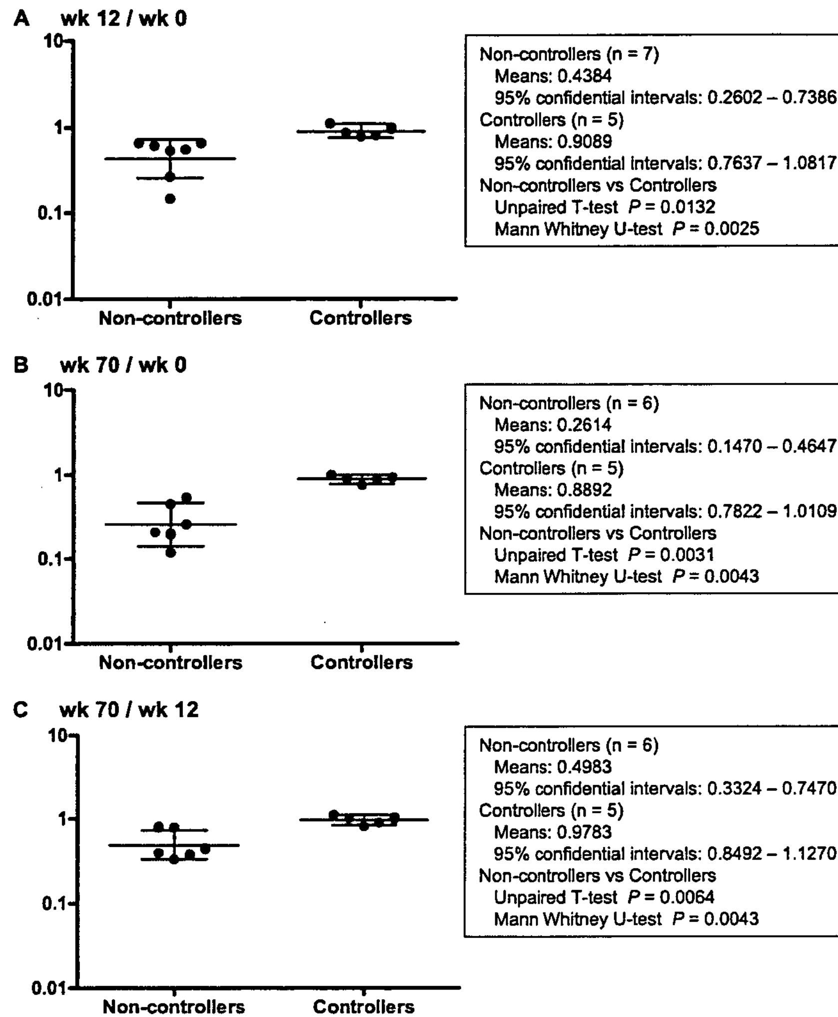

FIG. 6. Statistical analysis indicating preservation of central memory $CD4^+$ T-cell counts in the controllers. The ratios of central memory $CD4^+$ T-cell counts at week 12 to week 0 (A), week 70 to week 0 (B), and week 70 to week 12 (C) in the noncontrollers (except for rapid progressor V2 in panels B and C) and the controllers are plotted. The longer bars indicate geometric mean values, and the regions between the shorter bars indicate the 95% confidential intervals. Statistical analysis was performed with the t test and nonparametric Mann-Whitney U-test using the Prizm software.

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controllers (10). In contrast, Gag-specific CTL responses became undetectable and SIV non-Gag-specific CTL responses, instead, became predominant in macaques V6 and V8. The results obtained from a $CD8^+$ cell depletion experiment are consistent with involvement of these SIV non-Gag-specific CTL responses in the long-term viral control in both sustained controllers, although there might be involvement of other components, such as NK and $CD4^+$ memory T cells. Thus, it can be speculated that vaccine-based control of primary SIV replication can preserve the ability of the immune system to elicit functional CTL responses, leading to reinforcement or adaptation of protective immunity by postchallenge induction or expansion of effective CTL responses. This may contribute to stable viral containment in the chronic phase.

In the natural courses of HIV and SIV infections, the infected hosts exhibit acute, massive depletion of $CCR5^+$ $CD4^+$ effector memory T cells from mucosal effector sites, and the chronic immune activation with gradual immune disruption that follows leads to AIDS (7, 15, 20, 25). The former acute memory loss may influence the latter chronic disease progression (25, 26). The acute depletion results in compromised immune responses at the effector sites and systemic proliferative responses that partially compensate for the loss of mucosal memory $CD4^+$ T-cell populations. Recent reports indicating amelioration of acute mucosal memory $CD4^+$ T-cell depletion and associated central memory $CD4^+$ T-cell loss in the early phase by CTL-based vaccines have suggested that vaccine-based amelioration of acute memory $CD4^+$ T-cell depletion in mucosal effector sites can delay AIDS progression (13, 19, 35). However, this acute memory $CD4^+$ T-cell depletion is not the only cause of chronic disease progression and persistent viral replication-associated immune activation may be responsible for chronic immune disruption leading to AIDS (7). Indeed, in both of the transient controllers, V3 and V5, central memory $CD4^+$ T cells were preserved during the initial, transient period of viremia control but decreased after the reappearance of plasma viremia. This suggests that there may be an association between persistent viral con-

tainment and central memory $CD4^+$ T-cell preservation, even in the chronic phase.

Theoretically, protection by CTL-based AIDS vaccines is likely to be nonsterile, and it will be difficult to contain viral replication completely. Additionally, CTL-based viremia control would require CTL activation. Indeed, our $CD8^+$ cell depletion experiment indicated that persistent viral replication was inefficient but not completely contained in the absence of plasma viremia in sustained controllers V6 and V8. Transition of recognition of CTL epitopes from Gag to other non-Gag proteins in the chronic phase suggests that these "new" CTLs were either elicited or expanded by viral replication in the acute phase or by this inefficient persistent viral replication. Nevertheless, these macaques showed long-term viral control with central memory $CD4^+$ T-cell preservation, indicating that nonsterile protection by CTL-based vaccines can result in prevention of chronic central memory $CD4^+$ T-cell loss.

In summary, the present study shows that primary viral control by a CTL-based AIDS vaccine can result in long-term control of SIV replication by adapted CTL responses and preservation of central memory $CD4^+$ T cells without AIDS progression. Our results suggest that CTL-based vaccines can result in long-term viral containment and disease control.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology, grants from the Japan Health Sciences Foundation, and grants from the Ministry of Health, Labor, and Welfare in Japan.

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center, National Institute of Biomedical Innovation with the help of the Corporation for Production and Research of Laboratory Primates. We thank Centocor Inc. and K. A. Reimann for providing cM-T807 and DNAVEC Corp. and J. Lifson, Y. Ami, F. Ono, K. Komatsuzaki, A. Hiyaoka, A. Oyama, K. Oto, H. Oto, H. Akari, K. Terao, M. Miyazawa, M. Yasunami, A. Kimura, M. Takiguchi, A. Kato, K. Mori, N. Yamamoto, T. Takemori, T. Sata, T. Kurata, K. Koike, Y. Nagai, and A. Nomoto for their help.

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JOURNAL OF VIROLOGY, Nov. 2007, p. 11640–11649 Vol. 81, No. 21
0022-538X/07/$08.00+0 doi:10.1128/JVI.01475-07
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Induction of $CD8^+$ Cells Able To Suppress CCR5-Tropic Simian Immunodeficiency Virus SIVmac239 Replication by Controlled Infection of CXCR4-Tropic Simian-Human Immunodeficiency Virus in Vaccinated Rhesus Macaques[▽]

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Received 5 July 2007/Accepted 16 August 2007

Recent recombinant viral vector-based AIDS vaccine trials inducing cellular immune responses have shown control of CXCR4-tropic simian-human immunodeficiency virus (SHIV) replication but difficulty in containment of pathogenic CCR5-tropic simian immunodeficiency virus (SIV) in rhesus macaques. In contrast, controlled infection of live attenuated SIV/SHIV can confer the ability to contain SIV superchallenge in macaques. The specific immune responses responsible for this control may be induced by live virus infection but not consistently by viral vector vaccination, although those responses have not been determined. Here, we have examined in vitro anti-SIV efficacy of $CD8^+$ cells in rhesus macaques that showed prophylactic viral vector vaccine-based control of CXCR4-tropic SHIV89.6PD replication. Analysis of the effect of $CD8^+$ cells obtained at several time points from these macaques on CCR5-tropic SIVmac239 replication in vitro revealed that $CD8^+$ cells in the chronic phase after SHIV challenge suppressed SIV replication more efficiently than those before challenge. SIVmac239 superchallenge of two of these macaques at 3 or 4 years post-SHIV challenge was contained, and the following anti-CD8 antibody administration resulted in transient $CD8^+$ T-cell depletion and appearance of plasma SIVmac239 viremia in both of them. Our results indicate that $CD8^+$ cells acquired the ability to efficiently suppress SIV replication by controlled SHIV infection, suggesting the contribution of $CD8^+$ cell responses induced by controlled live virus infection to containment of HIV/SIV superinfection.

Live attenuated immunodeficiency virus infection can induce effective immune responses against pathogenic human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) replication, although concerns about conditions necessary for its safety as an AIDS vaccine have not been satisfied at present (3, 13, 19). In macaque AIDS models, infection of live attenuated viruses such as SIVmac239Δnef, SIVmac239Δ3, and simian-human immunodeficiency virus (SHIV) 89.6 have been shown to confer potent immune responses resulting in control of SIV superchallenge (7, 14, 35, 53). While involvement of virus-specific $CD8^+$ cytotoxic T-lymphocyte (CTL) responses has been indicated, it has remained unclear what immune responses play a key role in this control (19, 34).

Virus-specific cellular immune responses are crucial for control of HIV-1 and SIV infections (1, 4, 5, 10, 12, 20, 29, 38, 41, 42). Recombinant viral vector-based vaccines efficiently eliciting virus-specific cellular immune responses have been developed as promising AIDS vaccine candidates (32). These prophylactic vaccine trials in rhesus macaques have shown viral control and prevention of acute $CD4^+$ T-cell depletion after CXCR4-tropic SHIV challenge (2, 27, 36, 37, 40, 46). Unfortunately, however, trials of these vaccines have shown difficulty in containment of CCR5-tropic SIV infection that induces acute, massive depletion of $CCR5^+$ $CD4^+$ memory T cells and chronic disease progression like HIV-1 infection in humans (6, 8, 11, 21, 23, 28, 30, 31, 39, 49, 50, 52). Possibly, the specific immune responses responsible for SIV control might be induced by live SIV/SHIV infection but not consistently by recombinant viral vector vaccination. Previous $CD8^+$ cell-depletion experiments in macaques using a monoclonal anti-CD8 antibody have indicated the importance of $CD8^+$ cells in SIV control (12, 29, 42), but differences in antiviral efficacy between live SIV/SHIV infection-induced and recombinant viral vector vaccination-induced $CD8^+$ cells have not been determined.

Our previous trials of a prophylactic vaccine using a Gag-expressing Sendai virus (SeV-Gag) vector have shown control of CXCR4-tropic SHIV89.6PD replication in vaccinated rhesus macaques (27, 47). While this vaccination did not always result in CCR5-tropic SIVmac239 control (28), it was speculated that, after SHIV challenge, these vaccinees may possibly

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▽ Published ahead of print on 29 August 2007.

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TABLE 1. Virus challenge and antibody administration schedule

Macaque	Prophylactic vaccination	Time (wk) of:			
		SHIV89.6PD challenge	Anti-CD20 monoclonal antibody administration	SIVmac239 superchallenge	Anti-CD8 monoclonal antibody administration
R00-017	SeV-Gag	0	166	203	209
R00-020	DNA prime with SeV-Gag boost	0	140	151	163
R00-023	DNA prime with SeV-Gag boost	0			
R00-024	DNA prime with SeV-Gag boost	0			

acquire the potential for controlling SIVmac239 superchallenge. In the present study, we have examined whether these SHIV controllers acquired $CD8^+$ cells effective against SIVmac239 replication. Our analyses have suggested that $CD8^+$ cell responses capable of suppressing SIVmac239 replication in vitro were induced by controlled SHIV infection and that these responses might be crucial for control of superchallenged SIVmac239 replication.

MATERIALS AND METHODS

Animal experiments. Four Burmese rhesus macaques (*Macaca mulatta*) used in this study (Table 1) were maintained in accordance with the *Guides for Animal Experiments Performed at National Institute of Infectious Diseases* (35a). Blood collection, vaccination, virus challenge, and antibody administration were performed under ketamine anesthesia. These macaques received prophylactic vaccination and SHIV89.6PD challenge as described in our previous studies (27, 47). Macaque R00-017 was vaccinated intranasally with 1×10^8 cell infectious units (CIU) of replication-competent SeV-Gag vector (15, 16), whereas macaques R00-020, R00-023, and R00-024 were primed intramuscularly with 5 mg of cytomegalovirus (CMV)-SHIVdEN DNA and then boosted intranasally with 6×10^9 CIU of replication-defective F-deleted SeV-Gag vector (22). The CMV-SHIVdEN DNA was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA (45) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx; SIVmac239-HIV-1_{DH12} chimeric Vpr; and HIV-1_{DH12} Tat and Rev as described previously (28, 47). These vaccinees were challenged intravenously with 10 50% tissue culture infective doses ($TCID_{50}$) of SHIV89.6PD (25) 13 weeks (in R00-020, R00-023, and R00-024) or 14 weeks (in R00-017) after SeV-Gag vaccination.

Macaques R00-023 and R00-024 were euthanized around 2 years after SHIV89.6PD challenge, while macaques R00-017 and R00-020 were followed up for more than 2 years. The latter two animals received monoclonal anti-CD20 antibody administration for $CD20^+$ cell depletion (starting at week 166 in R00-017 and week 140 in R00-020), intravenous superchallenge with 1,000 $TCID_{50}$ of SIVmac239 (18) (at week 203 in R00-017 and week 151 in R00-020), and monoclonal anti-CD8 antibody administration for $CD8^+$ cell depletion (starting at week 209 in R00-017 and week 163 in R00-020) (Table 1). For $CD20^+$ cell depletion, animals were inoculated intravenously with 10 mg/kg of monoclonal anti-CD20 antibody (Rituximab; Zenyaku Kogyo, Tokyo, Japan) four times every other week. Peripheral B-cell depletion was confirmed by immunostaining using anti-human CD19 antibody and anti-human CD20 antibody (Becton Dickinson, Tokyo, Japan). For $CD8^+$ cell depletion, animals received a single subcutaneous inoculation of 10 mg/kg of monoclonal anti-CD8 antibody (cM-T807) provided by Centocor (Malvern, PA) followed by three intravenous inoculations of 5 mg/kg cM-T807 on days 3, 7, and 10 after the first inoculation. Peripheral $CD8^+$ T-cell depletion was confirmed by immunostaining using anti-human CD8 antibody (DK25; Dako, Kyoto, Japan). Macaques R00-017 and R00-020 were euthanized 3 months after the anti-CD8 antibody administration.

Quantitation of plasma viral loads. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). For quantitation of plasma SIV/SHIV RNA levels, serial fivefold dilutions of RNA samples were amplified in quadruplicate by reverse transcription (RT) and nested PCR to determine the endpoint. SIV *gag*-specific primers (AGAAACTCCGTCTTGT CAGG and TGATAATCTGCATAGCCGC for the first RT-PCR and GATTA GCAGAAAGCCTGTTGG and TGCAACCTTCTGACAGTGC for the second DNA PCR) (Sigma-Aldrich, Tokyo, Japan) that recognize the *gag* region shared by SHIV89.6PD and SIVmac239 were used. Plasma SIV/SHIV RNA levels were calculated according to the Reed-Muench method as described previously (28). The lower limit of detection in this assay is approximately 4×10^2 copies/ml. After SIVmac239 superchallenge, plasma SIVmac239 RNA levels were measured by the LightCycler system (Roche Diagnostics) using SIVmac239 *env*-specific primers (AAGAATTGTTGCGACTGACC and CAGTAGTGTGGCA GACTTGTC) and probes (CATTCAGCTGCGCCTGGTCCTTTAAGTAC-Flu and LcRed-TCTTCGATGGCAGTGACCCTAGTCTGGAGG) (Nihon Gene Research Laboratories, Inc., Sendai, Japan) that recognize SIVmac239 *env* but not SHIV89.6PD *env*. SHIV89.6PD RNA levels were also measured using SHIV89.6PD *env*-specific primers (GGATGTTGATGATCTGTAGTGC and CCAATACTACTTCTTGTGGGTT) and probes (CAGTCTATTATGGGG TACCTGTGTGGAGAGAAGCA-Flu and LcRed-CCACCACTCTATTTT GTGCATCAGATGCTAAAGCC) that recognize SHIV89.6PD *env* but not SIVmac239 *env*. The lower limit of detection in this assay is approximately 1×10^3 copies/ml.

In vitro viral suppression assay. We examined SIVmac239 replication on CD8-depleted peripheral blood mononuclear cells (PBMCs) in the presence of $CD8^+$ cells positively selected from PBMCs. Macaque PBMCs prepared from blood at several time points were frozen and stored until use. Thawed PBMCs were separated into $CD8^+$ cells and $CD8^-$ cells by using MACS CD8 MicroBeads (Miltenyi Biotec, Tokyo, Japan). The purity of the former was more than 96%, while the latter included less than 3% of $CD8^+$ cells. To prepare target cells, one fifth of $CD8^-$ cells negatively selected from PBMCs obtained before SHIV89.6PD challenge were infected with SIVmac239 at a multiplicity of infection (MOI) of 1:10^4, and these infected cells and the remaining uninfected $CD8^-$ cells were cultured separately in the presence of 2 μg/ml phytohemagglutinin-L (Roche Diagnostics). After a 48-h culture, both infected and uninfected $CD8^-$ cells were collected, washed three times, and mixed to be used as target cells. Then, 4×10^5 target cells were cultured alone or cocultured with 4×10^5 (effector/target [E:T] ratio of 1:1) or 4×10^4 (E:T ratio of 1:10) $CD8^+$ effector cells positively selected from PBMCs in a well of 96-well flat-bottom plate and the culture supernatants were harvested every other day for measurement of SIV Gag CA p27 concentration by SIV core antigen enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter, Tokyo, Japan). RPMI 1640 medium (Invitrogen, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 20 IU/ml recombinant human interleukin-2 (Roche Diagnostics) were used for cell culture. All of the cocultures were in duplicate, and the mean value of p27 concentrations at each time point is shown.

Measurement of virus-specific $CD8^+$ T-cell responses. We measured virus-specific T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously (27, 28). PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCL) (51) infected with vesicular stomatitis virus G (VSV-G)-pseudotyped SIVGP1 for SIVGP1-specific stimulation. The VSV-G-pseudotyped SIVGP1 was obtained by cotransfection of COS-1 cells with pVSV-G (Clontech, Otsu, Japan) and SIVGP1, an *env*- and *nef*-deleted $SHIV_{MD14}$ molecular clone DNA (28, 45). Intracellular IFN-γ staining was performed using a Cytofix-Cytoperm kit (Becton Dickinson). Peridinin chlorophyll-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting the IFN-γ T-cell frequencies after nonspecific stimulation from those after SIVGP1-specific stimulation.

Measurement of virus-specific neutralizing titers. We measured virus-specific neutralizing titers as described previously (17, 44). Serial twofold dilutions of heat-inactivated plasma were prepared in duplicate and mixed with 10 $TCID_{50}$ of SIVmac239 or SHIV89.6PD. In each mixture, 5 μl of diluted plasma was incubated with 5 μl of virus. After a 45-min incubation at room temperature, each 10-μl mixture was added to 5×10^4 MT-4 cells in a well of a 96-well flat-bottom

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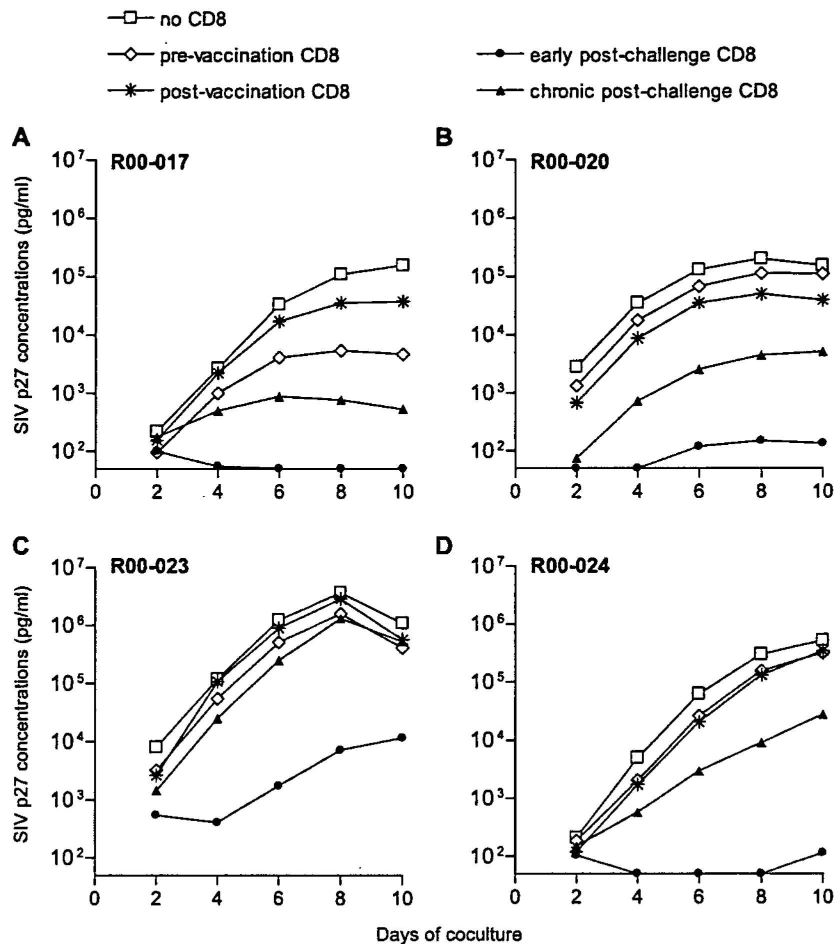

	R00-017	R00-020	R00-023	R00-024
post-vaccination	wks -6 & -4	wks -11 & -4	wk -7	wks -11 & -6
early post-challenge	wks 3 & 5	wks 5 & 8	wk 5	wks 5 & 13
chronic post-challenge	wk 67	wks 52 & 63	wk 30	wks 52 & 63

FIG. 1. SIVmac239 replication in vitro in the absence or the presence of $CD8^+$ cells in macaques R00-017 (A), R00-020 (B), R00-023 (C), and R00-024 (D). PBMC-derived $CD8^-$ (target) cells infected with SIVmac239 were cultured alone (no CD8) or cocultured with autologous PBMC-derived $CD8^+$ (effector) cells obtained prevaccination (pre-vaccination CD8), postvaccination and pre-SHIV challenge (post-vaccination CD8), in the early phase post-SHIV challenge (early post-challenge CD8), or in the chronic phase post-SHIV challenge (chronic post-challenge CD8) at an E:T ratio of 1:1. A representative result of two sets of experiments with similar patterns is shown in panels A and D, whereas the result of a single experiment is shown in panels B and C. Postvaccination and postchallenge $CD8^+$ cells were prepared from PBMCs obtained at different time points, as shown in the bottom table (weeks before [shown by minus] or after SHIV challenge), because of a limitation of available samples. SeV-Gag vaccination was performed 13 weeks (in R00-020, R00-023, and R00-024) or 14 weeks (in R00-017) before SHIV challenge. In some groups, $CD8^+$ cells at two time points were mixed to prepare enough cells. p27 concentrations in the culture supernatants were examined by ELISA.

plate. After 12 days of culture, supernatants were harvested. Progeny virus production in the supernatants was examined by SIV core antigen ELISA for detection of SIV p27 to determine the 100% neutralizing end point. The lower limit of detection is a titer of 1:2.

RESULTS

Potency of $CD8^+$ cells post-SHIV challenge for suppressing SIVmac239 replication in vitro. We established a method for examining SIVmac239 replication in vitro in the presence of $CD8^+$ cells and evaluated the effect of $CD8^+$ cells on SIVmac239 replication in vitro in four rhesus macaques that showed vaccine-based containment of SHIV89.6PD challenge (Table 1). One of them (R00-017) received a single intranasal SeV-Gag vaccination, while the other three (R00-020, R00-023, and R00-024) received a single intramuscular DNA priming followed by a single intranasal SeV-Gag booster before SHIV89.6PD challenge as described previously (27, 47). All four of these macaques controlled viral replication with undetectable plasma viremia after the acute phase for more than 2 years post-SHIV89.6PD challenge (54).

From each animal, we prepared four groups of bulk $CD8^+$ cells obtained prevaccination, post-SeV-Gag vaccination (pre-SHIV challenge), in the early phase post-SHIV challenge (weeks 3 to 8), and in the chronic phase post-SHIV challenge

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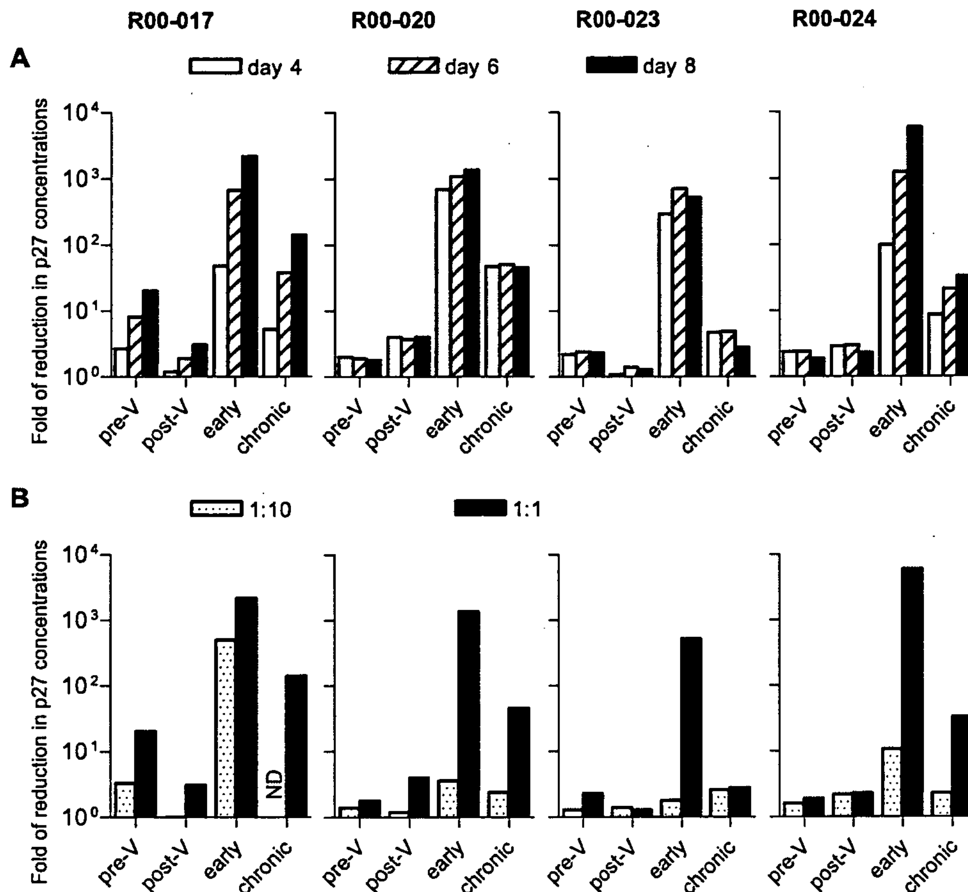

FIG. 2. Reduction in SIVmac239 production by addition of CD8$^+$ cells. The reduction (fold) in p27 concentration in the supernatant from coculture of SIV-infected CD8$^-$ cells with each group of CD8$^+$ cells compared to that from SIV-infected CD8$^-$ cell culture without CD8$^+$ cells is shown. (A) Reduction in p27 concentration on days 4, 6, and 8 of coculture at an E:T ratio of 1:1 (calculated from the data in Fig. 1). (B) Reduction in p27 concentration on day 8 of coculture at an E:T ratio of 1:1 (black bars) or 1:10 (dotted bars). pre-V, prevaccination CD8; post-V, postvaccination CD8; early, early postchallenge CD8; chronic, chronic postchallenge CD8 as described in the legend to Fig. 1. ND, not determined.

(weeks 30 to 67). These groups of effector CD8$^+$ cells were cocultured with SIVmac239-infected autologous target CD8$^-$ cells at the E:T ratio of 1:1, and p27 concentrations in the culture supernatants were measured for evaluation of SIVmac239 production (Fig. 1). Reduction in SIVmac239 production by addition of each group of CD8$^+$ cells was shown as reduction (fold) in p27 concentration compared to that in the supernatant from the SIVmac239-infected CD8$^-$ cell culture without CD8$^+$ cells (Fig. 2A).

Even addition of prevaccination CD8$^+$ cells resulted in reduction of SIV production. Especially, prevaccination CD8$^+$ cells derived from macaque R00-017 efficiently suppressed SIV replication, showing an approximately 20-fold reduction in viral production at day 8 of culture. In other three macaques, however, the reduction in SIV production by addition of prevaccination CD8$^+$ cells was less than threefold. In macaque R00-020, postvaccination/prechallenge CD8$^+$ cells suppressed SIV replication more efficiently than prevaccination ones, but in the other three macaques, the levels of suppression by postvaccination/prechallenge CD8$^+$ cells were not more than those by prevaccination cells.

In contrast, CD8$^+$ cells in the early phase postchallenge showed an efficient suppressive effect on SIV replication in all four macaques. Maximum reduction (fold) in SIV production by addition of these CD8$^+$ cells was more than 7×10^2. Addition of CD8$^+$ cells in the chronic phase postchallenge also resulted in efficient reduction of SIV production. The levels of reduction were lower than those by CD8$^+$ cells in the early phase postchallenge but higher than those by prechallenge CD8$^+$ cells. Thus, all four vaccinees, after SHIV challenge, acquired CD8$^+$ cells able to suppress SIVmac239 replication in vitro efficiently. Efficient reduction by early postchallenge CD8$^+$ cells was observed in some animals even at the E:T ratio of 1:10 (Fig. 2B).

We then measured SIVGP1-specific CD8$^+$ T-cell frequencies in PBMCs by detection of IFN-γ induction after stimulation with B-LCL expressing an *env*- and *nef*-deleted SHIV molecular clone (SIVGP1) DNA (27, 28) (Fig. 3). In all four macaques, SIVGP1-specific CD8$^+$ T-cell levels peaked during the acute phase post-SHIV challenge and gradually decreased after the set point. SIVGP1-specific CD8$^+$ T-cell frequencies after the acute phase were higher in macaques R00-017 and R00-023 compared to those post-SeV-Gag vaccination (prechallenge) but interestingly lower in macaque R00-020.

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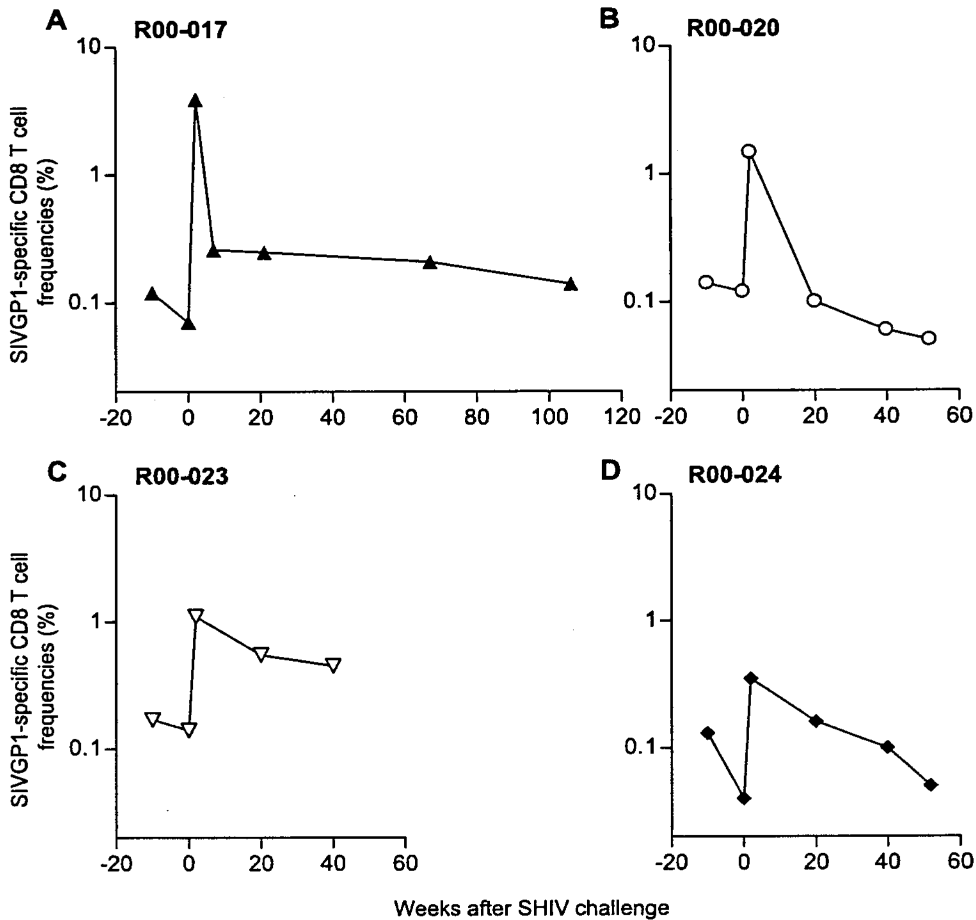

FIG. 3. SIVGP1-specific CD8$^+$ T-cell frequencies in macaques before and after SHIV89.6PD challenge. Frequencies of CD8$^+$ T cells showing SIVGP1-specific IFN-γ induction per total CD8$^+$ T cells in PBMCs are shown. The first time point prechallenge is 10 weeks before challenge.

CD20 depletion and SIVmac239 superchallenge in the SHIV controllers. Macaques R00-017 and R00-020 were further followed up and received monoclonal anti-CD20 antibody administration at week 166 (R00-017) or week 140 (R00-020) and SIVmac239 superchallenge at week 203 (R00-017) or week 151 (R00-020) (Table 1). Viral control was not abrogated, and plasma viremia remained undetectable after anti-CD20 antibody administration (Fig. 4). In both macaques, SHIV89.6PD-specific neutralizing antibodies (NAbs) were induced efficiently after SHIV89.6PD challenge and maintained at high levels in the chronic phase (54). The monoclonal anti-CD20 antibody administration resulted in rapid and prolonged depletion of peripheral CD20$^+$ lymphocytes, and more than a few months later, an approximately fourfold reduction in SHIV-specific NAb levels was observed (Fig. 5).

The following SIVmac239 superchallenge was contained in both macaques (Fig. 4). Macaque R00-017 did not show detectable plasma viremia even after SIVmac239 superchallenge, and macaque R00-020 showed only transient appearance of plasma viremia 1 week after SIVmac239 superchallenge. SIVmac239 *env* RNA but not SHIV89.6PD *env* RNA was detected in the transient plasma viremia (Fig. 6). SIVGP1-specific CD8$^+$ T-cell frequencies were at marginal levels just

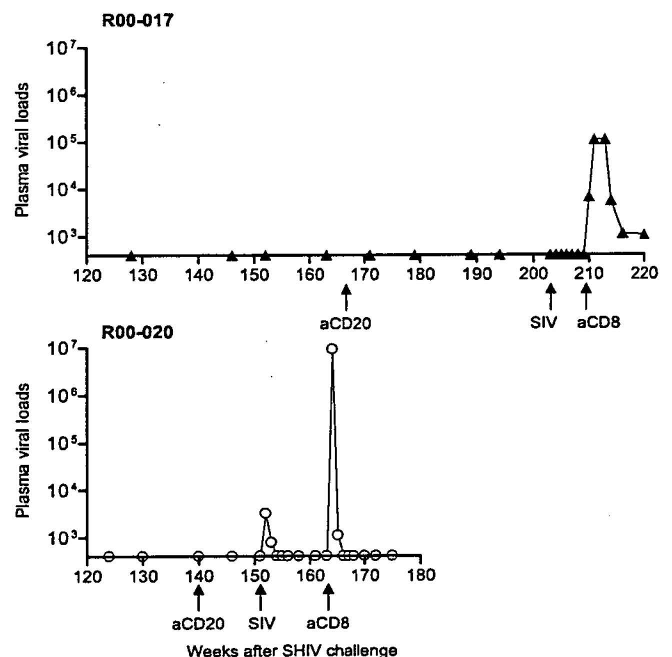

FIG. 4. Plasma viral loads (SIV *gag* RNA copies/ml plasma) in macaques R00-017 (upper panel) and R00-020 (lower panel) after week 120 post-SHIV challenge. aCD20 and aCD8, anti-CD20 and anti-CD8, respectively.

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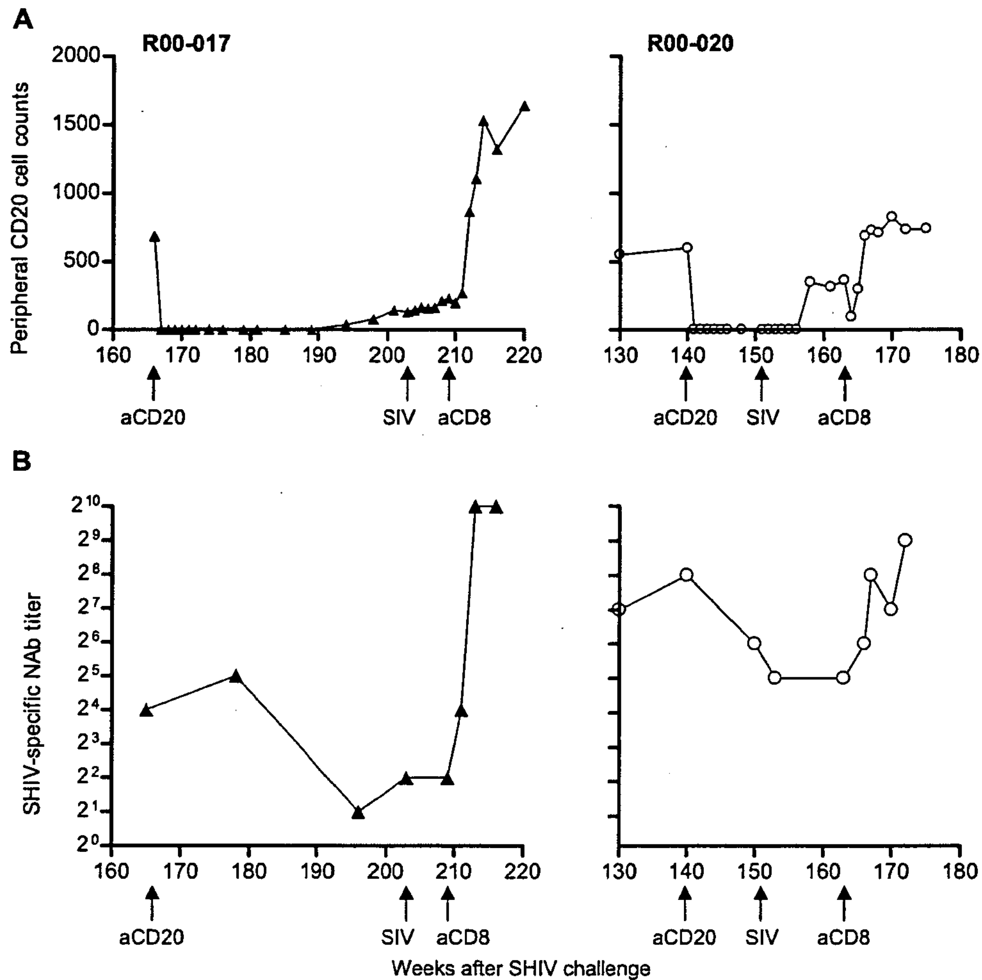

FIG. 5. Changes in SHIV89.6PD-specific NAb levels after monoclonal anti-CD20 antibody administration at week 166 in macaque R00-017 (left panels) and at week 140 in macaque R00-020 (right panels). (A) Peripheral CD20$^+$ cell counts (per μl). (B) SHIV89.6PD-specific neutralizing titers in plasma. aCD20 and aCD8, anti-CD20 and anti-CD8, respectively.

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before SIVmac239 superchallenge but increased after the superchallenge (Fig. 7).

CD8 depletion after SIVmac239 superchallenge. Macaques R00-017 and R00-020 received monoclonal anti-CD8 antibody administration at week 209 (6 weeks after superchallenge) and week 163 (12 weeks after superchallenge), respectively (Table 1). Both macaques showed transient depletion of peripheral CD8$^+$ T lymphocytes and appearance of plasma viremia after the anti-CD8 antibody administration (Fig. 6).

In macaque R00-020, exhibiting a shorter period of CD8$^+$ T-lymphocyte depletion, plasma viremia was transient and detectable only at weeks 164 and 165, 1 and 2 weeks after the initial anti-CD8 antibody treatment. SIVmac239 *env* RNA but not SHIV89.6PD *env* RNA was detected in the transient plasma viremia. In macaque R00-017, exhibiting a longer period of CD8$^+$ T-lymphocyte depletion, plasma viremia appeared at week 210, 1 week after the initial anti-CD8 antibody treatment, and remained detectable during the observation period of 3 months. Interestingly, both SIVmac239 *env* RNA and SHIV89.6PD *env* RNA were detected; the former became detectable at week 210 and was detected during the observation period, whereas the latter was detected only at weeks 211 and 212. The former SIVmac239 *env* RNA levels peaked at week 213, and the latter SHIV89.6PD *env* RNA levels peaked at week 211.

SIVmac239-specific NAb responses were undetectable even after SIVmac239 superchallenge and CD8 depletion in both of the macaques (data not shown). SHIV89.6PD-specific NAb titers increased after the CD8 depletion not only in macaque R00-017 showing SHIV89.6PD viremia but also in macaque R00-020 without SHIV89.6PD viremia (Fig. 5). Both macaques showed increases in SIVGP1-specific CD8$^+$ T-cell frequencies after recovery from peripheral CD8$^+$ T-lymphocyte depletion (Fig. 7).

DISCUSSION

Previous CD8$^+$ cell depletion experiments in macaques using a monoclonal anti-CD8 antibody have indicated the importance of CD8$^+$ cell responses in SIV control in vivo (12, 29, 42). The present study evaluated the anti-SIV efficacy of these bulk CD8$^+$ cells in the vaccinated macaques that exhibited prophylactic SeV-Gag vaccine-based control of viral replication and showed induction of CD8$^+$ cells able to efficiently

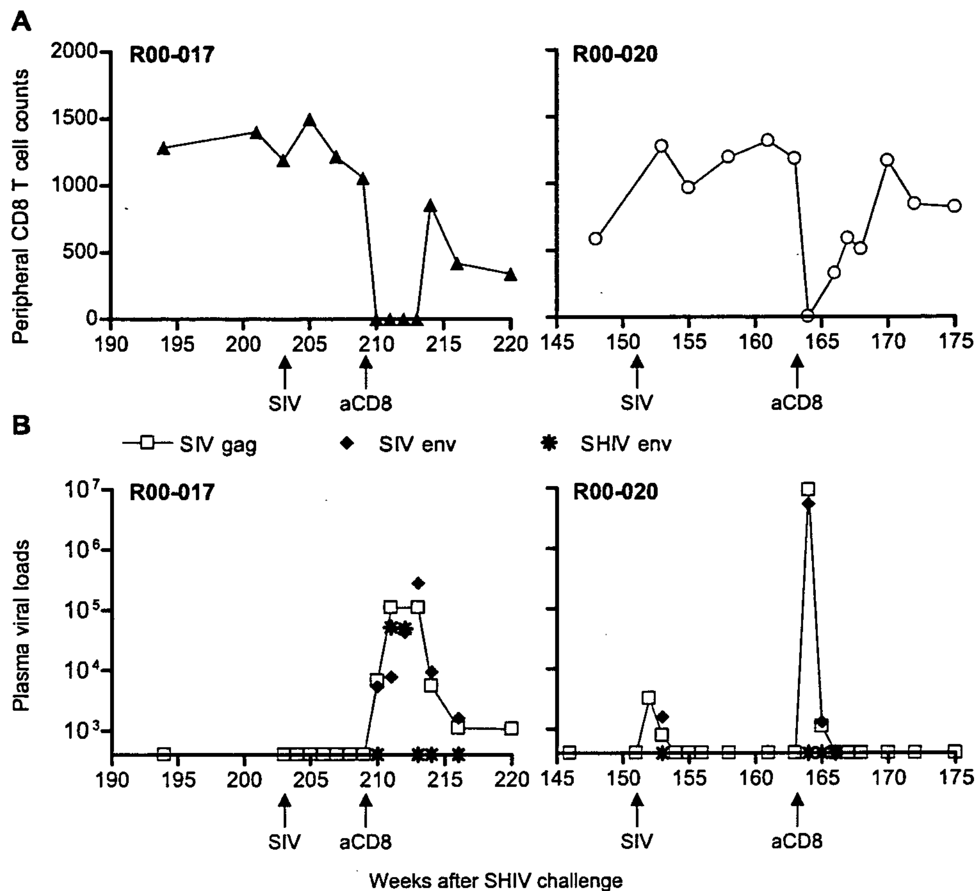

FIG. 6. SIVmac239 superchallenge and $CD8^+$ cell depletion in macaques R00-017 and R00-020. Macaque R00-017 received SIVmac239 superchallenge at week 203 and monoclonal anti-CD8 (aCD8) antibody administration starting at week 209, while macaque R00-020 received superchallenge at week 151 and anti-CD8 at week 163. (A) Peripheral $CD8^+$ T-cell counts (per μl) in macaques R00-017 (left panel) and R00-020 (right panel). (B) Plasma viral loads (copies/ml plasma) in macaques R00-017 (left panel) and R00-020 (right panel). In addition to SIV *gag* RNA levels, levels of SIV *env* RNA and SHIV *env* RNA at several time points are shown.

suppress SIV replication in vitro after SHIV challenge in these macaques. The difference in anti-SIV efficacies between postvaccination/prechallenge and postchallenge $CD8^+$ cells may explain why protective immune responses can be consistently induced not by current viral vector vaccination but by live virus infection.

These bulk $CD8^+$ cells are considered to include $CD8^+$ NK cells in addition to $CD8^+$ T lymphocytes. While previous studies using bulk $CD8^+$ cells or CTL clones (9, 24, 48, 55) have shown the importance of CTL activity on suppression of HIV/SIV replication, there may be a possibility that NK cells exert some suppressive effect on SIV replication, contributing to reductions in SIV production by prevaccination $CD8^+$ cells in the present study. The suppressive effect of postvaccination/prechallenge $CD8^+$ cells was not larger than that of prevaccination except for macaque R00-020. In contrast, postchallenge $CD8^+$ cells suppressed SIV replication more efficiently than those prevaccination and postvaccination. In the in vitro assay of SIV replication, individual macaques showed different sensitivities of target $CD8^-$ cells to SIV infection and different patterns of SIV replication kinetics in the absence of $CD8^+$ cells (Fig. 1). In macaque R00-023 showing higher levels of SIV production in the absence of $CD8^+$ cells, SIV infection at a lower MOI might exhibit a larger reduction in SIV production by addition of postchallenge $CD8^+$ cells.

Gag-specific $CD8^+$ T-cell levels peaked around 1 week after SeV-Gag vaccination and then decreased in the late phase after that (28). To prepare postvaccination/prechallenge $CD8^+$ cells, we used PBMCs in the late phase without those at week 1 post-SeV-Gag vaccination that include the peak levels of Gag-specific $CD8^+$ T cells. Thus, we compared anti-SIV efficacy of $CD8^+$ cells in the late phase postvaccination with that in the chronic phase post-SHIV challenge in this study. The postvaccination/prechallenge SIVGP1-specific $CD8^+$ T-cell frequencies roughly reflect Gag-specific $CD8^+$ T-cell ones because SIVGP1-specific $CD8^+$ T-cell responses were undetectable before SeV-Gag vaccination (data not shown). On the other hand, the postchallenge SIVGP1-specific $CD8^+$ T-cell responses are considered specific for SHIV antigens, including SIV-derived Gag, Pol, Vif, and partial Vpr. Therefore, our results shown in Fig. 3 suggest that SIV-specific $CD8^+$ T-cell frequencies in the chronic phase post-SHIV challenge were less than those post-SeV-Gag vaccination (prechallenge) in macaque R00-020. Interestingly, however, such postchallenge $CD8^+$ cells suppressed SIV replication more efficiently than postvaccination/prechallenge ones. Thus, SIV-specific $CD8^+$

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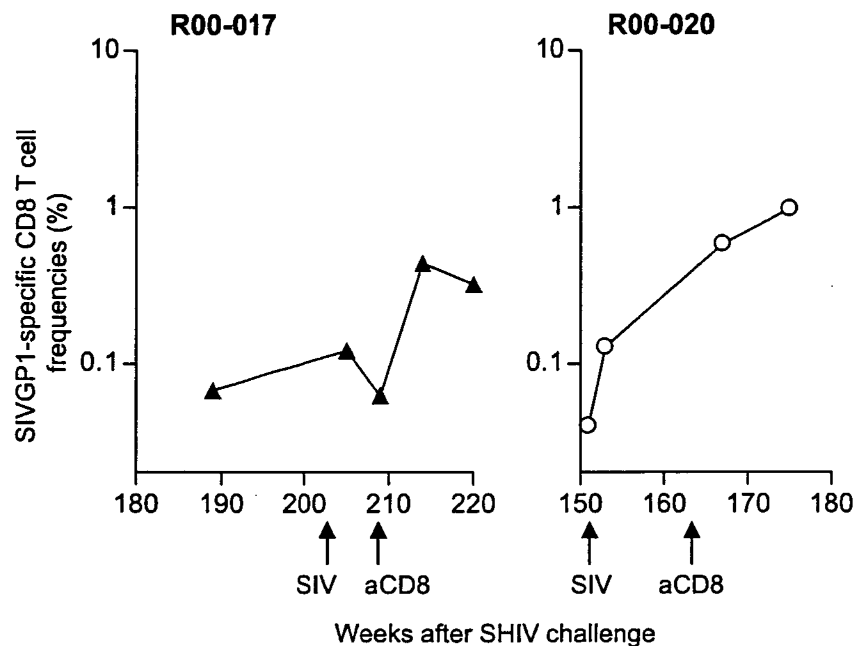

FIG. 7. SIVGP1-specific $CD8^+$ T-cell frequencies in macaques R00-017 (left panel) and R00-020 (right panel) before and after SIVmac239 superchallenge. Frequencies of $CD8^+$ T cells showing SIVGP1-specific IFN-γ induction per total $CD8^+$ T cells in PBMCs are shown. aCD8, anti-CD8.

T-cell frequencies may not always correlate with anti-SIV efficacy in vitro. It may be because postchallenge-induced, certain epitope-specific $CD8^+$ T cells may have higher anti-SIV efficacy in vitro than postvaccination/prechallenge $CD8^+$ T cells in this macaque. There may be a possibility of augmentation of anti-SIV efficacy by induction of broader $CD8^+$ T-cell responses after SHIV challenge.

A previous $CD8^+$ cell depletion study in macaques infected with live attenuated SIV has shown partial loss of superchallenged SIVmac251 control by monoclonal anti-CD8 antibody administration at the superchallenge and has suggested involvement of both cellular and humoral immune responses in this control (43). On the other hand, administration of monoclonal anti-CD8 antibody to macaques infected with live attenuated SIVmac239Δnef after SIVmac251 superchallenge resulted in the appearance of SIVmac239Δnef viremia without detectable SIVmac251 viremia (33). In contrast, the present study showed the appearance of superchallenged SIVmac239 viremia by monoclonal anti-CD8 antibody administration after superchallenge, suggesting that $CD8^+$ cells were crucial for the control of superchallenged SIVmac239 replication. It can be speculated that, in SIVmac239Δnef-infected animals, live virus replication levels before superchallenge were higher, resulting in more strict containment of superchallenge than that in our study. Additionally, neutralizing antibody responses may be involved in the containment of superchallenge in SIVmac239Δnef-infected animals but not in SHIV-infected ones. Thus, our results imply a more profound contribution of $CD8^+$ cells to control of SIV superchallenge in the absence of NAb help.

More than a few months after the anti-CD20 antibody administration, both macaques R00-017 and R00-020 showed fourfold reductions in SHIV-specific neutralizing titers, although it is unclear if these reductions were due to the $CD20^+$ cell depletion. Macaque R00-017 with a lower neutralizing titer showed transient appearance of SHIV viremia by $CD8^+$ cell depletion, but macaque R00-020 with a higher titer did not. These results were consistent with the previous study indicating involvement of humoral as well as cellular immune responses in the CXCR4-tropic SHIV control (26).

In summary, our results indicate that $CD8^+$ cells acquired the ability to efficiently suppress CCR5-tropic SIV replication in vitro by controlled CXCR4-tropic SHIV infection. While the levels of in vitro anti-SIV efficacy resulting in SIV control in vivo have not been determined, our results imply that such $CD8^+$ cell responses may be crucial for live attenuated vaccine-based containment of HIV/SIV superinfection.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology; grants from the Japan Health Sciences Foundation; and grants from the Ministry of Health, Labor, and Welfare in Japan.

The animal experiments were conducted through the Cooperative Research Program at the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates. We thank Centocor, Inc., and K. A. Reimann for providing cM-T807 and M. Takiguchi, F. Ono, K. Komatsuzaki, A. Hiyaoka, A. Oyama, K. Oto, H. Akari, K. Terao, M. Miyazawa, A. Kimura, K. Mori, N. Yamamoto, T. Sata, T. Kurata, Y. Nagai, and A. Nomoto for their help.

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Suppression of virus replication via down-modulation of mitochondrial short chain enoyl-CoA hydratase in human glioblastoma cells

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Received 17 August 2006; received in revised form 17 February 2007; accepted 21 February 2007

Abstract

Several viruses have been demonstrated to be the etiologic agent in chronic progressive diseases, associated with persistence; however, major questions concerning the pathogenic mechanisms of viral persistence are still unanswered. With the aim of identifying host cellular proteins that may play a role in viral replication, we established long-term persistently infected human glioblastoma cell lines with mutant measles virus (MV) and analyzed the host proteins by two-dimensional gel electrophoresis (2-DE) with mass spectrometry. We observed significant down-modulation in the expression of mitochondrial short chain enoyl-CoA hydratase (ECHS), which catalyzes the β-oxidation pathway of fatty acid. Knockdown of this gene by a short interference RNA (siRNA) apparently impaired wild-type MV replication and the cytopathic effects (CPEs) of MV were significantly reduced in siRNA-transfected cells. These findings will shed light upon a new important notion for the interaction between virus replication and lipid metabolism in host cells and might provide a new strategy for virus control.
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Keywords: Measles virus; Persistent infection; Mitochondrial short chain enoyl-CoA hydratase; β-Oxidation; Short interference RNA

1. Introduction

A persistent viral infection is one in which a virus in a replicating or non-replicating form persists in the host beyond the normal recovery and elimination period for that particular viral infection. Although the dynamics of immune responses after acute viral infection are well studied and very consistent, the patterns of responses noted during persistent infection are more complex and differ depending on the infection. Two essential ingredients have been identified in the current understanding of persistent virus infection. The first is an immune response that is ineffectual in recognizing and clearing a virus and/or virus-infected cells; the second is that viruses can regulate the expression of both their own genes and host genes to achieve residence in a non-lytic state within the cells they infect. However, knowledge of how viral genes and cellular factors interact to cause persistence is incomplete in most instances.

In our laboratory, we have established several monkey kidney cell lines persistently infected with temperature-sensitive mutants of measles virus (MV) (Watari et al., 1979, 2001). Many aspects of these cells such as interferon production have been investigated but fail to provide a coherent mechanistic explanation for viral persistence. In this study we established a human glioblastoma cell line persistently infected with mutant measles virus, because MV persistently infect and replicate in human cells of neuronal origin and elicit subacute sclerosing panencephalitis (SSPE) in humans (Horta-Barbosa et al., 1969; Payne et al., 1969). We analyzed these persistently infected cells using two-dimensional gel electrophoresis (2-DE) in combination with tandem mass spectrometry (MS/MS), which allows us to study the alterations of host proteins during virus adaptation to the cells.

Here, we found that the expression level of mitochondrial short chain enoyl-CoA hydratase (ECHS), which catalyzes the β-oxidation pathway of fatty acid, was specifically down-modulated in persistently infected cells. Moreover, knockdown of the gene by short interference RNA (siRNA) apparently impaired wild-type MV replication, and cytopathic effects (CPEs) by MV infection were significantly reduced in siRNA-transfected cells. If one of the hallmarks of persistent infection is stable and low-level virus replication, our findings suggest that some host cellular proteins associated with lipid metabolism

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doi:10.1016/j.antiviral.2007.02.002

might contribute to the regulation of virus replication followed by the establishment of persistent infection.

2. Materials and methods

2.1. Cell culture and viruses

Human glioblastoma cells, A172 and U373MG (Bender et al., 1992), were gifts from Dr. Hiroshi Takahashi (Department of Neurosurgery, Nippon Medical School, Tokyo, Japan) and were grown in Eagle's minimum essential medium (MEM, Nikken BioMedical Laboratory, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. As described recently (Watari et al., 2005), wild-type Edmonston strain measles virus (Rapp clone 5) was grown and titrated on Vero cells. A temperature-sensitive mutant virus P-448 was established from Rapp clone 5 described previously (Yamaji et al., 1975). To establish persistently infected cells, A172 cells grown as monolayers were infected with P-448 mutant virus at a multiplicity of 0.1. Infected cells were passaged for the first time at 24 days' post-infection. Since the first passage, cells have been passaged weekly and were termed 448-A172 cells. For virus titration, serial tenfold dilutions of cell supernatants and cell lysates were inoculated into each of four wells of Vero cells and then incubated for 5 days. After incubation, wells were scored for CPE and we determined the dilution as $TCID_{50}$/ml at which 50% of the wells were infected.

2.2. Cell staining

For morphological analysis, cells grown on the culture plate were washed with PBS and fixed in 4% paraformaldehyde or acetone for 10 min, then cells were stained with hematoxylin solution. For immunochemical analysis, a cytospin preparation of A172 cells infected with wild-type MV and 448-A172 cells was incubated with vaccinated human serum with MV. After washing, they were overlaid with fluorescein isothiocyanate-conjugated goat anti-human antibody (Tago, Inc., Burlingame, CA).

2.3. Preparation of protein samples

The cell pellet (5×10^6 cells/sample) was disrupted in sample re-hydration buffer (8 M urea, 2% CHAPS, 0.5% ZOOM Carrier Ampholytes, 20 mM dithiothreitol (DTT), 0.002% bromophenol blue; Invitrogen, Carlsbad, CA) at room temperature for 15 min. The lysate was separated by centrifugation at $10,000 \times g$ for 5 min to yield supernatant that was stored at −80 °C until use. To visualize low-abundance proteins more efficiently, we prepared sub-cellular fraction of cells using a proteome extraction kit (Calbiochem, Darmstadt, Germany).

2.4. Two-dimensional gel electrophoresis

Cell lysate in re-hydration buffer was applied to ZOOM strips (pH 4–7, Invitrogen) in a total volume of 155 μl. After re-hydration for 16 h at room temperature, proteins were separated by isoelectrofocusing (IEF) at room temperature and 50 mA/strip with the following linear voltage increases: 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2000 V for 30 min. The strips were equilibrated in 50 mM Tris containing 6 M urea, 30% glycerol, 2% sodium dodecylsulfate (SDS) and 2% DTT for 20 min. The second dimension was performed on 13% SDS-polyacrylamide gels. Separated protein spots were fixed and stained on the gel with a silver staining kit (Nacalai Tesque, Kyoto, Japan). Differential spots were excised from silver-stained gels and treated with 20 μg of trypsin/ml in 50 mM ammonium bicarbonate buffer at 37 °C overnight. After in-gel digestion, the digested solution was transferred into a clean tube and dried under vacuum. The resulting samples were dissolved in 20 μl of 2% acetonitrile and 0.1% trifluoroacetic acid, and applied to LC–MS/MS analysis.

2.5. Identification of protein spots

Analysis was performed using an LC–MS/MS system with RP-mLC composed of a Paradigm MS4 dual solvent delivery system (Michrom BioResources, Auburn, CA), a HTC PAL auto sampler with two 10-port injector valves (CTC Analytics), Finnigan LCQ Deca XP plus (Thermo Electron, Waltham, MA) equipped with NSI sources (AMR Inc., Tokyo, Japan). The mass spectrometer was operated in data-dependent acquisition mode in which MS acquisition with a mass range of m/z 450–2000 was automatically switched to MS/MS acquisition under the automated control of Xcalibur software. The capillary exit of the electrospray ion source was set at 70 V, the octapole at 3 V, and the capillary temperature at 250 °C. A counter flow of helium was used as nebulizing gas. Each sample was injected onto a capillary RP column, MAGIC C18 (3 mm, 200 Å, 50 m × 0.2 m i.d., Michrom BioResources) with an acetonitrile linear gradient of 3 ml/min in formic acid 0.1%, from 2 to 60%. The HPLC column was rinsed with 90% acetonitrile in 0.1% formic acid between each injection.

2.6. siRNA transfection

A172 cells were plated in 48-well tissue culture plates at 1×10^5 cells/well in 150 μl MEM on the day of transfection. Cells were transfected with 1 μl HiPerFect Transfection Reagent (Qiagen, Düsseldorf, Germany) and 1 μl siRNAs (5 nM) in a total volume of 100 μl DMEM (Sigma–Aldrich, St. Louis, MO) according to the manufacturer's protocol. At 48 h after transfection, cells were infected with viruses and their growth on transfected cells was analyzed using the $TCID_{50}$ protocol. The siRNA oligonucleotides targeted the ECHS gene at position 864–884 (termed #864: aaagagaaaggccaacttcaa), 865–885 (termed #865: aagagaaaggccaacttcaaa), 1088–1108 (termed #1088: ctggqgcqccttctaaatcta), and 1245–1265 (termed #1245: cagatgctgattaaagtgata). These siRNAs were synthesized by Qiagen. Non-silencing siRNA with no known homology to mammalian genes was a commercially available duplex (Qiagen) and was used as control siRNA.

2.7. Quantitative RT-PCR

RNA was prepared from siRNA-transfected A172 cells using RNAeasy (Qiagen). One microgram of RNA was incubated for 1 h at 42 °C after adding 20 U of RNase inhibitors (TaKaRa, Bio Inc., Otsu, Japan), 0.2 mM deoxynucleoside triphosphates, 2.5 nM random primers, 11 U of Rous associated virus 2 reverse transcriptase (TaKaRa) and reverse transcriptase buffer to a final volume of 20 μl. One microlitre of RT reaction mixture was used as a template for real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the following primers specific for ECHS (forward, cgctgctgtcaatggctatg, and reverse, cttggcgtcctgggctgaga), β-actin (forward, tcacccacactgcccatctacga, and reverse, cagcggaaccgctcattgccaatgg), MV-NP protein (forward, tcagtagagcggttggaccc, and reverse, ggcccggtttctctgtagct), MV-H protein (forward, ttcatcgggcagccatctac, and reverse, ctctgaggtgtcctcaggcc), MV-F protein (forward, gcgagcctggaaactactaatca, and reverse, ccctgaacagccaatatcatctc). The amount of each ECHS mRNA was normalized to that of β-actin mRNA in the same sample.

2.8. Measurement of cell growth

Single cell suspensions were seeded at a density of 5×10^4 cells/well on 96-well microtiter plates. After 1–3 days of incubation, the cells were pulse-labeled with 0.5 μCi methyl-^{3}H-thymidine/well for the last 8 h, and were then harvested and counted using a β-counter (1450 Microbeta Trilux; Wallac, Gaithersburg, MD).

2.9. Western blotting

A172 cells treated with or without siRNA were infected with MV. After 2 days' infection, cells were lysed in 30 μl of lysis buffer (1% Nonidet P-40, 140 mM NaCl, 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, and 50 mM monoiodoacetamide) on ice for 15 min. After centrifugation at $20,400 \times g$ for 15 min, proteins in cell lysates were separated by 10% SDS-PAGE under reducing conditions and transferred to a nylon membrane. The blots were probed with vaccinated human serum with MV or mouse anti-β-actin (clone AC-74, Sigma, St. Louis, MO) followed by peroxidase-

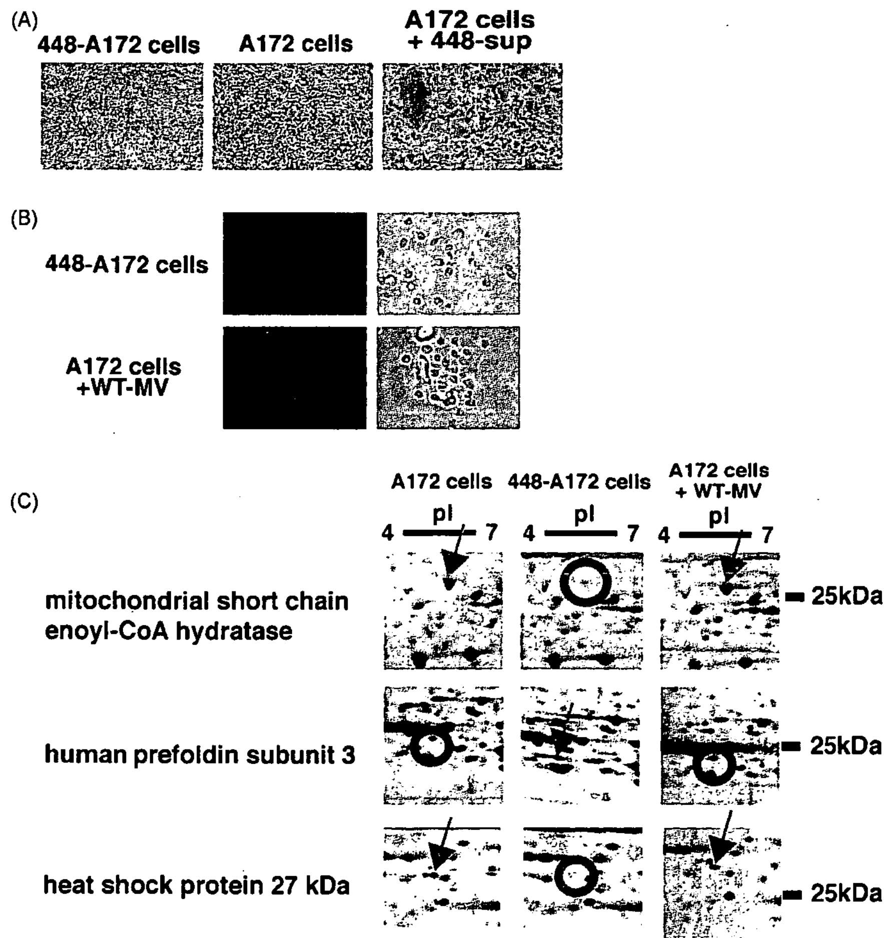

Fig. 1. Characterization of 448-A172 cells persistently infected mutant MV. (A) Morphology of 448-A172 cells (left), A172 cells (middle), and A172 cells treated with supernatants from 448-A172 cells. Cells were stained with hematoxylin solution. (B) Detection of virus antigens with vaccinated human serum with MV in 448-A172 cells (upper) or A172 cells infected with wild-type MV (lower). (C) Silver-stained two-dimensional gels of A172 cells (left column), 448-A172 cells (middle column), or A172 cells infected with wild-type MV (right column). Mitochondrial short-chain enoyl-CoA hydratase (ECHS) was detected in whole cell fraction, human prefoldin subunit 3 was detected in cytosolic fraction, and heat shock protein 27 kDa was detected in nucleus fraction.

conjugated rabbit anti-human IgG (MP Biomedicals, Irvine, CA) or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Bands were visualized using a tetramethylbenzidine substrate kit (Vector, Burlingame, CA).

3. Results

3.1. Establishment and analysis of a cell line persistently infected with temperature-sensitive mutant measles virus

To investigate the mechanisms underlying virus persistence, we established a human glioblastoma cell line persistently infected with a temperature-sensitive mutant MV named 448-A172 after about 50 days of infection. The appearance of the 448-A172 cell line was indistinguishable from intact uninfected A172 cells (Fig. 1A). Thus, the situation of persistent MV infection in 448-A172 cells was examined by the detection of intracellular viral antigens using an immunofluorescent technique. As shown in Fig. 1B, viral antigens were mainly observed in the cytoplasms of 448-A172 cells, and infectious virions from cells could be obtained and titrated on Vero cell monolayers. Indeed, culture supernatants harvested on day 4 contained measurable amounts of viruses (10^4 $TCID_{50}$/ml) that induced syncytial cell formation for intact A172 cells (Fig. 1A).

3.2. Identification of proteins crucial for persistent infection in the established 448-A172 cell line

These findings suggest that such persistent infection did not depend on the type of virion but rather on host cellular conditions; therefore, to explore the possible mechanisms involved in persistent infection, we precisely compared the cellular proteins between 448-A172 cells and A172 cells infected with or without wild-type MV using 2-DE with p*I* values in the range of 4–7 to obtain a greater resolution in protein separation. The 2-DE image of cellular proteins after silver staining is shown in Fig. 1C. For the assessment of differentially expressed proteins, protein spots clearly altered in 448-A172 cells were considered. We did find three altered proteins, which were then characterized by mass spectrometry and identified as mitochondrial short chain enoyl-CoA hydratase, human prefoldin subunit 3, and heat shock protein 27 kDa.

3.3. Inhibition of ECHS expression with specific siRNA

Among those three proteins, we focused on investigating the functional role of ECHS for viral replication, because ECHS protein is more abundant than other proteins in intact A172 cells and the amount of ECHS is obviously reduced in 448-A172 cells. Using quantitative RT-PCR analysis quantifying ECHS transcripts relative to that of β-actin from 448-A172 cells, we confirmed that the expression levels of ECHS mRNA in 448-A172 cells was decreased to less than 10% in comparison with intact A172 cells (data not shown). Then, ECHS-specific siRNAs were prepared to evaluate the potential involvement of ECHS in regulating the replication of MV. Four different siRNAs were used to specifically knockdown the expression of ECHS in intact A172 cells. Twenty-four or 48 h post-transfection with a siRNA, mRNA and protein levels of ECHS in A172 cells were reduced up to 30–45% of control siRNA-transfected cells (Fig. 2A and B). As ECHS catalyzes the second step in the β-oxidation pathway of fatty acid metabolism, down-modulation of ECHS might result in the deficient production of energy-yielding substrates via β-oxidation. Therefore, we examined the effect of ECHS suppression on cell proliferation using the ^{3}H-thymidine uptake method. Although siRNA had little effect on cell proliferation until 2 days after transfection, the reduction of ^{3}H-thymidine uptake was observed in three siRNA-transfected cells, but no dead cells were detected 3 days after transfection (Fig. 2C).

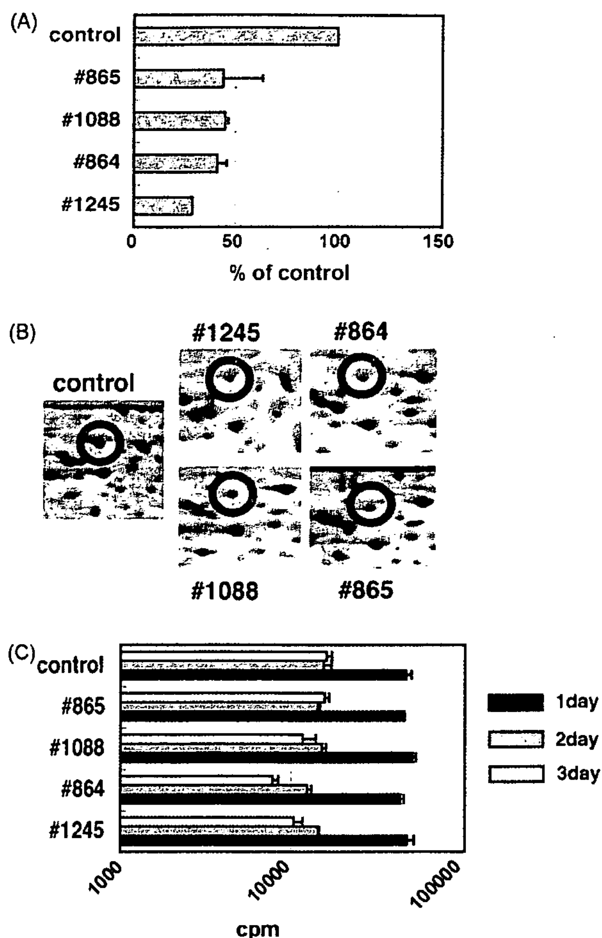

Fig. 2. Effect of siRNA transfection on A172 cells. (A) A172 cells were transfected with each siRNA. Total RNA was isolated from cells 24 h after transfection and was subjected to quantitative RT-PCR specific for ECHS or β-actin primer. Data were normalized to the amount of β-actin mRNA and are expressed as percentages of the normalized value for control siRNA-transfected cells. Values are the mean ± standard deviation (S.D.) of at least three experiments. (B) A172 cells were transfected with each siRNA. After 48 h incubation, cells were lysed and subjected to 2-DE analysis. Gels were visualized with silver staining. (C) A172 cells were transfected with each siRNA. After 1–3 days' incubation, the cells were pulse-labeled with 0.5 μCi/well methyl-^{3}H-thymidine for the last 8 h.

3.4. Inhibition of MV replication by ECHS siRNA in A172 cells

To see whether the treatment of cells with ECHS-specific siRNA can inhibit MV replication, we examined their inhibitory

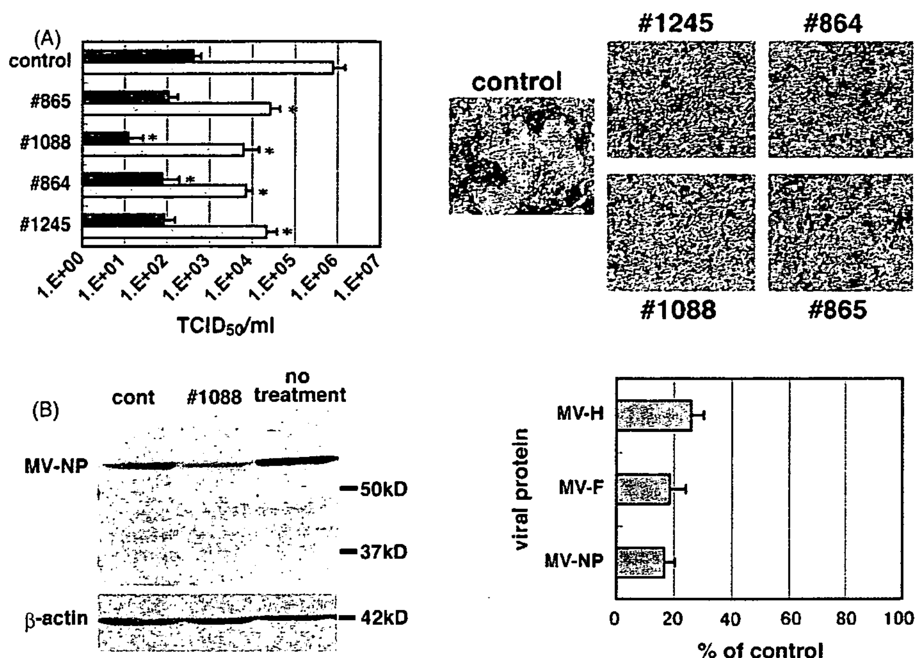

Fig. 3. Effect of siRNA transfection on MV replication. (A) A172 cells were transfected with each siRNA. After 48 h incubation, A172 cells were infected with wild-type MV, and then both cells (gray bars) and supernatants (charcoal bars) were harvested at 48 h after infection followed by determination of the virus titer (left). Data are expressed as the virus titer ($TCID_{50}$) and are the means ± S.D. of values of at least three independent experiments. $^{*}P<0.05$ vs. the value of cells transfected with control siRNA (Student's *t*-test). Cells were stained with hematoxylin solution after 48 h infection (right). (B, left) A172 cells treated with or without siRNA were infected with MV. After 2 days' infection, cells were lysed in 30 μl of lysis buffer, and proteins in lysates were separated in 10% polyacrylaminde gel and blotted on a nylon membrane. The blot was probed with vaccinated human serum with MV (upper column) or mouse anti-β-actin antibody (lower column). (B, right) A172 cells treated with or without siRNA were infected with MV. After 2 days' infection, total RNA was isolated from cells 24 h after transfection and subjected to quantitative RT-PCR specific for MV-NP protein, MV-F protein or MV-H protein primer. Data were normalized to the amount of β-actin mRNA and are expressed as percentages of the normalized value for control siRNA-transfected cells. Values are the mean ± S.D. of at least three experiments.

effect on intact A172 cells. Forty-eight hours post-transfection with siRNA, A172 cells were infected with wild-type MV at a multiplicity of infection (moi) of 0.1. At 48 h after infection, culture supernatants and cells were harvested, serially diluted, and the virus titer determined (expressed as $TCID_{50}$/ml). As shown in Fig. 3A, we observed the efficient inhibition of MV replication after transfection with four distinct siRNAs. Indeed, when #1088 siRNA was used, inhibition was so pronounced that culture supernatants contained only a few viruses. CPE by MV infection observed at 48 h post-infection in control and ECHS siRNA-transfected groups is also shown in Fig. 3A. There is an apparent marked reduction of CPE in all siRNA-transfected cells. This was confirmed by titrating the virions yielded between control and siRNA-transfected cells. Moreover, Western blot analysis showed that transfection of A172 cells with #1088 siRNA reduced the accumulation of viral protein compared to cells transfected with control siRNA or without siRNA. This protein reduction was due to a decrease in the expression of viral mRNA (Fig. 3B).

3.5. *Effect of ECHS siRNA on other virus replication*

Next, to determine if ECHS is also involved in the replication of other RNA viruses, siRNA-transfected A172 cells were infected with vesicular stomatitis virus (VSV) or semliki forest virus (SFV). As demonstrated in Fig. 4A, targeting ECHS mRNA also significantly inhibited both VSV and SFV replication in A172 cells. Similar to MV infection, CPEs were not detected in all siRNA-transfected A172 cells at 24 h after VSV or SFV infection (data not shown).

ECHS catalyzes the β-oxidation pathway of fatty acid. To further analyze the involvement of β-oxidation in virus replication, we examined the effect of etomoxir (Sigma), an inhibitor of carnitine palmitoyltransferase that inhibits mitochondrial β-oxidation, on MV replication in A172 cells. Treatment of A172 cells with etomoxir resulted in the suppression of MV replication in a dose-dependent manner (Fig. 4B). These results suggest that β-oxidation might be involved in MV replication.

We also observed that treatment of A172 cells with IFN-β (1000 IU/ml) effectively inhibited MV replication by approximately 100 times (Fig. 4B), indicating that down-modulation of ECHS potency with siRNA corresponds to treatment with a high titer of IFN-β to inhibit virus replication. Recently, it was reported that siRNA treatment could nonspecifically induce IFN-mediated innate immune responses (Sledz and Williams, 2004); however, it was unlikely that IFN mediated protection in our experiments, because a large amount of IFN-α was originally produced in intact A172 cells but IFN-β was not detected in culture supernatants from intact or siRNA-transfected A172 cells (data not shown).

To extend the results in A172 cells, we further tested the ability of siRNAs to inhibit virus replication in other glioblastoma cells, U373MG. When U373MG cells were transfected with each siRNA and then infected with MV at a moi of 0.1

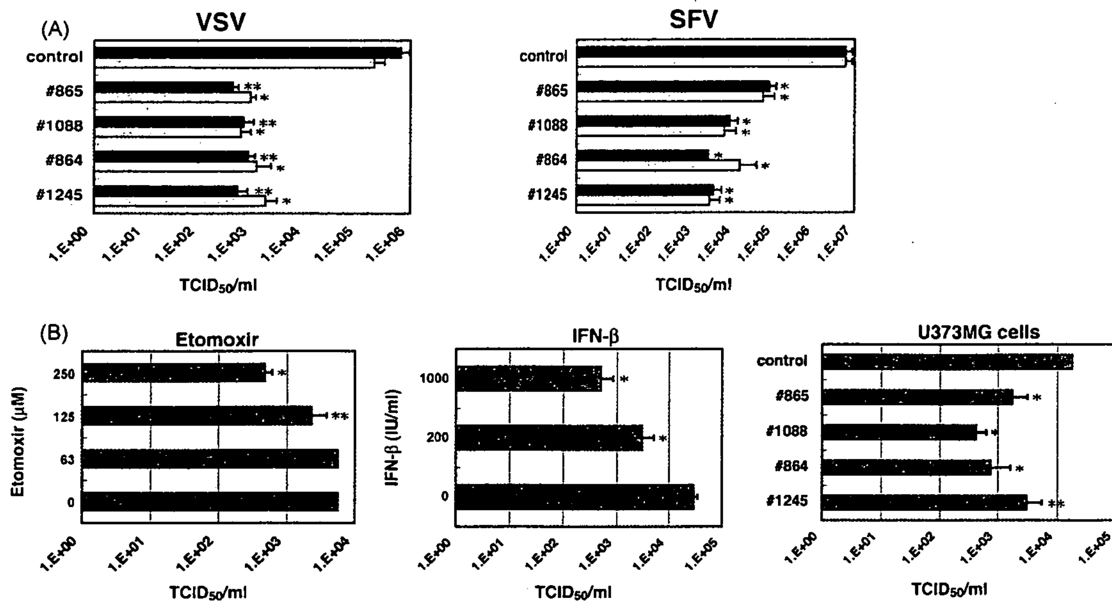

Fig. 4. Effect of siRNA transfection on other virus replication. (A) A172 cells were transfected with each siRNA. After 48 h incubation, A172 cells were infected with VSV (left) or SFV (right) at a moi of 0.1, and then cells (gray bars) and supernatants (charcoal bars) were harvested at 24 h after infection followed by determination of the virus titer. (B) A172 cells were treated with the indicated concentration of etomoxir (left). After 48 h incubation, A172 cells were infected with wild-type MV, and then supernatants were harvested at 72 h after infection followed by the assay for determination of the virus titer. A172 cells were treated with the indicated concentration of IFN-β (middle). After 24 h incubation, cells were infected with wild-type MV, and then supernatants were harvested at 48 h after infection followed by determination of the virus titer. U373MG cells were transfected with each siRNA (right). After 48 h incubation, U373MG cells were infected with wild-type MV, and then supernatants were harvested at 48 h after infection followed by determination of the virus titer. Data are expressed as the virus titer ($TCID_{50}$) and are the means ± standard deviations (S.D.) of values of at least three independent experiments. (A and B) $^{*}P<0.02$, and $^{**}P<0.05$ vs. the value of cells transfected with control siRNA or treated without reagents.

at 48 h post-transfection, siRNA, especially #864 and #1088, significantly inhibited virus replication (Fig. 4B). Moreover, to confirm whether stable infection with mutant MV in other cells induce the down-modulation of ECHS expression as well as 448-A172 cells, we are now attempting to establish a cell line persistently infected with mutant MV using U373MG cells.

4. Discussion

In this study, we have found that the expression of ECHS protein was significantly down-modulated in cells persistently infected with temperature-sensitive mutant MV. Moreover, similar to conventional MV, the mutant MV produced and secreted from persistently infected cells showed the capacity to induce syncytial formation upon infection to normal A172 human glioblastoma cells, indicating that such persistent infection is not due to the type of virion but rather to host cellular conditions. We thus speculate the ECHS gene as an essential host cellular gene utilized for virus replication, and conducted experiments to inhibit host cellular ECHS with siRNA by the gene-transfer technique. As expected, the replication of wild-type MV was specifically blocked by siRNA via knockdown of the ECHS gene.

ECHS catalyzes the second step in the physiologically important β-oxidation pathway of fatty acid metabolism (Agnihotri and Liu, 2003). In mammals, fatty acid oxidation occurs in mitochondria, peroxisomes, and smooth endoplasmic reticulum. Although mitochondria and peroxisomes oxidize fatty acids via β-oxidation, smooth endoplasmic reticulum metabolizes fatty acids by ω-oxidation. Such mitochondrial β-oxidation is responsible for oxidation in the major portion of short- (<C8), medium- (C8-12), and long- (C14-20) chain fatty acids and, in that process, constitutes the primary source of energy derived from fatty acids. The catalytic mechanism of ECHS has been studied in great depth through a combination of kinetic, spectroscopic, and structural techniques (Kim and Battaile, 2002); however, the expression and gene regulation of this enzyme have not been fully elucidated. Several reports have shown that the expression level of ECHS was decreased in cancer cell lines and carcinoma (Balabanov et al., 2001; Fratelli et al., 2003; Hwa et al., 2005; Sakata et al., 1998). Although the biological significance of ECHS in human cancer has not been confirmed, regulation of this enzyme by a carcinogen might have a role in the proliferation and differentiation of normal cells. On the other hand, very few reports refer to the interaction between ECHS and microbial infections. Only Yokoyama et al. have reported that the expression level of ECHS was decreased in hepatocellular carcinoma from patients infected with hepatitis C virus (Yokoyama et al., 2004).

Although we have not yet elucidated the molecular mechanism for the regulation of MV replication by ECHS, down-modulation of ECHS inevitably leads to the inhibition of β-oxidation. We also found that treatment of A172 cells with etomoxir, an inhibitor of β-oxidation, effectively suppressed virus replication. These findings suggest that β-oxidation appeared to be involved in MV replication; moreover, the fact that ECHS

siRNA also effectively interfered with VSV or SFV replication in A172 cells indicates that β-oxidation might be essential for the common replication cycles of various viruses.

Recently, there have been significant advances in identifying cellular factors that promote or inhibit viral replication. In the case of HIV-1, novel factors such as APOBEC or TRIM5α have been discovered (Sheehy et al., 2002; Stremlau et al., 2004). Both proteins show anti-viral activity; in particular, APOBEC family proteins have activity in a wide variety of viruses. Consequently, up-modulation of these proteins in host cells imparts resistance to viral replication. Taken together, there may be two concepts for suppressing virus replication in host cells: the lack of an essential factor for virus replication and the presence of an efficient mechanism for controlling replication. APOBEC proteins seem to correspond only to the latter mechanism, while ECHS might correspond to both, because the impairment of β-oxidation might result in the reduction of energy-yielding substrates (acetyl-CoA and ketone bodies), which eventually leads to a shortage of ATP, and adversely cause the accumulation of both free fatty acids and toxic acyl-CoA intermediates in cells.

Although the expression level of ECHS in 448-A172 cells seemed significantly lower than that in siRNA-transfected cells, culture supernatants from the 448-A172 cells still contained measurable amounts of viruses (10^4 $TCID_{50}$/ml) and their growth rate was almost the same as #864-siRNA-transfected cells. These data suggest that other host factors relating to viral replication besides ECHS might be stably disturbed in 448-A712 cells. Indeed, we found that human prefoldin subunit 3 and heat shock protein 27 kDa seemed to down-modulate virus replication and extend cell survival (Fig. 1C). Further investigation on another host proteins must be required to understand the precise mechanisms how viral genes and cellular factors interact to cause persistent viral infection.

One of the hallmarks of persistent infection is to create an excellent intracellular status for stable and low-level virus replication. The reduction of ECHS may contribute not only to low steady-state levels of virus replication but also to the survival of virus-infected cells. The suppression of virus replication might help to prolong the asymptomatic phase of virus infection; therefore, further precise analysis of the molecular regulation of our findings shown here might cast new light on the development of novel anti-viral drugs concerned with lipid metabolism. Indeed, Sakamoto et al. recently identified an HCV replication inhibitor which prevents the de novo synthesis of sphingolipids, a major lipid raft component (Sakamoto et al., 2005).

Acknowledgements

We thank Drs. Takahiro Isono, Noriyuki Nagahara and Masayasu Minami for experimental advice on the proteomic analysis. This work was supported in part by grants from the Ministry of Education, Science, Sport, and Culture, from the Ministry of Health and Labor and Welfare, Japan, from the Japanese Health Sciences Foundation, and from the Promotion and Mutual Aid Corporation for Private School of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2007.02.002.

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