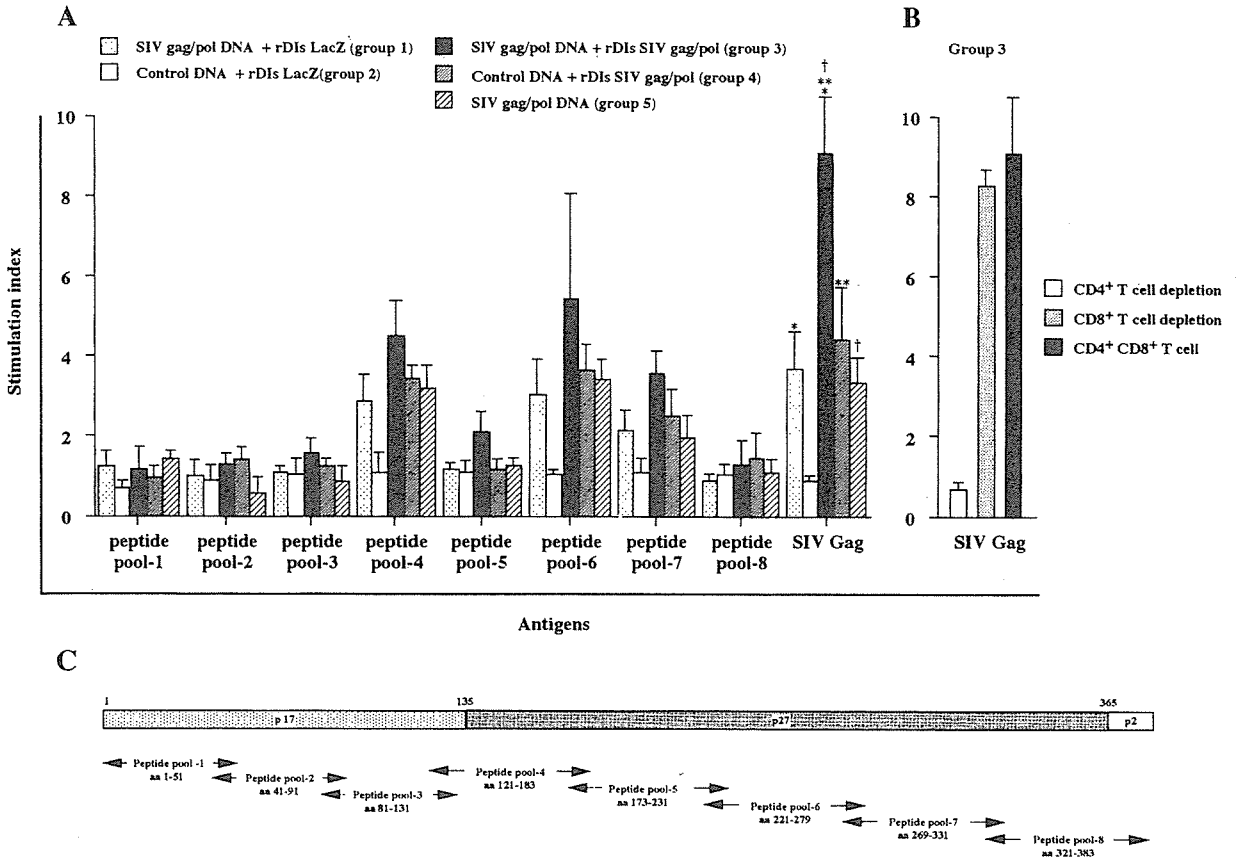

FIG. 3. Induction of SIV Gag-specific lymphocyte proliferative responses. (A) Spleen cells were cultured in the presence or absence of SIV Gag antigens, and [^{3}H]thymidine incorporation was measured as described in Materials and Methods. Proliferative responses are expressed as the SI. (B) Aliquots of spleen cells from mice vaccinated with the prime-boost regimen were depleted of either the CD4$^+$-T-cell, the CD8$^+$-T-cell, or the CD4$^+$ CD8$^+$-T-cell subpopulation prior to measuring SIV Gag-specific proliferative responses (C). Set of 15-mer overlapping peptides spanning the full-length Gag protein of SIV_{mac239}. The peptides were grouped into eight pools to evaluate cell-mediated immune responses. Values indicated by the single asterisk, double asterisks, and dagger symbol all showed a P value of <0.01, which were compared to give the indicated P values between groups 1 and 3, groups 3 and 4, and groups 3 and 5, respectively.

of immune responses induced by a prime-boost vaccination with SIV*gag/pol* DNA and rDIsSIV*gag/pol*, efforts were made to identify distinct antigen-specific CD4$^+$-T-helper-cell subsets. CD4$^+$ T cells were isolated from splenocytes and restimulated in vitro with purified SIV Gag protein. Culture supernatants were then examined for evidence of antigen-specific Th1- or Th2-type cytokine secretion. CD4$^+$ T cells from the immunized mice in group 3 (prime-boost regimen) generated the highest levels of Th1 cytokines, including IFN-γ and IL-2, whereas no evidence was found for the secretion of Th2 cytokines such as IL-4, IL-5, IL-6, or IL-10 (Fig. 4). The levels of cytokines generated by CD4$^+$ T cells from mice belonging to control group 2 were undetectable. These results demonstrate that priming with SIV*gag/pol* DNA, followed by boosting with rDIsSIV*gag/pol*, effectively induces predominantly Th1-type cytokine production in mice.

Characteristics of SIV-specific immunities in immunized animals at virus challenge. The induction of antigen-specific IFN-γ secretion and CTL was also evaluated in the immunized mice. ELISPOT assays were used to measure the number of SIV-specific SFC secreting IFN-γ in splenocytes from the immunized mice in each group (Fig. 5). Cells were restimulated in vitro with either SIV Gag p27 protein or pooled peptides spanning the full-length SIV Gag. SIV Gag-specific SFC were induced in mice receiving SIV*gag/pol* DNA alone, rDIsSIV*gag/pol* alone, or the combined prime-boost regimen. The number of SFC was higher in spleen cells from mice of group 3 immunized with the prime-boost regimen when stimulated with SIV Gag p27 than with Gag protein (735 ± 124 SFC per 10^6 splenocytes) than in those of mice immunized with either SIV*gag/pol* DNA or rDIsSIV*gag/pol* alone ($P < 0.01$). Stimulation of spleen cells of group 3 with whole Gag showed a stronger response than with Gag peptide pools 3, 5, and 8, with ELISPOT activities of 582 ± 121, 532 ± 117, and 394 ± 85 SFC per 10^6 splenocytes, respectively.

To determine whether the prime-boost regimen was able to induce antigen-specific CTL, ^{51}Cr-release assays were performed 1 week after the final inoculation. Spleen cells were isolated and restimulated in vitro for 7 days with each of eight different peptide pools. Cytotoxic activity was evaluated at effector/target (E:T) ratios of 100:1 to 12.5:1. SIV Gag-specific CTL activity was detected after stimulation with peptide pools 3, 5, and 8 (Fig. 6). The highest specific activity was induced by the prime-boost regimen after restimulation with peptide pool

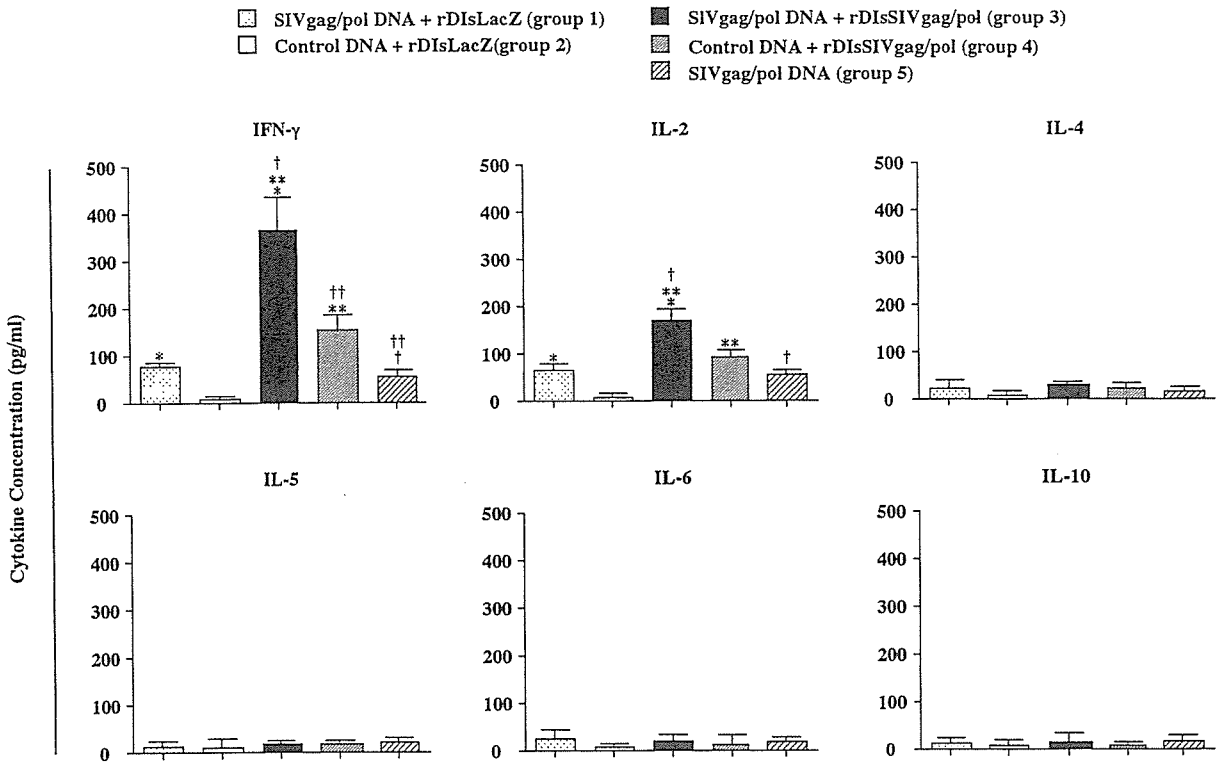

FIG. 4. In vitro production of IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 from spleen cells of immunized mice. Spleen cells were stimulated with recombinant SIV Gag protein, and secreted cytokines were quantified by cytokine-specific ELISA. The single asterisk, double asterisks, and dagger symbol all indicate a P value of <0.01, and the double dagger symbol (‡) indicates a P value <0.05, compared to give the indicated P values between groups 1 and 3, groups 3 and 4, groups 3 and 5, and groups 4 and 5, respectively.

3 (60% ± 11% specific lysis at an E:T ratio of 100:1). In contrast, spleen cells restimulated with peptides from pools 1, 2, 4, 6, and 7 showed little (<20%) or no specific lysis, as did cells from animals vaccinated with control DNA. CTL activity induction paralleled that of IFN-γ-secreting SFC (Fig. 5) and was dependent on the choice of immunizing and restimulating antigens.

We studied whether this regimen also enhances specific antibody responses (Fig. 7). Three consecutive inoculations of SIV*gag/pol* DNA, followed by two of rDIsLacZ in group 1 and five consecutive SIV*gag/pol* DNA vaccinations in group 5, showed low levels of Gag-specific antibody responses, with ELISA titers of <120 throughout the immunization period. Group 4 animals receiving three control DNAs, followed by two rDIsSIV*gag/pol* vaccinations, and group 5 animals with the prime-boost vaccination also elicited low levels of SIV Gag-specific antibody responses with titers of <180, showing that the induction of SIV Gag-specific humoral responses are very low in these vaccination regimens.

Elicitation of positive immunity by the prime-boost vaccine regimen against challenge with the wild-type SIV*gag/pol*-expressing vaccinia virus. Seven days after final vaccination, immunized mice were challenged i.p. with 10^7 PFU of the wild-type vaccinia virus strain vv9019, which expressed SIV*gag/pol*. At 6 days after the viral challenge, the mice were sacrificed, and ovaries were harvested to determine the viral load of the challenge virus in the organs. Among the five vaccinated groups, group 3 mice immunized by the prime-boost vaccine regimen showed a striking inhibition of viral infection into ovaries, with a fold reduction as high as 322 ± 48 (dark column in Fig. 8). The mice immunized with other regimens of group 1, 2, 4, or 5 (Fig. 2) showed fold reductions of 52 ± 23, 4 ± 5, 112 ± 21, and 41 ± 10, respectively, in the virus titer. By comparing the different groups with each other and with naive mice, we defined that the prime-boost vaccine group 3 showed statistically the most significant reduction of the viral load of the wild-type virus in tissues ($P < 0.01$). However, although vaccinated with SIV*gag/pol* prime and rDIsSIV*gag/pol* boost, group 3 exhibited no protection when challenged with wild-type vaccinia virus. The comparable vaccine efficacy to these animal groups were achieved by a second-series immunization experiment with 50 more animals (Fig. 9). These results suggest that vaccination with an SIV*gag/pol* DNA prime, followed by a rDIsSIV*gag/pol* boost, leads to a protective immunity against challenge with wild-type recombinant vaccinia virus in the immunized animals.

Gag-specific responses dominate in the positive immunity induced by the prime-boost regimen at the time of challenge. We then studied the immune responses by differentiating the gag-, pol-, and vaccinia virus antigen-specific responses in respective antigen-specific ELISPOT assays (Fig. 10). At the time of vv9019 challenge, we defined not only SIV Gag- but also SIV Pol-specific T-cell responses in immunized animals in groups 1, 3, 4 and 5. However, the Gag response was remarkably higher than the Pol response in group 3 of the prime-boost regimen ($P < 0.01$) with a less pronounced but similar ten-

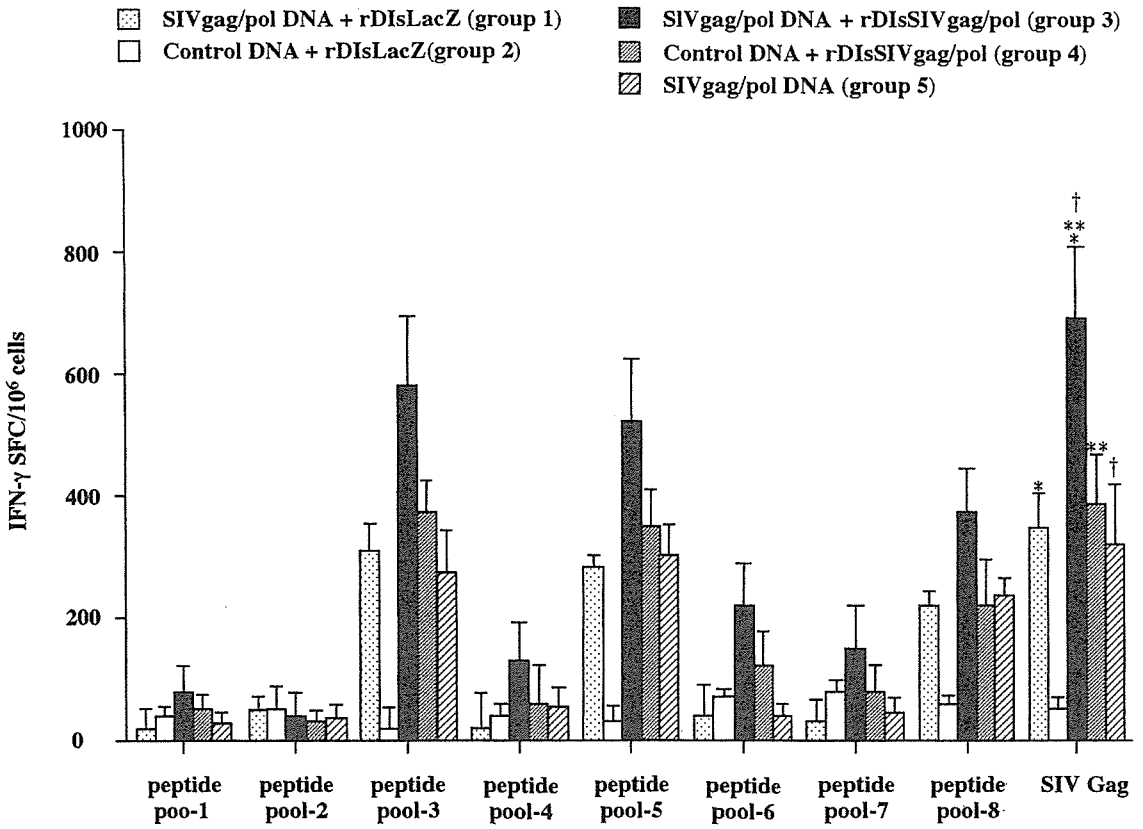

FIG. 5. Frequency of SIV Gag-specific IFN-γ-producing cells in immunized mice. Spleen cells were stimulated with either SIV Gag protein or pooled SIV Gag peptides. IFN-γ-producing cells were detected by IFN-γ-specific ELISPOT assays, and data are expressed as the number of SFC per 10^6 splenocytes. The single asterisk, double asterisks, and dagger symbol all showed a P value of <0.01, which were compared to give the indicated P values between groups 1 and 3, groups 3 and 4, and groups 3 and 5, respectively.

dency seen in groups 1, 4, and 5 ($P < 0.01$). In contrast, vaccinia virus antigen-specific ELISPOT activities were all extremely low, and the positive spots numbered fewer than 80 per million of spleen cells among vaccinia virus- and recombinant vaccinia virus-inoculated groups 2, 3, and 4. The results were not significantly different among the three groups of animals tested, suggesting that the very low levels of vaccinia virus antigen-specific immunity do not significantly contribute to the induction of positive immunity in the prime-boost regimen. These findings demonstrate that the SIVGag-specific T-cell responses dominate in the elicitation of positive immunity induced by the prime-boost vaccine regimen with vaccines expressing SIV*gag/pol*.

DISCUSSION

Previously, we demonstrated that DNA-based vaccination results in the induction of virus-specific immunity to several viral pathogens, including HIV-1 (3, 47, 48, 53, 64, 76). Furthermore, we recently established a system to express HIV-1 genes by inserting them into a deleted region of the attenuated vaccinia virus strain, DIs (25, 30, 71). Like the parental DIs strain, the recombinant DIs-HIV was shown to be completely replication deficient in mammalian cells. Moreover, the expression of SIVGag was sufficient to elicit positive immunity against pathogenic viral challenge in a SHIV-macaque model (26). In the present study, the prime-boost regimen with HIV-DNA and rDIs-HIV clearly enhanced the protective efficacy over that of rDIs-HIV alone or HIV-DNA alone. Although it is not possible to directly compare protective efficacy among different vector-based vaccine models, recombinant vaccinia virus strains (including MVA) (69), a substrain of Copenhagen (NYVAC) (73), and recombinant adenovirus-HIV strains (68), our results appear to be as effective for obtaining protective immunity as those achieved with vector-based vaccines. Taken together, these results suggest that a combination regimen of DNA and rDIs might be used as a safe and effective vaccine.

In the present study, we addressed whether a prime-boost regimen consisting of a plasmid DNA prime and rDIs boost could promote a strong Th1-type immune response capable of affecting the outcome of experimental challenge. It has been proposed that Th1-type responses are associated with protection against infection, including HIV-1 infection and AIDS. Individuals who control HIV-1 viremia in the absence of antiviral therapy respond to HIV-1 Gag protein and its helper epitopes with a Th1-like response, producing IFN-γ and β-chemokines (58). Moreover, a shift from Th1- to Th2-dominant cytokine production occurs during the course of HIV-1 disease progression (36, 39, 65), suggesting that the cytokine profile may be indicative of a T helper phenotype and represent a

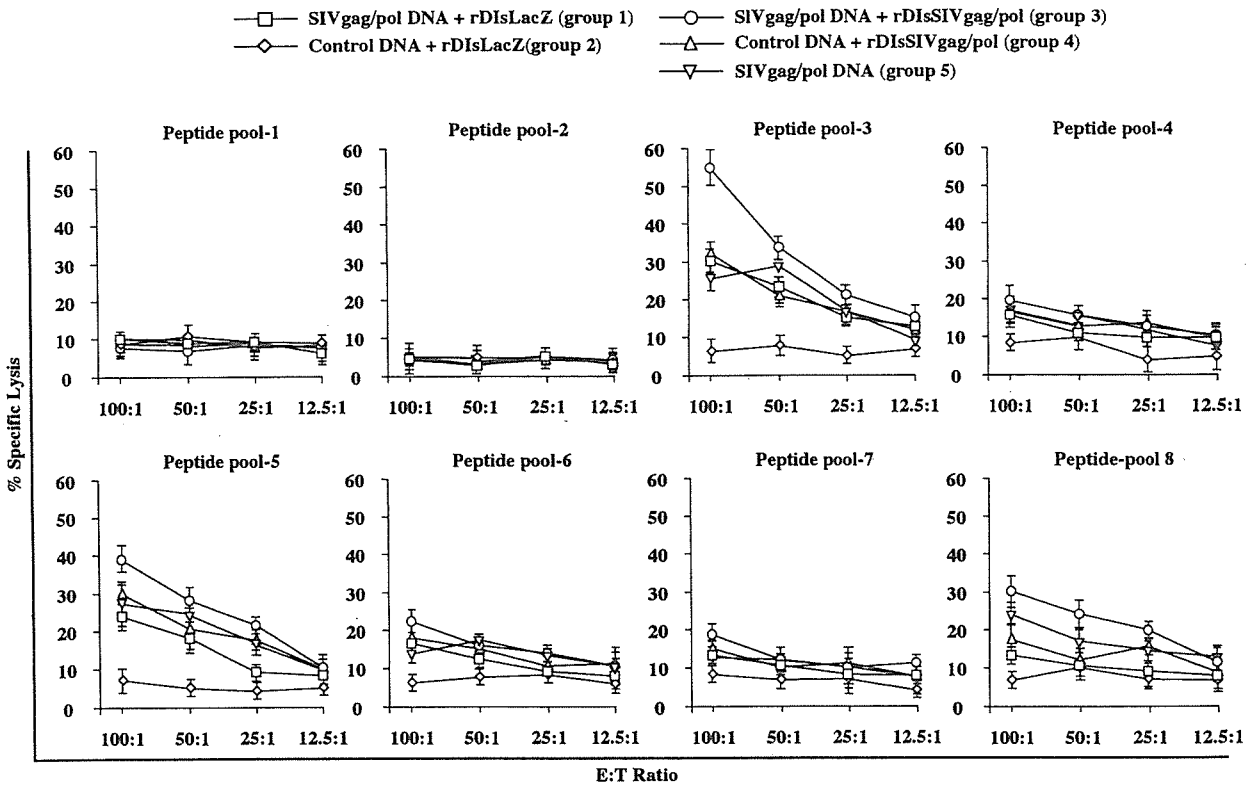

FIG. 6. Induction of SIV Gag-specific CTL in immunized mice. Spleen cells were stimulated with pooled SIV Gag peptides and tested in ^{51}Cr release assays with peptide pulsed M12.4.4 cells as targets cells at E:T ratios ranging from 100:1 to 12.5:1.

response to infection. Our results demonstrate that Th1-type cytokines, including IL-2 and IFN-γ, are secreted by $CD4^+$ T cells from mice immunized with a prime-boost regimen targeting the SIV*gag/pol* region. These Th1-type responses were associated with SIV Gag protein- or peptide-specific SFC activity and $CD8^+$ CTL. These observations are encouraging in light of the hypothesis that Th1-mediated immunity is associated with resistance to HIV infection and virus suppression (6, 31). In vitro restimulation of splenocytes from mice immunized with the prime-boost regimen generated high levels of Th1 cytokines, such as IL-2 (>100 pg/ml) and IFN-γ (>300 pg/ml), and lower levels of Th2 cytokines, such as IL-4, IL-5, IL-6, and IL-10 (<30 pg/ml). In contrast, immunization with either SIV*gag/pol* DNA or rDISSIV*gag/pol* alone led to lower levels of Th1-type cytokine production, suggesting that the prime-boost regimen is superior for the induction of SIV Gag-specific Th1-type T-cell responses.

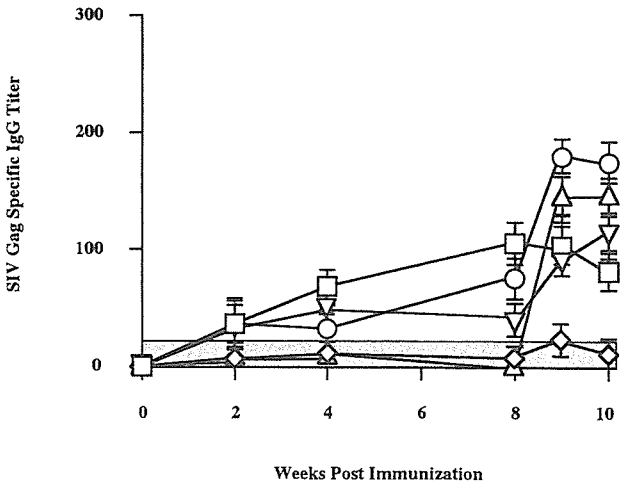

FIG. 7. Kinetics of binding antibody titer specific for SIV Gag in mice. The endpoint titers of immune sera were measured by the SIV Gag p27 antigen-ELISA at each time point. Bars represent the mean ± the SD value of four independent experiments. Symbols: □, SIV*gag/pol* DNA + rDIsLacZ (group 1); ◇, control DNA + rDIsLacZ (group 2); ○, SIV*gag/pol* DNA + rDISSIV*gag/pol* (group 3); △, control DNA + rDISSIV*gag/pol* (group 4); ▽, SIV*gag/pol* DNA (group 5).

Having observed an induction of SIV Gag-specific Th1-type responses in mice after immunization with the prime-boost regimen, we also detected significant levels of virus-specific proliferative responses in spleen cells from the immunized animals. Fractionation of the spleen cell population revealed that the SIV-specific lymphocyte responses were mediated by $CD4^+$ T cells. Several reports have demonstrated that HIV-1-specific $CD4^+$ T-cell proliferation inversely correlates with disease progression in infected individuals (37, 78). Moreover, HIV-1 Gag p24-specific $CD4^+$-lymphocyte proliferation has been shown to be inversely correlated with the HIV-1 load in plasma (58, 59). Although SIV-specific T-cell proliferative responses were induced in mice immunized with either SIV*gag/pol* DNA or rDISSIV*gag/pol* alone, the SI was generally not as high as that obtained by the combined prime-boost regimen. Our data showing the induction of $CD4^+$-T-cell proliferative responses to SIV Gag in mice immunized with the

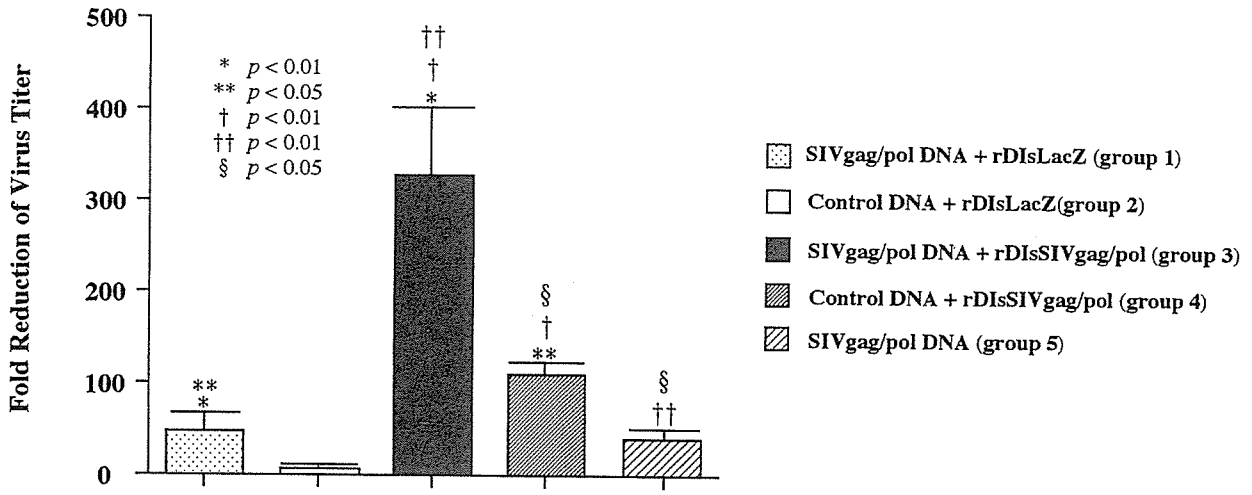

FIG. 8. The prime-boost vaccine regimen augmented protective immune responses. The animals immunized with five different strategies were challenged i.p. with 10^7 PFU of wild-type vaccinia virus strain vv9019 recombinant expressing SIV*gag/pol*. The bar shows the fold reduction of vaccinia virus titer in the ovaries of vaccinated mice versus naive mice. Bars show the geometric mean values of four mice per group.

prime-boost regimen suggests this vaccine approach may be effective at inducing strong virus-specific $CD4^+$-T-cell responses capable of controlling viral load in the immunized animals.

The importance of T helper responses is highlighted in reports that antigen-specific $CD4^+$ T helper cells may promote CTL activity either by a CD4-antigen-presenting cell-CD8 pathway and IL-2 secretion (18, 58, 79) or by an increased production of antiviral cytokines and chemokines. Furthermore, $CD4^+$ T cells promote other types of cell-mediated immunity, including activation of macrophages and cytokine secretion, which may also contribute to the control of HIV-1 and other intracellular pathogens. Recent reports have documented that a vaccine regimen consisting of a DNA prime and a recombinant poxvirus boost generates pathogen-specific protective immune responses (2). The protective role of CTL is also well documented in HIV-1 infection (9, 28, 41, 44, 50, 52, 56, 60, 61, 62), and the induction of an HIV-1-specific CTL population is considered an important goal for most current vaccine strategies. HIV-1 Gag-specific $CD8^+$ cytolysis has been highly correlated with IFN-γ synthesis by $CD8^+$ spleen T cells (49). In the present study, the prime-boost regimen induced significant levels of SIV Gag-specific IFN-γ-producing cells (>700 SFC/10^6 splenocytes). These responses were higher than those induced by immunization with either SIV*gag/pol* DNA or rDIsSIV*gag/pol* alone.

In conclusion, our data show that a new vaccine regimen consisting of SIV*gag/pol* DNA priming and rDIsSIV*gag/pol* boosting induces strong SIV Gag-specific and Th1-type cellular immune responses, which were associated with the control of viral challenge. Since the magnitude and phenotype of the induced immunity are believed to be associated with protection against viral infection and disease progression, this new priming-boosting vaccine regimen may be useful for the develop-

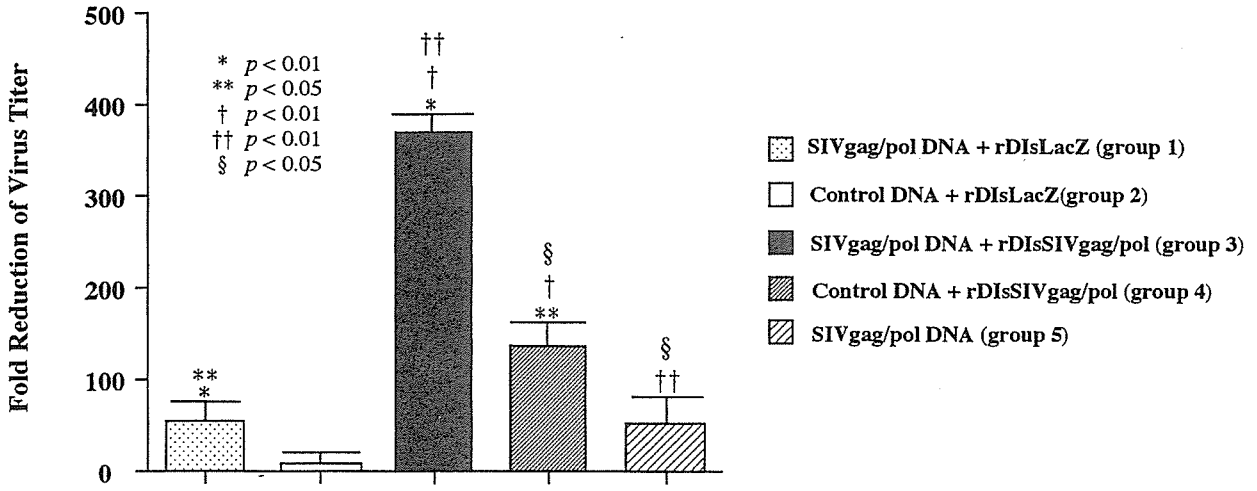

FIG. 9. A second series immunization with the prime-boost regimen resulted in a similar augmentation of protective immune responses. Fifty animals were divided into five groups of 10 animals each, and the animales were immunized by the five different strategies, respectively, described in Fig. 8.

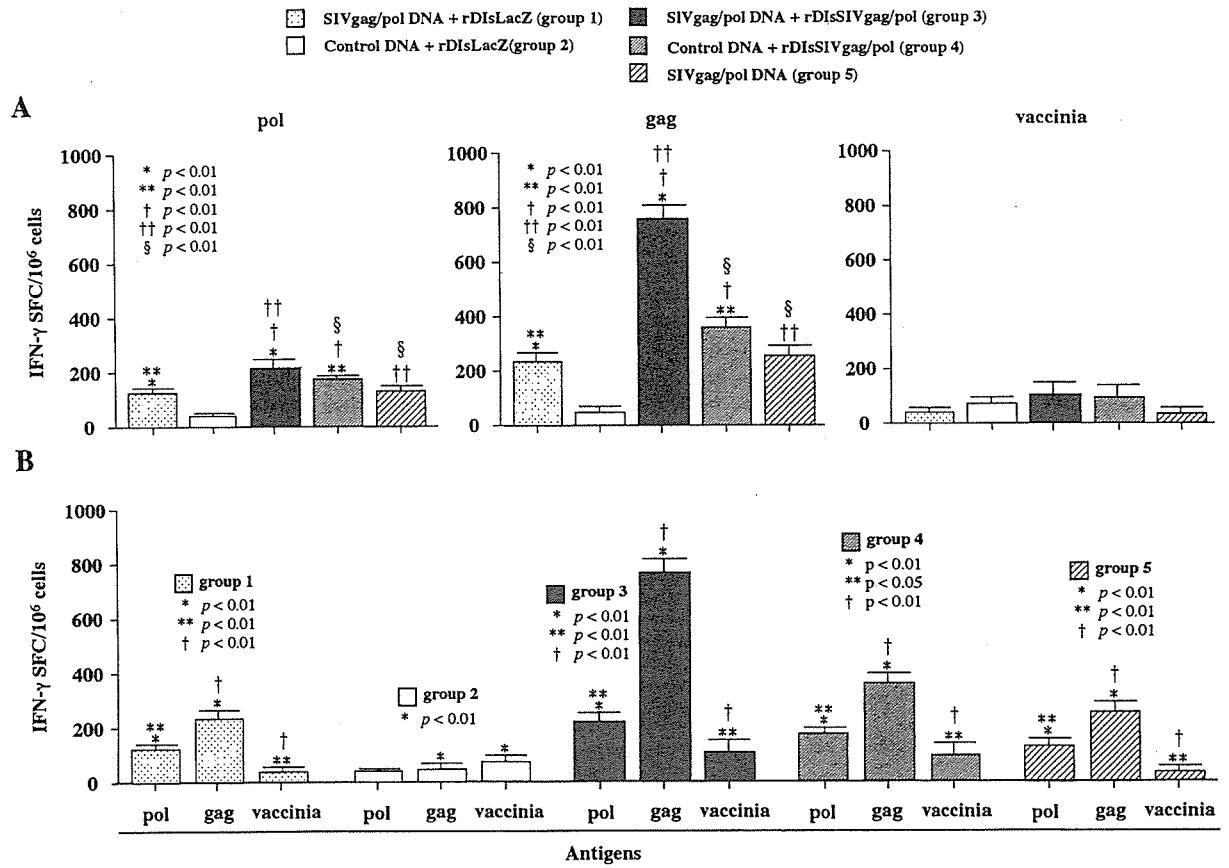

FIG. 10. Comparison of SIV*gag*-, SIV*pol*-, and vaccinia virus antigen-specific immunities in animals at the time of challenge. ELISPOT activities in vaccinated animals with different strategies. Each group of animals was immunized with different strategies and antigen-specific T-cell responses were 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.

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-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 RIHIGPGRAFYTTKN (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

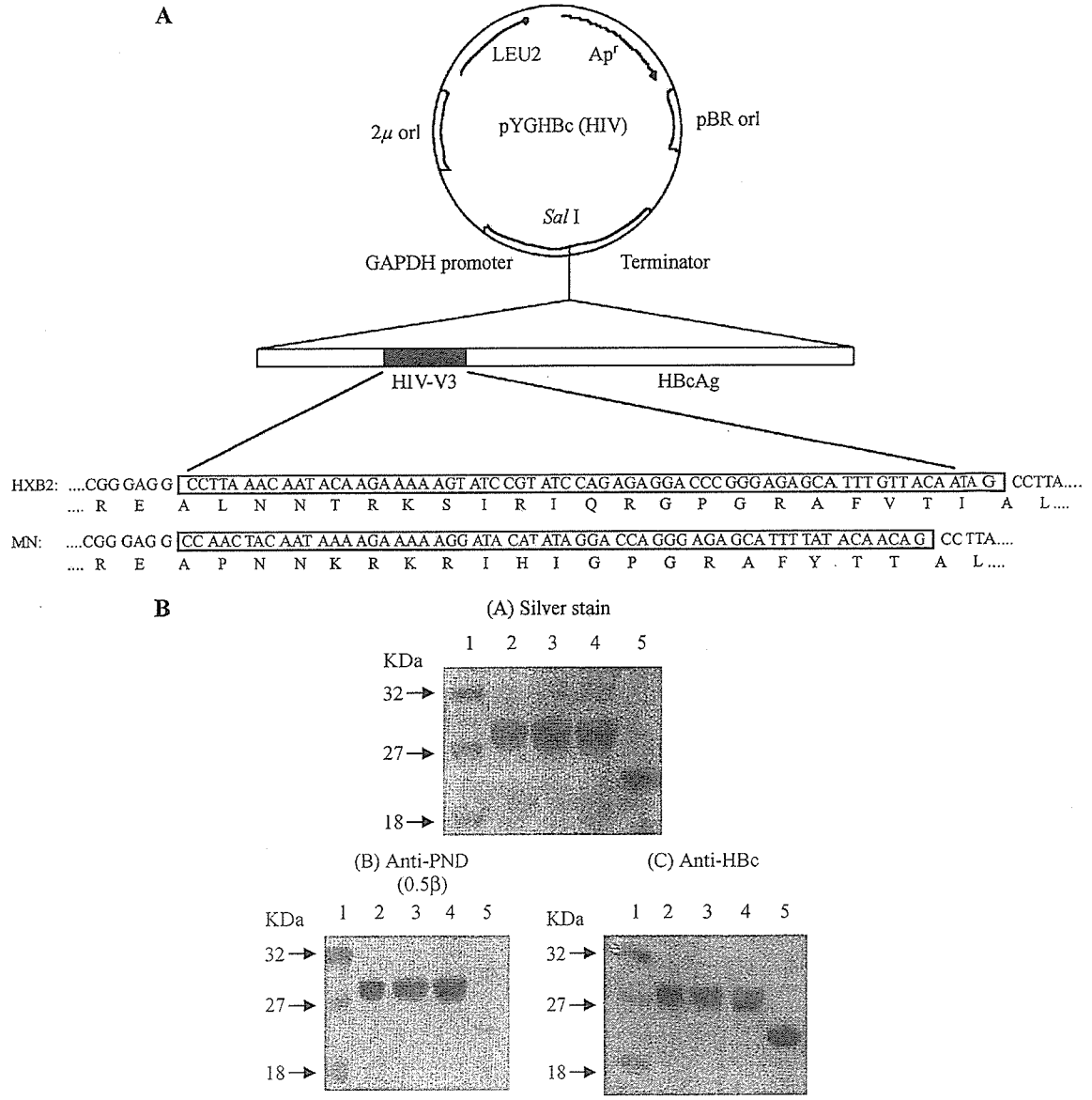

Fig. 1. Vector construction and expression of the HIV-HBc chimeric protein. (A) Vector construction of the HIV-HBc chimeric protein. DNA fragments encoding the V3-tip of the HIV V3 region from HIV-1_{HXB2}, and HIV-1_{MN} were inserted into the *SalI* restriction site in the gene for HBc antigen in plasmid 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-

A. Mouse experiment

(Group)	Week 0	1	2
1.	HIV-HBc (10μg S.C.)	HIV-HBc	
2.	HBc (10μg S.C.)	HBc	

(Group)	Week 0	1	2	3	4
1.	HIV-HBc (10μg S.C.)	HIV-HBc		HIV-HVJ-liposome (10μg S.C.)	
2.	HIV-HBc (10μg S.C.)	HIV-HBc		HIV-liposome (10μg S.C.)	
3.	NBc (10μg S.C.)	HBc		HIV-HVJ-liposom (10μg S.C.)	
4.	HBc (10μg S.C.)	HBc		HIV-liposome (10μg S.C.)	
5.	HIV peptide (10μg S.C.)	HIV peptide		HIV-HVJ-liposome (10μg S.C.)	
6.	HIV peptide (10μg S.C.)	HIV peptide		HIV-liposome (10μg S.C.)	
7.	HIV-HVJ-liposome (10μg S.C.)	HIV-HVJ-liposome		HIV-HVJ-liposome	

B. Guinea-pig experiment

(Group)	Week 0	2	6	10
1.	HIV-HBc (50μg S.C.)	HIV-HBc	HIV-HVJ-liposome (10μg S.C.)	
2.	HIV-HBc (50μg S.C.)	HIV-HBc	HIV-liposome (10μg S.C.)	
3.	HBc (50μg S.C.)	HBc	HIV-HVJ-liposome (10μg S.C.)	
4.	HIV-HVJ-liposome (10μg S.C.)	HIV-HVJ-liposome	HIV-HVJ-liposome	

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].

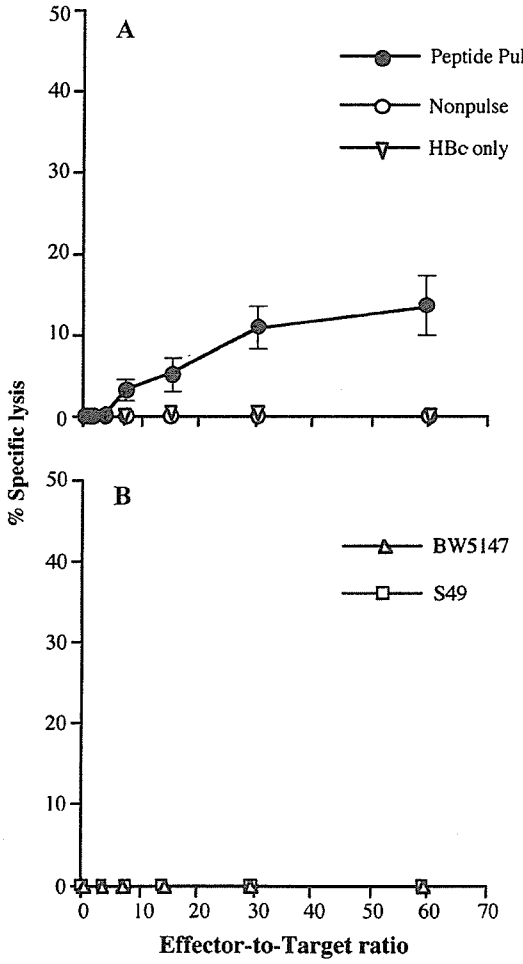

Fig. 3. Immunization of HIV-HBc chimeric antigen induces HIV-specific CTL in mice. (A) Cytolytic activity was measured against M12.4.5 target cells pulsed or not pulsed with the V3-tip peptide. The restimulated spleen cells from HIV-HBc-immunized mice were incubated with ^{51}Cr-labeled target cells 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

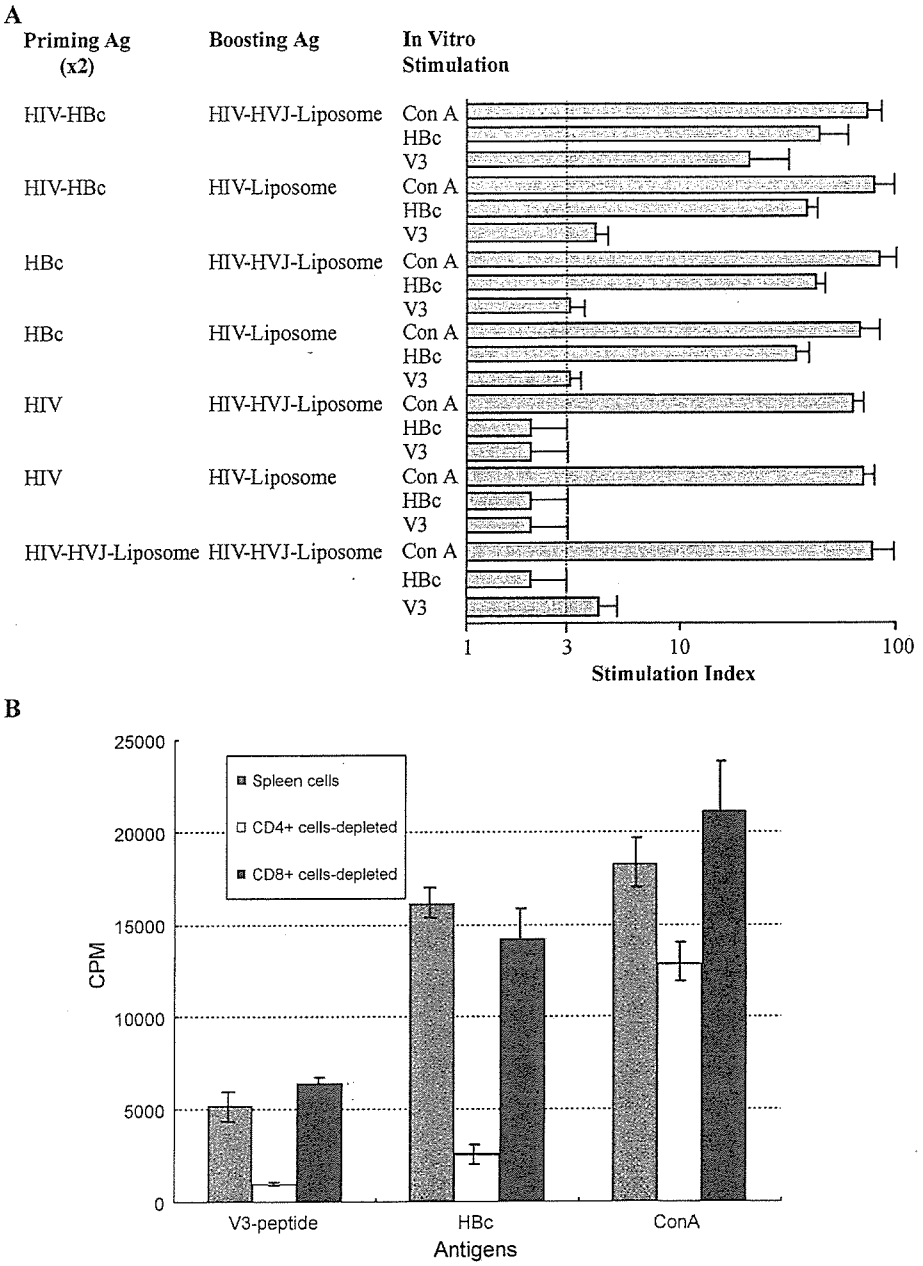

Fig. 4. $CD4^+$ T-cell proliferative responses specific for HIV-1 Env-V3 antigens. (A) The proliferative responses can be induced in animals immunized with the HIV-HVJ-liposome, which incorporated the HVJ protein into the HIV-liposome in a consecutive immunization regimen, involving priming with HIV-HBc 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 $HIV\text{-}1_{HXB2}$ V3 circular peptide, $HIV\text{-}1_{MN}$ V3 circular peptide, HBc protein, or medium alone. SIs are expressed as mean ± SEMs. Because results of SIs obtained by using $HIV\text{-}1_{HXB2}$ V3 circular peptide and $HIV\text{-}1_{MN}$ 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).

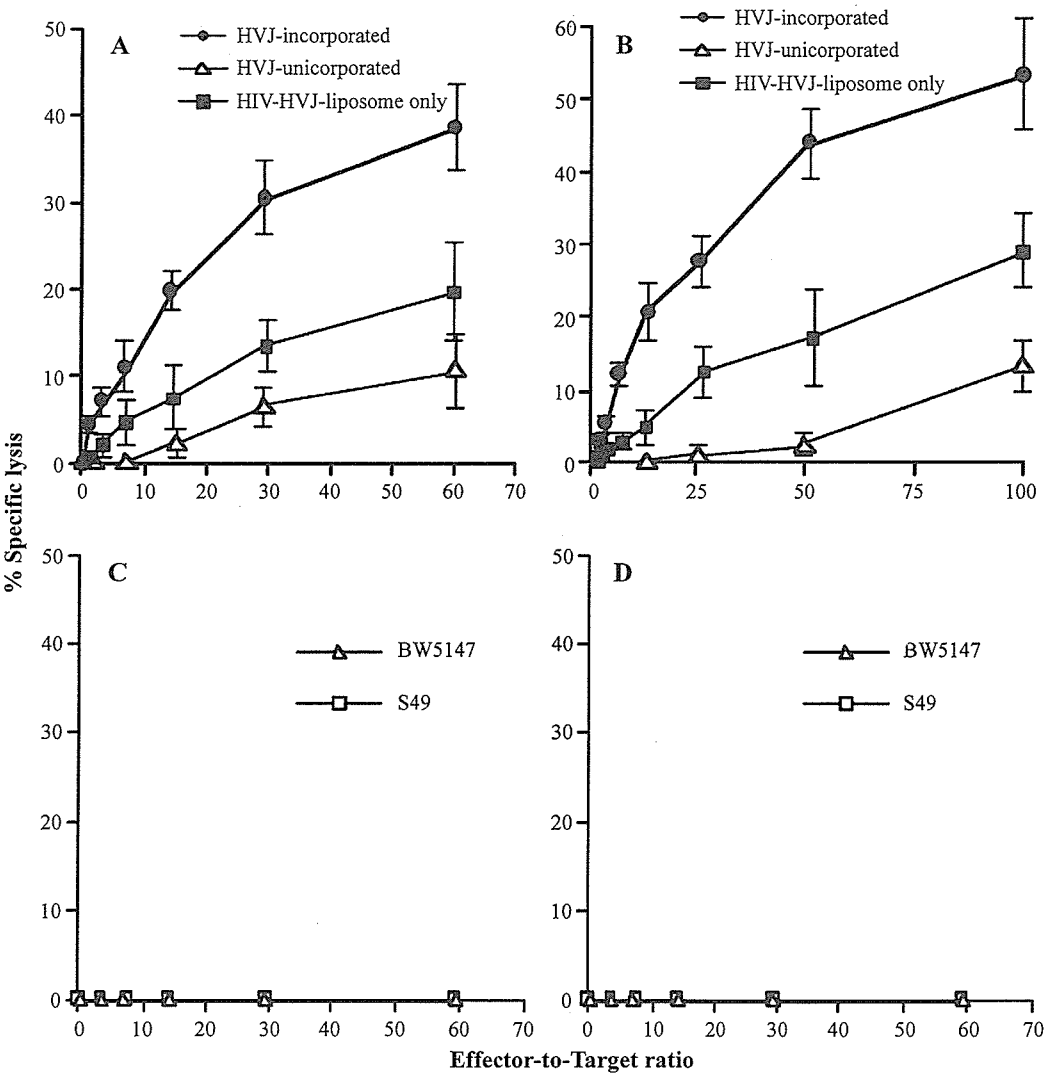

Fig. 5. Effect of the incorporation of the HVJ protein in terms of induction of HIV-1-specific CTL responses in the consecutively vaccinated mice primed with HIV-HBc followed by administration of HVJ-HIV-liposome. (A) Enhancement of HIV-1-specific CTL responses in the consecutively vaccinated mice with 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

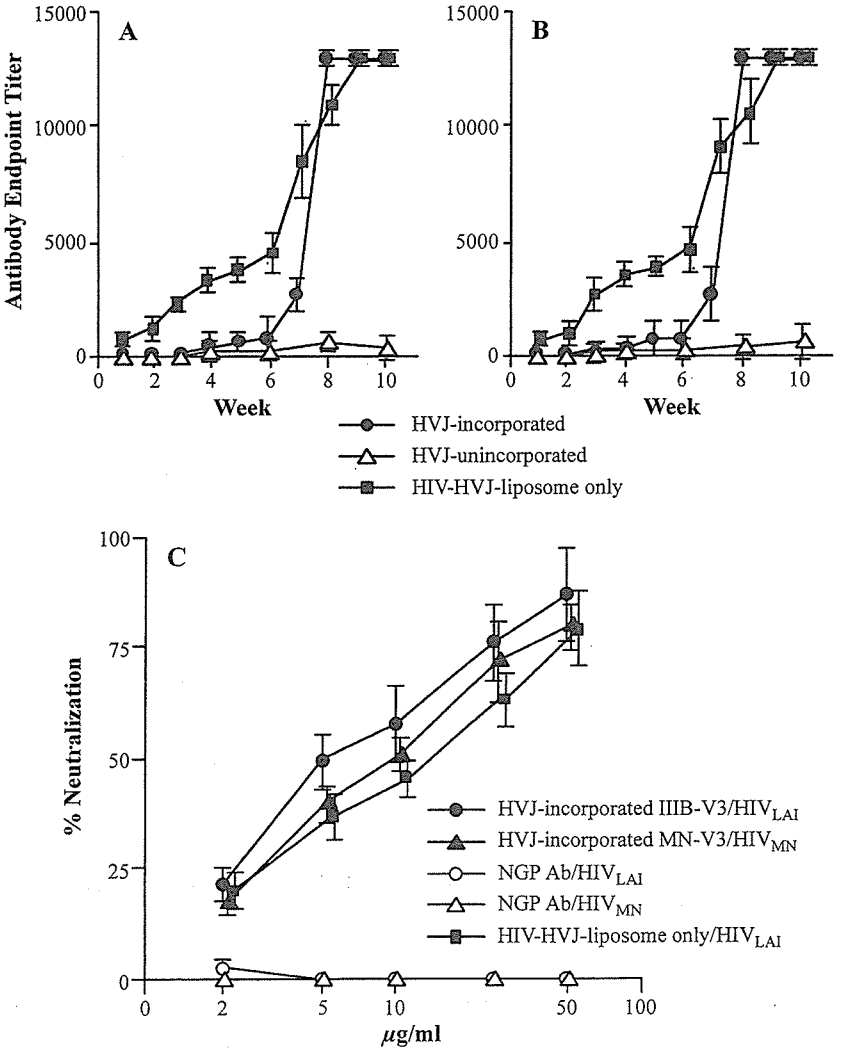

Fig. 6. HIV-1 antibody induction in a consecutive immunization regimen involving HIV-HBc immunization followed by the HIV-HVJ-liposome. HIV-1 antibody titers against HIV_{HXB2} V3 peptide (A) or HIV_{MN} V3 peptide (B) in the immunized animals were measured by ELISA with an endpoint dilution of immune 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; (○) 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

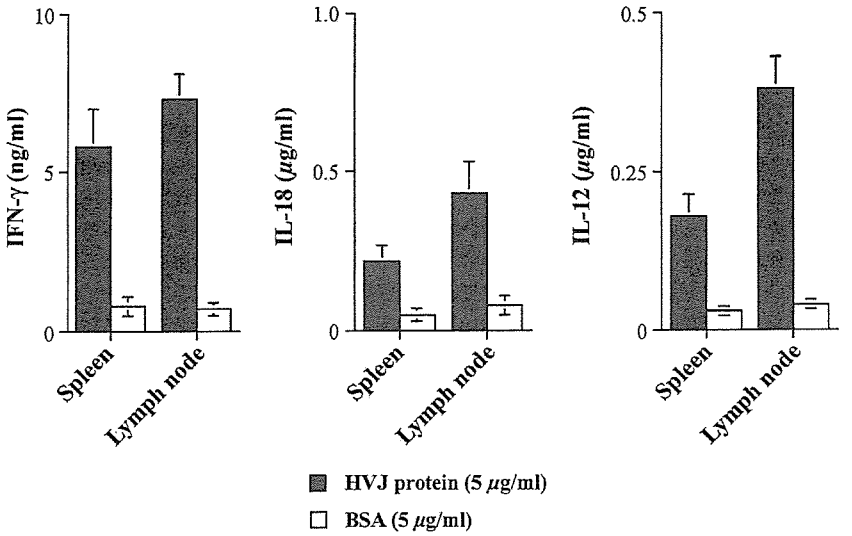

Fig. 7. IFN-γ, IL-18, and IL-12 secretion in HVJ extract-stimulated spleen cells from animals immunized with HBc-HIV priming followed by boosting with HVJ-HIV-liposome. Freshly isolated splenocytes were stimulated with 5.0 μg/ml of HVJ extract or same concentration of BSA for 24 h. Supernatants from 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.

Acknowledgments

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