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Recombinant vaccinia viruses expressing an immunodominant epitope of HIV-1 envelope protein within an influenza hemagglutinin cassette predominantly prime epitope-specific CD8$^+$ CTL

M. Chiba[1,4], **H. Takahashi**[2], **K. Kato**[1,4], **Y. Nakagawa**[2], **T. Fukushima**[2,4], **H. Iinuma**[3], and **K. Nerome**[4]

[1]Medical Development Department of the Nisshin Oil Mills, Ltd., Yokohama, Japan
[2]Department of Microbiology and Immunology, Nippon Medical School, Tokyo, Japan
[3]Second Department of Surgery, Teikyo University School of Medicine, Tokyo, Japan
[4]Department of Virology I, National Institute of Infectious Diseases, Tokyo, Japan

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Summary. We constructed recombinant vaccinia viruses (RVV) expressing a 15-residue peptide (P18IIIB; RIQRGPGRAFVTIGK) of gp160 envelope protein from a human immunodeficiency virus type-1 (HIV-1) IIIB isolate using an H1 influenza virus hemagglutinin (HA) gene cassette. Immunofluorescent tests with antisera against both H1N1 influenza virus and P18IIIB localized chimeric HA molecules comprising influenza virus HA and P18IIIB peptide intracellularly, but the P18IIIB could not be seen on the outer surfaces of infected cells though weak fluorescence was detected regarding HA molecule. Consistent with these findings, Western blotting confirmed the expression of a polypeptide of about 74-kDa protein representing chimeric HA molecule in the infected cells. These recombinants markedly primed CD8$^+$ cytotoxic T lymphocytes (CTL) specific for P18IIIB as well as HA protein of the influenza virus, but failed to elicit P18IIIB-specific antibody despite stimulating production of HA-specific antibody. In addition, the P18IIIB-specific CTL could strongly lyse target cells expressing the whole HIV-1 envelope gene of IIIB strain. Thus, the influenza virus chimeric HA cassette vector system used in the present study appeared to be a useful tool for constructing vaccine candidates which will predominantly prime CD8$^+$ CTL specific for immunodominant determinants of various infectious agents.

Introduction

Clinical studies on HIV-1 positive asymptomatic carriers or persistently infected individuals indicate that cellular immune responses, particularly $CD8^+$ cytotoxic T lymphocytes (CTL), play an important role in preventing both viral infection and disease progression. $CD8^+$ CTL can block HIV-1 entry by secreting chemokines [9] that may bind onto the chemokine receptors, such as CXCR4 [15] or CCR5 [11, 12], and may inhibit viral replication in $CD4^+$ lymphocytes probably by apoptosis or release of so-called cell anti-viral factors (CAF) [25, 41]. Also, Koup and Ho demonstrated that the initial viremia induced by HIV-1 infection is efficiently removed in the presence of HIV-1-specific CTL-precursors before the appearance of neutralizing antibodies [22]. In addition, a report describing seronegative prostitutes with HIV- specific $CD8^+$ CTL who had frequently been exposed to HIV-1 and 2 [28] but remained uninfected, suggests the importance of cellular immunity in conferring protection against HIV. This type of antiviral $CD8^+$ CTL response is also found at high levels in long-term non-progressors [24, 42]. Moreover, clinical studies on asymptomatic HIV-exposed, but otherwise healthy patients have revealed high levels of HIV-specific $CD8^+$ CTL in their peripheral blood mononuclear cells in the absence of unique humoral anti-HIV antibody responses [8, 27, 31]. Furthermore, elimination of $CD8^+$ T lymphocytes from monkeys during chronic SIV infection resulted in a rapid and marked increase in viremia that was again suppressed coincident with the appearance of SIV- specific $CD8^+$ T cells [30]. Therefore, vaccines that will efficiently elicit cellular immunity, in particular virus-specific $CD8^+$ CTL, might be important to prevent HIV infection and disease progression.

Several HIV candidate vaccines that can generate $CD8^+$ CTL, including live recombinants, have been developed [3, 5, 13, 17, 21, 32, 37, 38, 40]. In general, these candidates also tend to elicit strong humoral immunity producing various HIV-1 specific antibodies. It is now evident that active antibodies must bind well to the native, oligomeric gp120/gp41 complex on the surface of virions but not merely attached to purified gp120 in order to neutralize the virus [46]. Thus, to generate actual neutralizing antibodies for controlling HIV-infection by vaccines may be more complicated than expected.

In the present study, we have established an influenza hemagglutinin (HA) cassette which enables to efficiently insert a short length of DNA encoding an epitopic peptide within a variable loop domain of antigenic site A and generated two types of recombinant viruses, RVV-EA2 and RVV-EA4, expressing slightly different chimeric HA proteins with an insert of 15 amino acids, P18IIIB (RIQRGPGRAFVTIGK) [35], derived from an immunodominant CTL epitope of HIV-1 envelope protein in the V3 loop domain. Using these recombinant viruses, we would like to demonstrate their unique feature as immunogens that they could predominantly prime $CD8^+$ CTL response to the inserted gene products.

Materials and methods

Cells and viruses

RK13 and CV1 cells were cultured at 37 °C in Eagle's MEM medium supplemented with 5% fetal calf serum (FCS). Influenza virus A/sw/Ehime/1/80 (H1N2) was cultivated in the allantoic cavity of 11-day-old embryonated hen's eggs. Wild-type vaccinia virus (WR strain) and recombinant vaccinia viruses including vSC25 [6] expressing HIV-1 envelope protein gp160 of the IIIB isolate were expanded in RK13 cells and purified as described [19].

Preparation of recombinant vaccinia viruses

HA-IIIB2 or HA-IIIB4 chimeric genes were constructed by replacing the coding region of aa 137–149 or 125–134 within the a variable loop domain at antigenic site A in influenza HA by a synthesized oligonucleotide encoding a 15 amino acid epitope of HIV-1-IIIB gp160 (315–329) (P18IIIB; RIQRGPGRAFVTIGK) (Fig. 1). Each fragment or the authentic HA gene

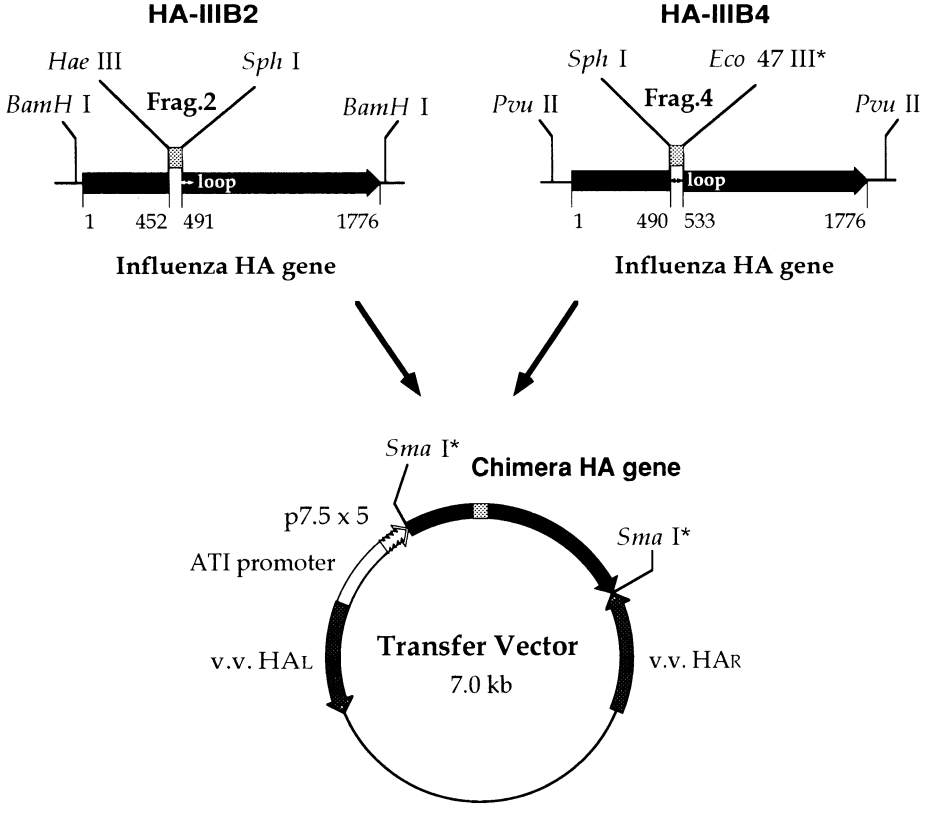

Fig. 1. Establishment of chimeric HA gene plasmid. A 53-mer oligonucleotides coding for a 15 amino acid HIV-1-IIIB gp160 epitope (P18IIIB) and a complimentary oligonucleotides were synthesized then annealed (fragment 2 and 4). Fragment 2 was replaced with the HA gene of A/sw/Ehime/1/80 (H1N2) influenza virus at position 453–490 nucleotides using HaeIII and SphI restriction site of the HA gene to obtain HA-IIIB2. Fragment 4 was also replaced at position 491–532 nucleotides of the HA using SphI and newly constructed Eco47IIIB site by point mutation to obtain HA-IIIB4. The chimeric HA genes were inserted into vaccinia virus transfer vector pSFB5 respectively. *Restriction sites were eliminated after ligation

was ligated into the vaccinia virus transfer vector pSFB5 [16]. The plasmids and the DNA of vaccinia virus (WR strain) were co-precipitated using calcium phosphate and the isatin-b-thiosemicarbazone (IBT^D) variant strain [14], then transfected into CV-1 cells infected with WR vaccinia virus. These homologous viruses were cloned by plaque assay that utilizes hemagglutinin ability and recombinants RVV-EA2 (HA-IIIB2), RVV-EA4 (HA-IIIB4) and RVV-EA0 (authentic HA) were obtained.

Indirect fluorescent antibody test

CV-1 cells were infected with recombinant vaccinia virus RVV-EA0, RVV-EA2, RVV-EA4, vSC25[6] or wild-type vaccinia virus at a multiplicity of infection (MOI) of 10 PFU/cell. 24 h post-infection, the cells were fixed with 2% paraformaldehyde, immersed in 0.05% Triton X-100 for the cells internal staining, then reacted with primary antibodies (anti-A/sw/Hong Kong/1/74 rabbit serum, anti-P18IIIB rabbit serum or anti-HIV-1 gp120 rabbit serum diluted at 1:100) for 1 h, followed by an incubation with secondary antibodies (1:200 dilution of FITC-conjugated goat anti-rabbit IgG) for 40 min. After extensive washing, the cells were examined by fluorescent microscopy.

SDS-PAGE and Western blotting (immunoblotting)

CV1 cells were infected with RVV-EA2, RVV-EA4 or WR vaccinia virus at an MOI of 10 plaque-forming units (PFU) per cell. After 24 h, the cells were lysed with 20 mM Tris-HCl, 0.1% SDS, 1%, Triton-X 100 and sodium desoxycholate. Lysates were resolved by SDS-PAGE on 4 to 20% gradient gels as described by Laemmli [23]. Electrophoretic transfer to a polyvinylidene fluoride membrane (Millipore, MA, USA) was performed using a semi-dry electroblotter at 2.0 mA/cm^2 for 90 min [4]. Proteins were immunodetected as described [39]. In brief, either A/sw/Ehime/1/80 immunized mica sera diluted 1:100 or P18IIIB immunized rabbit sera diluted 1:1500 were added to a blotted membrane and incubated overnight at 4 °C. The membrane was then washed and reacted with either peroxidase labeled goat anti-mouse IgG (KPL, MD, USA) or peroxidase labeled goat anti-rabbit IgG (KPL) diluted 1:1000 for 1 h at room temperature. Bands were visualized by reaction with H_2O_2 and 4-chloro-1-napthol substrates (Nakarai Tesque, Kyoto, Japan).

CTL generation and CTL assay

Immunization

Six–twelve week-old BALB/c (H-2^d) mice were intravenously immunized with 10^7 PFU/mouse of each recombinant vaccinia virus or PBS.

Induction of HIV-specific CTL and measurement of their cytolytic activity

Five weeks after infection, 5×10^6/ml immune spleen cells were restimulated in vitro with 1×10^6/ml mitomycin C (MMC)-treated BALB/c.3T3 fibroblasts expressing gp160 envelope gene of HIV-1-IIIB isolate [35]. The cells were cultured for 6 days in 24-well culture plates containing complete T cell medium (CTM, 1:1 mixture of RPMI 1640 and Eagle-Hank's amino acid (EHAA)) containing 10% heat inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes, and 50 μM2-ME) in presence or absence of 10% rat Con A supernatant (rat T-STIM) (Collaborative Research Inc., Bedford, MA, USA). Then the cytolytic activity of the cells was assayed as described [35] using ^{51}Cr-labeled targets indicated in the figure legends. The percent specific ^{51}Cr release was calculated as $100\times$(experimental release-spontaneous release)/(maximum release-spontaneous release).

Maximum release was determined from the supernatants of cells lysed with 5% Triton-X 100. Spontaneous release was determined from target cells incubated without effector cells.

Induction of HA specific CTL

The remaining spleen cells (5×10^6/ml) from the immune mice used for the HIV-specific CTL assay were restimulated in vitro for 5 days with A/sw/Ehime/1/80 influenza virus infected, MMC treated syngeneic splenocytes (5×10^6/ml) in CTM in presence or absence of 10% rat T-STIM. Cytotoxic activity was measured against ^{51}Cr-labeled M12.4.5 (H-2^d) B cells, infected with A/sw/Ehime/1/80 influenza virus (MOI 10) [18]. The percent specific ^{51}Cr release was calculated as described above.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 10 μg/ml synthetic peptide (RP135; NNTRKSIRIQRGPGRAFVTIGKIGC) [29], or 10 μg/ml of recombinant HIV-1 IIIB gp120 (Immuno Diagnostics, NY, USA), or 10 μg/ml of recombinant HIV-1 MN gp120 (Immuno Diagnostics), or with 1:100 diluted allantoic fluid (HA titer 512) of influenza virus A/sw/Ehime/1/80. The plates were incubated with immune sera for 1 h, washed and incubated with peroxidase-labeled goat anti-mouse IgG (Zymed, CA, USA). Color was developed using 2,2-azinobis (3-ethylbenzthiazoline) sulfonate (ABTS), then the optical density was measured at 405 nm with a microplate reader (Model 450, Bio-Rad, CA, USA).

Results

Expression of chimeric HA molecules in recombinant vaccinia virus infected cells

We examined the expression of the chimeric HA molecules and their biological properties by an indirect immunofluorescence (IF) assay using immune sera to A/sw/ Hong Kong/1/74 and P18IIIB peptide. Antisera to influenza virus (Figs. 2a and 2b) and P18IIIB peptide (Figs. 2c and 2d) showed intensive fluorescence in the cytoplasm of the cells infected with RVV-EA2 or RVV-EA4 treated with detergent (Fig. 2) as compared with controls stained with non immunized rabbits sera (Figs. 2e and 2f). In contrast, cells infected with recombinants but not exposed to detergent (Fig. 3) did not produce fluorescence when probed with P18IIIB immune sera (Figs. 3e and 3f), though weak fluorescence could be seen with influenza immune sera (Figs. 3c and 3d). These results suggest that chimeric HA molecules might be partially transported to the outer surface of the infected cells. However, recombinant vaccinia viruses harboring authentic influenza HA or HIV-1 gp160 genes (RVV-EA0 or vSC25) expressed the respective proteins on the cell surface (Figs. 3a and 3b). In contrast, non-permeabilized cells infected with wild-type vaccinia virus did not show any fluorescence in the cytoplasm or on the surface of the cells against either anti-HA antiserum or anti-HIV antiserum (Figs. 3g and 3h). In addition, Western blotting confirmed the expression of chimeric HA molecules, detected as a single band of approximately 74-kDa in cells infected with RVV-EA2 or RVV-EA4 when probed with the rabbit anti-HA serum (Fig. 4a). In contrast, a relatively weak signal of the chimeric HA protein

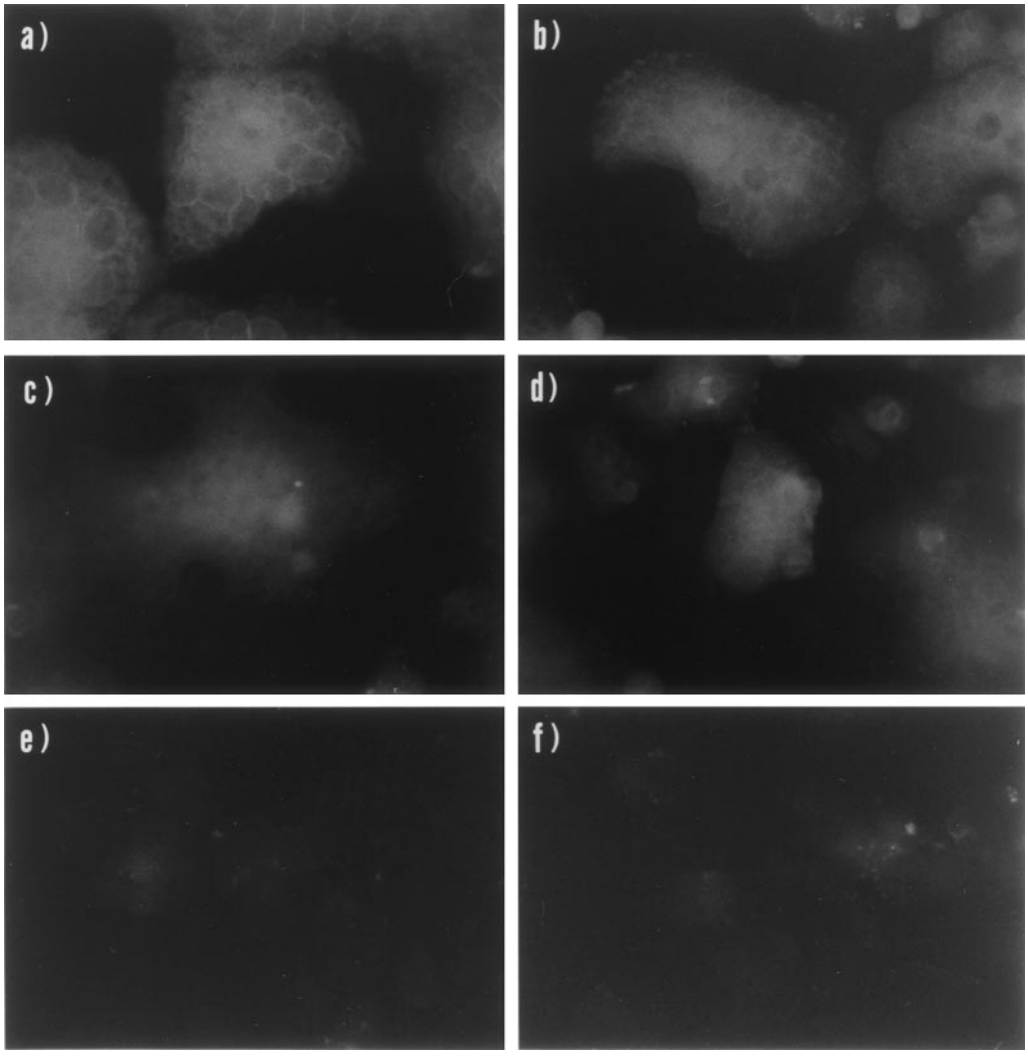

Fig. 2. Intracellular analysis with indirect immunofluorescent staining. RVV-EA2 (**a, c, e**) or RVV-EA4 (**b, d, f**) infected CV1 cells were treated with detergent and then stained with either anti-A/sw/Hong Kong/1/74 (H1N1) rabbit serum (**a, b**), anti-P18IIIB rabbit serum (**c, d**) or normal rabbit serum (**e, f**)

in lysates of RVV-EA4 infected cells was observed when stained with rabbit anti-P18IIIB serum (Fig. 4b).

Priming murine CD8$^+$ CTL specific for HIV-1 IIIB gp160 envelope protein using recombinant vaccinia viruses expressing P18IIIB

We compared the immunogenicity to prime CD8$^+$CTL by the recombinants (RVV-EA2 and RVV-EA4) expressing the chimeric HA encoding P18IIIB gene (HA-IIIB2 or HA-IIIB4), with that bearing the entire HIV-1 IIIB gp160 gene

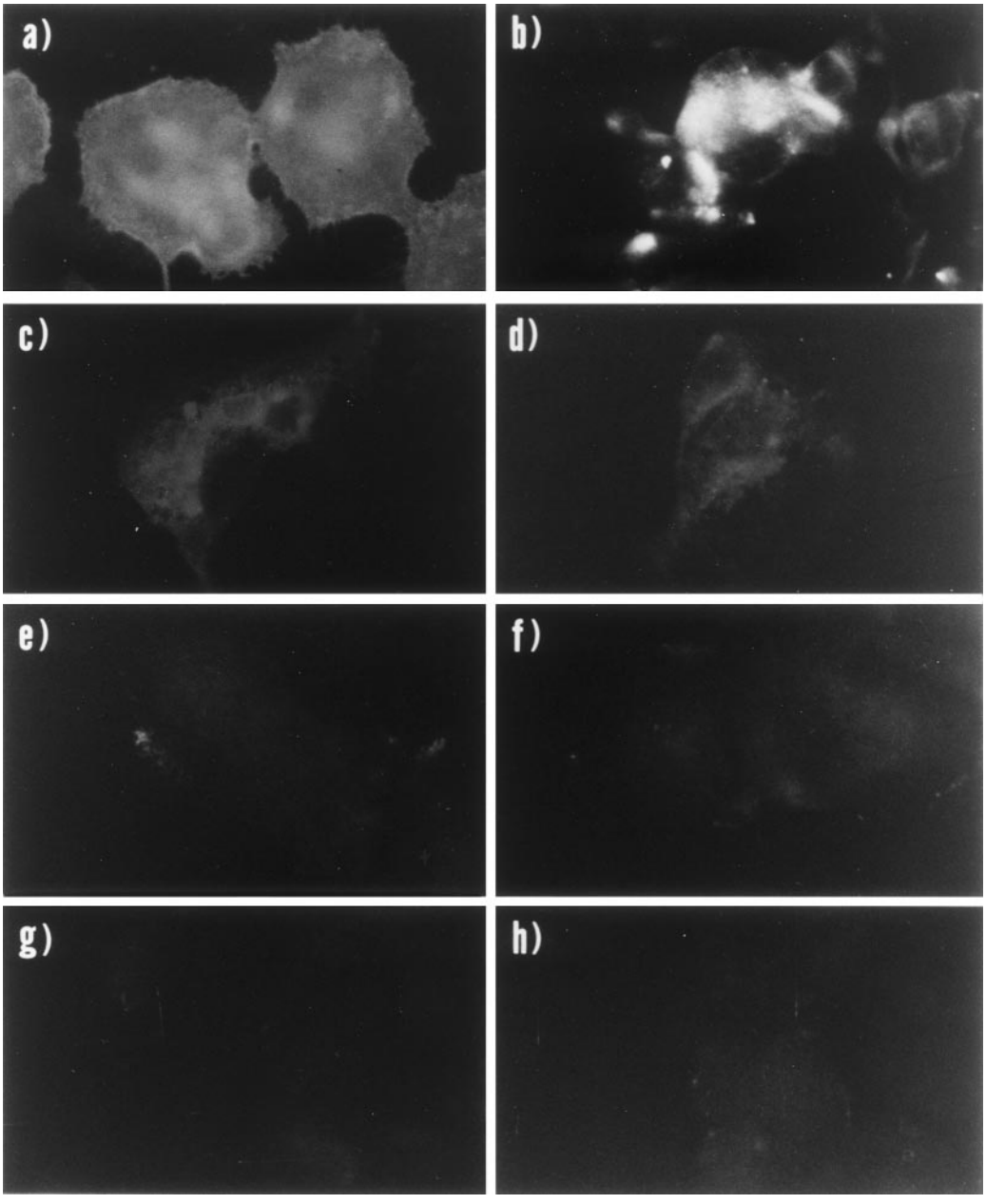

Fig. 3. Cell surface analysis with indirect immunofluorescent staining. RVV-EA2 (**c, d**), RVV-EA4 (**e, f**), RVV-EA0 (**a**) or vSC25 (**b**) or wild-type vaccinia virus (**g, h**) infected CV1 cells were stained with either anti-HA rabbit serum (**a, c, e, g**) or with anti-HIV-1 gp120 rabbit serum (**b, d, f, h**)

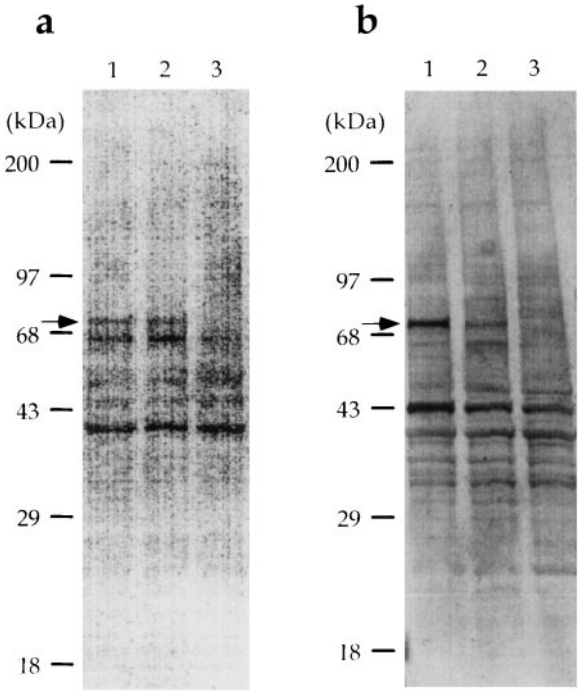

Fig. 4. Western blot analysis of recombinant virus infected CV1 cells. RVV-EA2 (*1*), RVV-EA4 (*2*) or wild-type vaccinia virus (*3*) infected CV1 cells. Whole-cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were developed after incubation with either mouse anti-HA serum (**a**) or with rabbit anti-P18IIIB serum (**b**). Arrows indicate positions of recombinant molecules. The original membranes were scanned and arranged by computer (IX Scan ver. 1.03 for Macintosh; Canon, Japan)

(vSC25). These recombinants were intravenously injected into mice and their CTL activity was examined. Cytolytic activity of the effector CTL generated from RVV-EA2 or RVV-EA4 immunized spleen cells was almost comparable to that from vSC25 against the whole gp160 gene of HIV-1 IIIB expressing BALB/c.3T3 fibroblast targets as well as P18IIIB-sensitized targets (Figs. 5a and 5b). These results demonstrate that the immunodominancy of P18IIIB and that P18IIIB-specific CTL can eliminate both P18IIIB-sensitized syngeneic targets and the whole HIV-1 IIIB gp160 gene-transfected targets. The results also clearly showed that there was almost no difference between RVV-EA2 which harbored P18IIIB in a location on the HA protein (amino acids 125–134) and RVV-EA4 (amino acids 137–149) in capacity for priming the CTL (Figs. 5a and 5b). Moreover, these cytotoxic activities were not significantly enhanced by restimulating 6-day cultures with mitomycin C-treated fibroblasts transfected with the gp160 gene cultured in supernatant medium containing 10% rat Con A (interleukin 2:IL2) (data not shown). Thus, IL2, a typical Th1 derived cytokine, appears to be endogenously produced by the RVV-EA2 or the RVV-EA4 immune spleen cells.

The CTL elicited by either RVV-EA2 or RVV-EA4 recombinant viruses were CD8 positive, because their activity was eliminated by anti-CD8 mAb plus complement, but not by anti-CD4 mAb plus complement or by complement alone (data not shown). Taken together, epitope P18IIIB gene-inserted recombinant vaccinia viruses are capable of priming $CD8^+$ CTL that kill targets expressing the whole envelope gene as well as targets sensitized with P18IIIB, and their ability to prime such $CD8^+$ CTLs appears to be similar to vSC25, expressing the whole HIV envelope gene.

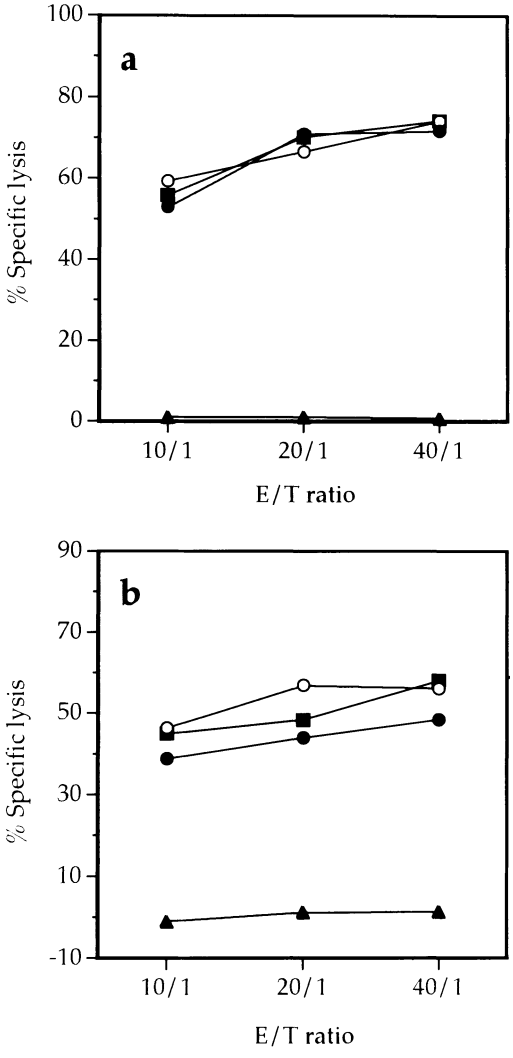

Fig. 5. Induction of HIV specific CTL. BALB/c (H-2^d) mice were immunized intravenously with 10^7 PFU/mouse of RVV-EA2 (●), RVV-EA4 (○), vSC25 (□) or PBS (▲) as a negative control. Cytolytic activity of the effector CTL generated from RVV-EA2 or RVV-EA4 immunized spleen cells and that from vSC25 were compared. Spleen cells (5×10^6/ml) were restimulated in vitro with mitomycin C(MMC)-treated HIV-1 of IIIB envelope gp160 gene transfected syngeneic BALB/c.3T3 fibroblasts (1×10^6/ml). CTL activities were measured against ^{51}Cr-labeled BALB/c.3T3 target cells preincubated overnight with 2μM of peptide P18IIIB (**a**) or ^{51}Cr-labeled gp160 gene transfected BALB/c.3T3 cells (**b**). CTL activities against ^{51}Cr-labeled BALB/C.3T3 targets were also examined in each effector CTL and confirmed negative. Standard errors of the mean of triplicate cultures were always less than 5% of the mean

Induction of CTL specific for influenza HA molecules

Since the chimeric protein constructed in the present study contains influenza HA, specific CTL activity against this protein should also be induced by the recombinants RVV-EA2 and RVV-EA4. Thus, we examined cytolytic activity using spleen cells from mice immunized with the recombinants and the results are presented in Fig. 6. As a positive control, spleen cells collected from mice primed with RVV-EA0 expressing authentic HA were used. The cytolytic activity induced by the recombinant RVV-EA2 was lower than that of RVV-EA4, and the CTL activity of EA0 was the highest over the entire E/T ratio (Fig. 6a). These findings were quite different from those of CTL specific for HIV-1 IIIB, which were similar to those obtained using authentic, complete gp160 of HIV-1. In addition, the HA-specific CTL responses of RVV-EA2 or RVV-EA4 were enhanced by adding 10% rat T-STIM (IL2) at the time of restimulation, however, the CTL levels of EA0 were still the highest (Fig. 6b).

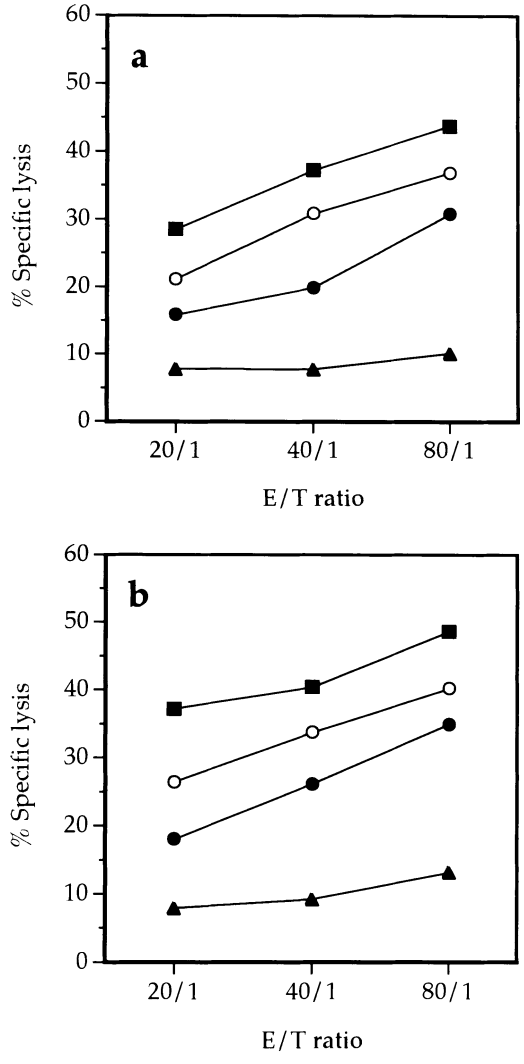

Fig. 6. Comparison of influenza virus specific CTL activities. RVV-EA2 (●) or RVV-EA4 (○) immunized spleen cells were prepared from the same mice used for the HIV assay. Spleen cells collected from mice administered RVV-EA0 (■) and PBS (▲) were used for positive and negative control respectively. Spleen cells (5×10^6/ml) were restimulated in vitro with either A/sw/Ehime/1/80 influenza virus infected BALB/c.3T3 cells (1×10^6/ml) (**a**) or influenza virus infected splenocytes plus 10% rat T-STIM (IL-2) (**b**). After 5 days of culture, CTL activities were measured against ^{51}Cr-labeled M12.4.5 target cells infected with the influenza virus A/sw/Ehime/1/80. Standard errors of the mean of triplicate cultures were always less than 5% of the mean

Humoral antibody responses in immune mice

Although vSC25 (expressing the whole envelope gp160 gene from HIV-1 IIIB isolate) immune serum from BALB/c mice contained a large amount of HIV-1 IIIB V3-specific IgG, pooled sera from the mice immunized with either RVV-EA2 or RVV-EA4 did not show any significant IgG antibody response ($p < 0.05$) to the V3 region using RP135, rgp120IIIB, and rgp120MN as antigens based on a minimum of three sets of experiments (Fig. 7b). In contrast, the RVV-EA2 and RVV-EA4 immune sera represented anti-influenza HA-specific IgG responses to some extent such that the activity was not as strong as that in RVV-EA0 primed mice, whereas the vSC25 immune sera did not produce any detectable level of anti-HA-specific IgG antibody response as expected (Fig. 7a). Thus, the newly established recombinant vaccinia viruses, RVV-EA2 and RVV-EA4, might not generate specific antibody responses to the inserted portions within the HA cassette.

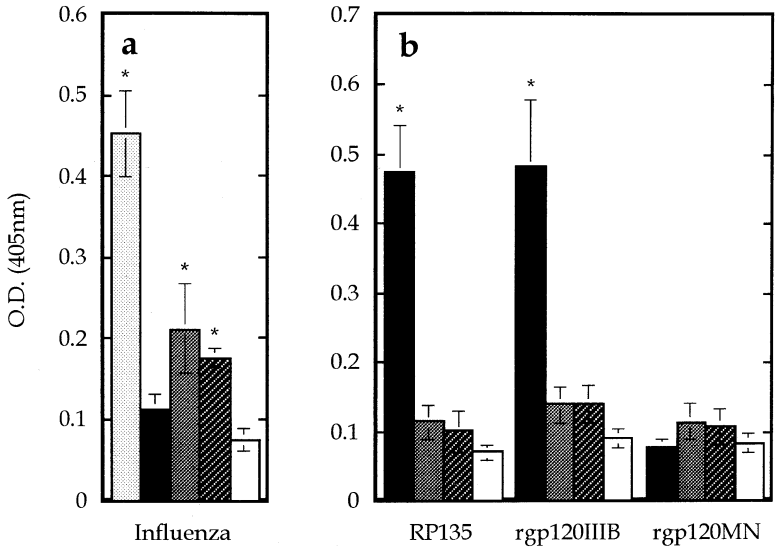

Fig. 7. Measurement of IgG antibody responses by ELISA. Plates were precoated with either 10 μg/ml synthetic peptide RP135 (NNTRKSIRIQRGPGRAFVTIGKIGC), or 10 μg/ml rgp120 IIIB, or 10 μg/ml rgp120 MN, or with 1:100 diluted allantoic fluid of influenza virus A/sw/Ehime/1/80 at 4 °C overnight. The plates were blocked with PBS containing 1% bovine serum albumin, then pooled sera from BALB/c mice immunized with either RVV-EA2 (▩), or RVV-EA4 (▨), or vSC25 (■), or RVV-EA0 (▣) or unimmunized control (□), were added to the plates for measuring their IgG antibody responses. The sera were diluted at 1:10 (**a**) or 1:30 (**b**) in each experiment. Data represent the mean ± standard deviation for three independent assays. *$P < 0.05$ compared with control serum

Discussion

The antigenic drift of influenza hemagglutinin caused by the accumulation of point mutations and associated mechanisms has been thoroughly investigated in terms of three dimensional structure [43, 45]. Amino acid substitutions frequently occur in the globular head domains of HA molecules composed of four distinct antigenic sites A, B, C and D [10, 44]. These domains seem to be less functionally constrained from the viewpoint of tertiary structure suggesting that these regions will be suitable for inserting small foreign epitopes such as peptide P18IIIB from the HIV-1 envelope protein and HA molecules of the influenza virus. We confirmed that two chimeric HA genes containing the HIV-1 IIIB gene were expressed in CV1 cells infected with recombinant vaccinia viruses (RVV-EA2 and RVV-EA4). However, the chimeric proteins produced by these recombinants were localized in the cytoplasm of the infected cells and they were not completely transported to the outer surface of the cells.

The recombinant vaccinia viruses, RVV-EA2 and RVV-EA4, containing the HIV derived 15-mer epitope P18IIIB within antigenic site A of influenza HA, strongly primed $CD8^+$ CTL specific for P18IIIB pulsed target cells as well as transfected target cells expressing the whole HIV-1 gp160 envelope glycoprotein. These high activity levels corresponded to those elicited by a recombinant

vaccinia virus expressing all epitopes of gp160 (vSC25) [6]. Also, the immune responses were further characterized by the absence of HIV-1 IIIB envelope-specific antibody responses either to the linear V3 peptide (RP 135) or to the conformational gp120 protein (rgp120IIIB) in the sera of mice immunized with RVV-EA2 or RVV-EA4. As far as we have done several intensive experiments, we could not detected any measurable amount of V3 or gp120-specific antibody responses in that sera. However, when recombinant vaccinia virus expressing the whole HIV-1 gp160 gene (vSC25) was applied as the immunogen, substantial humoral immune responses to the same antigens were elicited. Thus, the recombinant viruses described in the present study may predominantly prime $CD8^+$ CTL specific for the inserted site within the loop of HA cassette. These results are similar to our previous findings using P18IIIB-pulsed irradiated syngeneic dendritic cells as the immunogen; P18IIIB-specific $CD8^+$ CTL were predominantly primed [38].

Kalyan et al. demonstrated that chimeric HA protein carrying a 22 mer HIV-1) MN envelope V3 epitope peptide in site A elicited neutralizing antibody against a homologous strain of HIV-1 [20]. Li et al. have described a chimeric influenza virus having the HIV-1 MN envelope V3 gene at positions 312–323 corresponding to 12 amino acids in site B of the HA globular head domain, which had neutralization activity against the MN strain, as well as MN epitope-specific CTL in mice immunized with the virus [26]. These reports indicate that recombinant chimeric HA protein can induce both antibodies specific for the inserted fragment and cellular immune responses to an inserted V3 fragment. Our results differed from theirs in terms of antibody production towards the inserted epitope. Also, they are contrary to the above reports in that CTL activity was predominantly primed by the newly made two recombinants. This may be a favorable effect for the vaccines to HIV-1, since cellular immune responses seem to be more important for preventing early viral spread and disease progression.

Although P18IIIB-specific antibodies were undetectable in the sera of mice immunized with either RVV-EA2 or RVV-EA4, the sera included antibodies specific for HA to some extent. The titers of HA-specific IgG were lower than that of the sera of mice immunized with RVV-EA0 which did not contain an inserted fragment encoding P18IIIB in the HA cassette. These results are compatible with the level of surface expression of the chimeric HA product detected by immunofluorescence. Inserting a P18IIIB coding fragment may affect normal expression of the HA product on the surface of virus infected cells by partially changing the conformational structure of HA. The expression of recombinant molecules corresponding to the influenza HA polypeptide produced by RVV-EA2 or RVV-EA4 infection was confirmed by Western blotting of cells infected with the recombinant virus, with either mouse anti-HA antiserum or with rabbit anti-P18IIIB antiserum. It should be noted that the amount of HA-specific IgG production in the sera from mice immunized with RVV-EA2 was lager than that from RVV-EA4 immunized mice, whereas the stronger HA-specific CTL activity was found in immune spleen cells from the latter, than that from the former in three separate experiments. These results may have some relation to the lower produc-

tivity of chimeric HA protein in RVV-EA4 infected cells than in RVV-EA2 cells as observed in the Western blotting analysis. Thus, humoral antibody responses and cellular CTL responses toward HA protein seem to be cross-regulated in these immune mice.

Endogenously synthesized viral proteins are fragmented for processing inside of the cell and are presented on the surface in association with class I MHC molecules for recognition by T lymphocytes bearing CD8 molecules. Our results indicate that processed influenza HA fragments and P18IIIB are expressed on the cell surface within class I MHC molecules and cannot directly bind to their specific antibodies. Nevertheless, HA molecules were detectable with specific anti-HA antibodies on the surface of virus-infected cells by indirect immunofluorescent staining, though we failed to see P18IIIB using its specific antibody. This may be because internally synthesized influenza HA protein can be delivered by two ways. One route is the regular processing steps through the endoplasmic reticulum and the other is direct delivery to the cell surface for either secretion or assembly of viral components. The regularly processed fragment is presented in conjunction with class I MHC to be recognized by, and prime $CD8^+$ CTL, whereas the directly delivered product is captured by antibody-bearing B cells or macrophages and presented on the cell surface in association with class II MHC molecules, which may stimulate $CD4^+$ helper T cells to elicit humoral immune responses to produce specific antibodies. Since the amount of interleukin-2 in the culture supernatant of the restimulated RVV-EA2 or RVV-EA4 primed spleen cells was measurable (data not shown), class II MHC molecule-restricted Th1-type helper T cells may also be primed in these immunized animals. Therefore, such viral products together with HA and P18IIIB may stimulate both class I-restricted $CD8^+$ CTL and Th1-type of class II restricted $CD4^+$ helper T cells [36]. We are currently investigating the existence of HA or P18IIIB-specific Th1-type cells in primed spleen cells.

We have also found that the P18IIIB (amino acids 315–329) region appeared to be a highly promiscuous epitope that is presented by at least four different class I MHC molecules ($H\text{-}2^d$, $H\text{-}2^P$, $H\text{-}2^u$, and $H\text{-}2^q$) [33, 34] in mice out of 10 MHC types tested and at least three major class I MHC molecules (HLA-A2, A3, A11) [1, 2, 7] in humans. Thus, we might overcome the barrier of MHC restriction in most individuals by combining several promiscuous epitopes in this cassette system. Using recombinant vaccinia viruses expressing the promiscuous epitope, P18IIIB, within a HA cassette system, we have demonstrated here unique features of the recombinants that could predominantly prime a cellular immune response without enhancing a humoral response to the region. However, these live recombinant viral vectors may still have some unfavorable side effects as candidate vaccines particularly for immunocompromised hosts to enhance their immunity. Based on our previous findings [18], we are currently generating muramyldipeptide (MDP)- liposome bearing influenza hemagglutinin and neuraminidase containing HA cassette protein inserted P18IIIB and other immunodominant epitopes to establish a non-infectious safer vaccine candidate for HIV-1. Therefore, the influenza virus chimeric HA cassette vector system shown here may provide a useful tool for vaccine development for both protection from

viral infection and prevention of its disease progression such as HIV infection by predominantly enhancing cellular immunity.

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Authors' address: Dr. M. Chiba, Medical Development Department of the Nisshin Oil Mills, Ltd., 1-3 Chiwaka-cho, Kanagawa-ku, Yokohama 221-0 036, Japan.

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