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Early Bone Marrow Hematopoietic Defect in Simian/Human Immunodeficiency Virus C2/1-Infected Macaques and Relevance to Advance of Disease

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To clarify hematological abnormalities following infection with human immunodeficiency virus (HIV), we examined the hematopoietic capability of bone marrow by using cynomolgus monkeys infected with pathogenic simian/human immunodeficiency virus (SHIV) strain C2/1, an animal model of HIV infection. The relationship between the progress of the infection and the CD4/CD8 ratio of T lymphocytes or the amount of SHIV C2/1 viral load in the peripheral blood was also investigated. A colony assay was performed to assess the hematopoietic capability of bone marrow stem cells during the early and advanced phases of the infection. Colonies of granulocytes-macrophages (GM) were examined by PCR for the presence of the SIVmac239 *gag* region to reveal direct viral infection. There was a remarkable decrease in the CFU-GM growth on days 1 and 3 postinoculation, followed by recovery on day 56. During the more advanced stage, the CFU-GM growth decreased again. There was minimal evidence of direct viral infection of pooled cultured CFU-GM despite the continuously low CD4/CD8 ratios. These results indicate that the decrease in colony formation by bone marrow stem cells is reversible and fluctuates with the advance of the disease. This decrease was not due to direct viral infection of CFU-GM. Our data may support the concept that, in the early phase, production of inhibitory factors or deficiency of a stimulatory cytokine is responsible for some of the bone marrow defects described in the SHIV C2/1 model.

It is generally known as a feature of human immunodeficiency virus (HIV) infection that CD4-positive T lymphocytes and monocytes infected with pathogenic HIV or simian/human immunodeficiency virus (SHIV) decrease in number and disappear. Infected hosts will thus become immunodeficient. Moreover, it has been reported that, after HIV infection is contracted, hematological abnormalities in the bone marrow and the peripheral blood such as anemia, lymphopenia, and thrombocytopenia ensue and correlate with the advance of the illness (36). Several possibilities have been noted as the cause of such hematological abnormalities: the apoptosis of virus-infected cells, changes in the hematological environment, dysfunction of the thymus or the lymphoid system, change of cell division, or dysfunction of hematopoietic progenitor cells (1). Furthermore, a few reports have shown that the bone marrow of patients with AIDS displays morphological alterations similar to those of patients with myelodysplastic syndrome (2, 31). The term "HIV myelopathy" has been used for this bone marrow pathology by some investigators (10, 22).

Reduced numbers of CFU (burst-forming units–erythrocytes [BFU-E] or CFU–granulocytes-macrophages [CFU-GM]) have been reported in bone marrow samples from patients infected with HIV (9, 16, 27). Moreover, the reduction in CFU-GM resembles that of an animal model of AIDS experimentally induced by simian immunodeficiency virus (SIV) (13, 30, 32). While the precise mechanisms of such hematopoietic abnormalities remain unclear, several hypotheses have been proposed: (i) decreased levels of appropriate cytokines secondary to altered numbers of T-cell subsets or macrophages, which are commonly seen in HIV type 1 (HIV-1) infection (28); (ii) production of inhibitory factors (14, 29); (iii) cytotoxic elimination of the precursor cells by the antibody-dependent cell-mediated cytolytic mechanism (7); and (iv) infection of hematopoietic precursor cells with viruses, which leads to death of these cells or their metabolic alteration (7). On the other hand, it has been suggested that primitive bone marrow progenitor cells are most likely not a major reservoir for HIVs (6, 13, 28).

Despite mounting data supporting the above-mentioned hypotheses, a unifying explanation remains elusive. We studied bone marrow samples from cynomolgus monkeys (*Macaca fascicularis*) experimentally infected with an SHIV strain in order to evaluate possible cellular and molecular events that affect hematopoiesis in SHIV infection.

MATERIALS AND METHODS

Animals. Twenty cynomolgus monkeys (nine males and 11 females) used in this study were maintained in our facility according to the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), Tokyo, Japan. All treatments were performed according to the standard operating procedures for monkeys for evaluation of human vaccines in the Tsukuba Primate Center, NIID, Tsukuba, Ibaraki, Japan. Their ages were 3 to 6 years, and their weights were approximately 3 to 5 kg (Table 1). Four sham-inoculated monkeys were included as a control. They were inoculated with saline alone instead of virus-containing saline solution. Two additional monkeys without sham treatment also served as a negative control. Low-dose ketamine (intramuscular dose of 10 mg/kg of body weight) was used as an anesthetic for blood and bone marrow sampling.

Viruses. A highly pathogenic SHIV strain, designated C2/1, was obtained by serum passages in cynomolgus monkeys. The SHIV C2/1 strain contains the *env*

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TABLE 1. Protocol for control and infection of cynomolgus monkeys with SHIV C2/1 and subsequent bone marrow harvesting[a]

Monkey no.	Age (yr)	Sex	Day of bone marrow harvesting	Virus administration	Dose of inoculated virus ($TCID_{50}$)
13	5	Male			
44	4	Male			
181	6	Male			
1037	5	Male			
1091	5	Male			
759	4	Male			
4345	4	Male	1	Intravenous	20
1	5	Female	3	Intravenous	20
2	5	Female	3	Intravenous	20
90c	5	Female	56	Intravenous	20
560	4	Female	56	Intrarectal	2,000
430	4	Female	56	Intrarectal	2,000
442	3	Female	56	Intrarectal	2,000
200	5	Female	56	Intravenous	20
944	5	Male	56	Intravenous	20
520	5	Female	56	Intrarectal	20
844	4	Female	56	Intrarectal	20
0634	4	Female	56	Intravenous	10
054	4	Female	113	Intravenous	2,000
039	4	Male	380	Intravenous	20

[a] Monkeys 13 to 759 were controls that were not infected with SHIV. Monkeys 4345 to 039 were inoculated intravenously or intrarectally with the doses of SHIV C2/1 shown in the table. Bone marrow harvesting was performed on the indicated day after inoculation. $TCID_{50}$, 50% tissue culture infective dose.

gene of pathogenic HIV-1 strain 89.6. This chimeric virus was propagated in concanavalin A-activated peripheral blood mononuclear cells (PBMC) from healthy monkeys or in a cell line, M8166. Cell-free virus stocks were stored at −120°C (25).

Antibodies. The mouse monoclonal antibodies (MAbs) used in this study were fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated MAbs to monkey CD3 (NF-18; BioSource) and to human CD4 (Nu-T H/I; Nichirei), CD8 (Nu-T S/C; Nichirei), CD16 (3G8; Pharmingen), and CD20 (Leu-16; Becton Dickinson).

Preparation of bone marrow cells. Fourteen monkeys were infected with SHIV C2/1 at three 50% tissue culture infective doses by intravenous or intrarectal inoculation (Table 1). Bone marrow samples were aspirated from their femoral bone during autopsy. For a sham-inoculated control, monkeys received 0.5 ml of saline alone. One day or 3 days later, bone marrow samples were aspirated from their pelvic bones. Non-sham-control monkeys received only ketamine anesthesia for bone marrow aspiration.

Preparation of blood samples for cell surface antigen analysis by flow cytometry. Peripheral blood was mixed with lysis buffer (Becton Dickinson) and centrifuged at 300 × *g* for 5 min. Viable cells were counted by the trypan blue dye-exclusion method. The cell surface antigens CD3, CD4, CD8, CD16, and CD20 were stained with their respective MAbs. After being washed with staining buffer, 5×10^4 cells in each labeled sample tube were analyzed by a FACSCalibur flow cytometer (Becton Dickinson) with use of Cell Quest software (Becton Dickinson). Absolute PBMC count was determined as follows. Fifty milliliters of each whole-blood sample, containing FITC-conjugated anti-CD3 MAb (BioSource), PE-conjugated anti-CD4 MAb (Becton Dickinson), and peridinin-chlorophyll protein-conjugated anti-CD8 MAb (Becton Dickinson), was added to a TRUCOUNT tube and incubated at room temperature. Contaminating red blood cells were lysed, and each sample was analyzed by flow cytometry as described above. All measurements were made under the same instrumental setting.

Quantification of cell-associated and plasma viral load. Plasma viral RNA was extracted and purified using a QIAamp viral RNA minikit (Qiagen, Valencia, Calif.). For quantitative analysis of the RNA, reverse transcriptase-PCR (RT-PCR) was performed with primers and probes targeting the SIVmac239 *gag* region, designed by computer with the Primer Express software (PE Biosystems). The viral RNA was reverse transcribed and amplified using a Taqman EZ RT-PCR kit (PE Biosystems) with the designed primers (forward primer, 5′-AATGCAGAGCCCCAAGAAGAC-3′, and reverse primer, 5′-GGACCAAGGCCTAAAAAACCC-3′) and detected with a probe, FAM–5′-ACCATGTTATGGCCAAATGCCCAGAC-3′–TAMRA. Probed products were quantitatively monitored by their fluorescence intensity with ABI 7700 (PE Biosystems). For a positive-control RNA, SIVmac239 *gag* RNA was synthesized and purified using a MEGAscript kit (Ambion, Austin, Tex.) with template plasmid pKS460. This template contained the SIVmac239 *gag* sequence within the T7 promoter region. Plasma viral load, measured in duplicate, was estimated based on a standard curve of the control RNA and the RNA recovery rate (19).

Performance of colony assays on bone marrow specimens and detection of the SIVmac *gag* sequence by PCR in pooled cultured CFU-GM. Bone marrow samples (n = 20) were obtained by aspiration from the femoral or pelvic bones of monkeys. An approximately 10-ml bone marrow sample diluted with phosphate-buffered saline was slowly layered on top of 10 ml of sterile Ficoll-Hypaque in a 15-ml conical tube. The tubes were then centrifuged at 400 × *g* for 30 min at room temperature. With use of a pipette, a top plasma layer was removed, and a mononuclear cell layer was transferred in a small volume to a tube. After two washes with 2% fetal calf serum–Iscove's medium (code no. HBM-3160; Stem Cell Technologies Inc.), cell density was adjusted to 10^6 mononuclear cells/ml. The cell suspensions were then mixed with methylcellulose medium (Methocult HF4434; Stem Cell Technologies) so that it gave a final concentration of 10^5 cells per 1.1 ml for final plating. The cell culture was performed in duplicate in 35-mm-diameter plastic dishes at 37°C, 5% CO_2, and 100% humidity for 10 days, and colonies (BFU-E, CFU-GM, and CFU–granulocytes-erythroids-macrophages-megakaryocytes) were counted by inverted microscopy. CFU-GM were plucked from the methylcellulose culture and collected in pools and then subjected to PCR analysis by the method described above.

RESULTS

Figure 1 shows the relationship between the CD4/CD8 ratios of the peripheral blood T cells of infected monkeys and the postinoculation time. In general, the CD4/CD8 ratio decreased in 14 to 21 days after inoculation. It has been reported that monkeys inoculated with SHIV C2/1 had transient decreases of $CD4^+$ T lymphocytes within several days after infection (25). In this study, one monkey showed a decrease in CD4/CD8 ratio even within several hours: namely, the CD4/CD8 ratio of monkey 4345 decreased to 1.28 in 6 h after inoculation and went up to 1.80 in 24 h (Fig. 1). Control monkeys showed only negligible declines (Fig. 1).

The number of viral copies was estimated for four animals (200, 944, 520, and 844) by real-time PCR (Fig. 2). It has been

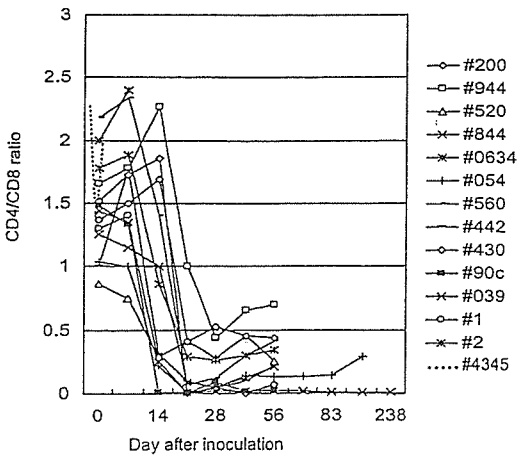

FIG. 1. Changes of CD4/CD8 ratio in monkeys inoculated with SHIV C2/1. All monkeys showed decreased CD4/CD8 ratios between day 14 and day 21 after inoculation. Monkey 4345 had a decrease in CD4/CD8 ratio in the first 24 h. Control monkeys showed only negligible declines in the first 24 h (preinoculation, 1.26 ± 0.400 [mean ± standard deviation]; 6 h, 1.24 ± 0.259; 24 h, 1.11 ± 0.323; n = 4).

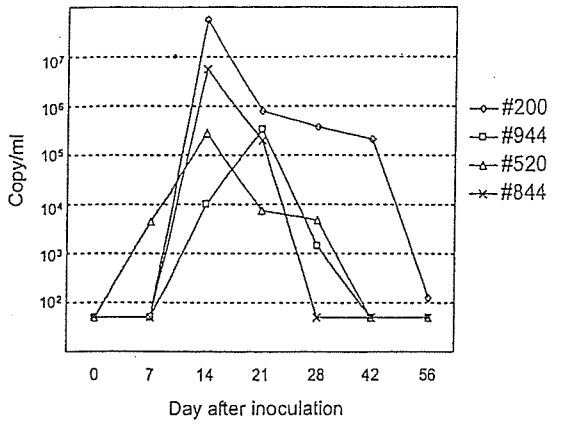

FIG. 2. Plasma viral load in the infected monkeys. Plasma viral RNA of four monkeys (200, 944, 520, and 844) was analyzed by PCR for the presence of the SIVmac239 *gag* region.

shown that the reduction of the CD4/CD8 ratio correlates with the increase of SHIV viral copies (15, 25). These four monkeys showed that the peak of viral copies occurred on the 14th day after inoculation and declined to 500 copies or less on the 56th day (Fig. 2).

We arbitrarily defined a postinoculation period of day 1 through 3 as "early stage" (covering monkeys 4345, 1, and 2 in Fig. 3) and that of day 56 or later as "advanced stage" (covering monkeys from 90c to 039 in Fig. 3). Monkey 4345 showed a remarkable reduction in the number of colonies in 24 h (Fig. 3). Monkeys 1 and 2 also had such a dramatic decline on day 3 (Fig. 3). However, nine monkeys (90c through 0634 in Table 1 and Fig. 3) maintained colony formation during the advanced stage at a level comparable to that of the control monkeys (Fig. 3). Compared with sham-inoculated controls, monkey 054 had a somewhat lower number of colonies on the 113th day. Monkey 039, which died of AIDS on day 238, showed more reduced colony formation, especially CFU-GM formation, than did monkey 054 or the sham-inoculated control monkeys (Fig. 3). At the advanced stage, no difference in the morphology or the number of colonies was noted between the noninfected and the infected monkeys (Fig. 4).

Taken together, a reduction of CD4/CD8 ratio and CFU-GM growth occurred in the early phase of the postinoculation period. However, the CFU-GM growth tended to increase following viremia while $CD4^+$ T lymphocytes continuously declined. The colony growth of the infected monkeys during the advanced stage recovered up to a level comparable to that of the control monkeys.

Infection of CFU-GM with SHIV C2/1 virus was tested by a PCR technique as described in Materials and Methods. Of the 14 cynomolgus monkeys infected with SHIV C2/1 virus, only three were positive, suggesting that the direct infection of bone marrow progenitor cells was minimal (Fig. 5). There was no positive case in the control monkey group.

DISCUSSION

Hematological abnormalities such as anemia, lymphopenia, and thrombocytopenia have been documented in a variety of retrovirus infections in both humans and experimental animals. While the precise mechanisms for such hematological abnormalities remain to be elucidated, several hypotheses have been postulated: (i) destruction of infected cells by a virus itself

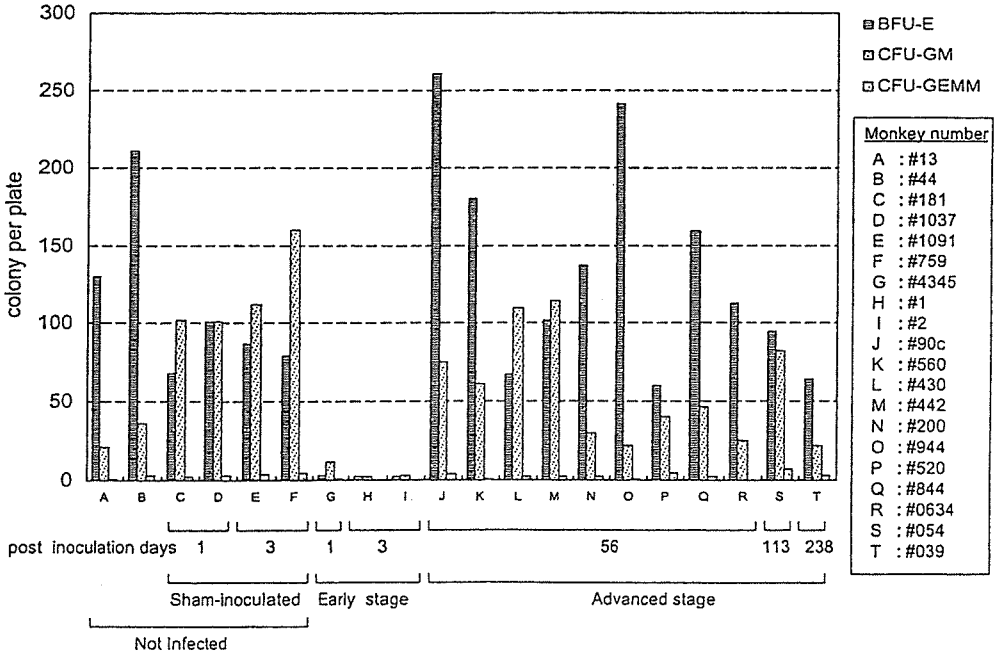

FIG. 3. Colony assay on monkeys inoculated with SHIV C2/1. A period of days 1 through 3 after inoculation was defined as early stage, whereas days 56 through 238 were defined as advanced stage. *P* was <0.005 for CFU-GM, and *P* was <0.02 for CFU-E in comparison of early stage and advanced stage and of virus-inoculated monkeys and sham-inoculated controls at days 1 and 3. *P* values were calculated according to Kruskal-Wallis analysis. There was no statistically significant difference between sham-inoculated controls and non-sham-treated controls by Mann-Whitney analysis.

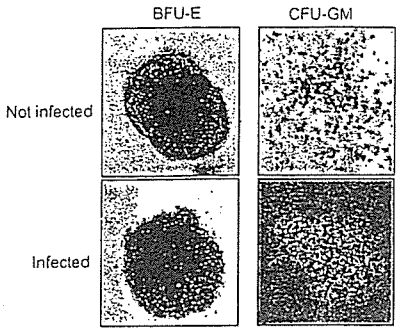

FIG. 4. Morphology of colonies produced by BFU-E and CFU-GM. Photographs of colonies cultured in nitrocellulose medium are shown at an ×75 magnification by a microscope. The left column shows BFU-E, and the right column shows CFU-GM. The upper section shows colonies from uninfected monkeys, while the lower shows colonies from monkeys infected with SHIV C2/1.

or by the antibody-dependent cell-mediated cytolytic mechanism, (ii) damage of the thymus or the lymphoid tissue, (iii) abnormal turnover of infected cells in the peripheral blood (i.e., apoptosis), and (iv) suppression of hematopoietic progenitor cells (23).

In this report, we showed that the remarkable decrease in the colony formation occurred during the early stage of infection with SHIV C2/1 (days 1 through 3 postinoculation). These results suggest that the hematopoietic progenitor cells are damaged or defective during such an early phase of infection. Furthermore, the CD4/CD8 ratio in monkey 4345 decreased within several hours, compared with controls (Fig. 1). We used ketamine for viral or saline inoculation, blood sampling, and autopsy. Ketamine has safely been used for bone marrow aspiration in humans and monkeys (21, 22, 26, 34, 35). It would be unlikely, therefore, that such bone marrow suppression occurred as a result of the anesthetic agent. However, our anesthetic procedure probably induces the release of corticosteroids in animals by the stress of capture and injection, which may have a negative impact on the colony formation. Our observation of the ability of bone marrow cultures from sham-inoculated controls to produce BFU-E and CFU-GM at days 1 and 3 proved otherwise.

After day 56, the ability of the bone marrow to form colonies recovered despite the preceding viremia and the continuing reduction of CD4/CD8 ratio. Furthermore, the colony formation was maintained at a level comparable to that of the control monkeys toward the terminal stage. Many reports have noted that CFU-GM growth continuously declines in SHIV infection, and such a decline appears to correlate with disease activity (20). However, CD4/CD8 ratio may not reflect the ongoing status of the bone marrow. A reason for the continuous reduction of CD4/CD8 ratio could be that infected T lymphocytes were destroyed in the peripheral blood more than they were produced in the bone marrow. This could be due to enhanced apoptosis or ongoing destruction of T cells by the antibody-dependent cell-mediated cytolytic mechanism in SHIV infection (24).

In contrast to previous reports, our results clearly showed that the decreasing CFU-GM growth recovered in the advanced stage, suggesting that the damage to colony formation during the early stage is reversible. We showed by PCR in this report that the direct infection of bone marrow progenitor cells with SHIV C2/1 was minimal (3, 5, 13, 18). It is possible, however, that the number of colonies was too low for detection of SHIV C2/1 virus or that SHIV C2/1 virus-infected cells were already removed by the host immune system before the assays (6, 8, 13).

It has been reported that the cellularity of the bone marrow from patients with HIV does not always correlate with the peripheral blood abnormalities (4). The commonly seen pancytopenia is often associated with hypercellular bone marrow where the increased number of lymphocytes, plasma cells, or histiocytes is seen. The latter finding suggests either dysmyelopoiesis or increased peripheral destruction of blood cells. Yoshino et al. have recently reported that atypical lymphocytes and monocytes were observed in the peripheral blood following intense viremia on the 10th to 14th days of SHIV infection (33). They further found erythroid multinucleation and atypical mononuclear cells in the hypercellular bone marrow, suggesting direct viral infection of hematopoietic progenitor cells (33).

As mentioned above, the colony formation in the bone marrow of the infected monkeys recovered spontaneously following viremia, suggesting that the reduced colony formation capability was reversible. It has been reported that inhibition of SIV replication in bone marrow macrophages resulted in increased colony growth of progenitor cells (32), and administration of recombinant human GM colony-stimulating factor could reverse leukocytopenia (11). Our data thus support the concept that, in the early phase, production of inhibitory factors or a lack or an inhibition of stimulatory cytokine production from lymphoid cells may be responsible for some of the bone marrow kinetic defects previously described in HIV (14, 28, 29). It is necessary to determine and verify what factor is participating in the regulation and recovery of the bone marrow CFU-GM growth.

The highly pathogenic SHIV C2/1 virus is an interesting tool to study the effect of HIV and SHIV infection on hematopoietic progenitor cells (15, 17, 25). Such studies will help us understand the pathophysiology of AIDS and contribute to the development of vaccines in humans (12).

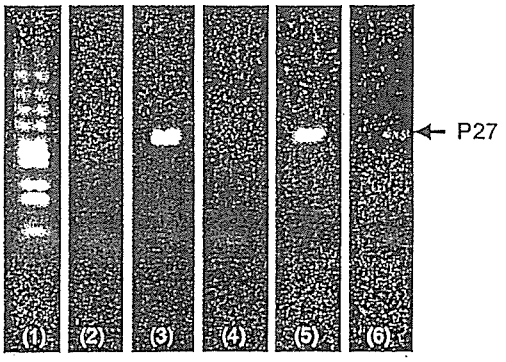

FIG. 5. Detection of SIVmac239 *gag* sequence in bone marrow colonies by PCR. Lanes: 1, a DNA ladder marker, HincII; 2, a negative control; 3, a positive control, a DNA sample from cell line M8166; 4, CFU-GM from monkey 4345 at the early stage; 5, CFU-GM from monkey 430 at the advanced stage; 6, CFU-GM from monkey 039 at the advanced stage (this monkey died of AIDS on day 238).

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A Novel Adjuvant for Mucosal Immunity to HIV-1 gp120 in Nonhuman Primates[1]

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The development of a safe and effective mucosal adjuvant is a crucial step toward a mucosal HIV/AIDS vaccine. This study seeks to determine the promise of a nontoxic mutant of cholera toxin (mCT; E112K) as a mucosal adjuvant in nonhuman primates. HIV-1 gp120 was nasally administered together with mCT E112K or native CT (nCT) as adjuvant on five to six occasions over a 6- to 8-wk period to groups of four rhesus macaques and alone to two monkeys that acted as controls. Macaques given nasal gp120 with either mCT E112K or nCT showed elevated gp120-specific IgG and IgA Ab responses with virus-neutralizing activity in both their plasma and mucosal external secretions, as well as higher numbers of gp120-specific IgA Ab-forming cells in their mucosal and peripheral lymphoid tissues and of IL-4-producing Th2-type CD4-positive ($CD4^+$) T cells than did controls. Even though significant mucosal adjuvanticity was seen with both mCT E112K and nCT, neuronal damage was observed only in the nCT-treated, but not in the control or mCT E112K-treated groups. These results clearly show that mCT E112K is an effective and safe mucosal adjuvant for the development of a nasal HIV/AIDS vaccine. *The Journal of Immunology,* **2004, 173: 6850–6857.**

It is well known that HIV-1 infections occur through contact with contaminated blood or during unprotected vaginal or anal intercourse. Indeed, it is estimated that 70–85% of HIV-1 infections are transmitted sexually (1–3). Given that fact, immune responses at mucosal surfaces in which the virus crosses the epithelium of the genital or rectal tracts are an essential component of vaccine-induced protection. The evidence for an association between mucosal immune responses and protection in humans has stemmed from studies on the immune system of women who remained seronegative despite a high rate of exposure to HIV-1. High levels of secretory IgA were detected in the genital secretions of the protected women (4–7). Because the mucosa of the small and large intestine are the largest source of lymphocytes and APCs in the host (8, 9), they act as a potential reservoir for HIV-1-infected cells in viral pathogenesis (10). Studies to develop a HIV/AIDS mucosal vaccine have been conducted in nonhuman primate (NHP)[4] models by using recombinant SIV proteins or peptides (11–17), live-attenuated SIVs (18–23), SIV-encoded virus or bacterial vectors (24–29), DNA vaccines (30–33), and a prime/boost regimen (34–36). Collectively, these studies point to the importance of a mucosal HIV/AIDS vaccine for the prevention of HIV-1 infection.

Recent studies have shown that nasal immunization is the most effective approach for the induction of both mucosal and systemic immune responses (37). For example, nasal immunization with protein/peptide vaccines together with mucosal adjuvant more effectively induces mucosal immunity in the female reproductive tract than does oral immunization (38). Like its gut-associated lymphoreticular tissue counterpart in the gastrointestinal tract, the nasopharyngeal-associated lymphoreticular tissue-based immune system is key to the induction of Ag-specific mucosal and systemic immune responses (39–41). In this regard, we have shown that nasal immunization of rhesus macaques with SIV $p55^{gag}$ together with native cholera toxin (nCT) as mucosal adjuvant induced $p55^{gag}$-specific IgA and IgG Ab responses in vaginal secretions (16).

Although a potent mucosal adjuvant, nCT is not practical for use in humans because of its toxicity. Nasal application of CT B subunit (CT-B) or nCT resulted in its accumulation in the olfactory bulbs of the CNS through GM1 binding and in its subsequent retrograde axonal transport into the olfactory neurons (39). Furthermore, nCT is known to induce high levels of total and Ag-specific IgE Ab responses due to the nature of IL-4-dependent adjuvanticity (40–43). To overcome these potent pathological problems of nCT, we have developed and characterized two nontoxic mutants of cholera toxin (mCT; E112K and S61F) that retain adjuvant properties despite lacking the ADP-ribosyltransferase enzyme activity associated with toxicity (42, 43). Studies by our own group and by others have shown that mutant CT E112K is one of the most effective, safe, and stable adjuvants among the toxin-based mutants that have been tested (41–43).

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[4] Abbreviations used in this paper: NHP, nonhuman primate; AFC, Ab-forming cell; LP, lamina propria; mCT, nontoxic mutant of cholera toxin; MLN, mesenteric lymph node; nCT, native cholera toxin; NGF, nerve growth factor; NP, nasal passage; SMG, submandibular gland.

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Because HIV-1 is most often transmitted via mucosal surfaces, a mucosal vaccine capable of inducing protective Abs and/or CTLs in mucosal tissues and external secretions would act as a first line of defense at the site of initial invasion. We take the first step toward the ultimate goal of developing a safe and effective mucosal adjuvant for a mucsoal HIV/AIDS vaccine in humans by assessing in this study the efficacy and safety of mCT E112K as a mucosal adjuvant in nonhuman primates.

Materials and Methods

HIV-1 immunogen and adjuvant used

HIV-1_{LAI} *Env* gp120 was kindly provided by Quality Biologicals (Gaithersburg, MD) through Contract N01-AI 65278 of the Vaccine Research and Development Branch, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. *Escherichia coli* strains containing the plasmids for the mCT E112K were grown in Luria-Bertani medium (10 mg/ml NaCl, 5 mg/ml yeast extract, 10 mg/ml tryptone) with 100 μg/ml ampicillin (42, 43). The mCT E112K was purified using a D-galactose-immobilized column (Pierce, Rockford, IL) from a cell suspension prepared by sonication of the recombinant *E. coli*, as described previously (42, 43). The purity of mCT E112K was assessed by SDS-PAGE, and no contaminating proteins were noted. The nCT was purchased from List Biological Laboratories (Campbell, CA).

Rhesus macaques

Five mature female and seven male rhesus macaques (*Macaca mulatta*), bred in captivity and reproductively cycling, were obtained from the California Regional Primate Research Center (Davis, CA). They were confirmed negative for Abs to HIV-2, SIV, type D retrovirus, and simian T cell lymphotropic virus-1 (STLV-1), and were maintained in conditions that fully complied with the standards of the American Association of Accreditation of Laboratory Animal Care at the California Regional Primate Research Center.

Immunization methods and schedule used

Rhesus macaques were divided into four groups and nasally immunized with vaccine containing: 1) 100 μg of gp120 alone, 2) 100 μg of gp120 plus 10 μg of nCT, 3) 100 μg of gp120 plus 25 μg of mCT E112K, or 4) 100 μg of gp120 plus 100 μg of mCT E112K. Macaques were anesthetized with ketamine and placed in dorsal recumbancy with head tilted back so that the nares were pointed upward (16). Vaccine solution (0.5 ml) was instilled dropwise into each nostril without inserting the syringe into the nasal cavity. Macaques were kept in that position for 10 min and then placed in lateral recumbancy until they recovered from anesthesia, as described previously (16). Nasal immunization was conducted on days 0, 7, 14, 28, 42, and 56.

Collection of peripheral blood, tissues, and external secretion samples and lymphocyte isolation

Tissues and peripheral blood were harvested using sterile techniques, and appropriate biohazard precautions were observed. The PBMCs were isolated from heparinized peripheral blood using Lymphocyte-Mammal (Cedarlane Laboratories, Hornby, Canada) (44). Plasma, vaginal washes consisting of a mixture of cervical and vaginal secretions, rectal washes, nasal washes, and saliva were collected, as previously described (16). These four external secretions along with the plasma were stored at −80°C until used for the analysis of gp120-specific Ab responses. For isolation of lymphocytes from different mucosal tissues, a modified enzymatic dissociation procedure was used (15, 16). Nasal passages (NP) and submandibular glands (SMG) were dissociated using collagenase type IV (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO) in RPMI 1640 (Mediatech, Washington, DC) for 30 min at 37°C. After removal of Peyer's patches, the small intestine was treated first with PBS containing 1 mM DTT and then with 1 mM EDTA, while lamina propria (LP) mononuclear cells were isolated using the same method as for the NPs. The lymphocytes from tissues were purified using a discontinuous 40 and 75% Percoll gradient (Amersham Biosciences, Piscataway, NJ), as described previously (15, 16).

Monoclonal Abs

The mAbs used for cell surface staining in flow cytometric analysis were as follows: FITC-, PE-, or PerCP-conjugated mAb to human CD3 (SP34; BD Biosciences, San Jose, CA), CD4 (SK3; BD Biosciences), and CD8 (SK1; BD Biosciences). Cross-reactivity of these mAbs for the rhesus macaque was determined using the method described previously (45). However, the observed cross-reactivity with IL-5, IL-10, and IL-13 is a new finding and has not been published previously.

HIV-1 env *gp120-specific ELISA and ELISPOT assays*

HIV-1 *env* gp120-specific IgG, IgM, and IgA Ab titers in plasma, saliva, nasal washes, as well as rectal and vaginal lavages were determined by ELISA, as described previously (15, 16). The HIV-1 *env* gp120-specific IgG, IgM, and IgA Ab-forming cells (AFCs) were also determined by ELISPOT assay, as described elsewhere (15, 16).

Cytokine-specific ELISPOT assay

The PBMCs or lymphoid cells from various tissues were cultured in 10% FCS containing RPMI 1640 (Mediatech) supplemented with HEPES buffer (10 mM), L-glutamine (2 mM), nonessential amino acid solution (10 ml/L), sodium pyruvate (10 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (80 μg/ml) (complete medium) with or without 5 μg/ml HIV-1 *env* gp120, 1 μg/ml anti-human CD28 (CD28.2; BD Biosciences), and anti-human CD49d (9F10; BD Biosciences) mAbs at 37°C with 5% CO_2. Nonadherent cells were harvested after 3 days of incubation and stained with anti-human CD3 and CD8 mAbs. The FACSVantage (BD Biosciences) was used to sort out a subset of $CD3^+CD8^-$ T cells. The frequencies of $CD4^+$ Th1- and Th2-type cytokine-producing cells were determined by using rhesus macaque cytokine-specific ELISPOT kits (U-CyTech, Utrecht, The Netherlands).

In vitro HIV-1 neutralization assay

The diluted plasma or appropriate mucosal secretion was heat inactivated (56°C for 30 min) and incubated with 20 $TCID_{50}$ (50% tissue culture infective dose) units of HIV-1_{LAI} overnight at 4°C. This mixture was then cocultured with 1×10^6 M8166 cells for 2 h (16, 46, 47). After being washed twice with PBS, the cells were cultured in complete medium for 4 days at 37°C. Following incubation, culture supernatants were subjected to Lumipulse (chemiluminescence enzyme immunoassay/full automatic analyzer; Fujirebio, Tokyo, Japan) for measurement of HIV p24. The results were expressed as the percent inhibition of p24 *gag* production in culture supernatants when compared with the cultures containing pre- or nonimmunized plasma or mucosal secretions (16, 46, 47).

Nerve growth factor-β1 (NGF-β1) production in macaque olfactory tissues

The nasal turbinate region of the olfactory tissues was obtained from each macaque at the time of sacrifice. At the termination of the study, the nasal turbinate was perfused with PBS at 25°C. This was followed by perfusion with 100 ml of Zamboni's fixative (4% paraformaldehyde, 15% picric acid) in 0.1 M phosphate buffer. The olfactory bulbs and tubinates were removed and placed in fresh 4% paraformaldehyde at 4°C overnight. The tissue was then transferred to a 30% sucrose solution at 4°C for 48 h to cryoprotect it before sectioning. The tissue was then frozen in OCT compound, and the frozen sections (6 μm) were placed on precoated microscope slides (10% BSA in saline). For staining of sections, all slides were pretreated with rabbit IgG Ab to block nonspecific binding, followed by a biotinylated rabbit anti-human NGF-β1 Ab (Chemicon International, Temecula, CA) used at a concentration of 2 μg. The Ab-stained sections were incubated at 4°C overnight. The slides were then rinsed in three changes of PBS for 2 min and then reacted with avidin-biotin conjugate for 30 min at 25°C. The tissues were rinsed three times with PBS, and then reacted with 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA) for 5–10 min before being again rinsed three times and having sections counterstained with hematoxylin for 30 s. After being washed in distilled water, the slides were dehydrated in 100% alcohol and xylene. In some experiments, the anti-NGF-β1 Ab-stained sections were incubated with HRP-conjugated streptavidin-Alexa Fluor 488 (Molecular Probes, Eugene, OR). Sections were examined with a fluorescence microscope (BX50/BXFLA; Olympus, Tokyo, Japan) equipped with a digital image capture system (Olympus).

Statistics

The results are expressed as the mean ± SEM. Immunized NHP groups were compared with the controls using a Mann-Whitney *U* test with Statview II software (Abacus Concepts, Berkeley, CA) designed for Macintosh computers. A p value of <0.05 or less was considered significant.

Results

Plasma anti-gp120-specific Ab responses

In this study, we have assessed the mucosal adjuvanticity of mCT E112K in rhesus macaques nasally immunized with HIV-1 gp120. Eleven macaques were given 100 μg of gp120 by the nasal route. In addition to the gp120, five macaques were given two doses of mCT E112K as nasal adjuvant, two (Rh09 and Rh91) receiving a 25 μg dose and three (NHPs Rh16, Rh39, and Rh85) receiving a 100 μg dose. As a positive control, and because our previous research showed that nCT is a potent nasal adjuvant for NHPs (16), four other macaques (Rh07, Rh35, Rh60, and Rh88) were given 10 μg of nCT along with gp120. As a negative control, the two remaining macaques (Rh43 and Rh51) were given gp120 alone. The gp120-specific IgG and IgA Abs in plasma of individual macaques were sequentially assessed by an endpoint ELISA. As expected based upon our previous studies (16), significant levels of gp120-specific IgG Ab responses were detected in plasma of all macaques given gp120 with nCT (Fig. 1; $p < 0.01$). Interestingly, comparable gp120-specific IgG Ab responses were observed in macaques receiving 100 μg of mCT E112K as nasal adjuvant (Fig. 1; $p < 0.01$), while much lower levels of these responses were noted in macaques receiving 25 μg of mCT E112K as nasal adjuvant ($p > 0.1$). Furthermore, the group receiving 100 μg of mCT E112K showed comparable gp120-specific plasma IgA Ab responses to those receiving nCT as mucosal adjuvant. In contrast, the two macaques given gp120 alone or those receiving only 25 μg of mCT E112K showed low to undetectable IgA Ab responses. When gp120-specific plasma Ab responses were compared between the two groups given 25 or 100 μg of mCT E112K groups, the group given the higher dose showed greater IgG ($p < 0.01$) and IgA Ab responses than did the group given 25 μg of mCT E112K. Taken together, these results show that 100 μg of mCT E112K is an appropriate dose for inducing HIV-1 gp120-specific plasma Ab responses.

Induction of gp120-specific mucosal immune responses

The gp120-specific IgA and IgG Ab titers were assessed in the mucosal secretions (saliva; nasal, vaginal, and rectal lavages) of macaques given nasal gp120 and mCT. The peak titers of IgG and IgA Abs occurred 7 or 14 days after the last nasal immunization

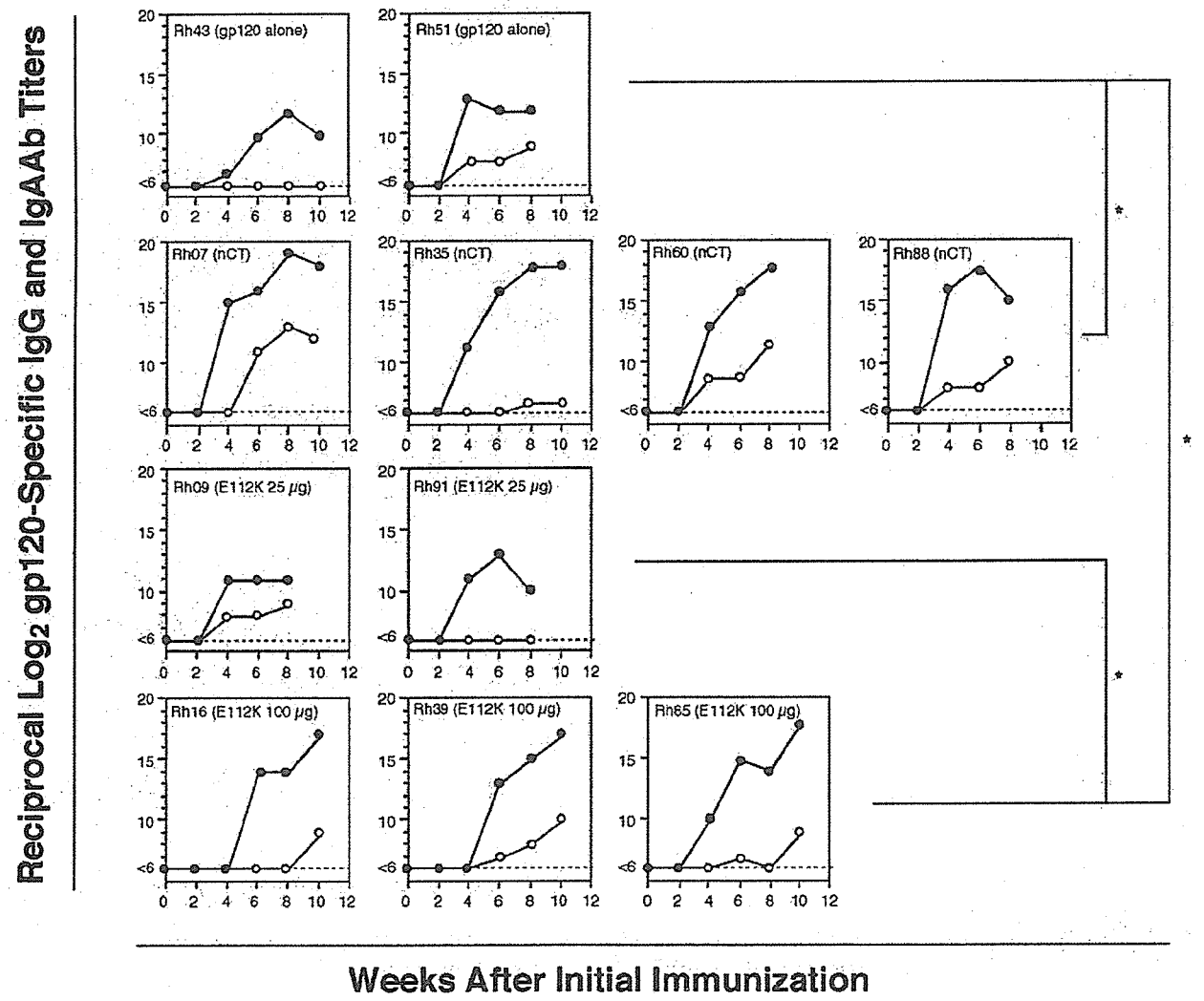

FIGURE 1. HIV-1 gp120-specific plasma IgG (●) and IgA (○) Ab titers were determined by endpoint ELISA. Rhesus macaques were nasally immunized with 100 μg of gp120 alone (Rh43 and Rh51), 100 μg of gp120 and 10 μg of nCT (Rh07, Rh35, Rh60, and Rh88), 100 μg of gp120 and 25 μg of mCT E112K (Rh09 and Rh91), or 100 μg of gp120 and 100 μg of mCT E112K (Rh16, Rh39, and Rh85). The data shown are endpoint titers for each macaque. *, $p < 0.01$ for IgG titers.

Table I. *gp120-specific Ab responses in mucosal secretions of rhesus macaques given a nasal vaccine*

Nasally Immunized with		Identification Number of Macaque	Anti-gp120-Specific Reciprocal Log_2 Ab Titers[a]							
			Saliva		Nasal washes		Vaginal washes		Rectal washes	
gp120	Adjuvant		IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
100 (μg)	E112K (25 μg)	Rh09	<1[b]	2	NA[c]	NA	<1	3	NA	NA
		Rh91	2	2	NA	NA	<1	<1	NA	NA
	E112K (100 μg)	Rh16	6	5	5	3	NA	NA	3	<1
		Rh39	8	7	5	4	NA	NA	2	3
		Rh85	4	6	4	<1	NA	NA	3	<1
100 (μg)	nCT (10 μg)	Rh60	6	6	NA	NA	2	6	NA	NA
		Rh88	2	<1	NA	NA	<1	4	NA	NA
		Rh07	7	7	7	4	NA	NA	3	<1
		Rh35	5	5	2	<1	NA	NA	<1	<1
100 (μg)	None	Rh51	<1	<1	NA	NA	<1	2	NA	NA
		Rh43	3	2	<1	<1	NA	NA	<1	<1

[a] Saliva, nasal, vaginal, and rectal washes were collected 7 or 14 days after final immunization and were then subjected to gp120-specific ELISA.
[b] Endpoint titers were expressed as the last dilution giving an OD_{460} of 0.1 U above samples obtained from nonimmunized controls.
[c] NA, Not available.

(Table I). The findings for mucosal secretions paralleled those for plasma described above, with a dose of 100 μg of mCT E112K inducing gp120-specific IgA and IgG Ab levels comparable to those seen in macaques receiving nCT, but with a dose of only 25 μg of mCT E112K failing to support induction of gp120-specific Ab responses (Table I). These findings further support the notion that 100 μg of mCT E112K is the optimal dose for nasal adjuvanticity. Furthermore, our results demonstrate that a nasal vaccine of HIV-1 gp120 and mCT E112K as mucosal adjuvant would be an effective regimen for induction of anti-HIV-1 immune responses in external secretions of NHPs.

Induction of gp120-specific AFCs in mucosal lymphoid tissues

The induction of gp120-specific Ab responses was further confirmed at the level of plasma cell AFC responses. Comparable numbers of HIV-1 gp120-specific IgA and IgG AFCs were seen in the nasal passages of macaques immunized with gp120 plus either the optimal dose of mCT E112K (Rh39) or nCT (Rh60). Similarly, the numbers of gp120-specific IgA AFCs in SMGs and intestinal LP of macaques given the optimal dose of mCT E112K were comparable to those seen in positive controls given nCT as mucosal adjuvant (Fig. 2). These findings show that nasally coadministered mCT possesses adjuvant activity for the induction of gp120-specific AFCs in mucosal effector tissues.

gp120-specific $CD4^+$ Th1 and Th2 cell responses

Because nasal mCT showed adjuvant activity in both mucosal and systemic lymphoid compartments, HIV-1 gp120-specific $CD4^+$ Th1- and Th2-type responses were assessed using a cytokine-specific ELISPOT assay. When restimulated with gp120 in vitro, mononuclear cells from spleen and mesenteric lymph nodes (MLNs) of macaques immunized with gp120 and either mCT E112K or nCT induced both Th1 (IFN-γ)- and Th2-type (IL-4, IL-10, and IL-13) cytokine-producing $CD4^+$ T cells (Fig. 3). Both the group given mCT E112K and that given nCT showed higher numbers of IL-4- and IL-13-producing $CD4^+$ T cells in MLNs than those observed in the two macaques nasally immunized with gp120 alone. The nCT-immunized group exhibited higher numbers of IL-4- and IL-13-producing $CD4^+$ T cells than did the mCT E112K-immunized macaques, but the latter group showed higher numbers of IL-10-producing $CD4^+$ T cells were noted in their MLNs. A similar pattern of Th2-type cytokine production was seen in the spleens of these two groups of macaques. The IFN-γ-producing $CD4^+$ T cells were also seen in both MLNs and spleens

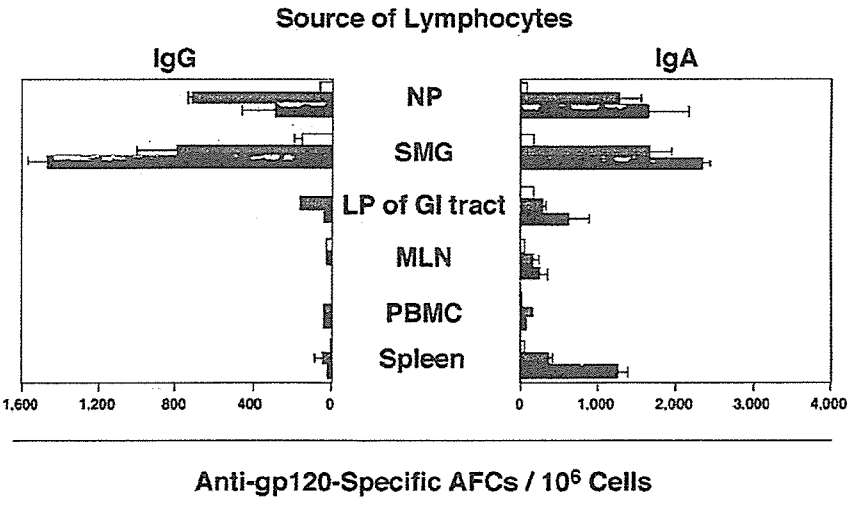

FIGURE 2. The gp120-specific IgG and IgA AFCs in mucosal and systemic lymphoid tissues of rhesus macaques (Rh51, □; Rh60, ▨; Rh39, ■) were determined by ELISPOT assay. Mononuclear cells were isolated from NPs, SMGs, the LP of the gastrointestinal tract; MLNs; spleen; and PBMC 2 wk following the final immunization. The results shown are the mean AFCs/10^6 cells ± SEM.

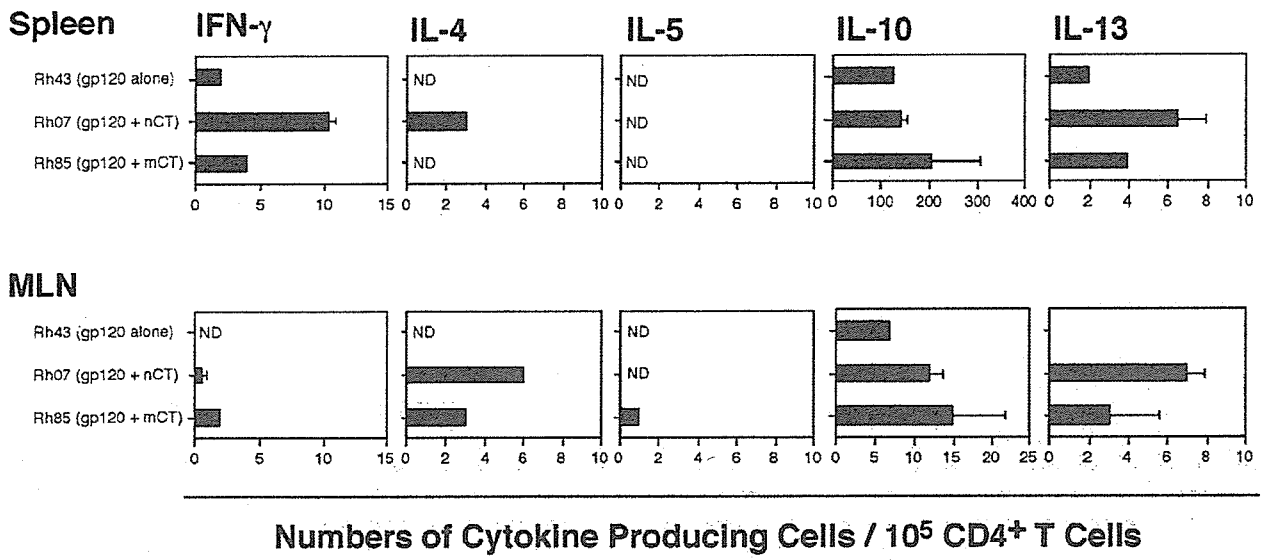

FIGURE 3. Th1 and Th2 cytokine production by gp120-stimulated $CD4^+$ T cells isolated from spleens and MLNs of rhesus macaques (Rh43, Rh07, and Rh85). Lymphocytes were cultured with or without gp120 for 3 days. Nonadherent cells were harvested and stained with anti-human CD3 and CD8 mAbs. A subset of $CD3^+$, $CD8^-$ T cells was purified using flow cytometry. The purified $CD4^+$ T cells were subjected to an array of macaque-specific cytokine (IFN-γ, IL-4, IL-5, IL-10, and IL-13) ELISPOT assays.

of macaques given either mCT E112K or nCT as mucosal adjuvant. Interestingly, the numbers of IFN-γ-producing $CD4^+$ T cells in the MLNs of both groups were lower than those seen in the spleens. These results suggest that mCT as nasal adjuvant preferentially induces Ag-specific Th2-type cytokine-producing $CD4^+$ T cells, while also somewhat enhancing the induction of Th1-type cytokine-producing $CD4^+$ T cells.

$HIV\text{-}1_{LAI}$-neutralizing Abs in external secretions and plasma

It was important to examine whether gp120-specific Abs in external secretions or plasma induced in NHPs given nasal gp120 and mCT E112K as mucosal adjuvant possessed HIV-neutralizing activity. To assess neutralizing activity, we performed an in vitro neutralization assay using $HIV\text{-}1_{LAI}$. The plasma (1/10 dilution) from macaques given nasal gp120 plus mCT E112K showed ~75–90% inhibition of $HIV\text{-}1_{LAI}$, a significantly higher rate than that seen in control plasma samples from either naive macaques or NHPs given gp120 only (Fig. 4*A*). Furthermore, the nasal lavages (1/10 dilution) from two rhesus macaques (Rh16 and Rh85) given nasal gp120 plus mCT E112K exhibited 35 and 55% inhibition of $HIV\text{-}1_{LAI}$, a rate of inhibition comparable to that seen in NHPs given nasal nCT as mucosal adjuvant. In contrast, control groups (naive macaques or those given gp120 alone) possessed little ability to inhibit $HIV\text{-}1_{LAI}$ (<20%) (Fig. 4*B*). These results clearly show that nontoxic mCT E112K can be used as a mucosal adjuvant for the induction of HIV-1-specific neutralizing immunity in both external secretions and plasma.

Safety of mCT E112K when used as a nasal adjuvant in NHPs

To assess the threat of neuronal damage posed by nasal vaccines containing gp120 and mCT E112K, NGF-β1 production in nasal turbinates of olfactory tissues was examined. Macaques given gp120 with nCT exhibited areas of intense NGF-β1 production in the olfactory region, which was associated with neuronal damage

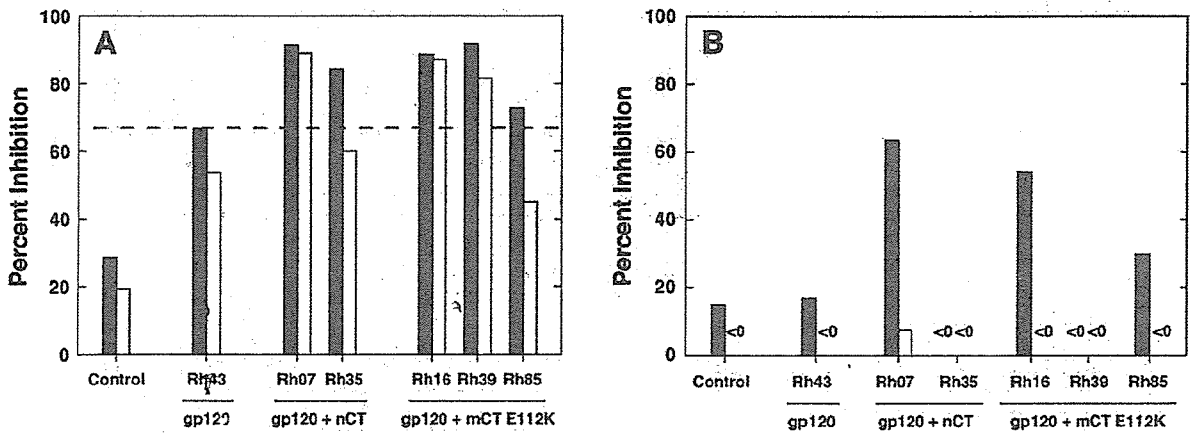

FIGURE 4. In vitro neutralization of HIV-1 was performed with a standard p24 release assay. The plasma (*A*) and nasal wash (*B*) samples were collected 2 wk after the final immunization. Samples were diluted 1/10 (■) or 1/100 (□) and were analyzed for the presence of neutralizing Abs against a homogenous laboratory strain ($HIV\text{-}1_{LAI}$). As controls, plasma samples were obtained from macaques before nasal immunization (preimmune sample). The results are the mean values of three separate assays.

and inhibition of apoptosis (Fig. 5*C*). In contrast, macaques given nasal gp120 plus mCT E112K (Fig. 5*B*) expressed very minimal levels of NGF-β1, which were essentially the same as those seen in olfactory tissues taken from the macaques given nasal gp120 alone (Fig. 5*A*). These results indicate that mCT E112K, although as effective a mucosal adjuvant as nCT, possesses none of its toxicity for neuronal tissues. As a safe and potent mucosal adjuvant, mCT E112K could speed the development of a nasal HIV-1 vaccine in humans.

Discussion

This study clearly provides direct evidence that mCT E112K is an effective mucosal adjuvant for the induction of HIV-1-specific immunity in the NHP model. When used as a nasal adjuvant, mCT E112K induced gp120-specific Abs possessing HIV-neutralizing activity in both external secretions and plasma, but showed negligible toxicity for the CNS-associated tissues of rhesus macaques. In contrast, nCT elicited increases in NGF-β production, a major manifestation of CNS inflammation. Thus, our study is the first to provide evidence establishing the efficacy and safety of an adjuvant for use in higher mammals. Collectively, our findings convincingly demonstrate the potential of mCT E112K as a mucosal adjuvant in humans and suggest that it may be time to take the next step toward the development of nasal vaccines, including those for HIV-1, by beginning clinical trials.

Our previous studies have already shown the efficacy and safety of mCT E112K as a nasal adjuvant in the murine system (43, 47, 48). In our earlier studies, we established that nasal immunization with pneumococcal surface protein A or diphtheria toxoid plus mCT E112K elicited sufficient Ag-specific immune responses to provide protection after lethal challenge with either *Streptococcus pneumoniae* bacteria or diphtheria exotoxin (48, 49). Furthermore, nasal application of mCT together with protein Ags elicited both Ag-specific IgA and IgG Ab responses in mucosal and systemic lymphoid tissue compartments (43, 47, 48). Among the different forms of mutant toxin-based adjuvants, mCT E112K was shown to be the safest and most effective in the murine model (41–43). However, until now, no studies assessing the mucosal adjuvanticity of different forms of toxin-based mutant adjuvants such as our mCT E112K had been performed in a large mammalian animal model, i.e., NHPs. Among the mammalian models, we chose the NHP experimental model as the most appropriate to and useful for the development of an HIV/AIDS mucosal vaccine.

AIDS is well known to be a sexually transmitted disease caused by HIV-1 infection via mucosal surfaces. The NHP experimental model of SIV infection has provided detailed evidence for the mucosal transmission of the virus, and has shown that the inhibition of its entry via the mucosa led to protection against disease development (50). Accordingly, an effective HIV/AIDS vaccine will be more readily developed if the potential of the common mucosal immune system is tapped, because mucosal immunization is known to induce effective protection against pathogens at mucosal surfaces as well as in lymphoid tissue compartments (37, 38, 51). Of note, our previous study showed that nasal immunization with SIV $p55^{gag}$ plus nCT as mucosal adjuvant induced in vaginal secretions of rhesus macaques Ag-specific Ab responses with virus-specific neutralizing Ab activity. In the case of the NHP experimental model, our studies have shown that mucosal (both oral or nasal) immunization with SIV $p55^{gag}$ plus nCT induced Ag-specific humoral and cellular immunity in both mucosal and systemic immune systems of rhesus macaques (15, 16, 52).

Despite its strong mucosal adjuvanticity, nCT is of little practical value as a mucosal adjuvant in humans because of its toxicity. Thus, much effort has been expended on the creation of genetically manipulated nontoxic mutants of CT that would retain adjuvanticity, but not toxicity. In the current study, we sought to examine the mucosal adjuvanticity of mCT E112K as nasal adjuvant when coadministered to rhesus macaques with HIV-1 gp120. In this study, we provide the first evidence that the nasal application of mCT E112K as a mucosal adjuvant effectively induces HIV-1 gp120-specific Ab responses in both mucosal and systemic lymphoid tissues of rhesus macaques. Furthermore, plasma and nasal

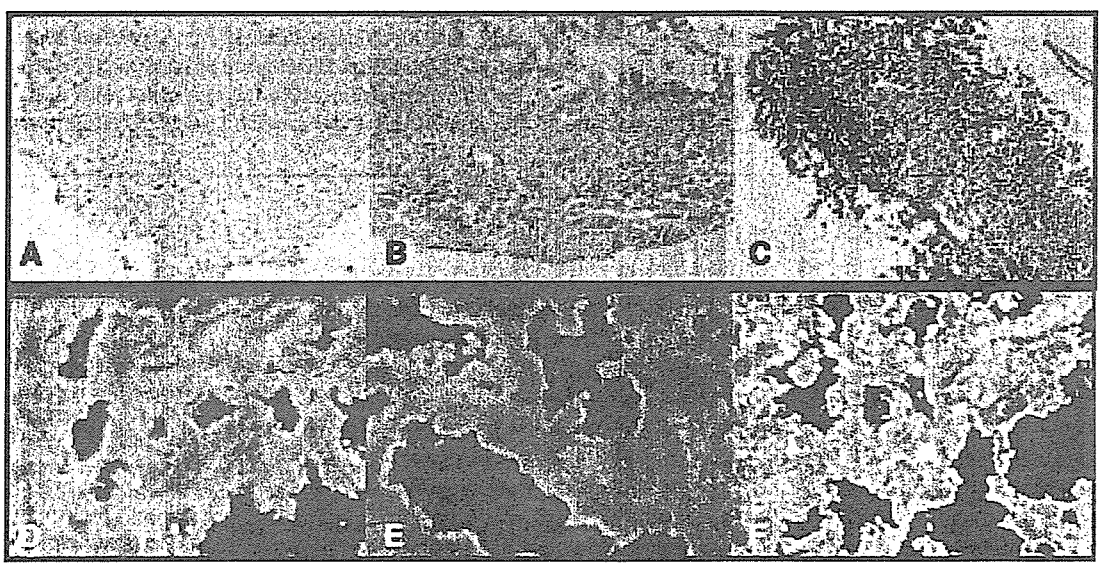

FIGURE 5. Detection of NGF-β1 expression in olfactory bulbs of rhesus macaques nasally immunized with gp120 and either nCT or mCT E112K as mucosal adjuvant. The anti-NGF-β1 Ab-stained sections were reacted with avidin-biotin conjugate, followed by 3,3′-diaminobenzidine (*A–C*), or incubated with HRP-conjugated streptavidin-Alexa Fluor 488 (*D–F*). *C* (×40) and *F* (×100), Show high expression of NGF-β1 along neuronal tracts when rhesus macaques were given nCT and gp120. *B* (×40) and *D* (×100), Show tissues from a macaque given gp120 plus mCT E112K. *A* (×40) and *D* (×100), Illustrate tissues from a macaque given gp120 alone.

washes from macaques given nasal gp120 plus mCT E112K contained HIV-1_{LAI}-neutralizing Abs. These findings clearly demonstrate the efficacy of mCT E112K as a mucosal adjuvant and suggest its potential for use in trial vaccines in humans.

However, nCT and even some of its nontoxic mutant forms pose additional, more specialized dangers when administered via the nasal route, the route of choice for mucosal vaccines because of its efficacy at inducing Ag-specific immune responses. Nasal vaccines using either nCT or one of its nontoxic mutants as adjuvant risk entering the CNS because of the proximity of the olfactory nerves/epithelium and olfactory bulbs to the brain. This potential for neurotoxicity has been a major obstacle for the use of enterotoxin-based mucosal adjuvants, even nontoxic mutant forms, in humans via the nasal route.

Our own studies have shown the potential toxicity of nCT for the olfactory nerves/epithelium and olfactory bulbs (39). Thus, neuronal association of CT-B through GM1 ganglioside binding appears to preclude efficient clearing of these enterotoxin-based mucosal adjuvants and to cause extended accumulation of them in neuronal tissues associated with the olfactory tract (39). These results show that nasally administered CT derivatives retain some toxicity and are targeted to the CNS, posing a serious obstacle to human use. Indeed, recent reports showed that a human vaccine containing inactivated influenza and native labile toxin as an adjuvant resulted in a very high incidence of Bell's palsy (53, 54). These results strongly indicate that it is essential to develop a more safe and effective nasal vaccine for human use.

Our current findings demonstrate the promise of the nontoxic form of mCT E112K as a safe and effective mucosal adjuvant and so point the way to the development of better nasal vaccines. The nontoxic form of mCT E112K did not elicit any increase in NGF-β expression by the olfactory tissues of NHPs. Only minimal NGF-β1 synthesis, comparable to that seen in NHP given nasal gp120 alone, was detected in the olfactory CNS tissues of rhesus macaques given nasal mCT E112K as nasal adjuvant.

Our previous study showed that nasal immunization with $p55^{gag}$ plus nCT induced $p55^{gag}$-specific T cell responses in both mucosal and systemic lymphoid tissue compartments (16). Thus, it was shown that both IFN-γ and IL-2 (Th1-type) expression as well as IL-5, IL-6, and IL-10 (Th2-type) production were seen in Ag-stimulated $CD4^+$ T cells isolated from NHPs given nasal $p55^{gag}$ and nCT. In this regard, our current study has shown that both Th1 (IFN-γ)- and Th2 (IL-10 and IL-13)-type cytokine-producing $CD4^+$ T cells were present in the MLNs and spleens of rhesus macaques given either mCT E112K or nCT as a nasal adjuvant. Although the viral Ags used in the current study are different from those in the previous report, our results also showed that mCT E112K provided adjuvanticity in NHPs through the generation of both Th1- and Th2-type cytokine responses by $CD4^+$ T cells. Induction of IFN-γ-producing $CD4^+$ T cells by nasally coadministered mCT E112K may be an additional benefit because it may lead to the generation of Ag-specific cell-mediated immunity responses. In viral infections including HIV and SIV, CTL activity has been shown to be of central importance for host defense and to correlate well with IFN-γ production (44). In this regard, we postulate that nasally coadministered mCT E112K would also induce CTL activity in various mucosal tissues. Confirming this prediction, rhesus macaques given nasal nCT as mucosal adjuvant showed SIV-specific CTL activity (16). We are currently testing Ag-specific CTL activity in macaques given nasal mCT E112K as mucosal adjuvant.

In conclusion, the current study has provided significant new information for a potential human phase I clinical trial using the nontoxic form of toxin mucosal adjuvant mCT E112K. Thus, nasal immunization of rhesus macaques with gp120 and mCT E112K resulted in the induction of Ab-neutralizing immunity against HIV-1 by inducing gp120-specific IgA and IgG Abs in both mucosal and systemic lymphoid tissue compartments, respectively. Furthermore, the safety of nasal mCT E112K was confirmed by the lack of CNS damage in this NHP model. This important new evidence supports the candidacy of mCT E112K as a potentially important mucosal adjuvant for use in humans.

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Vaccination of Rhesus Macaques with Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin Env V3 Elicits Neutralizing Antibody-Mediated Protection against Simian-Human Immunodeficiency Virus with a Homologous but Not a Heterologous V3 Motif

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Although the correlates of vaccine-induced protection against human immunodeficiency virus type 1 (HIV-1) are not fully known, it is presumed that neutralizing antibodies (NAb) play a role in controlling virus infection. In this study, we examined immune responses elicited in rhesus macaques following vaccination with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin expressing an HIV-1 Env V3 antigen (rBCG Env V3). We also determined the effect of vaccination on protection against challenge with either a simian-human immunodeficiency virus (SHIV-MN) or a highly pathogenic SHIV strain (SHIV-89.6PD). Immunization with rBCG Env V3 elicited significant levels of NAb for the 24 weeks tested that were predominantly HIV-1 type specific. Sera from the immunized macaques neutralized primary HIV-1 isolates in vitro, including HIV-$1_{BZ167/X4}$, HIV-$1_{SF2/X4}$, HIV-$1_{CI2/X4}$, and, to a lesser extent, HIV-$1_{MNp/X4}$, all of which contain a V3 sequence homologous to that of rBCG Env V3. In contrast, neutralization was not observed against HIV-$1_{SF33/X4}$, which has a heterologous V3 sequence, nor was it found against primary HIV-1 R5 isolates from either clade A or B. Furthermore, the viral load in the vaccinated macaques was significantly reduced following low-dose challenge with SHIV-MN, and early plasma viremia was markedly decreased after high-dose SHIV-MN challenge. In contrast, replication of pathogenic SHIV-89.6PD was not affected by vaccination in any of the macaques. Thus, we have shown that immunization with an rBCG Env V3 vaccine elicits a strong, type-specific V3 NAb response in rhesus macaques. While this response was not sufficient to provide protection against a pathogenic SHIV challenge, it was able to significantly reduce the viral load in macaques following challenge with a nonpathogenic SHIV. These observations suggest that rBCG vectors have the potential to deliver an appropriate virus immunogen for desirable immune elicitations.

Development of a preventive vaccine against human immunodeficiency virus type 1 (HIV-1) is urgently needed to control the spread of the virus worldwide. Although the immunological parameters that correlate with protective immunity against natural infection with HIV-1 are not fully known, it is assumed that a preventive vaccine must elicit potent, broadly reactive immunity against divergent strains of HIV-1 (25, 36, 42). Several recent studies have demonstrated that induction of virus-specific T-cell responses can confer protective immunity in nonhuman primate models, and these responses may also play a role in controlling HIV-1 replication in humans (6, 18, 19, 31, 33, 34, 38, 45, 48). Vaccine constructs containing viral *env* genes, in addition to *gag* and *pol*, have been shown to effectively control replication of challenge viruses (2, 5, 10), suggesting that neutralizing antibody (NAb) responses might also contribute to protection against pathogenic infection or disease progression. Passive transfer of serum immunoglobulin from chimpanzees experimentally infected with several different HIV-1 isolates has been shown to block the establishment of a simian immunodeficiency virus (SIV)-HIV chimeric simian-human immunodeficiency virus (SHIV) infection in pig-tailed macaques (37, 46). It is not known, however, whether vaccines that actively elicit a potent NAb response can provide protection in nonhuman primates challenged with SHIV.

Previously, we demonstrated that recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (rBCG), which secretes a chimeric protein consisting of the V3-neutralizing epitope of HIV-1 and α-antigen (rBCG Env V3), can induce HIV-1-specific NAb in a small-animal model (9, 15, 16). BCG was selected as a vaccine vehicle because it has several characteristics that are considered efficacious for developing a candidate

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HIV-1 vaccine (1, 49), including the ability to induce long-lasting immune responses (7). It is generally accepted that a candidate vaccine against HIV-1 must also be easily administered and affordable in developing countries, and it must be compatible with other commonly administered vaccines (35). If effective, a BCG-based recombinant HIV-1 (rBCG–HIV-1) vaccine would fulfill many of these critical requirements.

Results using other vaccine modalities, in particular, live attenuated SIV vaccines, have raised concerns about the potential for reversion to pathogenicity (3, 4), suggesting that many SIV strains may be potentially virulent. In this study, we used two distinct strains of challenge virus: SHIV-MN (29), which contains V3 sequences homologous to rBCG Env V3, and SHIV-89.6PD (12, 20, 28, 41), which is heterologous in the V3 region and highly pathogenic. We examined whether vaccination with rBCG Env V3 could effectively elicit NAb responses in rhesus macaques and whether it might effectively induce protective immunity against challenge with either SHIV-MN or SHIV-89.6PD.

MATERIALS AND METHODS

Animals. The macaques (*Macaca mulatta*) used in this study originated from China and were purchased through Japan SLC Ltd., Shizuoka, Japan. The animals were maintained according to standard operating procedures established for the evaluation of human vaccines at the Tsukuba Primate Center, National Institute of Infectious Diseases, Tsukuba, Ibaragi, Japan. The study was conducted in the P3 facility for monkeys in the Murayama Branch, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan, and in accordance with requirements specified in the laboratory biosafety manual of the World Health Organization.

Construction of the rBCG Env V3 immunogen. rBCG substrain Tokyo was produced by transfection of BCG-Tokyo 172 cells with plasmid pSO246 as described previously (21, 22, 30). The XhoI site of this plasmid was used to insert a mycobacterial codon-optimized DNA fragment encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence (NTRKSIHIGPGRAFYATGS), which has a neutralization sequence identical to that of HIV-1_{MN} (16, 23, 39, 52). The resulting rBCG vector was designated rBCG Env V3. By semiquantitation of a chimeric protein consisting of the V3 peptides and α-K protein (9), the concentration of the secreted protein was estimated to range from 1 to 3 μg/ml in the culture filtrate of rBCG Env V3 (data not shown).

Viruses. Viruses used in challenge experiments were kindly provided by Y. Lu, Harvard AIDS Institute, Cambridge, Mass. The SHIV-MN virus stock was prepared in concanavalin A-activated macaque peripheral blood mononuclear cells (PBMC) from normal animals, and the amount of virus was quantified by SIV p27 antigen enzyme-linked immunosorbent assay (ELISA) (Coulter Co., Hialeah, Fla.). The tissue culture infective dose (TCID) of the stock was measured on CEMx174 cells (AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, Md.). Stocks of HIV-1_{MN} and HIV-1_{IIIB} (AIDS Research and Reference Reagent Program) were prepared by propagating 100 50% TCID ($TCID_{50}$) of each virus in phytohemagglutinin-activated normal human PBMC, as described previously (17). The primary isolate, HIV-1_{MNp}, was kindly provided by J. Sullivan, University of Massachusetts Medical School, Worcester, Mass. All other viruses were obtained from the AIDS Research and Reference Reagent Program. Cell-free virus stocks were stored at −130°C until they were used.

V3-specific ELISA. HIV-1 V3 peptide-based ELISAs were used for titration and quantification of serum antibodies for detection as described previously (14). In brief, 96-well ELISA plates (MaxiSorp; Nunc A/S, Roskilde, Denmark) were coated with 100 μl of peptide MN (DKRIHIGPGRAFYTT)/well in 50 mM carbonate buffer (pH 9.3) at 5 μg/ml overnight at 4°C. The wells were washed and treated with 5% nonfat milk in phosphate-buffered saline for 1 h at 37°C. Duplicate samples containing either control or test macaque serum at appropriate dilutions were then added at 100 μl/well, and the plates were incubated for 1 h at 37°C. The wells were washed and incubated with a detection antibody solution consisting of peroxidase-conjugated goat anti-monkey immunoglobulin G (IgG) antibody (EY laboratories Inc., San Mateo, Calif.) at 100 μl/well for 1 h at 37°C. After final washes with 0.05% Tween-20–phosphate-buffered saline (PBST), peroxidase substrate was added, and the reaction was stopped by the addition of 0.5 M H_2SO_4.

IFN-γ ELISPOT assay. Enzyme-linked immunospot (ELISPOT) assays were performed using the method developed by Mothe and Watkins of the Wisconsin University Primate Center and described elsewhere (18, 33). In brief, 96-well flat-bottom plates (U-CyTech-BV, Utrecht, The Netherlands) were coated with anti-gamma interferon (IFN-γ) monoclonal antibody before being washed with PBST and blocked with bovine serum albumin. Freshly isolated PBMC were mixed with either concanavalin A or 2 μM V3 peptide and were then incubated for 16 h at 37°C in 5% CO_2 in anti-IFN-γ-coated plates. Once the plates had been washed, rabbit anti-IFN-γ polyclonal biotinylated detector antibodies were added, and the plates were incubated. Gold-labeled anti-biotin IgG solution (U-CyTech-BV) was added to the plates after they were washed with PBST. The plates were then incubated for 1 h at 37°C. Developed wells were imaged, and spot-forming cells (SFC) were counted using the KS ELISPOT compact system (Carl Zeiss, Oberkochen, Germany). An SFC was defined as a large black spot with a fuzzy border (33).

In vitro virus neutralization assays. GHOST cell neutralization assays were performed as previously described (8). Briefly, GHOST cells expressing either CXCR4 or CCR5 were used as targets for HIV-1 infection (50, 54). The cells were analyzed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, Calif.), and 15,000 events were scored. The mean number of fluorescent GHOST cells determined from negative controls plus 2 standard deviations was considered the cutoff for a positive sample. Purified human immunoglobulin (Nihon Pharmaceutical Co., Tokyo, Japan) and saline were included as additional controls.

M8166 cell-based virus neutralization assays were also performed as described previously (16, 47). In brief, the in vitro neutralization activity of purified macaque IgG was determined using 100 $TCID_{50}$ of either HIV-1_{MN} or SHIV-MN in cultures of M8166 cells. The results were compared with parallel cultures to which preimmune serum IgG was added. Neutralization was expressed as percent inhibition of HIV-1 p24 or SIV p27 antigen production in the culture supernatants. Purified normal macaque IgG was used as a control.

Quantification of cell-associated viral load. Levels of cell-associated virus were quantified by limiting dilution of PBMC (from 10^6 to 1 cells), and the virus was cocultured with M8166 cells as described previously (17). Virus released into the culture supernatant was measured by SIV p27 antigen ELISA (Coulter). The smallest number of PBMC required to produce a positive culture was considered the end point, and the titer of infectious virus was expressed as $TCID_{50}$ per 10^6 PBMC.

PCR detection of proviral HIV-1 infection of rhesus macaques. PBMC with SHIV were detected by DNA PCR using a primer pair that spans the C2-V3 sequence of HIV-1_{IIIB}, followed by Southern blotting with an SE1 probe, 5′-GCAGAAGAAGAGGTAGTAATTAGAT-3′ (nucleotides 7019 to 7043) (47). The positions of the oligonucleotides are numbered relative to the HIV-1_{HXB2} isolate in the ENTREZ database (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.). Viral DNA was quantified by comparison with standards derived from 8E5/LAV cells, which contain one copy of HIV-1 proviral DNA per cell (AIDS Research and Reference Reagent Program).

Competitive PCR quantitation of SHIV RNA in plasma. Quantitative, competitive reverse transcription-PCR was performed using a competitor RNA and a DNA template as previously described (18, 32, 44). The detection limit of this assay was 500 RNA copies/ml in monkey plasma (18, 32).

Sequencing of HIV-1 Env C2-V3 sequence. To determine the sequence of the HIV-1 Env C2-V3 region, mRNA was extracted from stock virus and cDNA was synthesized using a Micro-FastTrack version 2.0 kit (Invitrogen, Carlsbad, Calif.) and a cDNA cycle kit (Invitrogen) according to the manufacturer's instructions. The PCR products were cloned into a pCR II vector with a dual promoter using a TA cloning kit (Invitrogen) (47). Sequence analysis was performed using a Big Dye terminator cycle-sequencing FS kit (Perkin-Elmer, Foster City, Calif.) and automated ABI 310 sequencer (Perkin-Elmer) with Sp6 and T7 sequence primers (Invitrogen). Sequence data were compared with published HIV-1 sequences in GenBank (National Center for Biotechnology Information, National Institutes of Health).

Statistical analysis. Calculations of the geometric mean ± standard deviation (SD) were carried out with a microcomputer. Significance was defined as a P value of <0.05.

RESULTS

Vaccination protocol. Twenty-four male rhesus macaques (R-01 through R-24) were enrolled in the study. Of these, 15 were subcutaneously immunized for 24 weeks with 10 mg of

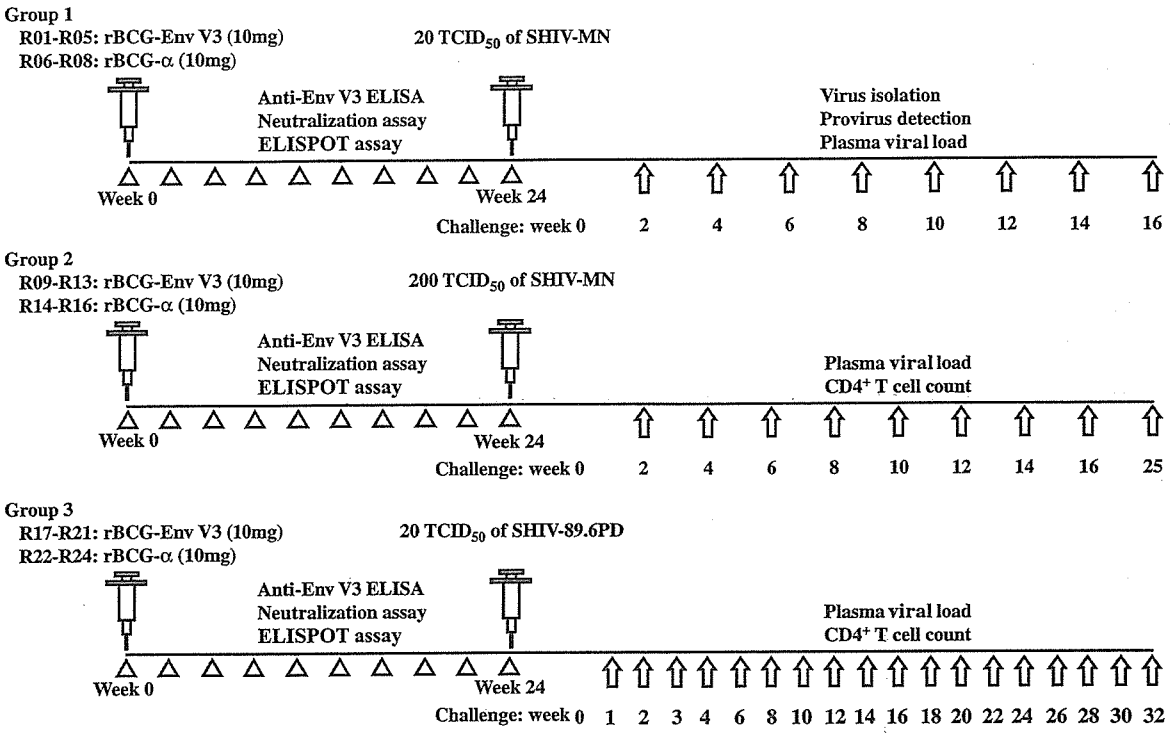

FIG. 1. Schematic representation of the experimental protocol for immunization of rhesus macaques with rBCG Env V3 and challenge with either SHIV-MN or SHIV-89.6PD. A total of 24 macaques were assigned to either the rBCG Env V3 vaccine or rBCG vector control group. The animals each received a single subcutaneous injection and were then split into three groups prior to challenge with either low-dose SHIV-MN, high-dose SHIV-MN, or SHIV-98.6PD.

rBCG Env V3 (16), which expresses and secretes a chimeric protein consisting of α-antigen and the Env V3 region of HIV-1_{MN}. The remaining nine macaques were immunized by the same route and with the same dose of rBCG α-antigen and served as vector controls. All macaques inoculated with rBCG Env V3 remained in good health following vaccination. Three of the 15 immunized macaques experienced transient redness with slight erosion localized at the injection site; however, the reaction spontaneously resolved within 3 months. Following immunization, the 24 macaques were divided into three groups, each group consisting of five immunized animals and three vector controls. The macaques within each group received an intravenous challenge with either SHIV-MN (20 or 200 $TCID_{50}$) or SHIV-89.6PD (20 $TCID_{50}$) (Fig. 1).

Vaccine-induced HIV-specific immune responses following rBCG Env V3 immunization. (i) Neutralizing antibodies. As described above, 15 rhesus macaques were vaccinated with a single subcutaneous inoculation of 10 mg of rBCG Env V3. Induction of HIV-1-specific immunity was measured 24 weeks later in blood samples obtained pre- and postvaccination. All 15 immunized macaques exhibited HIV-1 Env V3 peptide-binding antibody activity by ELISA at serum dilutions ranging from 1:640 to 1:10,240 (Fig. 2). Antibody responses were monophasic, peaking at 4 to 6 weeks and then gradually declining. Serum samples obtained from naïve macaques were consistently negative by ELISA, while postvaccination sera did not react with a control fusion peptide of HIV gp41 (data not shown).

Antibodies were purified from the macaque sera to remove factors that might interfere with certain bioassays (51). The purified antibodies were then tested in vitro for the ability to neutralize SHIV-MN infection in M1866 cells (Fig. 3). Antibodies induced in macaques vaccinated with rBCG Env V3 strongly neutralized both the challenge SHIV-MN (grown in rhesus PBMC) and a T-cell line-adapted (TCLA) laboratory strain, HIV-1_{MN}. A mean 50% inhibitory concentration (IC_{50}) of 0.05 to 0.5 μg of IgG/ml was measured against SHIV-MN, and a mean IC_{90} of ~3.0 μg of IgG/ml was observed against HIV-1_{MN}. Neutralizing activity was detected in serum samples obtained 4 to 6 weeks after vaccination and was maintained for at least 24 weeks. Preimmune serum IgG from nine macaques immunized with vector alone, and IgG from three additional naive macaques (data not shown), did not neutralize either virus.

(ii) Neutralization responses against primary HIV-1 isolates. To further assess the specificity of antibodies in immune sera, neutralizing activity was evaluated against a panel of seven primary HIV-1 isolates using GHOST cells expressing either CCR5 or CXCR4 (Table 1). Purified IgG from macaques in each of the three immunization groups was able to effectively neutralize HIV-$1_{BZ167/X4}$, HIV-$1_{SF2/X4}$, and HIV-$1_{CI2/X4}$ (Table 1 and Fig. 4), with mean IC_{50} values of 5 to 7, 4 to 7, and 5 to 15 μg/ml, respectively. By comparison, neutralization of HIV-$1_{MNp/X4}$ required ~10-fold more serum IgG, with a mean IC_{50} of 50 μg/ml. Three additional isolates, HIV-$1_{SF33/X4}$, HIV-$1_{SF33/R5}$, and the clade A isolate HIV-$1_{VI313/R5}$,

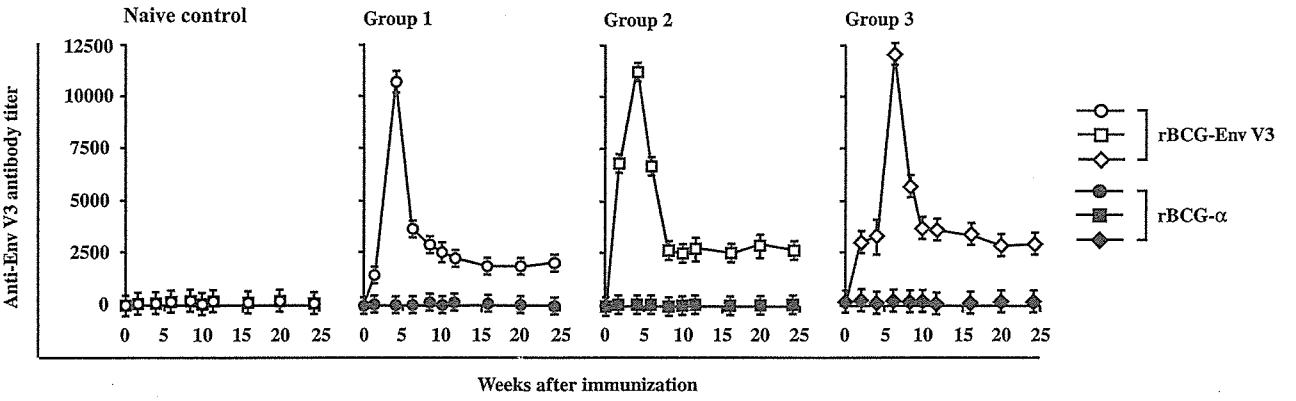

FIG. 2. Serum anti-V3 antibody titers determined by peptide-based ELISA. Preimmune and immune sera from macaques inoculated with rBCG Env V3 were collected and stored at −80°C until they were used. Sera from naïve macaques were used as controls. Data using preimmune sera were within the control levels (data not shown). The results are expressed as the means ± SD of four independent assays.

were not neutralized with serum IgG concentrations up to 50 μg/ml (Table 1). Preimmune sera had no neutralizing activity against any of the isolates. Thus, antibodies present in sera from the immunized macaques were able to neutralize primary HIV-1 isolates, including HIV-1_{BZ167}, HIV-1_{SF2}, and HIV-1_{CI2}, in assays using GHOST cells that express CXCR4 with 10- to 50-fold-higher sensitivity than that of the dual-tropic (X4-R5) TCLA strain HIV-1_{MNp}. Among the neutralization-sensitive viruses, the V3 sequence motifs of HIV-1_{BZ167} and HIV-1_{SF2} shown in Fig. 5 did not correlate with the observed neutralization profiles of HIV-1 Env V3.

(iii) V3 peptide-specific T-cell responses. Table 2 offers a comparison of the virus-specific T-cell response levels determined by IFN-γ ELISPOT analysis in immunized animals with the neutralization data provided in Fig. 2. Of the 15 animals immunized with rBCG Env V3 (180 and 160 SFC/10^6 PBMC at 6 weeks postimmunization [p.i.], respectively), only R-09 and R-10 showed very low levels of SFC activities at the time of

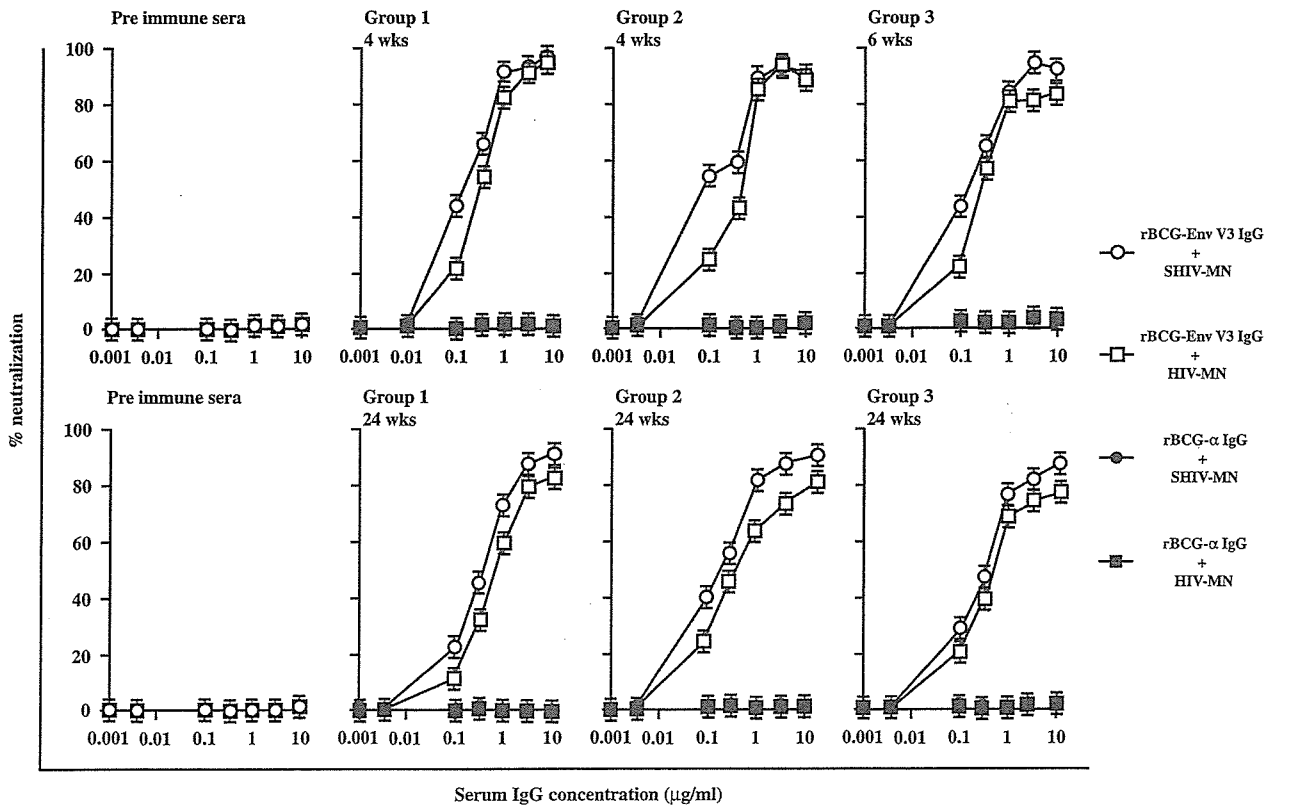

FIG. 3. HIV-1-specific neutralization antibody responses in macaques vaccinated with rBCG Env V3. Analysis of in vitro neutralization of SHIV-MN by anti-rBCG-HIV-1 antibodies using M8166 cell-based virus neutralization assays. Serum IgG was purified from preimmune or immune sera of macaques inoculated with rBCG Env V3 at the indicated times. The results are expressed as the means ± SD of four independent assays.

TABLE 1. 50% neutralization calculated on the basis of neutralization curves[a]

Serum sample	Neutralizing activity (μg)						
	BZ167/X4	MNp/X4	SF2/X4	SF33/X4	SF33/R5	V1313/R5	C12/X4
Group 1	6.5	50	7	>50	>50	>50	10
Group 2	5	50	4	>50	>50	>50	5
Group 3	7	50	6.5	>50	>50	>50	15
Pre immunization sera of groups 1, 2, and 3	>50	>50	>50	>50	>50	>50	>50

[a] The neutralization assays with the various viruses were carried out in GHOST cells expressing either CXCR4 (X4) or CCR5 (R5) as indicated in Fig. 4. BZ167, MNp, SF2, SF33, and CI-2 are HIV-1 clade B viruses. VI313 is an HIV-1 clade A virus.

SHIV challenge (120 and 110 SFC/10^6 PBMC at 24 weeks p.i., respectively) (Table 2). In contrast, <100 SFC/10^6 PBMC were observed in other immunized animals, and <20 SFC/10^6 PBMC were observed in controls. Thus, the V3 region antigen in the rBCG Env V3 proved unable to induce significant levels of virus-specific T-cell responses in immunized animals.

Challenge with low-dose SHIV-MN. The first group of eight macaques (R-01 through R-08), consisting of five animals that received rBCG Env V3 and three that received control rBCG α-antigen, were intravenously challenged with low-dose SHIV-MN (20 $TCID_{50}$) at 24 weeks p.i. The cell-associated virus load was measured in PBMC cocultures, and proviral copy numbers were estimated by DNA PCR using primary PBMC genomic DNA. The level of plasma viremia in each macaque was quantified by competitive reverse transcription-PCR to assess infection and virus replication for 16 weeks after virus challenge (Table 3).

Control macaques vaccinated with the vector alone (R-06 through R-08) were positive in all three viral-load assays 2 weeks after SHIV-MN challenge and remained positive for a follow-up period of 10 weeks. Because only low levels of viral RNA (<10^4 RNA copies/ml) were transiently detected 2 weeks postchallenge, all three assays (virus isolation, plasma RNA, and proviral DNA) were used for virus detection. Using these criteria, we observed that all three parameters remained negative after low-dose SHIV-MN challenge in three of five macaques vaccinated with rBCG Env V3 (R-02, R-04, and R-05). However, macaque R-01 was transiently positive in all three assays for virus infection at 4 weeks. Another macaque immunized with rBCG Env V3 (R-03) exhibited a sharp in-

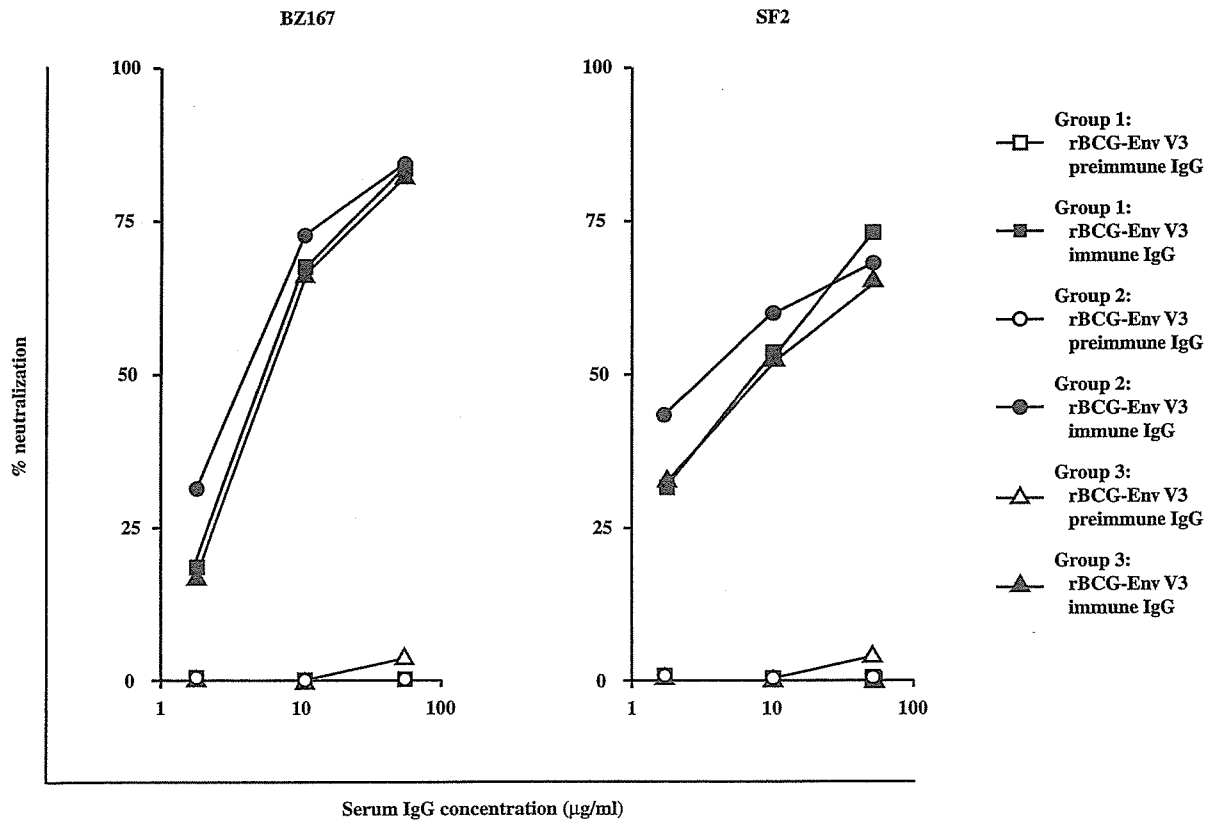

FIG. 4. Neutralization of HIV-1_{BZ167} and HIV-1_{SF2} in GHOST-X4 cells by immune sera from macaques vaccinated with rBCG Env V3. Dilutions of immune sera (closed symbols) and preimmune sera (open symbols) were tested in duplicate, and the percent neutralization was calculated using the mean value. The dose-response curves represent the means of three independent assays.