR9 mRNA expression. Equality of the RT reaction of isolated 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*).

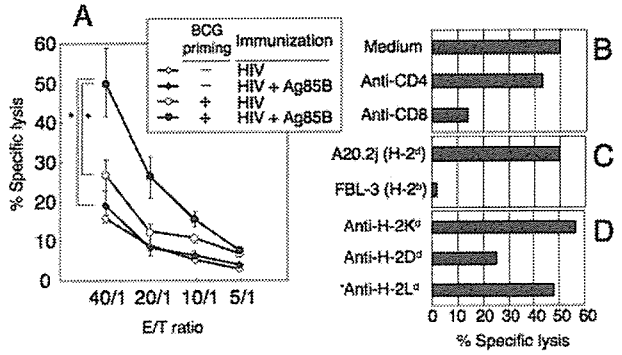

FIGURE 5. Spleen cells from BCG-primed mice coadministered pJWNL432 and pcDNA-Ag85B showed high levels of HIV gp120-specific MHC class I-restricted lytic activity. *A*, CD8$^+$ T cells were purified from spleens of BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B, and the 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

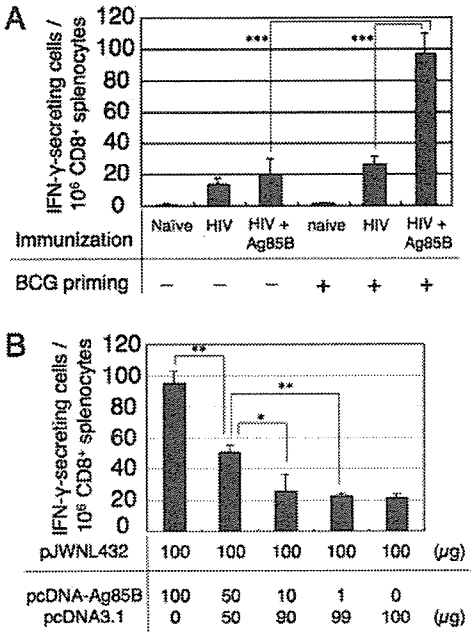

FIGURE 6. pcDNA-Ag85B coadministration in BCG-primed mice enhances HIV gp120-specific, IFN-γ-secreting cell frequency. CD8$^+$ T cells were purified from spleens of BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B (*A*) or with various doses of pcDNA-Ag85B (*B*) and were 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

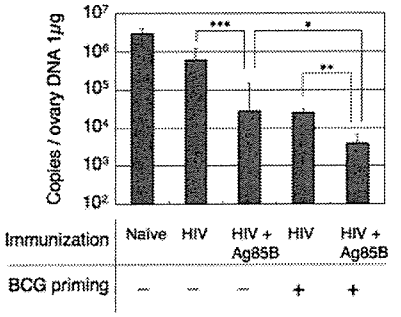

FIGURE 7. pcDNA-Ag85B coadministration in BCG-primed mice enhances HIV gp120-specific protective immunity. BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were challenged with 5×10^7 PFU of rVV-HIV-gp120. The bars show the virus as the log of the number of virus copies 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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Rapid Communication

Ubiquitination of APOBEC3 proteins by the Vif–Cullin5–ElonginB–ElonginC complex

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Abstract

APOBEC3 proteins are antiviral host factors for a wide variety of retroviruses. HIV-1 Vif overcomes the antiviral activity of APOBEC3G by ubiquitinating the protein. In this study, we examined the ability of Vif to antagonize other family members of APOBEC3 proteins, together with its mechanism. Using HIV infectivity, virion incorporation, immunoprecipitation, and in vitro ubiquitin conjugation assays, we show that the ability of Vif to inhibit antiviral activity of APOBEC3 proteins positively correlates with its ability to bind and ubiquitinate these proteins by a Vif–Cullin5–ElonginB–ElonginC (Vif–BC–Cul5) complex. These results suggest that Vif exhibits its anti-APOBEC3 activity by the ubiquitin ligase activity of the Vif–BC–Cul5 complex.

Keywords: HIV-1; Cytidine deaminase; Virion incorporation; Ubiquitin; Cullin5–ElonginB–ElonginC complex

Introduction

APOBEC3G (A3G) is a potent antiretroviral host factor (Sheehy et al., 2002). It deaminates cytidine to uridine in nascent minus-strand viral DNA, inducing G-to-A hypermutation in the plus-strand viral DNA (Harris et al., 2003; Mangeat et al., 2003; Shindo et al., 2003; Zhang et al., 2003). HIV-1 Vif protein overcomes the antiviral activity of A3G by targeting it for ubiquitin-dependent degradation (Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003). Vif interacts with cellular proteins, Cullin5 (Cul5), Elongin B (EloB), Elongin C (EloC), and Rbx1 through its novel SOCS-box motif to form a ubiquitin ligase (E3) complex (Vif–BC–Cul5) and functions as a substrate recognition subunit of the complex (Kobayashi et al., 2005; Mehle et al., 2004; Yu et al., 2003, 2004b). A3G belongs to the APOBEC superfamily of cytidine deaminases (Jarmuz et al., 2002), and several studies have reported that other members of this family such as APOBEC3B (A3B) and APOBEC3F (A3F) also have an antiviral activity on HIV-1, while the involvement of Vif in antagonizing these enzymes remains controversial (Bishop et al., 2004; Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004). We have recently reported that the Vif–BC–Cul5 complex could indeed work as an E3 ligase by an in vitro ubiquitin conjugation assay using the purified Vif–BC–Cul5 complex (Kobayashi et al., 2005). Using the assay, here, we show that the Vif–BC–Cul5 complex ubiquitinates A3F as well as A3G, but not A3B. We also demonstrate that the ability of Vif to inhibit antiviral activity of APOBEC3 proteins positively correlates with its ability to bind and ubiquitinate these proteins by the Vif–BC–Cul5 complex, indicating that Vif exhibits its anti-APOBEC3 activity by the ubiquitin ligase activity of the Vif–BC–Cul5 complex.

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Results

We first tested the antiviral activity of A3G, A3B, and A3F on HIV-1 as well as their incorporation into HIV-1 virions. As shown in Fig. 1A, expression of APOBEC3 proteins suppressed the infectivity of ΔVif virions to various extents. HIV-1

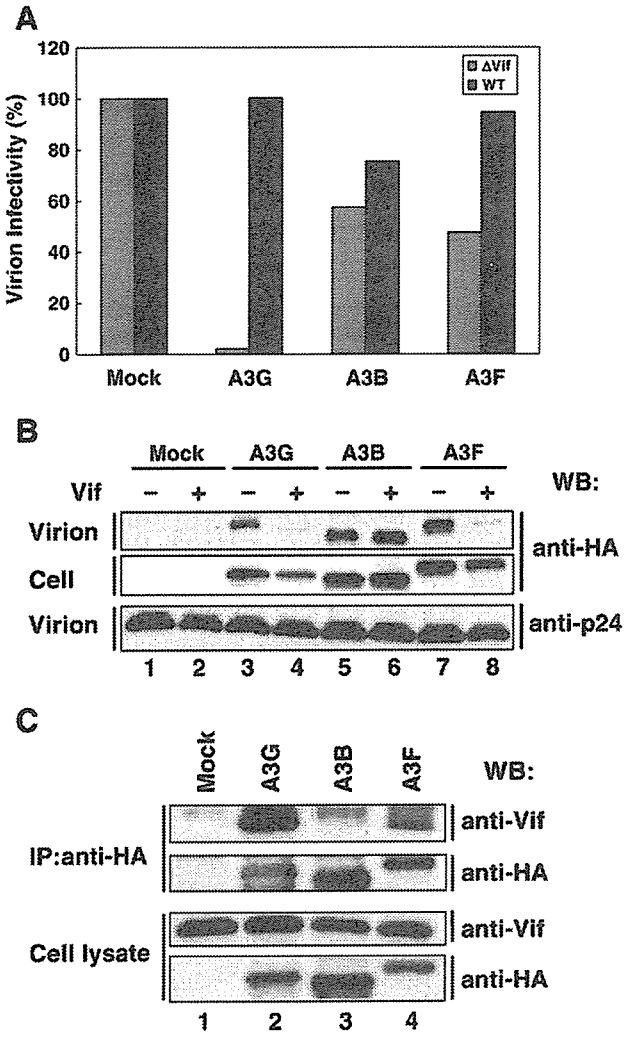

Fig. 1. Antiviral activity of APOBEC3 proteins on HIV-1. (A) A3B is resistant to HIV-1 Vif. We transfected HEK293T cells with pNL43/ΔEnv-Luc (WT) or pNL43/ΔEnvΔvif-Luc (ΔVif) plus pVSV-G in the presence of pcDNA3/HA-based vectors (a mock, A3G, A3B, and A3F). Viruses from these cells were challenged to M8166 cells, and productive infection was measured by luciferase activity. Values are presented as the percent infectivity relative to the values of each virus without expression of APOBEC3 proteins. Expression of APOBEC proteins suppressed the infectivity of ΔVif virions, and HIV-1 Vif overcame the antiviral activity of A3F as well as A3G, but not that of A3B. (B) Vif inhibited virion incorporation of A3G and A3F, but not that of A3B. HIV-1 virions prepared as described above were precipitated by ultracentrifugation and subjected to immunoblot with anti-HA (top panel) and anti-p24 (bottom panel) mAbs. Cell lysates of producer cells were also subjected to immunoblot with anti-HA mAb (middle panel). (C) Vif could bind to A3G and A3F, but not to A3B. HEK293T cells were co-transfected with expression vectors for APOBEC3 proteins and Vif. The lysates were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-Vif mAb (top panel) or anti-HA mAb (2nd top panel). Vif was coprecipitated with A3G and, to a lesser extent, with A3F, but not with A3B. Cell lysates were also subjected to immunoblot with anti-Vif mAb (3rd top panel) or anti-HA mAb (bottom panel).

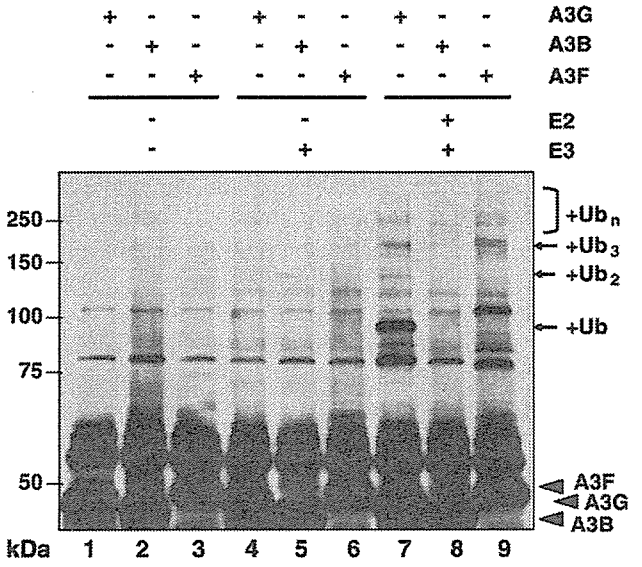

Fig. 2. In vitro ubiquitination of APOBEC3 proteins. An in vitro ubiquitin conjugation assay was performed as described in Materials and methods. GST-ubiquitin-conjugated A3G and A3F proteins were specifically detected as ladder (arrows) by immunoblotting with anti-HA mAb.

Vif overcame the antiviral activity of A3G and A3F, but not that of A3B, suggesting that APOBEC3B was resistant to HIV-1 Vif. The infectivity of the wild type (WT) virion was 8 to 47 times higher than that of ΔVif virion with A3G, and 2 to 3 times higher with A3F, suggesting that HIV-1 Vif antagonized A3G more effectively as compared to A3F. An immunoblotting of APOBEC3 proteins in virions and producer cells (Fig. 1B) revealed that all APOBEC proteins were incorporated into ΔVif virions (Fig. 1B, top panel, lanes 3, 5, and 7). An immunoblot with anti-β-actin mAb revealed no incorporation of this abundant cellular protein into virions, suggesting the specific incorporation of APOBEC3 proteins (data not shown). Vif could inhibit virion incorporation of A3G and A3F effectively (top panel, lanes 4 and 8, respectively), but not that of A3B (top panel, lane 6). In parallel, Vif reduced intracellular levels of A3G and A3F, but not that of A3B (Fig. 1B, middle panel), and reduction of the intracellular level of A3G by Vif was stronger than that of A3F. We next examined the physical interaction of Vif with APOBEC3 proteins by an immunoprecipitation assay. HIV-1 Vif was co-immunoprecipitated with A3G and, to a lesser extent, with A3F (Fig. 1C, top panel, lanes 2 and 4, respectively), but not with A3B (lane 3). These results suggested that Vif inhibited the virion incorporation of A3F as well as A3G, but not that of A3B because Vif could not bind to A3B. This also suggested that the ability of Vif to bind these proteins corresponded to the extent of reduction of their intracellular levels. Finally, we tested the E3 activity of Vif–BC–Cul5 ligase complex on APOBEC3 proteins by an in vitro ubiquitin conjugation assay using the purified Vif–BC–Cul5 as previously reported (Kobayashi et al., 2005). As shown in Fig. 2, the Vif–BC–Cul5 (E3) complex specifically ubiquitinated A3G (lane 7) and, to a lesser extent, A3F (lane 9) since it did not ubiquitinate these when E2 was omitted. The magnitude of ubiquitination of these proteins corresponded to the extent to which Vif overcame the antiviral

activity of these proteins. In contrast, the assay showed no ubiquitination of A3B (lane 8).

Discussion

HIV Vif is known to antagonize the antiviral activity of A3G by excluding the protein from HIV virion, which is attributed to the ubiquitination of A3G by the Vif–BC–Cul5 complex as previously reported (Kobayashi et al., 2005). In this study, we show the clear correlation between the function of Vif to antagonize APOBEC3 proteins and the ubiquitination of these by Vif–BC–Cul5 ubiquitin ligase complex using the in vitro ubiquitin conjugation assay. Vif overcomes the antiviral activity of A3F by ubiquitinating it through the Vif–BC–Cul5 complex as reported with A3G although to a lesser extent. However, Vif cannot overcome the antiviral activity of A3B because it cannot bind to A3B. The magnitude of inhibitory activity of Vif against the proteins corresponds to the extent of ubiquitination of APOBEC3 proteins by the Vif–BC–Cul5 complex as well as the binding ability of Vif to APOBEC3 proteins. This suggests two possibilities. One is that the binding of Vif to APOBEC3 proteins might induce the changes in its conformation or subcellular localization leading to unpackaging into virions as reported by the Strebel laboratory (Kao et al., 2004) because the binding ability of Vif to APOBEC3 proteins correlates to the inhibitory activity on APOBEC3. The other is, as we reported previously, that the ubiquitination of APOBEC3 proteins by the Vif–BC–Cul5 complex is essential for Vif function against the proteins. Although we cannot fully exclude the former possibility at this time, we believe that the latter is more likely because our in vitro ubiquitination assay showed the clear correlation between the in vitro ubiquitination of APOBEC3 proteins and the inhibitory effect of Vif on the proteins. This could not be fully explained by the former mechanism alone. Further study is necessary to fully elucidate this mechanism.

The antiviral activity of A3B on HIV-1 has been controversial. Some groups reported a weak inhibitory effect of A3B on HIV-1 (Bishop et al., 2004; Yu et al., 2004a), while others recently reported a strong inhibition (Doehle et al., 2005). In this study, we found only a weak antiviral activity of A3B on HIV-1. By sequencing, we found some SNPs in the coding region of A3B according to National Center for Biotechnology Information database. Although we could not fully explain the discrepancies of the anti-HIV-1 activities of A3B among studies, one explanation might be that SNPs in the coding region of A3B might affect its antiviral activity. Further study on this matter is also be warranted.

We previously demonstrated that ubiquitination of A3G by the Vif–BC–Cul5 complex is essential for Vif function against A3G. In this study, we further extend this notion by showing that the ability of Vif to inhibit antiviral activity of APOBEC3 positively correlates with its ability to bind and ubiquitinate APOBEC3 by Vif–BC–Cul5. This will provide us with new insights into the mechanism of Vif function to antagonize APOBEC proteins and to identify new targets for therapeutic strategy.

Materials and methods

Plasmids and cell lines

Expression vector for hemagglutinin (HA)-tagged human A3G, pcDNA3/HA-A3G, was constructed as previously described (Kobayashi et al., 2004). pcDNA3/HA-A3F was constructed in the same way. pNLA1-43Vif was constructed by inserting a Vif fragment from NL4-3 into the subgenomic expression vector pNL-A1 (a kind gift from Dr. K. Strebel), which expresses all HIV-1 proteins except for *gag* and *pol* products (Strebel et al., 1987). pcDNA3/HA-A3B was a kind gift from Dr. K Imada (Kyoto University) (Hishizawa et al., 2005). pNL43/ΔEnv-Luc and pNL43/ΔEnvΔvif-Luc were constructed as previously described (Shindo et al., 2003). HEK293T and M8166 cells were maintained as previously described (Shindo et al., 2003).

Infectivity assay with luciferase reporter viruses

Luciferase reporter viruses with or without Vif were prepared by cotransfection of pNL43/ΔEnv-Luc (WT) or pNL43/ΔEnvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or expression vectors for A3G, A3B, and A3F by calcium phosphate method as previously described (Shindo et al., 2003). Productive infection was measured by luciferase activity. Values were presented as percent infectivity relative to the value of each virus without expression of APOBEC3 proteins.

Co-immunoprecipitation assay

To see protein–protein interaction in vivo, we performed an immunoprecipitation assay as described previously (Shindo et al., 2003). pcDNA3/HA-A3G, A3B, or A3F was co-transfected with pNLA1-43Vif into HEK293T cells by calcium phosphate method. Two days after transfection, cells were lysed in lysis buffer (25 mM HEPES pH 7.4/150 mM NaCl/1 mM $MgCl_2$/0.5% TritonX-100/10% Glycerol), and complexes were immunoprecipitated with anti-HA monoclonal antibody (mAb) (12CA5) (F. Hoffmann-La Roche Ltd.) and protein A–Sepharose beads (Amersham Biosciences Corp., Piscataway, NJ) at 4 °C. The beads were washed with lysis buffer and analyzed on immunoblot with anti-HA mAb or anti-Vif mAb (#319) (A kind gift from Dr. M. Malim through the AIDS Research and Reference Reagent Program) (Simon et al., 1995).

In vitro ubiquitin conjugation assay

In vitro ubiquitin conjugation assay was performed as previously described (Kobayashi et al., 2005). In brief, a Vif–BC–Cul5 complex was purified from insect cells and incubated with immunopurified HA-A3G, A3B, or A3F from 293T cells in reaction buffer containing E1, E2, GST-ubiquitin, NEDD8, Ubc12 (E2 for NEDD8), and APP-BP/Uba3 (E1 for NEDD8) at 37 °C for 1 h. Samples were subjected to

immunoblot to detect GST-ubiquitin-conjugated HA-APOBEC3 proteins.

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Cytotoxic T Lymphocyte–based Control of Simian Immunodeficiency Virus Replication in a Preclinical AIDS Vaccine Trial

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Abstract

Recently, encouraging AIDS vaccine trials in macaques have implicated cytotoxic T lymphocytes (CTLs) in the control of the simian human immunodeficiency virus SHIV89.6P that induces acute $CD4^+$ T cell depletion. However, none of these vaccine regimens have been successful in the containment of replication of the pathogenic simian immunodeficiency viruses (SIVs) that induce chronic disease progression. Indeed, it has remained unclear if vaccine-induced CTL can control SIV replication. Here, we show evidence suggesting that vaccine-induced CTLs control SIVmac239 replication in rhesus macaques. Eight macaques vaccinated with DNA-prime/Gag-expressing Sendai virus vector boost were challenged intravenously with SIVmac239. Five of the vaccinees controlled viral replication and had undetectable plasma viremia after 5 wk of infection. CTLs from all of these five macaques rapidly selected for escape mutations in Gag, indicating that vaccine-induced CTLs successfully contained replication of the challenge virus. Interestingly, analysis of the escape variant selected in three vaccinees that share a major histocompatibility complex class I haplotype revealed that the escape variant virus was at a replicative disadvantage compared with SIVmac239. These findings suggested that the vaccine-induced CTLs had "crippled" the challenge virus. Our results indicate that vaccine induction of highly effective CTLs can result in the containment of replication of a highly pathogenic immunodeficiency virus.

Key words: $CD8^+$ T lymphocytes • selection • MHC • SIV • Sendai virus

Introduction

Virus-specific $CD8^+$ CTL responses are critical for the control of immunodeficiency virus infections. The importance of CTLs in the control has been indicated by several clinical correlations in HIV-1–infected humans (1–3) and $CD8^+$ T cell depletion experiments in macaque AIDS

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Abbreviations used in this paper: aa, amino acid(s); B-LCL, B lymphoblastoid cell line; DGGE, denaturing gradient gel electrophoresis; L, leucine; nt, nucleotide; RSCA, reference strand–mediated conformation analysis; S, serine; SeV, Sendai virus; SHIV, simian HIV; SIV, simian immunodeficiency virus; VSV-G, vesicular stomatitis virus G; Vv, vaccinia virus.

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models (4–6). Therefore, recent vaccine approaches have focused on eliciting CTL responses (7, 8). However, HIV-1–infected individuals often have high plasma virus concentrations despite the presence of high frequencies of CTLs (9) and it has remained unclear if HIV-1 replication can be contained by vaccine-elicited CTL responses.

DNA vaccines, recombinant viral vector–based vaccines, and their combinations are promising delivery methods for AIDS vaccine because of their potential for inducing CTL responses. Recently, encouraging trials of these vaccines in macaques have implicated vaccine-induced CTLs in the control of the simian HIV (SHIV)89.6P that induces acute $CD4^+$ T cell depletion (10–14). However, most of these vaccine regimens used Env as an immunogen and it is likely that Env-specific antibodies played a role in control of this chimeric virus. Additionally, it has been suggested that SHIV89.6P may not be an appropriate challenge virus (15) and none of these vaccine regimens have been successful in the containment of the more realistic challenge of the pathogenic simian immunodeficiency viruses (SIVs) smE660, mac251, or mac239 (16–19). Thus, it is quite important to know if vaccine induction of CTL responses can lead to the containment of replication of these SIVs that induce chronic disease progression.

We previously developed a DNA-prime/Gag-expressing Sendai virus (SeV) vector boost vaccine system and showed its potential for efficiently inducing Gag-specific cellular immune responses (13, 20). In the preclinical trial, all the vaccinated macaques controlled viremia and were protected from acute AIDS progression after SHIV challenge (13, 21). In this study, we examined if CTL induction by our vaccine system can result in the containment of SIVmac239 replication.

Materials and Methods

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

Vaccination and Challenge. An *env*- and *nef*-deleted SHIV DNA, SIVGP1, was constructed from an infectious $SHIV_{MD14YE}$ clone DNA as described previously (13, 22). The DNA is deleted with a gene fragment encoding Env surface protein (SU; nucleotide [nt] 6211 to nt 7726 in $HIV\text{-}1_{DH12}$; these sequence data are available from GenBank/EMBL/DDBJ under accession no. AF069140), the 3′ portion of the *env* gene (nt 8628 to nt 8764 in $HIV\text{-}1_{DH12}$), and the 5′ quarter of the *nef* gene (nt 9333 to nt 9481 in SIVmac239; GenBank/EMBL/DDBJ accession no. M33262). From SIVGP1 DNA, the 5′ long terminal repeat region was replaced with a CMV promoter with immediate early enhancer and the 3′ portion containing the remaining *nef* and the 3′ long terminal repeat was replaced with Simian virus 40 poly A to obtain CMV-SHIVdEN DNA. Therefore, the CMV-SHIVdEN DNA has SIV-derived *gag*, *pol*, *vif*, *vpx*, and partial *vpr* sequences and HIV-1–derived partial *vpr*, *tat*, *rev*, and partial env (nt 7726 to nt 8628 containing the second exon of *tat*, the second exon of *rev*, and RRE) sequences. At DNA vaccination, animals received 5 mg CMV-SHIVdEN DNA intramuscularly. We used two kinds of SeV vectors, a transmissible one (SeV-Gag) and an F-deleted nontransmissible one (F[−]SeV-Gag), for the boost. Recombinant SeV-Gag and F(−)SeV-Gag were constructed and recovered as described previously (20, 23, 24). 6 wk after the DNA prime, animals received 10^8 cell-infectious units of SeV-Gag or 6×10^9 cell-infectious units of F(−)SeV-Gag intranasally as a boost. Four macaques (V1, V2, V3, and V4) were vaccinated with DNA-prime/SeV-Gag-boost, and the other four (V5, V6, V7, and V8) were vaccinated with DNA-prime/F(−)SeV-Gag-boost. 13 wk after the boost, animals were challenged intravenously with 1,000 $TCID_{50}$ (50% tissue culture–infective dose) of SIVmac239 (25). An SIVmac239 molecular clone DNA, pBRmac239, was provided by T. Kodama (University of Pittsburg, Pittsburgh, PA) and R.C. Desrosiers (New England Primate Research Center, Southborough, MA), and the virus obtained from COS1 cells transfected with pBRmac239 was propagated on rhesus macaque PBMCs to prepare the SIVmac239 challenge stock.

Flow Cytometric Analysis of Virus-specific IFN-γ Induction. We measured virus-specific T cell levels by flow cytometric analysis of IFN-γ induction after specific stimulation as described previously (13). In brief, PBMCs were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs; reference 26) infected with a vaccinia virus (Vv) vector (27) for nonspecific Vv control stimulation and B-LCLs infected with a Vv vector expressing SIVmac239 Gag for Gag-specific Vv Gag stimulation, respectively. Intracellular IFN-γ staining was performed by using CytofixCytoperm kit (Becton Dickinson) according to the manufacturer's instructions. FITC-conjugated anti–human CD4, peridinin chlorophyll protein–conjugated anti–human CD8, allophycocyanin-conjugated anti–human CD3, and anti–human PE-conjugated IFN-γ antibodies (Becton Dickinson) were used. Gag-specific T cell levels were calculated by subtracting the IFN-γ^+ T cell frequencies after nonspecific Vv control stimulation from those after Gag-specific Vv Gag stimulation. Alternatively, for measurement of SIV-specific T cell levels, lymphocytes were cocultured with B-LCLs infected with a vesicular stomatitis virus G (VSV-G)–pseudotyped murine leukemia virus for nonspecific stimulation and B-LCLs infected with a VSV-G–pseudotyped SIVGP1 for SIV-specific stimulation, respectively. In the case of examining peptide-specific T cell levels, B-LCLs were pulsed with each peptide (at a final concentration of 1 μM) or peptide mixture (final concentration of each peptide was 1–10 μM) for peptide-specific stimulation or incubated without peptide for nonspecific stimulation. The peptides, including a panel of 117 overlapping peptides (15–17 amino acids [aa] in length and overlapping by 10 to 12 aa) spanning the entire SIVmac239 Gag sequence, were purchased from Sigma Genosys Japan. Specific T cell levels <100 cells per million PBMCs were considered negative, those between 100 and 200 borderline, and those >200 positive. Gag-specific T cells were undetectable before the vaccination in all of the vaccinees and before the challenge in all of the naive controls.

Quantitation of Plasma Viral Loads. Plasma RNA was extracted using High Pure Viral RNA kit (Roche Diagnostics). For quantitation of plasma SIV RNA levels, serial fivefold dilutions of RNA samples were amplified in quadruplicate by RT and nested PCR using SIV *gag*-specific primers to determine the end point as described previously (22). For preparing the RNA standard, we first set up the method for quantitation of SHIV RNA copy

number by using HIV-1 *vpu*-specific primers and an HIV-1 standard quantitated by Amplicor HIV-1 Monitor (Roche Diagnostics). By using this method, we prepared an SHIV standard for the present assay. The lower limit of detection in this assay is $\sim 4 \times 10^2$ copies/ml. The plasma viral loads at several time points were confirmed by real time PCR (28).

Sequencing. Plasma RNA was extracted using High Pure Viral RNA kit or RNA extraction system in Amplicor HIV-1 Monitor. The fragment spanning from nt 1231 to nt 2958 in SIVmac239 containing all of the *gag* region was amplified from plasma RNA by nested RT-PCR. In case of the plasma with low viral loads (<2,000 copies/ml), 8–16 tubes of nested RT-PCR amplifications were performed for each plasma to avoid obtaining only unrepresentative clones. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems). Alternatively, the PCR products were subcloned into a plasmid DNA by using the TOPO cloning system (Invitrogen) and sequenced.

Isolation of Mamu-A/B cDNA Clones. Total cellular RNA was used to synthesize oligo(dT)-primed cDNA with reverse transcriptase (Superscript II; Invitrogen). Full-length cDNAs of *Mamu-A* and *Mamu-B* were amplified by PCR with locus-specific primer pairs (*Mamu-A* forward: 5′-ATGGCGCCCCGAACCCTCCTCCTG-3′, *Mamu-A* reverse: 5′-TCACACTTTACAAGCCGTGAGAGA-3′; *Mamu-B* forward: 5′-ATGGCGCCCCGAACCCTCCTCCTG-3′, *Mamu-B* reverse: 5′-TCAAGCCGTGAGAGACACATC-3′) and cloned in pGEM-T Easy vector (Promega). The integrity of the clones was verified by reference strand–mediated conformation analysis (RSCA; 29) as the following and then sequenced.

Determination of Mamu MHC-I Haplotype. Locus-specific RT-PCR products were subjected to second round PCR to obtain 725-bp-long DNA fragments encoding Mamu-A/B extracellular domains using *Mamu-A/B* universal forward (5A: 5′-ATGGCGCCCCGAACCCTC-3′) and reverse (4R: 5′-CCAGGTCAGTGTGATCTCCG-3′) primers. The product was analyzed by RSCA conformation analysis essentially as described previously (31). In brief, the second round PCR products and "a reference strand," a fragment derived from the same PCR condition except for using 5′ Cy5-labeled forward primer and a certain cloned DNA template (its sequence is available upon request), were mixed together in a reaction tube, heat denatured, and then cooled down to form heteroduplex DNA. The mobility of heteroduplex DNA molecules in 6% nondenaturing Long Ranger gel (BioWhittaker Molecular Applications) was measured by ALF express II automated sequencing apparatus (Amersham Biosciences). Fluorescence electropherograms showed multiple peak patterns corresponding to multiple, different kinds of sequences expressed in individual macaques. The identity of each peak was determined by comparison of its mobility with those of heteroduplexes derived from parallel PCR using *Mamu-A/B* cDNA clones as templates. Alleles that were shared by a breeder macaque and subset of his sons were thought to be transmitted together and assigned to a single haplotype. The number of expressed alleles on one MHC-I haplotype ranged from one *A* and two *B* alleles to no less than three *A* and five *B* alleles.

Typing of MHC-II (Mamu-DRB and Mamu-DQA). MHC-II alleles and haplotype compositions of macaques were analyzed by sequencing of cloned cDNA and denaturing gradient gel electrophoresis (DGGE; reference 30). Total RNA was extracted from B-LCLs and cDNA was generated by using SuperScript II reverse transcriptase. The entire DRB cDNA and the DQA exon 2 fragments were amplified by PCR using the following primer sets designed to hybridize with the conserved monomorphic regions: 5′-CGCGAATTCTCAGCTCAGGAGTCC-3′ and 5′-GCGGGATCCATGGTGTGTCTG-3′ for DRB, and 5′-CGCGAATTCGGTAGCAGCGGTAGAGT-3′ and 5′-GCGGGATCCGTGTAAACTTGTACCAGTT-3′ for DQA. The PCR products were subcloned into pUC19 and sequenced. When more than four clones with an identical sequence were obtained for an allele, the allele was considered to be expressed in the animal (see Table I).

The number of DRB alleles expressed in macaques and their haplotype relationships were analyzed by comparing the patterns of DGGE and by cloning and sequencing of the DNA extracted from each band in the gel. For DGGE analyses, the DRB exon 2 fragment was amplified by PCR using the forward (5′-CACTGGCTTTGGCTGGGGACAC-3′) and the GC-clamped reverse (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCGCCCCCGCCCGCAGGATACACAGTCACCTTAG-3′) primers. DGGE was performed in 6% polyacrylamide gel containing a gradient of 36–50% of the denaturant mixture (7 M urea and 40% formamide) at 100 V at 60°C for 2.5 h in a DCode system (Bio-Rad Laboratories). DNA eluted from each separate band was subcloned into a plasmid by using the TOPO cloning system and sequenced.

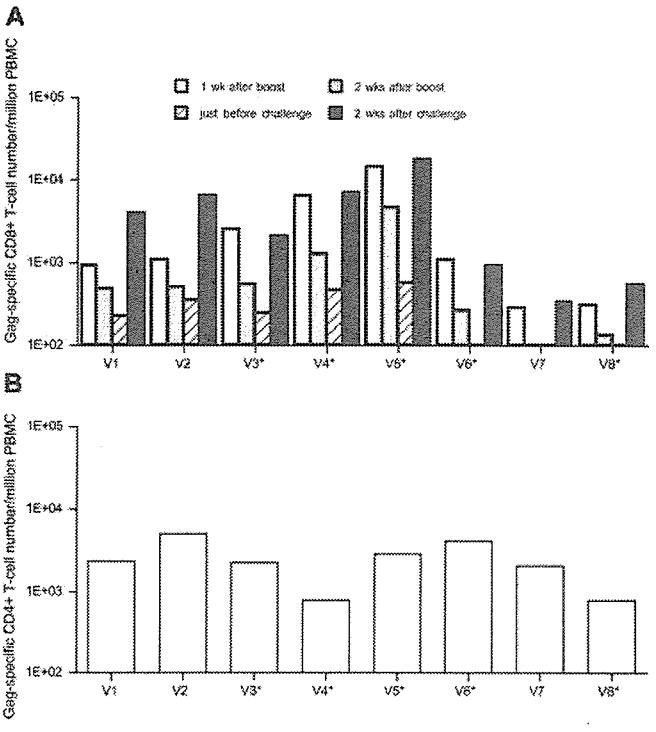


Figure 1. Gag-specific T cell frequencies in vaccinated macaques. Macaques V1, V2, V3, and V4 were boosted with a replication-competent SeV-Gag, whereas macaques V5, V6, V7, and V8 were boosted with a replication-defective F(−)SeV-Gag. *, macaques that controlled SIV replication after challenge. (A) Gag-specific $CD8^+$ T cell frequencies per million PBMCs. The frequencies at week 7 after vaccination (1 wk after boost), at week 8 after vaccination (2 wk after boost), at week 19 after vaccination (just before challenge), and at week 2 after challenge (2 wk after challenge) are shown. (B) Gag-specific $CD4^+$ T cell frequencies per million PBMCs at week 7 after vaccination (1 wk after boost). The frequencies were calculated by subtracting the IFN-γ^+ T cell frequencies after nonspecific Vv control stimulation from those after Gag-specific Vv Gag stimulation. The background IFN-γ^+ T cell frequencies after nonspecific stimulation were $<2.0 \times 10^2$.

Results

Gag-specific T Cell Induction after SeV-Gag-Boost. Our extremely simple vaccine protocol consisted of a single prime with DNA followed by a single boost with a recombinant SeV vector expressing SIVmac239 Gag 6 wk after the prime. Eight rhesus macaques (V1, V2, V3, V4, V5, V6, V7, and V8) were vaccinated with the prime/boost, and four naive controls (N1, N2, N3, and N4) received no vaccination before an intravenous SIVmac239 challenge.

We measured virus-specific T cell levels in the vaccinated macaques by flow cytometric detection of antigen-specific IFN-γ induction. SIV- and Gag-specific T cell responses were examined in PBMCs at weeks 2 and 6 after the DNA vaccination, respectively, but no responses to either SIV or Gag were detectable in any of the vaccinated macaques (not depicted). After the SeV boost, however, we found induction of Gag-specific $CD8^+$ T cells in all of the vaccinees (Fig. 1 A). The levels differed among the macaques, with five (V1, V2, V3, V4, and V5) maintaining detectable levels of Gag-specific $CD8^+$ T cells until challenge. The SeV boost also induced Gag-specific $CD4^+$ T cells in all eight vaccinees (Fig. 1 B).

Control of SIVmac239 Replication in Five of Eight Vaccinees. These vaccinated macaques were challenged intravenously with 1,000 $TCID_{50}$ of SIVmac239 at week 19 after the DNA prime (13 wk after the SeV boost). The unvaccinated control macaques had high peak viremia ($>10^7$ SIV RNA copies/ml plasma) on day 10 after challenge and maintained relatively high plasma viral concentrations (10^4–10^6 SIV RNA copies/ml plasma; Fig. 2). Three of them showed gradual loss of percent CD4 in peripheral T lymphocytes. In contrast, five vaccinated macaques (V3, V4, V5, V6, and V8) controlled replication of this highly pathogenic challenge virus. In these macaques, plasma viremia became undetectable after week 5 and peripheral $CD4^+$ T cells were maintained. The other three vaccinees (V1, V2, and V7) failed to control virus replication and

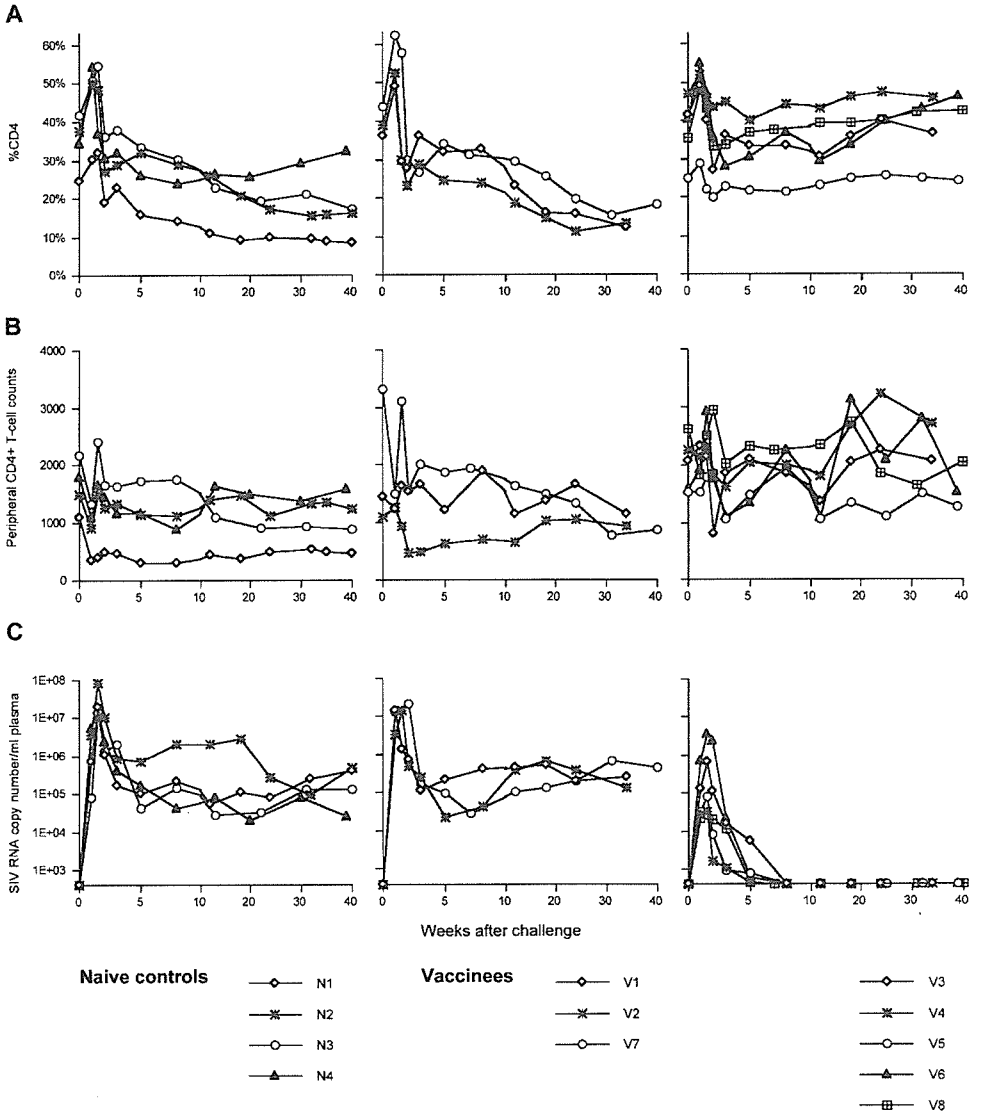
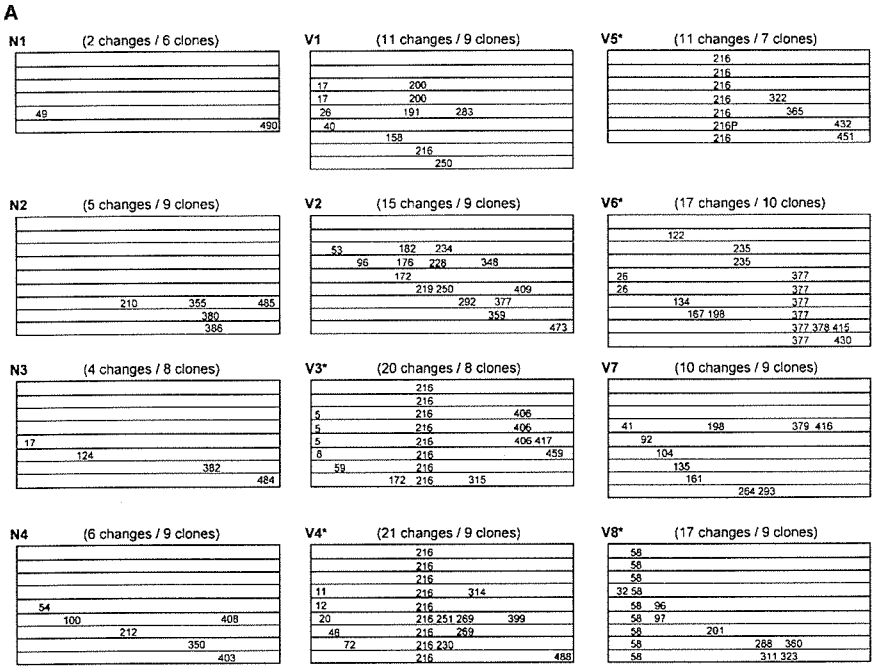


Figure 2. Changes in peripheral $CD4^+$ T cell levels and plasma viral loads after SIVmac239 challenge. (A) Percents of $CD4^+$ T cells in peripheral blood. (B) $CD4^+$ T cell counts in peripheral blood. (C) Plasma viral loads (SIV RNA copy number/ml). The left panels show the naive controls (N1, N2, N3, and N4), the middle panels show the vaccinees that failed to control SIV replication (V1, V2, and V7), and the right panels show the vaccinees that controlled SIV replication (V3, V4, V5, V6, and V8). The portion until week 10 after challenge is enlarged.



B

Animal	CTL escape mutant	Frequencies of the mutant		
		week 2	week 3	week 5
V3	mutation at the 216th aa	1/9	5/9	8/8
V4	mutation at the 216th aa	0/8	9/9	9/9
V5	mutation at the 216th aa	0/7	3/9	7/7
V6	mutation at the 377th aa	ND	1/10	6/10
V8	mutation at the 58th aa	ND	0/9	9/9

Figure 3. Mutations in SIV *gag*. (A) Schematic representation of the positions of aa changes in SIV Gag in each macaque after challenge. 6–10 clones of plasmids carrying the whole *gag* region amplified from plasma RNA at week 5 after challenge were obtained from each macaque and sequenced. Each lane represents the whole *gag* sequence derived from each clone and the positions of aa changes detected are indicated. Total number of aa changes and number of clones sequenced are shown in the parentheses. All the changes at aa 58 were glutamine to lysine, and all at aa 377 were isoleucine to threonine. All the changes at aa 216 other than the one indicated as 216P were L to S. The 216P represents L to P change at aa 216. (B) Frequencies of the CTL escape mutants at weeks 2, 3, and 5 in the vaccinees that controlled SIV replication. The ratio of the number of the clones with the escape mutation to the number of the sequenced clones is shown.

showed gradual loss of percent CD4 in peripheral T lymphocytes similar to the naive control animals. One of them (macaque V2) was killed at week 42 because of dyspnea, loss of body weight, and loss of peripheral $CD4^+$ T cells (4.4%, 97 cells/μl at week 42). Autopsy revealed that this animal developed AIDS with *Pneumocystis carinii* pneumonia.

At week 2 after challenge, we detected anamnestic Gag-specific $CD8^+$ T cell responses in all of the vaccinated macaques, indicating efficient secondary responses during the acute phase of infection (Fig. 1 A). These levels varied from macaque to macaque. Macaque V5 showed the highest level of Gag-specific $CD8^+$ T cells and macaque V7 showed the lowest. No significant difference in the levels was observed between the macaques that controlled viral replication and those that did not. The magnitude of the total prechallenge Gag-specific $CD8^+$ T cell or $CD4^+$ T cell responses did not appear to correlate with the level of control. We examined plasma-neutralizing activities against SIVmac239 as described previously (31), but found no neutralizing activities in any of the controls or the vaccinees at weeks 5 or 12 after challenge (not depicted), indicating that neutralizing antibodies were not essential for the control of SIV replication observed in this experiment.

Rapid Selection of CTL Escape Variants in the Vaccinees That Controlled SIVmac239 Replication. To determine whether vaccine-induced Gag-specific T cell responses exerted a selective pressure on the virus, we sequenced the SIV *gag* region in the viral genomes obtained from plasma RNA at week 5 after challenge (Fig. 3 A). The numbers of aa changes per clone in the vaccinated macaques were significantly higher than those in the unvaccinated (mean: unvaccinated, 0.51; vaccinated, 1.75; $P = 0.0006$ by t test). This may reflect the immune pressure by vaccine-induced Gag-specific T cell responses. Interestingly, all of the macaques that controlled SIVmac239 replication (V3, V4, V5, V6, and V8), but not those unable to control the virus, showed consistent aa changes in Gag (Fig. 3 A). Among them, three macaques (V3, V4, and V5) had a common aa change, leucine (L) to serine (S) at the 216th aa in Gag. We then examined peptide-specific T cell responses after the SeV boost and found, in these three macaques but not in the other vaccinees, efficient expansion of $CD8^+$ T cells specific for an epitope ($Gag_{206-216}$; IINEEAADWDL) spanning from the 206th to the 216th aa in SIVmac239 Gag. Interestingly, these three macaques showed no or diminished recognition of the mutant peptide, IINEEAADWDS ($Gag_{206-216}$L216S; Fig. 4 A), indicating that this mutant likely represents an escape variant. Sequence analysis of vi-

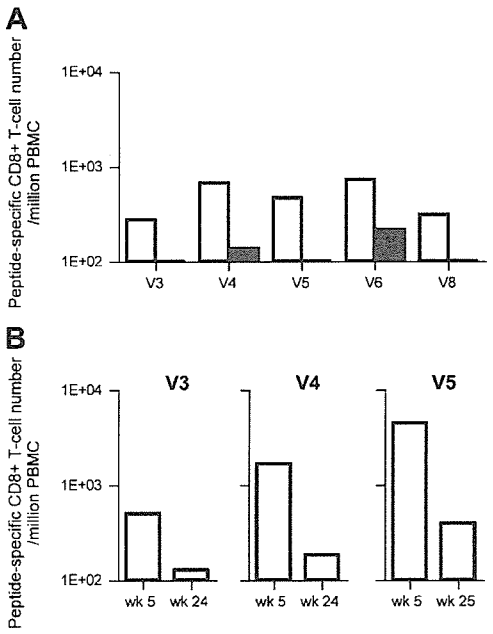
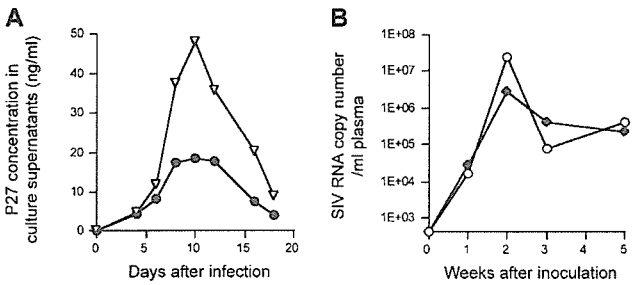


Figure 4. Peptide-specific T cell frequencies in the vaccinees that controlled SIV replications. (A) Comparison between the epitope peptide–specific and the variant peptide–specific $CD8^+$ T cell responses. PBMCs at week 10 after vaccination in macaque V3, at week 10 after vaccination in V4, at week 15 after vaccination in V5, at week 3 after challenge in V6, and at week 3 after challenge in V8 were used. The open bars indicate the levels of $CD8^+$ T cells specific for $Gag_{206-216}$ peptide in V3, V4, and V5, $Gag_{367-381}$ peptide in V6, and Gag_{50-65} peptide in V8, respectively. The solid bars indicate the levels of $CD8^+$ T cells specific for $Gag_{206-216}$L216S peptide in V3, V4, and V5, $Gag_{367-381}$I377T peptide in V6, and Gag_{50-65}Q58K peptide in V8, respectively. (B) $Gag_{206-216}$-specific $CD8^+$ T cell levels in macaques V3, V4, and V5 after challenge. The background IFN-γ^+ $CD8^+$ T cell frequencies after nonspecific stimulation were $<1.0 \times 10^2$.

ral genomes from the three macaques that made responses to this epitope at weeks 2 and 3 revealed that this CTL escape mutant became dominant around week 3 after challenge (Fig. 3 B). Thus, in these three macaques with high levels of $Gag_{206-216}$-specific $CD8^+$ T cells, the wild-type challenge virus disappeared quickly and only the CTL escape mutant was detectable in plasma at week 5. These three macaques had high levels of $Gag_{206-216}$-specific $CD8^+$ T cells 3 wk after challenge. However, these levels were considerably reduced in the chronic phase (Fig. 4 B).

We further examined epitope-specific $CD8^+$ T cell responses in the other two macaques that controlled viral replication. In macaque V6, a mutation leading to a change at the 377th aa (isoleucine to threonine) was observed at week 5 (Fig. 3 A). Analysis of peptide-specific responses revealed that this macaque had a high level of $CD8^+$ T cells specific for a 15-mer peptide corresponding to aa 367–381 in SIVmac239 Gag ($Gag_{367-381}$) at week 3 after challenge. Stimulation by the mutant $Gag_{367-381}$ peptide with the substitution ($Gag_{367-381}$I377T) induced IFN-γ^+ $CD8^+$ T cells, but their frequency was lower than that after stimulation by the wild-type $Gag_{367-381}$ peptide (Fig. 4 A). Additionally, viruses from macaque V8 had a mutation leading to a change at the 58th aa (glutamine to lysine; Fig. 3 A). In this animal, $CD8^+$ T cell responses specific for a 16-mer peptide corresponding to aa 50–65 in SIVmac239 Gag (Gag_{50-65}) were observed at week 3 after challenge. Stimulation by the mutant Gag_{50-65} peptide with the substitution (Gag_{50-65}Q58K) failed to induce IFN-γ^+ $CD8^+$ T cells (Fig. 4 A). Each of these mutants became dominant at approximately week 5 after challenge in the corresponding macaque (Fig. 3 B).

Among the 12 macaques used in the challenge experiment, 8 macaques (2 naive controls and 6 vaccinees) descended from a single male, macaque R90-120 (its sons: N2, V2, and V3; its grandsons: N3, V4, V5, V6, and V7; Table I). Analysis of MHC-I *Mamu-A* and *Mamu-B* alleles indicated that four macaques of the eight R90-120 descendants, N2, V3, V4, and V5, share an MHC-I haplotype (90–120-Ia) derived from macaque R90-120. Analysis of MHC-II also suggested that these macaques possibly share an MHC-II haplotype derived from macaque R90-120. Among these macaques possessing the 90–120-Ia haplotype, three (V3, V4, and V5) were vaccinees that controlled SIV replication with high levels of $Gag_{206-216}$-spe-



C

Animal	Sequencing	Frequencies of the L216S mutant			
		week 1	week 2	week 3	week 5
M1	direct	++	+	-	-
	clones	ND	3/10	0/10	ND
M2	direct	++	-	-	-
	clones	ND	0/10	0/10	ND

Figure 5. Comparison of replication efficiencies between the wild-type SIVmac239 and the escape variant SIVmac239G216S. (A) Replication kinetics of SIVmac239 (▽) and SIVmac239G216S (●) in macaque PBMCs. MT4 cells were transfected with pBRmac239 and pBRmac239G216S to obtain SIVmac239 and SIVmac239G216S, respectively. PBMCs were infected with the viruses at a multiplicity of infection of 0.0002 and concentrations of SIV Gag p27 in their culture supernatants were measured by ELISA (Beckman Coulter). A representative result from three independent experiments is shown. (B) Plasma viral loads (SIV RNA copy number/ml) in macaques M1 (○) and M2 (◆) after inoculation with both of the wild-type SIVmac239 molecular clone DNA and the mutant SIVmac239G216S molecular clone DNA. (C) Frequencies of the mutant viral genome in plasma in the macaques inoculated with both of the wild-type SIVmac239 molecular clone DNA and the mutant SIVmac239G216S molecular clone DNA. In case of direct sequencing of the PCR products (indicated by direct), ++ indicates detection of both the wild-type and the mutant at comparable levels, + indicates detection of the wild-type predominantly and the mutant slightly, and − indicates detection of the wild-type only. In case of sequencing clones (indicated by clones), the ratio of the number of the mutant clones to the number of the sequenced clones is shown.

Table I. *MHC-I and MHC-II Alleles of Macaques Used in This Study*

Animal[a]	Father	MHC-I *Mamu-A & B* RSCA pattern[b]	MHC-II *Mamu-DRB & DQA* alleles[c]
Naive control			
N1	R90-088	*1	*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
			*DRB(AB112040), DRB*W2603, DRB*W402, DQA1*0502*
N2	R90-120	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			*DRB(Z26165), DRB(AB112039), DRB(AB112043), DQA1*06(MM76195)*
N3	R94-027[d]	*2	*DRB1*0316, DRB*W2507, DQA1*01(M76202)*
			*DRB*W2104, DRB*W2603, DRB*W606, DQA1*0502*
N4	R90-010	ND	*DRB*W2104, DRB*W2603, DQA1*0502*
			*DRB*0321, DRB*0323, DRB*W606, DQA1*05(M76227)*
Vaccinee			
V1	R90-088	*1	*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
			*DRB*W2505, DRB(AB112046), DRB(AB124813), DQA(AB124814)*
V2	R20-120	90-120-Ib	*DRB*W2002, DRB*W2501, DQA1*0502*
			*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
V3	R90-120	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
V4	R94-027	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			*DRB*W2505, DRB(AB112046), DRB(AB124813), DQA(AB124814)*
V5	R94-027	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			DRB(AB112043), DRB(AB112047)
V6	R94-027	*2	*DRB1*0316, DRB*W2507, DQA1*01(M76202)*
			*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
V7	R94-027	*2	*DRB1*0316, DRB*W2507, DQA1*01(M76202)*
			*DRB*W2104, DRB*W2603, DRB*W606, DQA1*0502*
V8	R90-010	ND	*DRB*W2104, DRB*W2603, DQA1*0502*
			*DRB1*0316, DRB*W2507, DQA1*09(M76200)*
Breeder			
R90-088	unknown	*1	*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
R90-120	unknown	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
		90-120-Ib	*DRB*W2002, DRB*W2501, DQA1*0502*
R90-010	unknown	ND	*DRB*W2104, DRB*W2603, DQA1*0502*
			*DRB1*0316, DRB*2507, DQA1*01(M76202)*

[a]The underlined macaques showed control of SIV replication.
[b]MHC-I *Mamu-A* and *Mamu-B* alleles and haplotype compositions of macaques were examined by RSCA and sequencing of cloned cDNA. The haplotype 90-120-Ia derived from macaque R90-120 consists of three *Mamu-A* alleles (*Mamu-A120-1*, *Mamu-A120-4*, and *Mamu-A120-5*) and four *Mamu-B* alleles (*Mamu-B120-1*, *Mamu-B120-6*, *Mamu-B120-8*, and *Mamu-B120-9*). The haplotype 90-120-Ib derived from macaque R90-120 consists of two *Mamu-A* alleles (*Mamu-A120-2* and *Mamu-A120-3* [= *Mamu-A*05*]) and five *Mamu-B* alleles (*Mamu-B120-2*, *Mamu-B120-3*, *Mamu-B120-4*, *Mamu-B120-5* [= *Mamu-B*36*], and *Mamu-B120-7*). Macaques N1 and V1 shared an RSCA pattern of a haplotype derived from R90-088 (*1). Macaques N3, V6, and V7 shared an RSCA pattern of a haplotype not derived from R90-120 (*2).
[c]MHC-II *Mamu-DRB* and *Mamu-DQA* alleles were analyzed by DGGE and sequencing of cDNA. The determined alleles are shown. Each number in parentheses indicates the corresponding accession number for the nt sequence of the allele that has not yet been designated.
[d]The father of macaque R94-027 is macaque R90-120.

cific $CD8^+$ T cell responses. The remaining one (naive control macaque N2) showed a detectable level of $Gag_{206-216}$-specific $CD8^+$ T cell responses at week 3 after challenge, although the level was low (2.5×10^2 cells/million PBMCs). These results strongly suggest that the $Gag_{206-216}$ epitope is restricted by an MHC-I molecule derived from the 90–120-Ia haplotype.

Diminished Replicative Ability of the CTL Escape Variant SIV. We then explored the hypothesis that the escape mutation selected by the vaccine-induced $Gag_{206-216}$-spe-

cific CTL resulted in a loss of viral fitness. We constructed a molecular clone of the escape mutant SIV, referred to as SIVmac239G216S, with a mutation resulting in the L to S substitution at the 216th aa in Gag. The mutant SIV was replication competent in vitro but showed lower levels of proliferation kinetics in PBMC culture compared with the wild-type SIVmac239 (Fig. 5 A). To compare the SIVmac239G216S replication kinetics with the wild-type in macaques, two macaques (M1 and M2, neither of them descended from macaque R90-120) were coinoculated intramuscularly with 5 mg of the SIVmac239 molecular clone DNA (pBRmac239) and 5 mg of the SIVmac239G216S molecular clone DNA (pBRmac239G216S; Fig. 5, B and C). Both viral genomes were detected at comparable levels in plasma from both of the macaques at week 1 after the inoculation. After that, however, the mutant SIVmac239G216S disappeared and the wild-type SIVmac239 became dominant. Neither of the macaques showed $Gag_{206-216}$-specific $CD8^+$ T cell responses at week 3 (not depicted). These results indicate that the L to S change at the 216th aa in Gag is disadvantageous for SIV replication in the absence of $Gag_{206-216}$-specific $CD8^+$ T cell responses in macaques.

Discussion

In this study, we present evidence indicating that vaccine-induced CTLs control SIVmac239 replication in rhesus macaques. Each of the macaques that controlled viral replication had a mutation in Gag leading to an aa change in a CTL epitope by week 5 after challenge, reflecting strong CTL-induced selective pressure. This finding lends support to the notion that epitope-specific CTL responses played a central role in the control of replication of the SIVmac239 challenge virus because it was difficult to detect the challenge virus at week 5 after challenge.

Among the 12 macaques used in the challenge experiment, 8 macaques descended from macaque R90-120 and 4 of them shared an MHC-I haplotype, 90–120-Ia. Among the four, not the naive (N2) but the three vaccinees (V3, V4, and V5) controlled SIV replication and selected for the same $Gag_{206-216}$-specific CTL escape variant with L to S change at the 216th aa in Gag. Therefore, we examined the reproducibly selected escape variant SIVmac239G216S intensively and found that in the absence of $Gag_{206-216}$-specific $CD8^+$ T cell responses, its replication efficiency is diminished compared with the wild-type SIVmac239 in vivo as well as in vitro. The rapid selection of the escape variant with lower viral fitness in the vaccinees with $Gag_{206-216}$-specific CTLs indicates that the vaccine-induced CTLs exerted strong immune pressure leading to clearance of the wild-type SIVmac239.

The emergence of escape variants depends on the balance between CTL-induced immune pressure and viral fitness costs (32). Viral escape from CTLs during the acute phase of natural immunodeficiency virus infections has been observed in Tat, Nef, Vpr, and Env (33–36). Escape variants with mutations in the structural protein Gag have been also reported (37), but it has been shown that they mostly diminish viral fitness and require multiple additional compensatory mutations to restore their replicative competence (38–41). Indeed, the $Gag_{206-216}$-specific CTL escape variant selected in macaques V3, V4, and V5 diminished viral replication. Therefore, our results suggest that the vaccine-induced CTLs were crucial to the rapid containment of replication of the challenge virus and selected for the virus with diminished replicative ability. Without compensatory mutations, the crippled virus might be easily controlled by the immune system.

The macaques used in our challenge experiment were non-Indian rhesus and the setpoint plasma viral loads in the naive control group might be lower than those usually observed in SIVmac239-infected Indian rhesus. However, the viral loads are higher than those typically observed in untreated humans infected with HIV-1 and equivalent to viral loads seen in SIVsmE660-infected Indian rhesus (16, 42). Indeed, all of the naive animals failed to control the virus replication after SIVmac239 challenge, indicating that CTLs are unable to contain and clear the virus in natural SIVmac239 infections of our non-Indian rhesus macaques. Thus, this study provides clear evidence demonstrating that vaccine induction of effective CTLs that can cripple the virus can result in the containment of replication of a neutralization-resistant, highly pathogenic immunodeficiency virus that is unable to be contained in the natural chronic course of infections. In conclusion, our results show that vaccine-induced CTLs can control SIVmac239 replication and indicate that induction of highly effective CTLs might be critical for the vaccine-based containment of immunodeficiency virus replication.

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