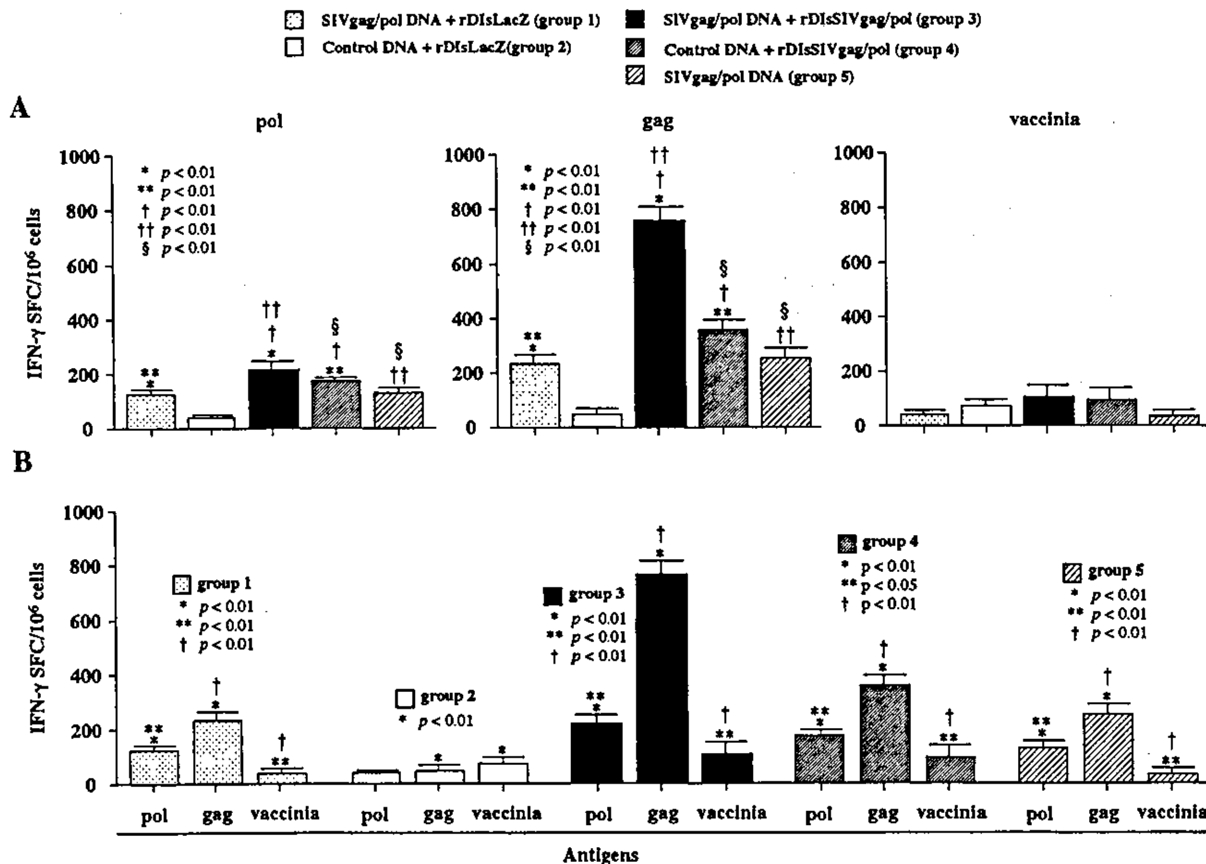

FIG. 10. Comparison of SIV*gag*-, SIV*pol*-, and vaccinia virus antigen-specific immunities in animals at the time of challenge. ELISPOT activities in vaccinated animals with different strategies. Each group of animals was immunized with different strategies and antigen-specific T-cell responses were analyzed by differentiating the SIV*gag*-, SIV*pol*-, and vaccinia virus antigen-specificities at the time of challenge infection by using protein antigen-specific ELISPOT assays. (A) SIV*gag*-, SIV*pol*-, and vaccinia virus antigen-specific analyses; (B) vaccine strategy-specific analysis of each group of five.

ment of an HIV-1 candidate vaccine. This strategy will be further evaluated to determine its efficacy against viral challenge in a nonhuman primate model.

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Hemagglutinating virus of Japan protein is efficient for induction of CD4$^+$ T-cell response by a hepatitis B core particle-based HIV vaccine

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Abstract

By using the hepatitis B core (HBc) protein gene as a carrier, HIV-1 *env V3* gene was inserted into the carrier gene, and the HIV gene was expressed inside a chimeric HIV-HBc particle (HIV-HBc), which was a unique candidate for induction of HIV-specific CTL activity. This was seen significantly in mice without the need of an adjuvant, because other responses specific for the HIV peptide such as T-cell proliferation and antibody production were not induced. However, when hemagglutinating virus of Japan (HVJ) protein was incorporated into an anionic liposome containing HIV peptide (HIV-HVJ-liposome) and was used as a booster immunization in HIV-HBc primed animals, the HIV-specific T-cell response and enhanced CTL activity were clearly induced in consecutively immunized animals. Furthermore, the HIV-specific humoral immune response was also induced and a neutralization activity was detected in the immune sera. Thus, when an HIV peptide antigen is expressed inside the virus like a particle of HBc, it can induce both cellular and humoral immunities when an HVJ-HIV-liposome, but not an HIV-liposome, is inoculated as the booster antigen. The HVJ-stimulated splenocytes secreted IL-18 and IL-12 to synergistically enhance the secretion of IFN-γ in vitro. These findings suggest that the HVJ protein is effective at inducing the HIV-specific immunities, if used as part of a booster antigen in the consecutive immunization regimen.
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Keywords: HVJ protein-incorporated liposome; HIV-hepatitis B core chimeric protein; HIV-specific immunity; Synergy of IL-18 and -12 to secrete IFN-γ

Introduction

A variety of novel approaches are currently being investigated to promote effective immunity against HIV-1. Such approaches often include attenuated, recombinant bacterial vectors that express antigenic epitopes from those of HIV [1–4], recombinant adenovirus vectors [5], recombinant vaccinia virus [6], DNA vaccines expressing gp120 [7], and synthetic peptides containing T- and B-cell epitopes of HIV as immunogens [8,9]. These candidate vaccines rely on the induction of both cellular and humoral immunities. It has been suggested that such immunities help protect individuals from HIV infection and from the subsequent development of AIDS [10]. Furthermore, HIV-specific CD4$^+$ T-cell response may play a critical role in vaccine development and immunother-

Abbreviations: HVJ, hemagglutinating virus of Japan; HBc, hepatitis B core protein; HIV-HBc, chimeric HIV-V3-HBc particle; HVJ-liposome, liposome with incorporated HVJ protein; HIV-liposome, liposome which encapsulated circular HIV-V3 peptide; HIV-HVJ-liposome, HVJ-liposome which encapsulated HIV-V3 circular peptide; HIV_{HXB2}-HVJ-liposome, HVJ-liposome encapsulated circular HIV-1_{HXB2} V3 peptide; V3, principal neutralizing determinant; KLH, keyhole limpet hemocyanin; KLH-IIIB, KLH-conjugated HIV-1_{HXB2} V3 peptide; BSA-IIIB, BSA-conjugated HIV-1_{HXB2} V3 peptide; Chol, Cholesterol; PC, phosphatidylcholine; Sph, shingomyelin; DOPE, dioleoylphosphatidylethanolamine; DC-chol, dimethylaminoethane carbamoyl-cholesterol; HAU, hemagglutinating unit; SI, stimulation index.

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apeutic interventions that aim to maintain effective immunity to HIV infection [11].

To render subunits of viral antigen or synthetic peptides immunogenic, a T helper cell's peptide or protein is fused to a target peptide [8,12], because free synthetic peptides or proteins are usually poor immunogens. For several reasons, the hepatitis B core (HBc) protein is a potential target carrier peptide [13–17]: (i) HBc can be assembled and can form particles that can induce immunity without the use of an adjuvant [18]. (ii) HBc Ag is a strong T-cell immunogen and is recognized over a wide range of MHC haplotypes [19]. (iii) The HBc Ag gene has been fused with respective target epitope genes to the N terminus [20–24], to the C terminus [14], and to internal sites [16,23,25,26]. In the present study, the HIV gene was inserted at the internal site of the HBc gene and the antigen was expressed inside the particles of the HBc chimeric protein (HIV-HBc) that spontaneously aggregated to a rigid particle of approximately 30 nm in diameter. This type of antigen inside the particle induced antigen-specific CTL but could not induce the antigen-specific $CD4^+$ T-cell response. These findings suggest that the HIV-HBc antigen may not be a suitable immunogen when used alone. However, the HIV-specific T-cell response is effectively inducible when the hemagglutinating virus of Japan (HVJ) protein was incorporated into anionic-type HIV-liposomes encapsulated by circular HIV-V3 peptides (HIV-HVJ-liposome). The HIV-HVJ-liposome was used as a booster injection in HIV-HBc primed animals.

In this paper, we chose the third variable domain (V3) of HIV-1 isolates' gp120 as an immunogen, because it evokes neutralizing antibody recognizing V3-tip region with a low efficiency by itself [27]. The V3 region is suggested to be immunodominant and so of importance in vaccine development [28]. The site is also assumed to be a chemokine receptor-binding site by the crystal structure analysis [29–31]. Furthermore, strong cellular immune responses and high HIV-specific neutralizing activity may account for long-term nonprogression in different individuals [32,33]. To improve immunogenicity of the V3 site for immunization of experimental animals, we designed to enhance immune induction of the HIV V3-specific immunity by using the HVJ protein-incorporated anionic liposome.

Materials and methods

Animals

Female eight-week-old BALB/c mice ($H\text{-}2^d$), and 6-week-old Hartley strain guinea pigs (400 g), were purchased from the Japan SLC Co., Ltd., Hamamatsu, Japan and were used within 10 days. All animal care and housing requirements determined by the National Institute of Infectious Diseases (NIID) committee for the care and use of laboratory animals were followed. Animal protocols were reviewed and approved by an institutional animal care and use committee.

Construction of expression vectors and preparation of HIV-HBc chimeric particles

A synthetic DNA fragment encoding 21-aa or 19-aa V3 tip sequence of HIV-1_{HXB2} or HIV-1_{MN}, respectively, was inserted into plasmid pYGHBc [34], which are seen in yeast cells. The product of HIV-HBc chimeric particles were purified and prepared as a vaccine antigen by using the methods described by Shiosaki et al. [15] and Miyanohara et al. [18]; however, different oligonucleotides were used for the present study. The monoclonal antibodies used for the antigen analysis by ELISA, Western immunoblot, and immuno-electron microscopy, were anti-HBc antibody [18], anti-HIV_{HXB2} V3 mAb 0.5β [35], and anti-HIV_{MN} V3 mAb μ5.5 [36].

Preparation of both anionic and cationic HIV-HVJ-liposomes

Lipids

Cholesterol (Chol), egg yolk phosphatidylcholine (PC), and egg yolk sphingomyelin (Sph) were purchased from Sigma (St. Louis, MO). Bovine brain phosphatidylserine (PS) was purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Dioleoylphosphatidylethanolamine (DOPE) and dimethylaminoethane carbamoyl-cholesterol (DC-chol) were obtained from NOF Corporation (Tsukuba, Ibaraki, Japan).

Preparation of HVJ

HVJ (Z strain) was grown in chorioallantoic fluid of 10-day-old embryonated chicken eggs at 36.5 °C. HVJ was collected as a pellet by centrifugation at 27,000 × *g* for 30 min at 4 °C and was suspended with a balanced salt solution (BSS; 10 mM Tris–HCl pH 7.5, 137 mM NaCl, 5.4 mM KCl). RNA genome of HVJ was inactivated by UV irradiation (198 mJ/cm^2) just before use.

Preparation of anionic-type and cationic-type liposomes

First, lipid mixtures were prepared by dissolving PC (1.63 mg), DOPE (1.53 mg), Sph (1.47 mg), and Chol (3 mg) in 0.5 ml chloroform. PS (1.25 mg) or DC-chol (0.75 mg) was added to the lipid mixtures to prepare the anionic-type or cationic-type HVJ-liposomes, respectively. The lipids in chloroform was transferred to a glass tube and dried as a thin lipid film by evaporation, as described elsewhere [37]. Both of the HIV Env V3 synthetic circular peptides; circular IIIB-V3, VEINCTRPLNNTRKSIRIQRGPGRAFVTIGSIIGDIRQAHCNLSR; and circular MN-V3, VEINCTRPNNNTRKSIHIGPGRAFYTTGSIIGDIRQAHCNLSR (1.67 mg each, Takara Shuzo Co., Ltd., Kusatsu, Shiga, Japan) were dissolved in 200 μl of distilled water. The suspension was then added to the dried lipid mixture. Liposomes were prepared by vigorous shaking, as described previously [38]. In the case of the anionic-type liposomes, they were sonicated for 3 s and 300 μl of BSS

was added to the liposomes followed by gentle shaking at 37 °C for 30 min. For cationic-type HVJ-liposomes, the liposome suspension was extruded through cellulose acetate membrane filters (pore size 0.45 μm and 0.20 μm) as described previously [39].

Preparation of HVJ-liposomes

The liposome suspension prepared above was mixed with a UV-inactivated HVJ suspension [15,000 hemagglutinating unit (HAU)] for 10 min on ice and incubated at 37 °C for 1 h while shaking the suspension in a water bath. The HVJ-liposome complexes were then separated from free HVJ by sucrose density gradient centrifugation (62,800 × *g* at 4 °C for 1.5 h). The HVJ-liposomes between BSS and 30% sucrose solution were collected. The volume of HVJ-cationic liposome was adjusted to 300 μl with BSS. The HVJ-anionic liposomes were diluted 4 times with BSS and centrifuged at 27,000 × *g* for 30 min at 4 °C. The pellets were suspended with 300 μl of BSS by vortexing.

Enzyme-linked immunosorbent assay

Peptide-based ELISA, as described previously [40], was performed to detect antigen-specific antibodies within the guinea pig.

Cytotoxicity assays

The procedure for in vitro CTL activation and in vitro effector cell assay has been described previously [28,41–43]. In brief, spleen cells were isolated from mice immunized with vaccine antigens. Primed and washed cells (1×10^7) were incubated for 6 days with 10 μg of synthetic V3 peptide per milliliter. The restimulated spleen cells were incubated for 4 h with ^{51}Cr-labeled M12.4.5 (H-2^d), BW5147 (H-2^k), and S49 (H-2^s) cell lines used as target cells. The target cells were treated with ^{51}Cr at a concentration of 100 μCi for 90 min, and were then pulsed with 10 μg of the synthetic V3 peptide for 60 min. The BW5147 (H-2^k) and S49 (H-2^s) cell lines were kindly provided by Dr. Ethan M. Shevach, National Institutes of Health, Bethesda, MD; these cells were also used as target cells. The percentage of specific release was calculated as follows: % specific release = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. The sequences of synthetic peptides of HIV_{HXB2} and HIV_{MN} V3 region of envelop proteins used for effector cells induction were RIQRGPGRAFVTIGK (P18IIIB) [42] and RIHIGPGRA-FYTTKN (P18MN) [42], respectively (Takara Shuzou).

T-cell proliferation assay

Lymphocyte proliferative assays were performed as previously described [44]. Briefly, isolated spleen cells were pooled and the $CD4^+$ or $CD8^+$ fraction was then depleted using magnetic cell sorting (MACS, Militenyi Biotec., Bergisch Gladbach, Germany) [40]. Results are expressed as the stimulation index (SI), which was calculated as a ratio of the counts per minute (cpm) in the presence and absence of an antigen.

PBMC-based virus neutralization assay of HIV-1

The serum antibody of the guinea pigs inoculated with HIV-HBc following a booster injection of HIV-HVJ-liposome or a booster injection of HIV-liposome was purified from the whole sera from 15 immunized guinea pigs with Protein A Sepharose (Amersham Pharmacia Biotech, AB, Uppsala, Sweden). Serum IgG from guinea pigs injected with the HIV-HVJ-liposome and normal guinea pig IgG were also purified by the same method. The diluted serum antibodies were incubated with 100 $TCID_{50}$ units of HIV-1_{LAI}, HIV-1_{MN} and HIV-1_{Th22} (AIDS Research and Reference Reagent Program, NIH, Rockville, MD). The mixtures were incubated with PHA-activated peripheral blood mononuclear cell (PBMC). After being washed three times with PBS, the cells were cultured in the presence of recombinant human IL-2 (40 units/ml, Shionogi and Co., Ltd., Osaka, Japan) for 7 days. The amount of HIV in the supernatant was measured by HIV-1 p24 antigen ELISA (Dinabot, Ltd., Tokyo, Japan) [43,45]. The in vitro neutralization activity of the immune IgG against HIV-1 was determined by using 100 $TCID_{50}$ of the stock virus [43] and was expressed as percentage inhibition of p24 antigen production in the culture supernatants compared with that of the cultures to which serum IgG from normal guinea pigs was added. For the neutralization assays, virus stocks were titrated on PHA-activated normal PBMC and the $TCID_{50}$ of each virus was determined [43,46].

Cytokine ELISAs

Specific ELISAs determined the amounts of IL-12, IL-18, and IFN-γ in culture supernatants. IL-18 ELISA kit was purchased from Fujisaki Institute, Hayashibara Biochemical Laboratories, Okayama, Japan and IL-12 and IFN-γ ELISAs from R&D Systems Inc., Minneapolis, MN.

Statistical analysis

Calculations of the geometric mean ± SD were carried out with a microcomputer. Significance was defined as $P < 0.05$.

Results

Construction of pYGHBc-HIV and preparation of HIV-HBc chimeric particles

Each V3 peptide gene from HIV-1_{HXB2} or HIV-1_{MN} was inserted into an internal position of pYGHBc (pYGHBc-

HIV) and the amino acid sequences of the V3 regions of HIV-1_{HXB2} and HIV-1_{MN} genes were as follows (name, sequence): synthetic IIIB-V3 peptide, LNNTRKSIRIQRGP-GRAFVTI; and synthetic MN-V3 peptide, PNNKRKR IHIGPGRAFYTT (Fig. 1A). Protein particles were prepared by purification from the extract of yeast cells that expressed the pYGHBc-HIV V3 as described by Shiosaki et al. [15] and Miyanohara et al. [18] to be a single protein band (upper panel of Fig. 1B). To determine the antigen capability of the purified protein particle, we analyzed whether the purified protein particle was the fusion protein of HBc and V3 peptide of HIV-1_{HXB2} by Western blot assay (lower left panel of Fig. 1B). The protein showed reactive behavior with both anti-V3 0.5β mAb and anti-HBc Yc-3 mAb (lower left and lower right panels of Fig. 1B, respectively), indicating that the protein is a chimeric protein, which is composed of HBc protein and HIV Env V3 peptide antigen of HIV-1_{HXB2}. HIV_{MN}-HBc chimeric particle was similarly prepared.

Furthermore, the HIV-HBc chimeric proteins spontaneously aggregated to form a rigid particle of approximately 30 nm in diameter. This was seen by electron microscopy and sucrose-density ultracentrifugation analysis, and was stable at 4 °C for 2 months (data not shown). The binding

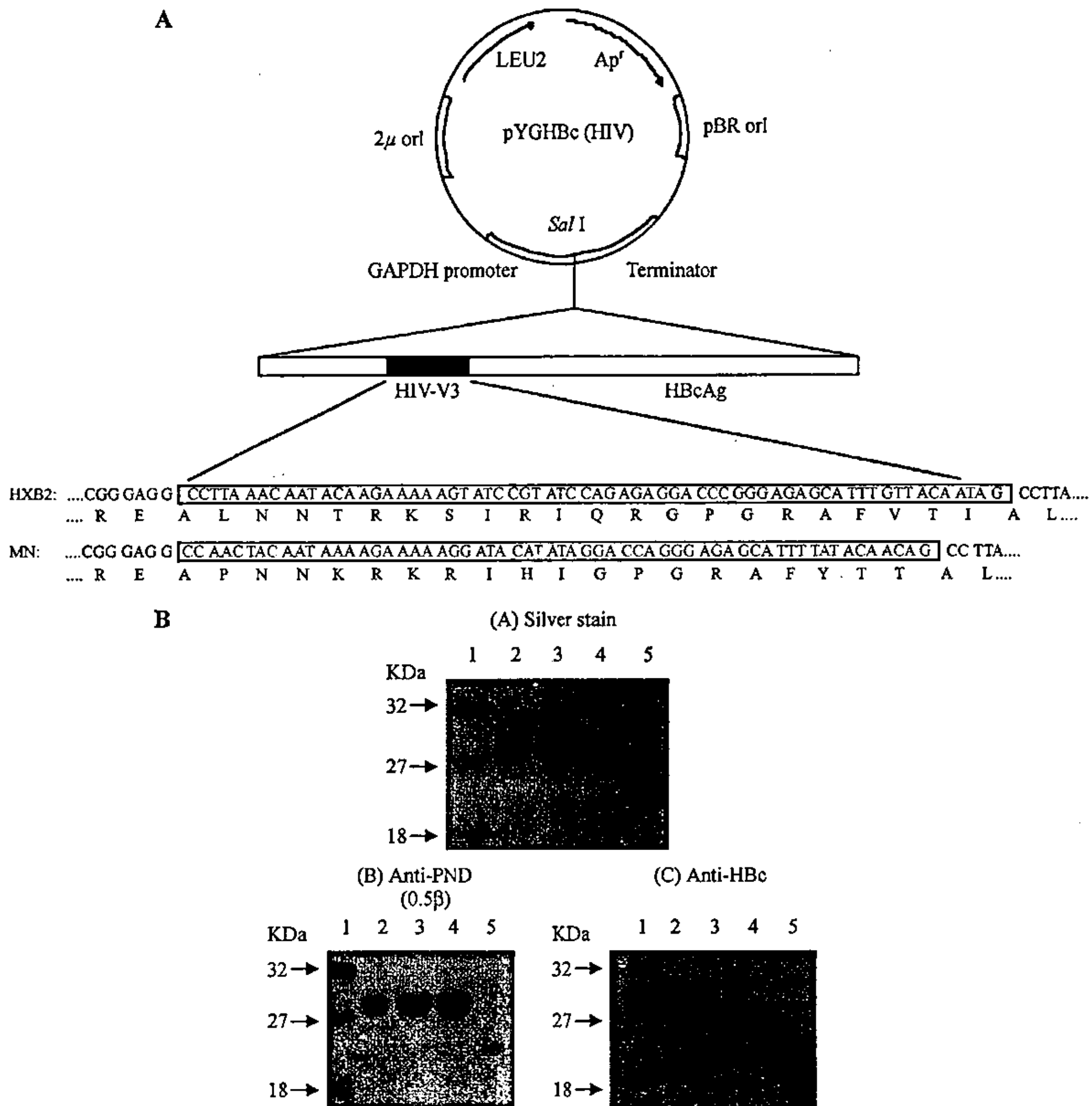
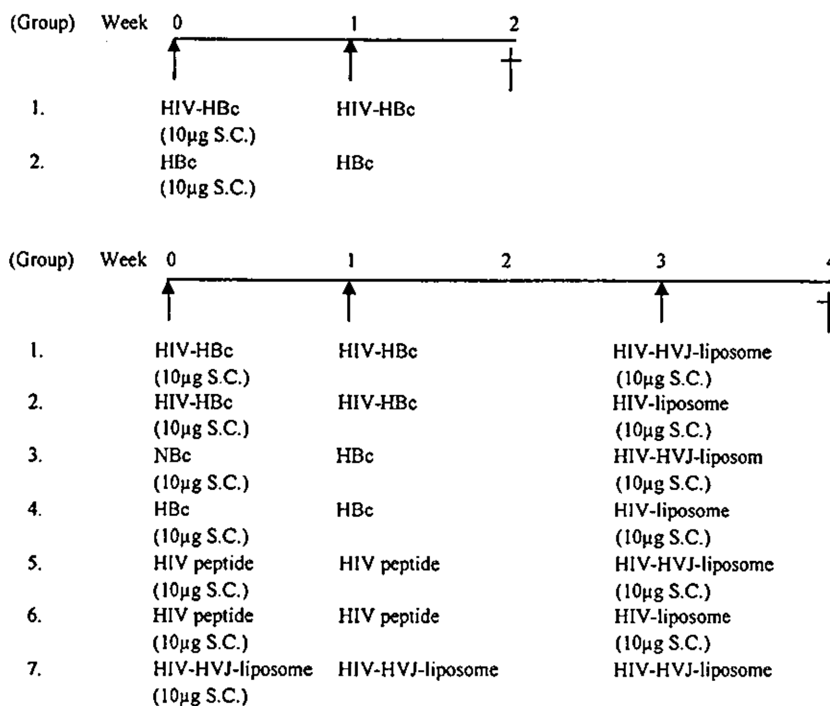


Fig. 1. Vector construction and expression of the HIV-HBc chimeric protein. (A) Vector construction of the HIV-HBc chimeric protein. DNA fragments encoding the V3-tip of the HIV V3 region from HIV-1_{HXB2}, and HIV-1_{MN} were inserted into the *SalI* restriction site in the gene for HBc antigen in plasmid pYGHBc. Apr, pBR ori, GAPDH promoter, 2μ ori, and LEU indicate genes for resistance to the drug marker, promoter, and initiation sites. DNA sequences of inserted fragments are in boxes and their deduced amino acid sequences are aligned. (B) Detection of the HIV-HBc chimeric protein by SDS-PAGE (upper panel) and Western blotting. A purified chimeric particle was separated by SDS-PAGE and detected by Western blotting with an anti-HIV Env V3 0.5β mAb or an anti-HBc Yc-3 antibody (lower left and lower right panels). Lane 1, molecular weight marker; lanes 2–4, HIV-HBc chimeric protein; lane 5, HBc protein.

ability of the HIV-HBc chimeric particle with anti-HIV_{HXB2} V3 mAb 0.5β, and anti-HIV_{MN} V3 mAb μ5.5 was examined using the ELISA antigen and immuno-electron microscopy, resulting in that the bindings were negative for anti-V3 antibodies, in contrast the binding was positive for anti-HBc antibody (data not shown). Thus, these data suggested that the HIV peptide antigens were assumed to be inside the HIV-HBc chimeric particle but not on the surface of the particle.

Experimental protocol

As shown in Fig. 2, in the first series of experiments, mice were intradermally injected in the neck with 10 μg of HIV-HBc chimeric protein within 100 μl of saline solution. The mice were given identical booster injections s.c. 7 days later. In the next series of experiments, we determined whether booster injections of the HIV-HVJ-liposome were able to elicit or enhance both cell-mediated and humoral immunity against the HIV antigen in mice and guinea pigs. Mice were immunized twice with 10 μg of HIV-HBc in saline. Two weeks later after the second immunization, the immunized animals were given an HIV-HVJ-liposome that included 10 μg of circular HIV-V3 peptides. Guinea pigs were immunized (50 μg per animal) with the HIV-HBc in saline and followed by an administration with the HVJ-liposome that included 10 μg of HIV-V3 circular peptides. As controls, the HIV-HBc-immunized animals were boosted with liposomes that had not incorporated HVJ protein but which did include 10 μg of circular HIV-V3 peptides (HIV-liposome); HBc-immunized animals were boosted with HIV-HVJ-liposome with 10 μg of circular HIV-V3 peptides. Normal animals were also injected with the HIV-HVJ-



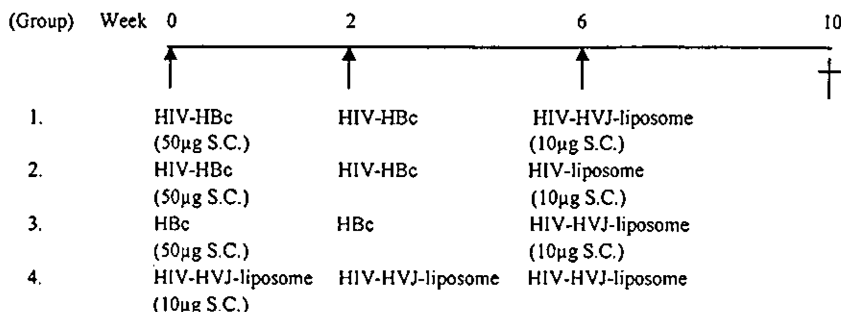


Fig. 2. Immunization schedule for induction of HIV-specific immunity.

liposome. In all experiments, five animals were used for each condition in all three of the experiments.

Characteristic immunogenicity of the HIV-HBc in mice and guinea pigs

To study the immune responses in mice, BALB/c mice were injected with an HIV-HBc chimeric protein and an HBc protein. Effector cells from the spleens of the mice immunized with HIV-HBc were generated by incubation with the V3 peptide in vitro. The stimulated effector cells significantly lysed target cells coated with the identical peptide but they did not lyse cells that were not coated (Fig. 3A). The induction of cytolytic response was specific to the HIV-antigen when it was expressed inside the chimeric protein particle; cytolytic activity was not detected when mice were immunized with an HBc protein alone and assayed for V3-specific CTL under the same condition (open triangle in Fig. 3A). Furthermore, the reactivity was restricted to the class I major histocompatibility complex, H-2^d, because cytolytic activity was not seen against allogeneic cells of BW5147 (H-2^k) and S49 (H-2^s) coated with the peptide (Fig. 3B) and the recognition of peptide 18IIIB and peptide 18MN are restricted by class I D^d molecule [28,42].

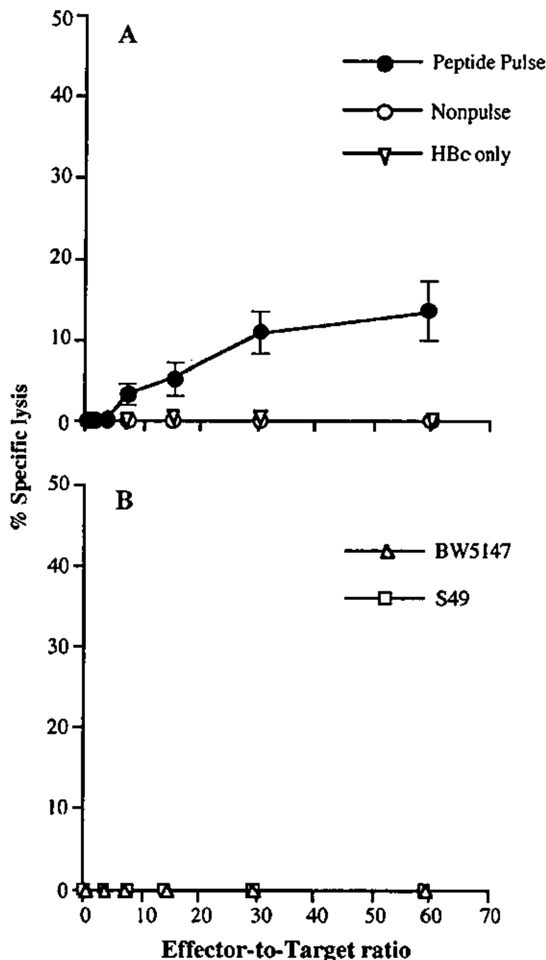


Fig. 3. Immunization of HIV-HBc chimeric antigen induces HIV-specific CTL in mice. (A) Cytolytic activity was measured against M12.4.5 target cells pulsed or not pulsed with the V3-tip peptide. The restimulated spleen cells from HIV-HBc-immunized mice were incubated with ^{51}Cr-labeled target cells that were either pretreated with the synthetic peptide (●), or untreated (○). As a control, the effector cell was prepared from HBc-immunized animals, and was incubated with ^{51}Cr-labeled target cells pretreated with the same synthetic peptide (▽). (B) Cytolysis was restricted to the major histocompatibility complex class I. Cytolysis was measured against BW5147 (△) and S49 (□).

The stimulatory effects of the HIV V3 peptide on the proliferative responses of spleen cells from the HIV-HBc immunized mice were tested 7 days after re-immunization with the same antigen. The V3 peptide stimulation did not enhance proliferations of the immune spleen cells at concentrations from 1 to 100 μg/ml, whereas HBc elicited more than 10 SI in all HIV-HBc- or HBc-immunized animals (data not shown).

Similarly, antibody titers of the sera from the above two groups specific for HIV-V3-tip antigen were all less than 10, which a value of 10 reflected an undetectable level of the antibody titer by the assay. However, HBc specific antibody titers were detected at 1:870–1150 in both groups of animals tested (data not shown).

Taken together, although these results demonstrate that immunization with a confined antigen (such as an HIV-V3 peptide expressed inside HIV-HBc particle) is able to induce HIV-specific CTL activity, neither antigen-specific $CD4^+$ T-cell nor humoral responses were observed. These results suggest that the HIV-HBc chimeric particle may induce HIV antigen-specific memory cells, but not induce effector cells effectively.

Booster injection of HVJ protein including HIV-liposome (HIV-HVJ-liposome) makes it possible to elicit $CD4^+$ T-cell response, enhanced CTL, and neutralization antibody production specific for the HIV-antigen

To study whether we could elicit marked HIV-specific immune responses to animals primed with the HIV-HBc chimeric protein, HVJ-protein was incorporated into HIV-liposome, which were used in antigen-primed animals as a booster injection. We initially characterized the effect of the incorporation of the HVJ protein into the liposome in a consecutive immunization strategy involving priming with HIV-HBc and boosting with anionic HIV_{HXB2}-HVJ-liposome. When HIV_{HXB2} V3-peptide was used, peptide-specific proliferative responses were detected with the addition of 5 μg/ml of the peptide to the culture of spleen cells from the immunized animals with the consecutive prime/boost regimen (Fig. 4). However, a lack of incorporation of the HVJ protein in HIV_{HXB2}-liposome in the booster antigen in the immunization strategy resulted in a marked decrease in the intensity of the proliferative response. In control animals that only had received a booster injection of HIV-HVJ-liposome, proliferative responses were not detected. Con A was used at a concentration of 2 μg/ml in spleen cell

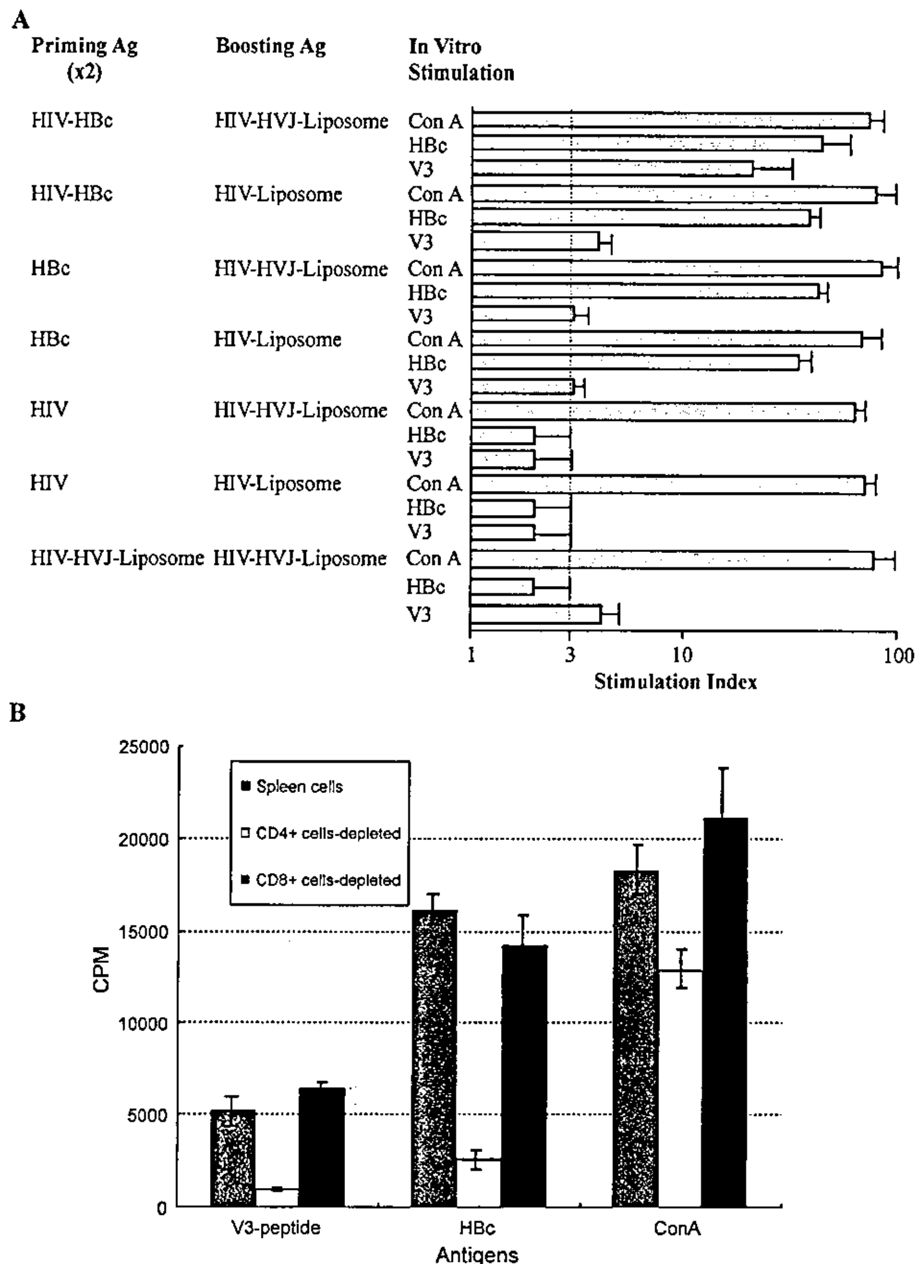


Fig. 4. $CD4^+$ T-cell proliferative responses specific for HIV-1 Env-V3 antigens. (A) The proliferative responses can be induced in animals immunized with the HIV-HVJ-liposome, which incorporated the HVJ protein into the HIV-liposome in a consecutive immunization regimen, involving priming with HIV-HBc and boosting with the HIV-HVJ-liposome. An anionic HIV-HVJ-liposome booster injection into HIV-HBc-primed guinea pigs resulted in a rise in lymphoproliferative responses to HIV-1 Env V3 antigens. The SIs of PBMC obtained from five guinea pigs in each group with various immunization regimens are shown in comparison to those obtained by stimulation with HIV-1_{HXB2} V3 circular peptide, HIV-1_{MN} V3 circular peptide, HBc protein, or medium alone. SIs are expressed as mean ± SEMs. Because results of SIs obtained by using HIV-1_{HXB2} V3 circular peptide and HIV-1_{MN} V3 circular peptide were roughly similar, the former results were shown. (B) Aliquots of spleen cells from mice vaccinated with the prime-boost regimen were depleted of $CD4^+$ or $CD8^+$ population before measuring the V3 peptide-specific proliferative responses.

cultures of normal animals and SI was always defined as more than 50. These results provide evidence that the incorporation of the HVJ protein into liposomes affects induction of a strong HIV-specific proliferative response in animals that had been immunized with the prime/boost regimen. Among the six groups, splenocytes from the mice in Group 1 (immunized with a prime-boost regimen) showed the highest levels of T-cell proliferative responses against the V3-loop peptides. The mean SI of each of the seven groups was 23.6 ± 12, 3.8 ± 2.4, 3.3 ± 1.3, 3.1 ± 1.5, 2.1 ± 1.3, 2.3 ± 1.4, and 4.4 ± 2.8, respectively (Fig. 4A). Depletion of the $CD4^+$ T-cell fraction dramatically reduced the proliferative responses from Group 1 to <10% (Fig. 4B). In contrast, proliferative activity was not affected by the depletion of the $CD8^+$ fraction from the cell suspensions.

We evaluated the effect of the anionic-type HIV-HVJ-liposome on induction of HIV-V3-specific CTL. The HIV-HVJ-liposome was administered to mice 3 weeks after immunization with HIV-HBc. CTL activity was clearly induced against syngeneic target cells pulsed with the HIV-1_{HXB2} V3 peptide (Figs. 5A and C) or HIV_{MN} V3 peptide (Figs. 5B and D) at an effector-to-target ratio from <1:6.25 in the mice inoculated with the booster injection of the HVJ-liposome that encapsulated the circular V3 peptide. However, in the booster injection of liposome that did not incorporate HVJ, the induction of HIV-specific CTL activities in the HIV-HBc primed animals was detected at an E/T ratio of 1:50–100. Furthermore, the CTL activity in the animals immunized with HVJ-HIV-liposome only was 18 ± 8.5% at 1:60 which is significantly less than that of prime-boost regimen consisting of HBc-HIV and HVJ-HIV-liposome (closed square in Figs. 5A and B). This result demonstrates that the incorporation of HVJ into liposome enhances CTL activity approximately 10-fold that of animals immunized with HVJ-unincorporated liposome (Figs. 5A and B). The enhanced induction of CTL activity by the HIV-HVJ-liposome is HIV-antigen specific and the reactivity was restricted to the class I-major histocompatibility complex, as is also shown in Fig. 3 (Figs. 5C and D).

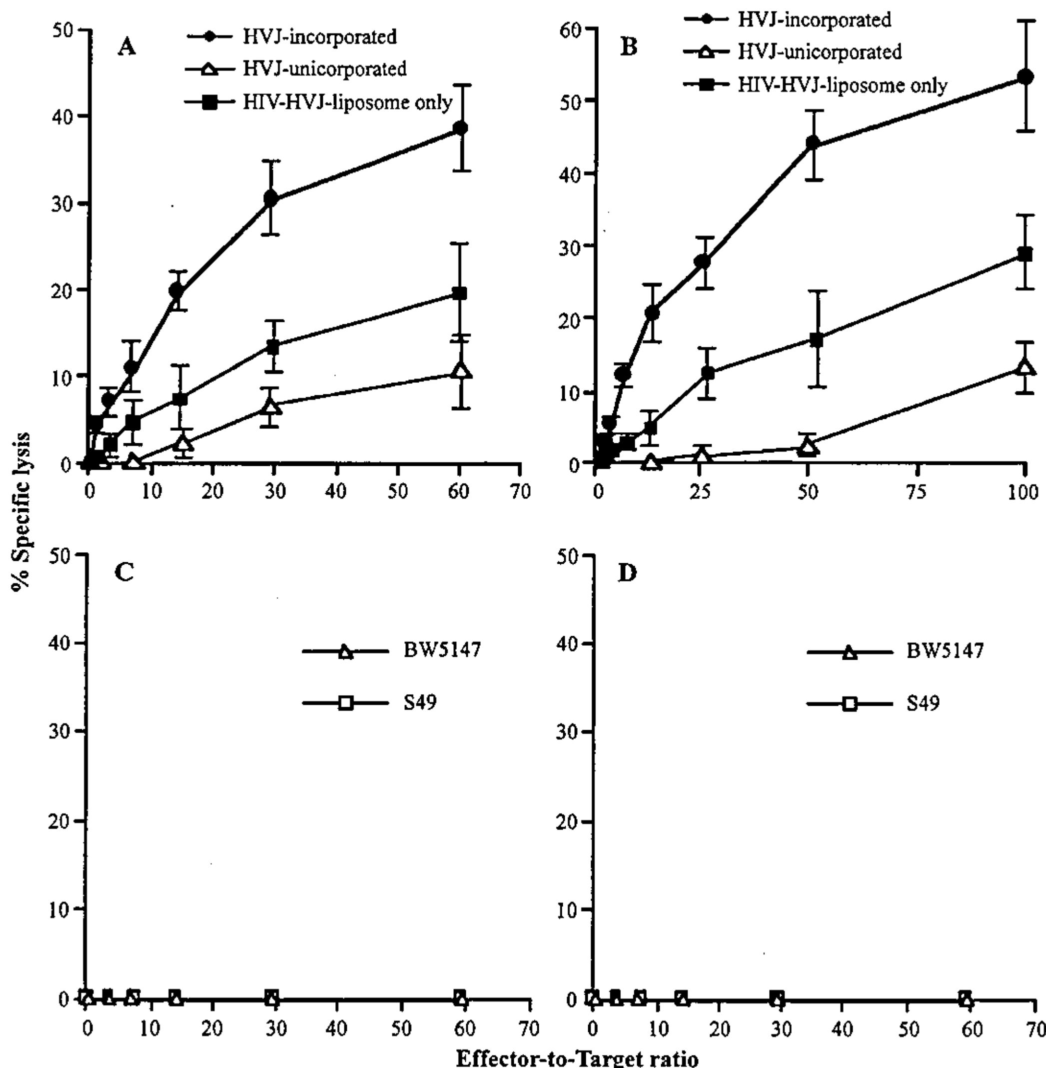


Fig. 5. Effect of the incorporation of the HVJ protein in terms of induction of HIV-1-specific CTL responses in the consecutively vaccinated mice primed with HIV-HBc followed by administration of HVJ-HIV-liposome. (A) Enhancement of HIV-1-specific CTL responses in the consecutively vaccinated mice with HIV-1_{HXB2}-HBc immunization followed by HIV-1_{HXB2}-HBc-liposome. Cytolytic activity was measured against M12.4.5 target cells pulsed or not pulsed with the V3-TIP peptide. The restimulated spleen cells from HIV-HBc-immunized mice followed by HIV-HVJ-liposome or followed by HIV-liposome were incubated with ^{51}Cr-labeled target cells. (●) cytolytic activity of V3-peptide-stimulated spleen cells from animals immunized with HIV-HBc priming followed by boosting with HVJ-incorporated HIV-liposome; (△) cytolytic activity of V3-peptide-stimulated spleen cells from animals immunized with HBc-HIV priming followed by boosting with HVJ-unincorporated HIV-liposome; and (■) cytolytic activity of V3-peptide-stimulated spleen cells from animals immunized with HVJ-incorporated HIV-liposome alone. (B) Similar enhancement of CTL activities was detected by a booster injection of HIV-1_{MN}-HVJ-liposome in animals with a prior immunization with HIV-1_{MN}-HBc injection. The results are expressed as the mean of three different experiments using five mice in each group. (C and D) Cytolysis was restricted to the major histocompatibility complex class I. Cytolysis was measured against BW5147 (△) and S49 (□).

HIV-V3-specific antibody responses were also induced in the HIV-HBc-primed animals boosting of the HIV-HVJ-liposome in the consecutive immunization regimen as well as HIV-HVJ-liposome immunization only. The guinea pigs of the two immunized groups similarly exhibited V3-binding antibody activity at serum dilutions more than 12,800 by HIV_{HXB2} V3 (Fig. 6A) or HIV_{MN} V3 (Fig. 6B) ELISA at 10 weeks after immunization. The serum antibody was purified from the HIV-HBc-immunized guinea pigs followed by the booster injection with HIV-HVJ-liposome or with HIV-liposome without HVJ, or from the animals immunized with HVJ-HIV-liposome only. PBMC-based virus neutralization assay with PBMC-passaged HIV_{LAI}, HIV_{MN} and HIV_{Th22} was used for the analysis (Fig. 6C). Measurements of inhibitory dose of 50% reduction of virus neutralization (ID_{50}) showed that the antibodies type-specifically neutralized the laboratory strain of clade B HIV_{LAI} and HIV_{MN2} with ID_{50} of serum antibodies from the HIV_{HXB2}- or HIV_{MN}-HVJ-liposome-boostered guinea pigs as well as from animals immunized with HIV_{MN}-HVJ-liposome only were approximately 6.5–15.5 μg/ml (Fig. 6C) but not HIV CRF01 AE, HIV_{Th22} (data not shown). However, virus neutralizations were not detected in sera from the HIV-HBc-immunized guinea pigs

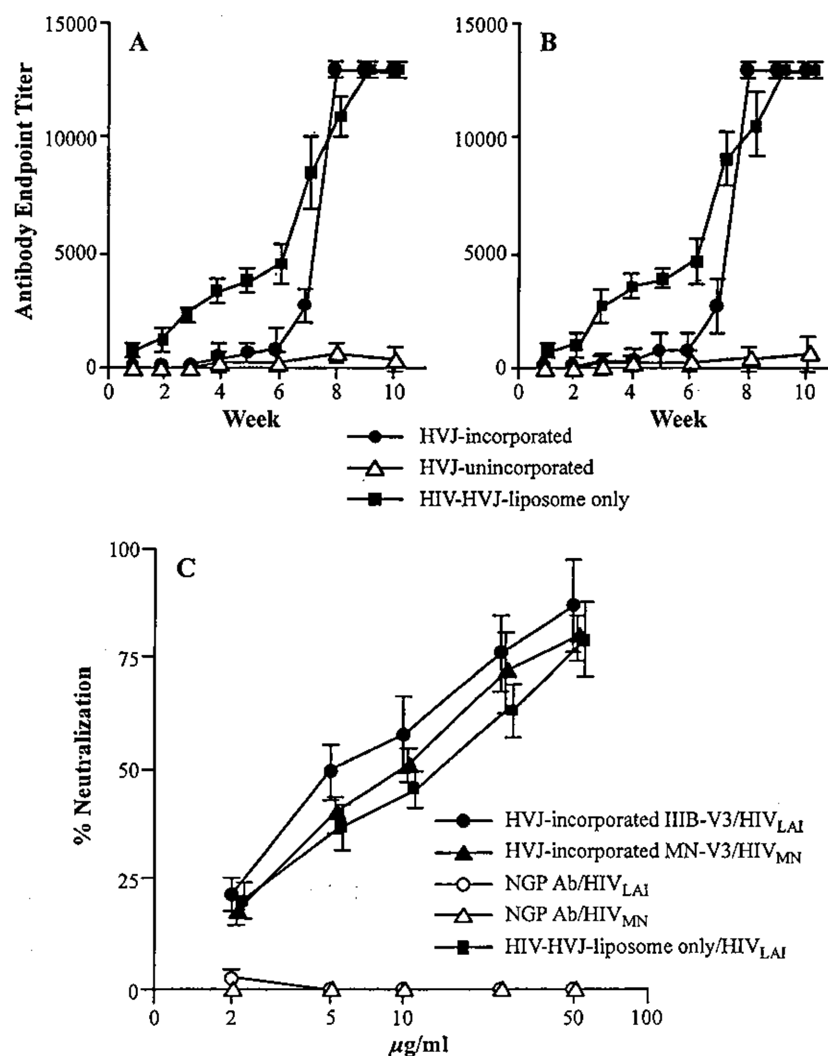


Fig. 6. HIV-1 antibody induction in a consecutive immunization regimen involving HIV-HBc immunization followed by the HIV-HVJ-liposome. HIV-1 antibody titers against HIV_{HXB2} V3 peptide (A) or HIV_{MN} V3 peptide (B) in the immunized animals were measured by ELISA with an endpoint dilution of immune sera. (●), sera from animals immunized with HBc-HIV priming followed by boosting with HVJ-incorporated HIV-liposome; (△) sera from animals immunized with HBc-HIV priming followed by boosting with HVJ-unincorporated HIV-liposome; and (■) sera from animals immunized with HVJ-incorporated HIV-liposome alone. (C) Detection of neutralization activity in the immune sera from the consecutively vaccinated animals. Neutralization activities are expressed as percentage inhibition, compared with control activity, and the mean of four different assays. (●), neutralization activity of serum IgG from animals immunized with HBc-HIV_{IIIB} priming followed by boosting with HVJ-incorporated HIV_{IIIB}-liposome; (▲) neutralization activity of serum IgG from animals immunized with HBc-HIV_{MN} priming followed by boosting with HVJ-incorporated HIV_{MN}-liposome; (O) neutralization activity of normal serum IgG against HIV_{IIIB}; (△) neutralization activity of normal serum IgG against HIV_{MN}; and (■) neutralization activity of serum IgG from animals immunized with HVJ-incorporated HIV-liposome alone.

following booster injections with HVJ-unicorporated HIV-liposome and in the preimmune animals or in naïve animals.

IL-12, IL-18, and IFN-γ productions are induced by stimulation with HVJ protein

We confirmed the HVJ protein-induced enhancement of IL-12, IL-18, and IFN-γ productions of spleen cells from animals immunized with HBc-HIV priming followed by HVJ-HIV-liposome boosting (Fig. 7). The enhanced production was specific to stimulation with HVJ protein for 24 h, but not with nonspecific culture with BSA. The result suggests that IL-18 may synergistically act with IL-12 to enhance IFN-γ production.

Discussion

This report describes a study, when an HVJ protein was incorporated into an HIV-liposome and used as a booster immunization in HIV-HBc-primed animals, the immunized animals demonstrated the induction of a strong HIV-specific $CD4^+$ T-cell response. The animals immunized with a consecutive immunization strategy were characterized. The analysis revealed enhanced cellular and humoral immunities. The findings thus suggest that the incorporation of the HVJ protein into the HIV-liposome significantly affects immunity in animals primed with HIV antigen encapsulated inside an HBc particle. Furthermore, the present results suggest that the HBc particle-based vaccine seems to be a suitable immunogen for an HIV-1 vaccine; this protocol effectively uses a booster immunization of an HIV antigen incorporated in an anionic HVJ-liposome.

In the present study, the HIV-liposome did not induce an HIV-specific proliferative response in HIV-HBc primed animals. However, the HVJ protein induced an immune response when it was incorporated into an HIV-liposome and used as a booster antigen. Moreover, a comparison of the T-cell proliferative responses inducing activity of both anionic-type and cationic-type HIV-HVJ-liposome demonstrated that the anionic liposome was more effective at inducing such activity than the cationic HIV-HVJ-liposome, when both are administered subcutaneously (data not shown). The effectiveness of the HVJ protein incorporation into the HIV-liposome at inducing the T-cell proliferative cell response does not seem to depend solely on the electrical charge, however. Instead, the effect depended on the route of antigen administration. This assumption was made because when the HIV-HVJ-liposome was administered nasally to mice, the HIV-HVJ-liposome was seen to induce antigen-specific CTLs and neutralizing antibody responses [47]. The different effects of HVJ-anionic and -cationic liposomes allow some inferences to be made about the antigen uptake rate into immune-competent cells. We previously developed a highly efficient method of gene transfer involving the entrapment of RNA or DNA using the HVJ protein to enhance the uptake of genes into target cells [48]. In that method, cationic lipids were used for the preparation of the liposome; the transgene expression level thereby significantly improved in cultured cells using this cationic-liposome gene delivery system [39,49]. Cellular uptake to targeted RNA complexed with an HVJ-cationic liposome was measured to be approximately 5 times higher than that of an HVJ-anionic liposome in cultured cell line cells [49]. In this study, we developed a highly efficient method for antigen immunization by delivering the antigen into cells using HVJ-anionic liposomes in experimental

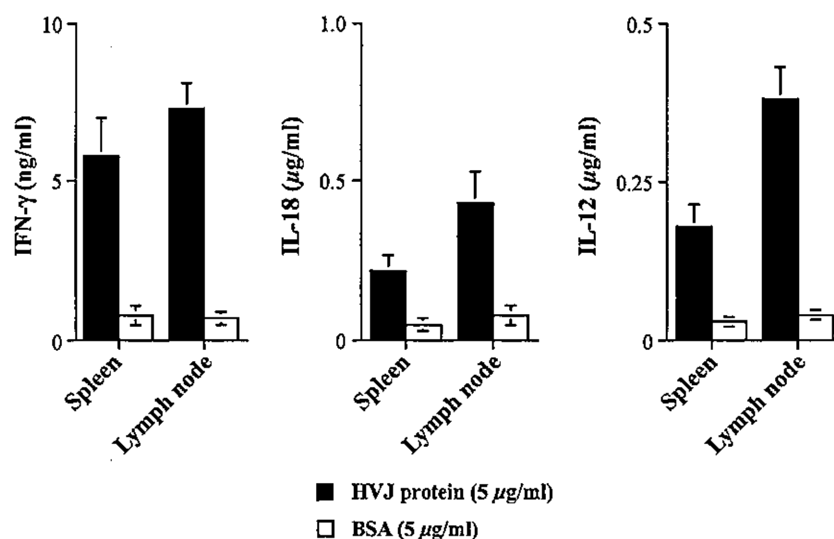


Fig. 7. IFN-γ, IL-18, and IL-12 secretion in HVJ extract-stimulated spleen cells from animals immunized with HBc-HIV priming followed by boosting with HVJ-HIV-liposome. Freshly isolated splenocytes were stimulated with 5.0 μg/ml of HVJ extract or same concentration of BSA for 24 h. Supernatants from separately cultured cells of five different animals were harvested and each cytokine amounts in supernatants were measured by ELISAs. The mean ± SD of three separate experiments is shown.

small animals. According to the present method, the anionic-liposome fused with HVJ was significantly effective at inducing an antigen-specific T-cell proliferative response. In fact, this method proved more effective than the use of antigen-primed animals that had received a booster injection of an HVJ cationic-liposome that was fused with an antigen (data not shown). Collectively, our results indicate that when the induction of antigen-specific T-cell immunity was targeted systemically, the use of an HVJ anionic liposome is suitable, because the antigen may not be localized at the injection site but rather be delivered throughout the body. Furthermore, antigen entrapment was enhanced by the effect of HVJ. However, when the immune induction is targeted locally, the cationic type of HVJ-liposome seems to be more effective as an antigen-delivery system, because the cationic HVJ-liposome effectively fused with the antigen and delivered the target antigen into cells located in a relatively limited region. Our observation also shows that HVJ protein is effective for the induction of an immune response. These findings suggest that enhancement of antigen uptake might be responsible for the induction of effective immune responses.

Concerning the adjuvant effect of the liposomes with HVJ protein, the HVJ protein appears to have the ability to enhance the secretion of immune enhancing cytokines, such as IL-12, IL-18, and IFN-γ, because those cytokines are released form splenocytes by in vitro culture with the HVJ-protein. Our results suggest that IL-18 released from stimulated macrophages may synergistically act with secreted IL-12 to stimulate enhanced production of IFN-γ. Stimulation of spleen cells or T-cell clones with HVJ protein-induced IFNs, TNF-α, and -β in vitro has been reported [50,51]. Thus, stimulation of lymphoid cells by HVJ protein might play a role in the enhanced induction of immunity. Pirhonen et al. [52] reported that the Sendai virus was able to enhance IL-18 level in macrophages, and that the data suggested that indirect immune activation by the effect of IL-18 produced by the stimulation of HVJ protein may also play a role in helper cell induction. This was thought to be because IL-18 can stimulate Th0 cells and promote the differentiation of cells to induce IFN-γ or IL-4 production in the presence or absence of IL-12 [53–55]. This co-stimulation of the immune system by a viral protein or component is commonly seen in the presence of other viruses or bacteria, for example, influenza virus and mycobacterium. The influenza fusion protein was similarly incorporated in fusigenic liposomes and used as a liposome-type adjuvant, namely, Virosome [56]. The advantage of the use of the HVJ protein is that it is known to be a highly fusigenic protein and has even previously been used for cell fusion to produce hybridoma. This method is expected to enhance the uptake of antigens into cells. The results show that repeated inoculations may be acceptable for in vivo use. In conclusion, it seems likely that HVJ protein-incorporated liposomes fused with antigen may enhance antigen uptake to the immunocompetent cells via the HVJ protein. Thus, the HVJ protein may also stimulate helper cells to differentiate and produce cytokines, thus enhancing immune responses.

As described above, the use of the HVJ protein for the preparation of HIV-liposomes allowed us to overcome the difficulty of immune induction induced by immunization of an antigen within the particles. Specifically, we were able to substantially induce a $CD4^+$ T-cell proliferative response. Furthermore, we observed a significant association among the induction of HIV-specific humoral response, and the enhancement of an HIV-specific CTL response due to immunization of HIV-HBc primed animals with the HVJ protein-incorporated HIV-liposome. These marked inductions of immunity are obtained by incorporating the HVJ protein into the anionic HIV-liposome, which were used as a booster antigen in a consecutive immunization regimen. The reason why the HVJ protein was able to induce HIV-specific immunity so effectively in the animal model may be that the consecutive immunization strategy, which employed the HVJ protein, can induce a marked $CD4^+$ T-cell response specific for HIV in the animal. We did not test a group immunized with the HVJ protein mixed with but not incorporated into the HIV-liposome. We speculate, however, that the HVJ protein was incorporated into the HIV-liposomes, and that after the incorporated HVJ peptide and HIV circular V3 peptide were mixed and trapped together on/in the liposome, the mixture was effective. If this is the case, the HVJ protein might be beneficial for forming the protein mixture that will work for the T-cell epitope. This speculation about the mechanism of helper response induction is also supported by the observation that when the HIV-V3 peptide was covalently constructed with an overlapping T-cell epitope peptide, it induced CTL in a non-emulsion adjuvant [57]. When the unlinked but mixed peptides were trapped together, the mixture also worked to some extent in a water-in-oil emulsion adjuvant [58,59]. The significance of the helper T-cell response has been demonstrated in other chronic viral infections [60]. The induction of a $CD4^+$ T-cell response in controlling HIV generation has recently shown that virus-specific T helper lymphocytes are critical for the maintenance of effective immunity in chronic viral infections [11]. The HIV-specific T-cell response is likely to be important in immunotherapeutic interventions [61] and vaccine development [62]. One of the explanations of the role of the $CD4^+$ T cells in CTL induction is that the $CD4^+$ T-cell help was mediated by binding with CD4 ligand on dendritic cells in the expansion of HIV-specific $CD8^+$ memory T-cell responses [63]. In contrast, in the absence of $CD4^+$ T-cell help, adequate CTL activity was not maintained and revealed the persistence of activated virus-specific CTL without effector function [64].

In conclusion, induction of a strong T-cell proliferative response has been obtained in a small animal model by using a consecutive immunization strategy that involved priming with HIV-HBc and boosting with the HIV-HVJ-liposome. Furthermore, it might be demonstrated that an efficacy test for viral challenges may be available.

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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 adminis-tration	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

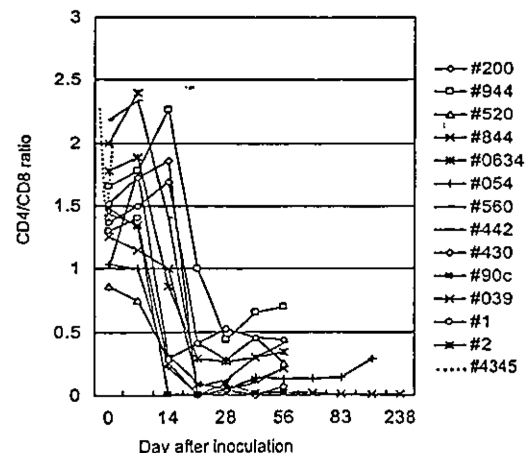

FIG. 1. Changes of CD4/CD8 ratio in monkeys inoculated with SHIV C2/1. All monkeys showed decreased CD4/CD8 ratios between day 14 and day 21 after inoculation. Monkey 4345 had a decrease in CD4/CD8 ratio in the first 24 h. Control monkeys showed only negligible declines in the first 24 h (preinoculation,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

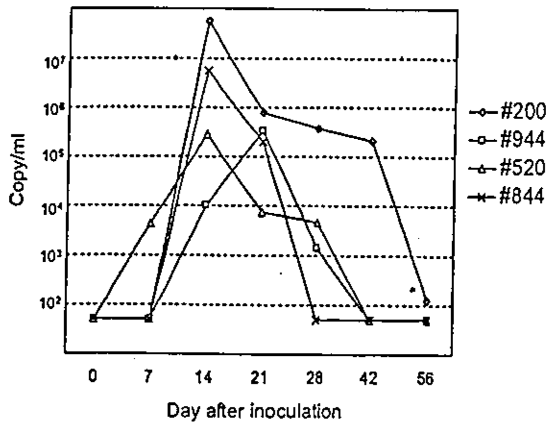

FIG. 2. Plasma viral load in the infected monkeys. Plasma viral RNA of four monkeys (200, 944, 520, and 844) was analyzed by PCR for the presence of the SIVmac239 *gag* region.

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

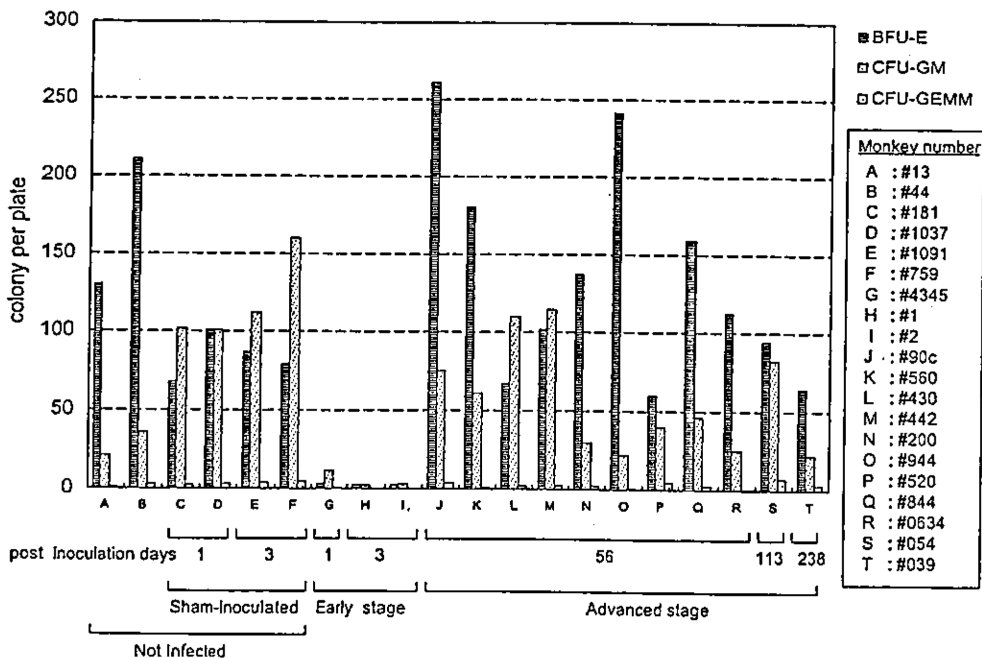

FIG. 3. Colony assay on monkeys inoculated with SHIV C2/1. A period of days 1 through 3 after inoculation was defined as early stage, whereas days 56 through 238 were defined as advanced stage. *P* was <0.005 for CFU-GM, and *P* was <0.02 for CFU-E in comparison of early stage and advanced stage and 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.