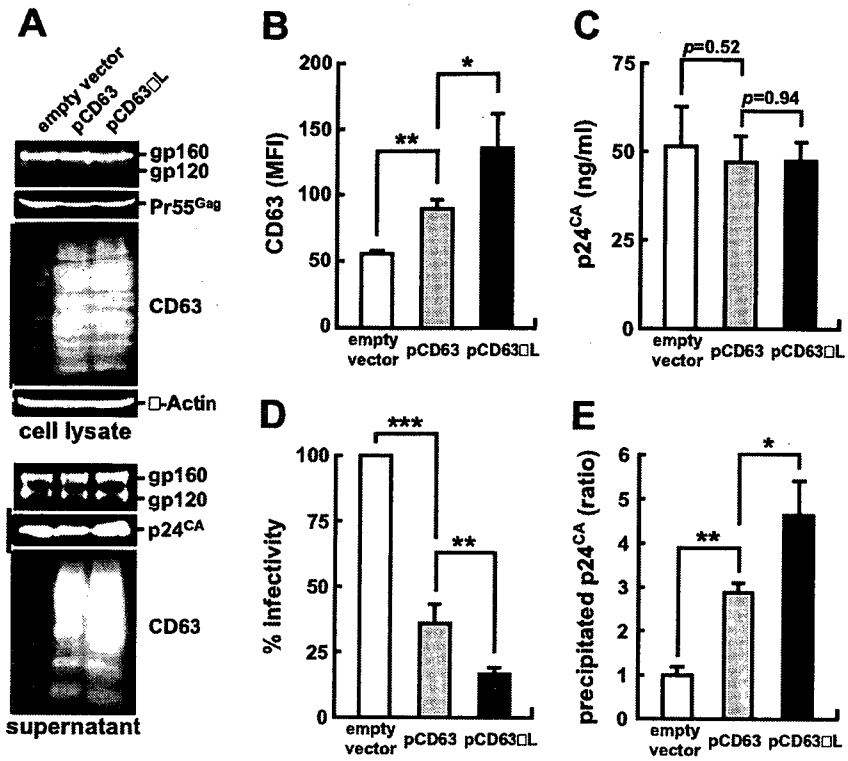

FIG. 4. Augmented reduction of the infectivity of released HIV-1 virions by CD63 L. Empty vector, pCD63, and pCD63 L were each cotransfected with pNL4-3 into 293T cells. (A) The expression of viral protein and CD63 in the transfected 293T cells and the components of released HIV-1_{NL4-3} particles were analyzed by Western blotting, and a representative result is shown. The cell number was normalized in comparison with -actin, and the released virions were harvested as described in Materials and Methods. (B) The surface expression of CD63 on the transfected 293T cells was analyzed by flow cytometry. (C) The amount of released HIV-1_{NL4-3} particles was quantitated by $p24^{CA}$ ELISA. (D) IU of HIV-1_{NL4-3} was measured by Magi assay. The IU were normalized to the amount of $p24^{CA}$, and infectivity is shown as a percentage of the empty-vector value. (E) HIV-1_{NL4-3} particles (100 ng of $p24^{CA}$) were used for immunoprecipitation by anti-CD63 antibody. The ratio of precipitated virions to empty vector is shown. Experiments were performed in triplicate. Statistical significance (Student's *t* test) is shown as follows: *, P 0.05; **, P 0.01; ***, P 0.001. Error bars indicate standard deviations. MFI, mean fluorescence intensity.

binding affinity of conventional and CD63-enriched virions. As shown in Fig. 7A, the binding affinity of CD63-enriched virions was not attenuated. To investigate the effect of CD63 on the HIV-1 fusion, we employed an enzyme-based HIV-1 fusion assay, involving preparation of BlaM-Vpr-containing virions (NL4-$3^{BlaM\text{-}Vpr}$), as described previously (8). Exogenous expression of CD63 had no effect on the amount of either virion-incorporated BlaM-Vpr or released virions, and CD63 was also successfully incorporated (data not shown). Using these virions, we studied the fusion of CD63-enriched virions by measuring the enzymatic activity of BlaM, which is taken up into the cytoplasm of target cells as a result of viral fusion. We observed that the uptake of BlaM, which reflects the fusion activity of viruses with target cells, was remarkably reduced in assays using CD63-enriched virions (Fig. 7B). The reduction in fusion efficiency

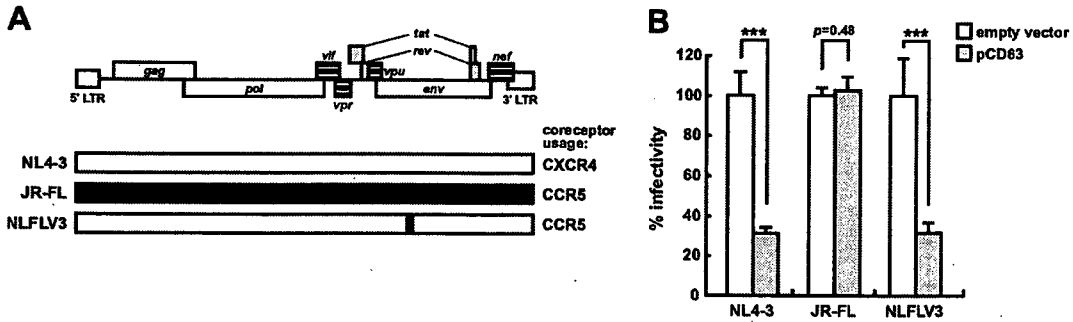

FIG. 5. Effect of virion-incorporated CD63 relative to other HIV-1 strains. (A) HIV-1 strains are schematically shown. (B) IU of respective viruses released from empty vector-cotransfected or pCD63-cotransfected 293T cells were measured by Magi assay. The IU were normalized to the amounts of $p24^{CA}$, and infectivity is shown as a percentage of the empty-vector value. Experiments were performed in triplicate. ***, P 0.001 by Student's *t* test. Error bars indicate standard deviations.

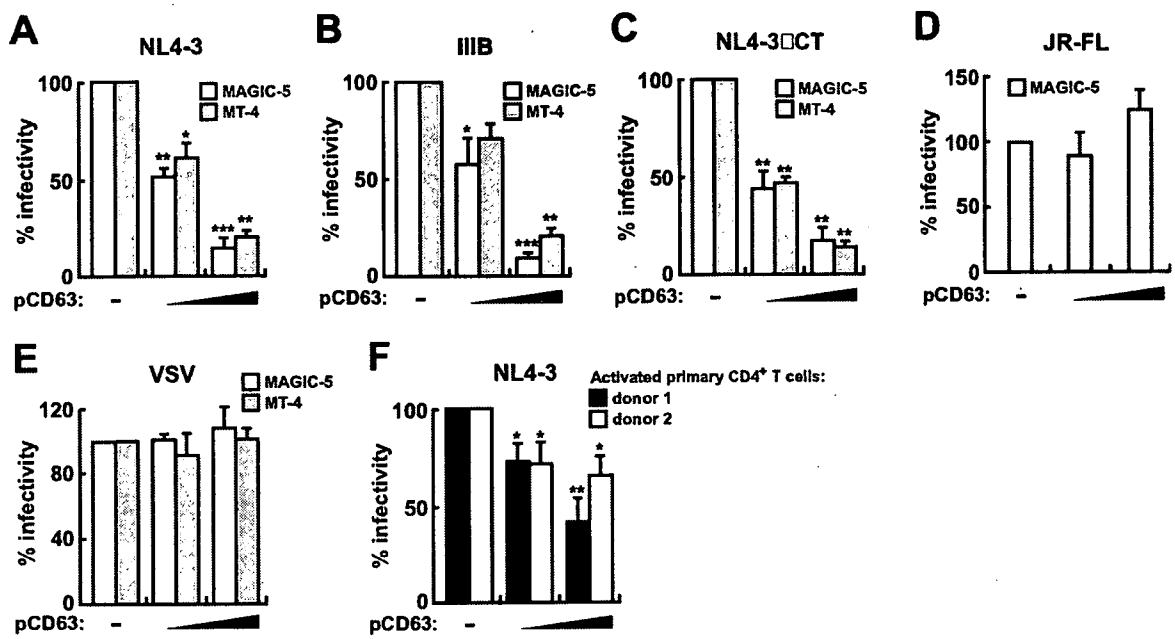

FIG. 6. Pseudotyped virus infection and luciferase assay. The prepared viruses were pseudotyped with Env of NL4-3 (A and F), IIIB (B), NL4-3 CT (C), JR-FL (D), or VSV (E). Each pseudotyped virus was prepared by cotransfection with pNLLuc, individual Env expression plasmids, and either empty vector or different doses of pCD63, as described in Materials and Methods. (A to E) MAGIC-5 cells and/or MT-4 cells were used as target cells. (F) NL4-3 Env-pseudotyped viruses were inoculated with CD3/CD28-activated primary CD4 T cells, and representative results are shown. The average luciferase activities per 1 g of protein was calculated as relative light units, and infectivity is shown as a percentage of the empty-vector value. Experiments were performed in triplicate. Statistical significance (Student's *t* test) versus empty-vector values is shown as follows: *, P 0.05; **, P 0.01; ***; P 0.001. Error bars indicate standard deviations.

corresponded closely to the reduction in infectivity (Fig. 2H). We also quantified HIV-1 RT by real-time PCR. As shown in Fig. 7C and D, both early and late RTs were decreased by CD63 enrichment. We concluded that virion-incorporated CD63 has the potential to prevent a postattachment step mediated by HIV-1 Env leading to the virus-to-cell fusion, and it causes the reduction in infectivity.

Tetraspanin proteins commonly have the potential to suppress HIV-1 infectivity. We analyzed the modulation of cell surface expression of other tetraspanins, CD9, CD81, CD82, and CD231, through cell activation. As shown in Fig. 8A, we observed that surface expression of other tetraspanins on Molt4/IIIB cells was also significantly down-modulated by PHA/PMA activation. Correlating with the surface expression, the amounts of virion-incorporated CD81, CD82, and CD231 were commonly decreased (Fig. 8B), although the amount of virion-incorporated CD9 was not significantly changed because of its lower level of incorporation.

Next, we cotransfected cells with pNL4-3 and individual tetraspanin (CD9, CD81, CD82, or CD231) expression plasmids. In addition, as a control protein, we also prepared an L6 expression plasmid. L6 has four transmembrane domains and is topologically similar to tetraspanins but does not belong to the genuine tetraspanin superfamily because of its structural

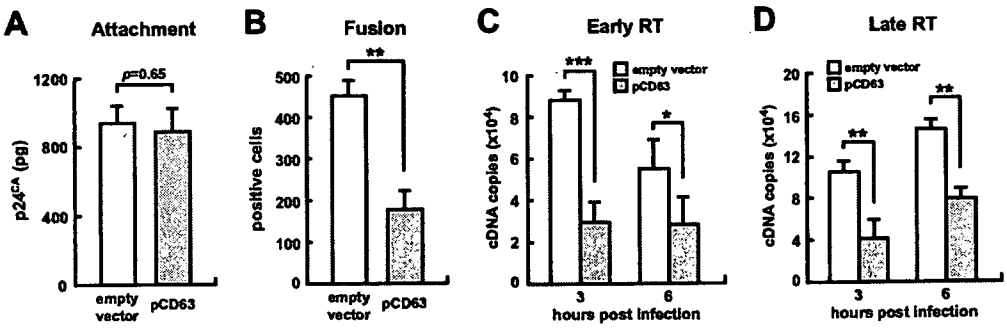

FIG. 7. Effect of virion-incorporated CD63 at early steps of HIV-1 infection. (A) Virus attachment assay. Aliquots of NL4-3 (10 ng of $p24^{CA}$) were incubated with MT-4 cells for 2 h at 4°C. After washing, the cells were lysed, and the amount of bound virions was quantitated by $p24^{CA}$ ELISA. A representative result is shown. (B) Virus fusion assay was performed as described in Materials and Methods, and the number of fused cells in 10,000 cells is shown. (C and D) Real-time PCR was performed as described in Materials and Methods, and the cDNA copy numbers of early (C) and late (D) RT in 100,000 cells are shown. Assays were performed in triplicate. Statistical significance (Student's *t* test) versus empty-vector values is shown as follows: *, P 0.05; **, P 0.01; ***, P 0.001. Error bars indicate standard deviations.

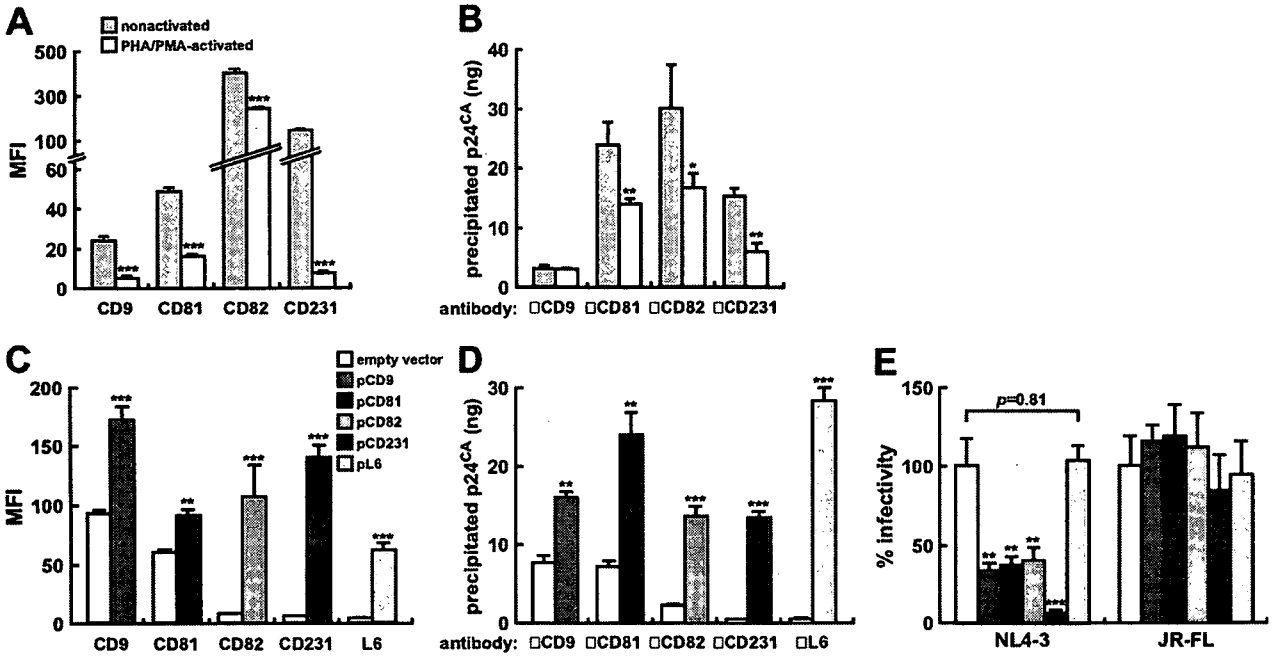

FIG. 8. Correlation between HIV-1 infectivity and the levels of tetraspanins in the released virions. (A and B) Molt4/IIIB cells were activated by PHA and PMA as described for Fig. 1. The surface expression of CD9, CD81, CD82, and CD231 on nonactivated and PHA/PMA-activated Molt4/IIIB cells was analyzed by flow cytometry. (B) The virus precipitation assay was performed as described in Materials and Methods. HIV-1_{IIIB} particles (100 ng of $p24^{CA}$) released from nonactivated or PHA/PMA-activated Molt4/IIIB cells were used for immunoprecipitation by respective antibodies. (C to E) Empty vector or individual tetraspanin-expression plasmids were each cotransfected with pNL4-3 into 293T cells. (C) The surface expression of tetraspanins on the 293T cells cotransfected with pNL4-3 and either empty vector or individual tetraspanin expression plasmids was analyzed by flow cytometry. (D) The virus precipitation assay was performed as described in Materials and Methods. HIV-1_{NL4-3} particles (100 ng of $p24^{CA}$) released from the 293T cells cotransfected with pNL4-3 and either empty vector or individual tetraspanin expression plasmids were used for immunoprecipitation by the respective antibodies. (E) IU of HIV-1_{NL4-3} or HIV-1_{JR-FL} released from the 293T cells cotransfected with pNL4-3 or pJR-FL and either empty vector or individual tetraspanin expression plasmids was measured by Magi assay. The IU were normalized to the amounts of $p24^{CA}$, and infectivity is shown as a percentage of empty-vector values. Experiments were performed in triplicate. Statistical significance compared to nonactivated (A and B) or empty vector (C to E) values (Student's *t* test) is shown as follows: *, P 0.05; **, P 0.01; ***, P 0.001. Error bars indicate standard deviations. MFI, mean fluorescence intensity.

and evolutional divergence (68, 71). As shown in Fig. 8C, tetraspanins and L6 were expressed on the surface of 293T cells and did not modulate the surface expression of the other tetraspanin proteins (data not shown). Correlating with their surface expression, they were also efficiently incorporated into the released virions (Fig. 8D), as in the case of CD63 (Fig. 2). Furthermore, Magi assay revealed that CD9-, CD81-, CD82-, and CD231-enriched NL4-3 also had less infectivity (Fig. 8E). However, L6-enriched NL4-3 had infectivity comparable to that of typical NL4-3 (Fig. 8E). Interestingly, as found in the case of CD63, we also observed that virion-incorporated tetraspanins did not affect JR-FL infectivity (Fig. 8E). These results suggest that many tetraspanins have the potential to attenuate NL4-3 infectivity and that tetraspanins may cooperatively modulate its infectivity.

DISCUSSION

Various host membrane proteins, including CD63 and tetraspanin proteins, exist on HIV-1 particles (7, 9, 45, 46, 55), and some of them affect viral infection. For example, HLAs (6, 13, 44), costimulatory molecules (21, 25) and ICAM-1 (2, 4) enhance HIV-1 infectivity, while CD4 has the potential to suppress HIV-1 infection through incorporation into the released progeny virions (66). However, most of their functions for or against HIV-1 infection are still unclear. In this study, we quantitatively assessed CD63 incorporation into HIV-1 particles released from T cells and epithelial cell lines (Fig. 1 and 2) and examined its influence on the infectivity of the virions. In Molt4/IIIB cells, we found that a large quantity of CD63 was incorporated into the released particles (Fig. 1D). CD63 incorporation was reduced upon cellular activation (Fig. 1D), and this reduction was accompanied by increased infectivity (Fig. 1B). We showed that our CD63 exogenous expression model in 293T cells is useful for studying the relationship between CD63 incorporation and virion infectivity by demonstrating that pCD63-transfected 293T cells expressed amounts of surface CD63 (Fig. 2B) similar to those in nonactivated Molt4/IIIB cells (Fig. 1F) and that exogenous CD63 was also successfully incorporated into the released virions (Fig. 2D). Using this system, we showed that the infectivity of NL4-3 virions was not affected by released CD63 not associated with virus particles (Fig. 3). Rather, the infectivity of NL4-3 was inversely correlated with both the amount of virion-incorporated CD63 and the level of surface expression of CD63 (Fig. 4). In contrast to NL4-3 and IIIB, we found that JR-FL was resistant to CD63-mediated infectivity reduction (Fig. 5 and 6) and that Env determined the susceptibility to CD63 (Fig. 6). In

addition, virion-incorporated CD63 had the ability to impair HIV-1 entry without affecting the binding of Env to CD4 (Fig. 7). Furthermore, we found that other tetraspanin proteins, such as CD9, CD81, CD82, and CD231, all had the potential to be incorporated into released HIV-1 particles and to interfere with NL4-3 infection, as in the case of CD63, and that this potential is unique in tetraspanin proteins (Fig. 8). Taken together, these finding are the first indication that some host membrane proteins have the potential to modulate HIV-1 infectivity in a strain-specific manner through incorporation into released particles.

As shown in Fig. 1B, the infectivity of released HIV-1 virion was enhanced by Molt4/IIIB activation. Although the amounts of mature Env in released virions were comparable (Fig. 1C), we detected a decrease in the amount of virion-incorporated CD63 (Fig. 1D) and suspected that CD63 on virions has the potential to regulate HIV-1 infectivity. To confirm this possibility, we first attempted to decrease endogenous expression of CD63 through transfection with small interfering RNA against *cd63* in 293T cells. However, we could not detect significant differences in the infectivity of released HIV-1 virions (data not shown). We suspected that the lack of difference was due to the low level of endogenous CD63 in HIV-1 particles released from 293T cells (Fig. 2D), and we next planned to study its exogenous expression. Exogenous CD63 was successfully incorporated into the released particles (Fig. 2D). This level corresponded closely to the level of CD63 on IIIB virions released from nonactivated Molt4/IIIB cells (Fig. 1D; compare Fig. 2D) and also to the level described in a previous report (35). In addition, the amount of endogenous CD63 in NL4-3 particles released from 293T cells (Fig. 2D) also corresponded with that in IIIB particles released from PHA/PMA-activated Molt4/IIIB cells (Fig. 1D; compare Fig. 2D). Because the level of surface CD63 as well as incorporated CD63 was comparable between two systems (Fig. 1F and 2B), this 293T system was adequate to simulate the physiological phenomenon observed in Molt4/IIIB cells.

As shown in Fig. 2H, we detected attenuation of NL4-3 infectivity released from pCD63-transfected 293T cells. It has been reported that surface CD4 impairs Env incorporation and reduces the infectivity of the released virions (12, 42). Surface CD63 might also reduce the infectivity by affecting incorporation of Env. However, exogenous CD63 did not impair either Env maturation (Fig. 2A, top) or Env incorporation (Fig. 2A, bottom). On the other hand, Wyma et al. reported that immature HIV-1 particles are less active for fusion with target cell than mature particles (72). Exogenous CD63 might attenuate the maturation of HIV-1 particles. However, we found that Gag cleavage was successful (Fig. 2A, bottom). Recently, Ho et al. reported that a soluble recombinant LEL of CD63 has an ability to prevent HIV-1 infection (33), and we detected release of non-virion-associated CD63 into the culture supernatant of pCD63-transfected cells (Fig. 2A, bottom, rightmost lane). It was suspected that non-virion-associated CD63 released into the culture supernatant affected HIV-1 infection. However, non-virion-associated CD63 had no effect on HIV-1 infection (Fig. 3). Non-virion-associated CD63, which would be embedded on exosome/microvesicle-like components, may be inaccessible, as previously seen in the soluble recombinant LEL (33). As shown quantitatively (Fig. 2D) and visually (Fig. 2F), we detected the existence of CD63 on the released particles, and the amount was increased through exogenous expression (Fig. 2D and G). In addition, we observed that CD63 L preferentially localized at the cell surface (Fig. 4B) (34), while the whole amount was comparable to that of wild-type CD63 (Fig. 4A). Furthermore, CD63 L was additively incorporated (Fig. 4E) and then severely suppressed the infectivity of released virions (Fig. 4D). From these results, the amount of CD63 at the surfaces of HIV-1-producing cells clearly correlated with the level of CD63 in virions and inversely correlated with the infectivity of progeny NL4-3 virions. Accordingly, it appears that CD63 at the cell surface has a greater potential to be efficiently incorporated into released virions, which leads to the reduction in infectivity. This preference is reminiscent of positive correlation between the level of ICAM-1 surface expression on HIV-1-producing cells and the level of ICAM-1 in HIV-1 virions (54), although the effects elicited by respective embedded proteins were completely opposite.

It is known that the tetraspanin proteins have high homology in their structures and amino acid sequences (32, 62). Consistent with this observation, we found that exogenous tetraspanin proteins, such as CD9, CD81, CD82, and CD231, were also efficiently expressed at the cell surface (Fig. 8C) and can be incorporated into released NL4-3 particles and interfere with their infection (Fig. 8D and E). In contrast, a transmembrane protein called L6, which has four transmembrane domains but does not belong to the tetraspanin superfamily (68, 71), was also expressed at the cell surface and incorporated into the released particles (Fig. 8C and D) but did not affect HIV-1 infection (Fig. 8E). Actually, it has been reported that soluble LELs of other tetraspanin proteins, such as CD9, CD81, and CD82, also have the potential to suppress HIV-1 infection (33). Therefore, our findings clearly suggest that this is a common role for tetraspanins.

We observed that virion-incorporated CD63 did not affect JR-FL infectivity (Fig. 5B). In addition, through a pseudotyped-virus infection assay, we found that exogenous CD63 impaired the infection mediated by NL4-3 Env (Fig. 6A) and IIIB Env (Fig. 6B) in a dose-dependent manner and independently of the target cells (as shown in Fig. 6F, it was also confirmed in primary activated CD4 T cells). In contrast, there were no effects on the infection mediated by JR-FL Env (Fig. 6D) and VSV-G (Fig. 6E), although exogenous CD63 was incorporated into released particles in an Env-independent manner (Fig. 2E). These results indicate that susceptibility to CD63 is determined in Env and that there is strain specificity. There is a well-known difference between NL4-3 and JR-FL in their coreceptor usage: the former uses CXCR4 and the latter uses CCR5, and coreceptor preference is determined by the V3 region of Env (66). Since the infectivity reduction could be V3 region dependent, we further used NLFLV3 that contains the V3 region of JR-FL Env in NL4-3 Env and uses CCR5 as the coreceptor. However, the difference in the susceptibility for virion-incorporated CD63 between NL4-3 and JR-FL was not caused by the difference in coreceptor usage (Fig. 5B). Actually, other R5 viruses, such as JR-CSF and several chimeras, were also susceptible to CD63 (data not shown). In this regard, it is interesting that there is a difference in CD63 susceptibility between two R5 HIV-1 strains, JR-FL and JR-CSF, which were simultaneously isolated from the brain of an HIV-1 en-

cephalopathy patient (40). While the determinant region(s) in Env for the vulnerability to virion-incorporated CD63 has not been identified, these results indicate that there is some kind of strain specificity, or that JR-FL Env has some resistant property against virion-incorporated tetraspanins. It has been reported that a trivial change in Env conformation caused by mutation of one or more amino acid residues is responsible for the resistance to neutralizing antibodies and anti-HIV-1 drugs and that there are trivial differences in Env conformation between HIV-1 strains (5, 58). However, the crystal structure of the entire HIV-1 Env is not yet resolved, and it is difficult to speculate accurately on the invisible conformation of Env from its amino acid sequence. Our findings shed light on an unknown difference(s) between HIV-1 Envs, which is possibly conformational. Therefore, our findings may provide a clue for elucidating the ambiguous conformation of HIV-1 Env, which may lead to a novel target of anti-HIV drugs.

It has been reported that CD4 has the potential to inhibit HIV-1 infection at the attachment step through incorporation into the released HIV-1 particles (66). In contrast, as shown in Fig. 7A, virion-incorporated CD63 did not affect the attachment step. Rather, a postattachment step was attenuated by CD63 enrichment (Fig. 7B to D). It has been reported that the correct interaction between gp41 CT and $p17^{MA}$ in progeny virions is important to elicit efficient conformational changes of HIV-1 Env, leading to infection (14, 16, 72, 73). Virion-incorporated CD63 may disturb the interaction between gp41 CT and $p17^{MA}$ at the lining of virions. However, the infectivity of a CT-deleted NL4-3 Env-pseudotyped virus was also decreased by exogenous CD63 (Fig. 6C), suggesting that virion-incorporated CD63 had no or little effect on the interior of progeny virions. Interestingly, there are several reports showing that tetraspanin proteins are associated with physiological membrane fusion events. For example, murine CD9 on oocytes contributes to sperm-egg fusion (43, 47), and CD9 and CD81 on mononuclear phagocytes prevent their mutual fusion (65). In the case of human retroviruses, it was recently reported that the syncytium formation mediated by HIV-1 Env is suppressed by overexpression of CD9 and CD81 on the target cells (26). We think this is yet another phenomenon in which tetraspanin proteins are involved. Actually, tetraspanin proteins are able to interact laterally with each other through their LELs (62). Hence, CD63 on HIV-1 virion may laterally interact with endogenous CD9 and CD81 on target cells and may constitute an obstacle that impairs the stable interaction between gp120 and CD4/coreceptors following attachment.

Interestingly, PHA/PMA activation of Molt4/IIIB cells enhanced the infectivity of released IIIB virions (Fig. 1B), while the amount of incorporated Env was not changed (Fig. 1C). In this situation, it was interesting that the amount of virion-incorporated tetraspanins was decreased (Fig. 1D and 8B) and that their surface expression was significantly down-regulated (Fig. 1F and 8A). There was a clear relationship between the infectivity of released HIV-1 virions and the amount of tetraspanin proteins on both the virion and the surfaces of HIV-1-producing cells. Therefore, the enhancement of IIIB infectivity upon PHA/PMA activation of Molt4/IIIB cells should be at least partially due to the down-regulation of tetraspanin proteins.

Recently, the importance of tetraspanin proteins in HIV-1 replication has been recognized (3, 26, 50). The so-called "Trojan exosome hypothesis" proposes that HIV-1 applies TEMs and the machineries of exosome biosynthesis to its extracellular egress (27). However, the results we present here suggest that CD63 and other tetraspanins on viral membranes also have the potential to interfere with viral infection. The fundamental roles of tetraspanins and cellular membrane proteins in HIV-1 virions are intriguing, and further studies will be needed to uncover their mechanisms of action.

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Neutralizing antibodies decrease the envelope fluidity of HIV-1

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Abstract

For successful penetration of HIV-1, the formation of a fusion pore may be required in order to accumulate critical numbers of fusion-activated gp41 with the help of fluidization of the plasma membrane and viral envelope. An increase in temperature to 40 °C after viral adsorption at 25 °C enhanced the infectivity by 1.4-fold. The enhanced infectivity was inhibited by an anti-CXCR4 peptide, T140, and anti-V3 monoclonal antibodies (0.5 and 694/98-D) by post-attachment neutralization, but not by non-neutralizing antibodies (670-30D and 246-D) specific for the C5 of gp120 and cluster I of gp41, respectively. Anti-HLA-II and an anti-HTLV-I gp46 antibody, LAT27, neutralized the molecule-carrying HIV-$1_{C\text{-}2(MT\text{-}2)}$. The anti-V3 antibodies suppressed the fluidity of the HIV-$1_{C\text{-}2}$ envelope, whereas the non-neutralizing antibodies did not. The anti-HLA-II antibody decreased the envelope fluidity of HIV-$1_{C\text{-}2(MT\text{-}2)}$, but not that of HIV-$1_{C\text{-}2}$. Therefore, fluidity suppression by these antibodies represents an important neutralization mechanism, in addition to inhibition of viral attachment.

Keywords: HIV-1; Neutralizing antibody; Membrane/envelope fluidity; Fusion pore

Introduction

Viral neutralization consists of a decrease in the infectious titer of a viral preparation following its exposure to antibodies. The loss of infectivity is brought about by interference of the antibodies bound to the viruses during any step from viral attachment to the cells to release of the viral genome into the host cells. The potent and broad capacity of the available neutralizing antibodies against human immunodeficiency virus type 1 (HIV-1) has been attributed to inhibition of fusion and/or virus attachment to the cell surface (Klasse and Sattentau, 2002; Zwart et al., 1991). Thus, the neutralization effects are currently well explained by antibody coating of the virions (high-occupancy theory of neutralization) (Klasse and Sattentau, 2002). However, the high-occupancy theory cannot solely account for the post-attachment neutralization (PAN) (Armstrong et al., 1996; Edwards and Dimmock, 2001), synergistic (Zwick et al., 2001) and additive (Verrier et al., 2001) effects of neutralizing antibodies, and HIV-1 neutralization by one or a few antibody molecules (Harada, 2002). Therefore, there must be additional mechanisms involved in HIV-1 neutralization.

Recently, we reported that small changes in the fluidity of the plasma membrane and/or viral envelope would be indispensable for modifying HIV-1 infectivity (Harada et al., 2005). We hypothesized that fluidity-mediated accumulation of fusion-activated domains was required to form a wide fusion pore large enough for an enveloped viral core to pass through. In fact, the increased or decreased fluidity changes observed after different treatments with the same fluidity modulator, such as glycyrrhizin (Harada, 2005) or fattiviracin FV-8 (Harada et al., 2007), were parallel with the high or low susceptibility of the treated cells to HIV-1 infection and fusion. These modulators are broad antiviral agents, which are especially effective against enveloped viruses. Furthermore, they were found to reduce the infectivity of virions when the viruses were pretreated (Harada, 2005; Harada et al., 2007). Notably, this latter finding reminded us of the actions of neutralizing antibodies against viral particles.

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In order to gain further insights into the functions of membrane/envelope fluidity in HIV-1 infection and neutralization, we designed a series of experiments to examine the effects of neutralizing antibodies on the fluidization of the HIV-1 envelope and plasma membrane. We found that these antibodies suppressed the fluidization of the highly ordered lipid bilayer of the viral envelope when they exerted neutralization against the respective virus. These studies provide novel insights into the cell biology of retroviral penetration and demonstrate distinct roles for membrane fluidity at early stages of HIV-1 penetration.

Results

Neutralization and PAN by the anti-V3, anti-C5 and anti-gp41 monoclonal antibodies

Conventional neutralization experiments were conducted by pre-incubating viruses with the antibodies prior to their addition to cell culture. At 1 g/ml, the 0.5 (anti-V3) monoclonal antibody (mAb) neutralized more than 90% of plaque-cloned HIV-1_{C-2} (C-2 viruses) (Masuda et al., 1990) and X4 envelope-pseudotyped viruses with a luciferase reporter gene (NL43-luc viruses) (Harada et al., 2004b). Next, the neutralization activities of human mAbs against gp120 V3 (694/98-D), gp120 C5 (670-30D) and gp41 cluster I (246-D) were examined. All of these mAbs were previously reported to bind to intact clade B (III_B) virions (Nyambi et al., 2000). As shown in Fig. 1A, 1 g/ml 694/98-D neutralized more than 90% of NL43-luc viruses, similar to 0.5 , whereas 670-30D and 246-D mAbs rather enhanced the infectivity by around 1.3-fold at all concentrations examined. Thus, 0.5 and 694/98-D mAbs are neutralizing antibodies, whereas 670-30D and 246-D are non-neutralizing antibodies.

NL43-luc viruses were incubated with GHOST/CXCR4 cells at 25 °C for 1 h. After this viral adsorption, the post-adsorption steps were carried out at either 37 °C or 40 °C in the presence or absence of the anti-CXCR4 peptide T140 or mAbs (Fig. 1B). T140 inhibited 95% of the infection when GHOST/CXCR4 cells were treated with 1 M T140 before infection with NL43-luc pseudoviruses (Harada et al., 2005). Increasing the temperature to 40 °C during the post-adsorption steps augmented the level of infection by 1.4-fold (Fig. 1B). This phenomenon is hereafter referred to as post-attachment enhancement (PAE). The PAEs were repeatedly observed by 1.5- to 2.0-fold (Harada et al., 2004a, 2005). The PAEs from 25 °C to 40 °C were strongly abrogated by 1 M T140 (Fig. 1B), indicating that T140 could restrain the viruses from forming multiple-site binding or accumulating fusion-activated gp41 domains by occupying CXCR4. The PAE of 1.4-fold observed at 40 °C was also significantly inhibited by 1 g/ml 0.5 or 4 g/ml 694/98-D mAbs (Fig. 1B), suggesting that these anti-V3 mAbs could mediate PAN when the viruses were loosely bound to the cells at 25 °C. An increased amount (10 g/ml) of 0.5 mAb showed the same inhibitory effect (about 30%) on the PAE as 1 g/ml, indicating that the PAE inhibition by this antibody could not be solely explained by

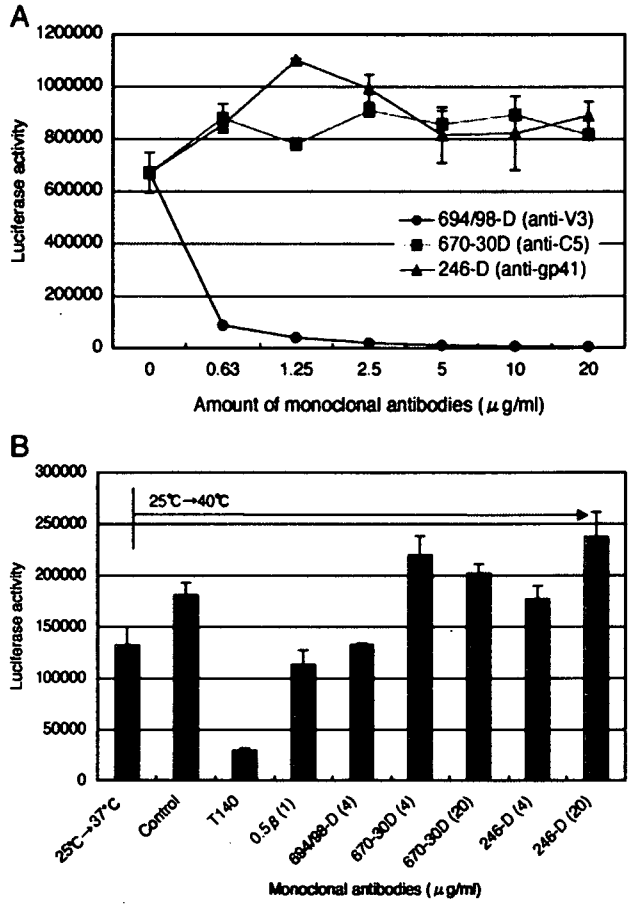

Fig. 1. Conventional neutralization of NL43-luc pseudoviruses by 694/98-D (circles), 670-30D (squares), and 246-D (triangles) mAbs (A), and effects of 1 M T140 and HIV-1-specific mAbs (0.5 , 694/98-D, 670-30D, and 246-D) on PAE at 40 °C for 1 h after viral adsorption at 25 °C (B). All experiments in panels A and B were carried out in triplicate, and solid lines and bars show the mean±SD.

covering the V3 loop of gp120 with antibody molecules, as was the case for T140 occupation of CXCR4. Anti-C5 (670-30D) and anti-gp41 (246-D) mAbs had little effects on the PAN, but tended to enhance the PAE (Fig. 1B).

Suppression of viral envelope fluidization by neutralizing anti-V3 antibodies

The structural and dynamic modification of envelope lipids was examined using a spin-label method (Figs. 2 and 3). As a negative control for C-2, 0.5 -escaped C-2 (esc.C-2) viruses were used. More than 90% of the C-2 viruses were neutralized by 1 g/ml 0.5 , whereas the esc.C-2 viruses were not affected (Masuda et al., 1990). The esc.C-2 viruses have one point mutation in the crown of the V3-loop, resulting in exchange of GPG to GQG. The order parameters of 0.5 -treated C-2 viruses were higher at 25 °C, 37 °C and 40 °C than those of control IgG (MOPC21)-treated viruses (Figs. 2A and B; Fig. 3A), indicating that 1 g/ml 0.5 mAb suppressed the fluidity of the viral envelope. At 37 °C, 0.5 mAb suppressed the fluidity by 7%. However, no such effect of 0.5 was observed in the case of the esc.C-2 viruses (Fig. 3B). Thus, the fluidity suppression by this antibody is consistent

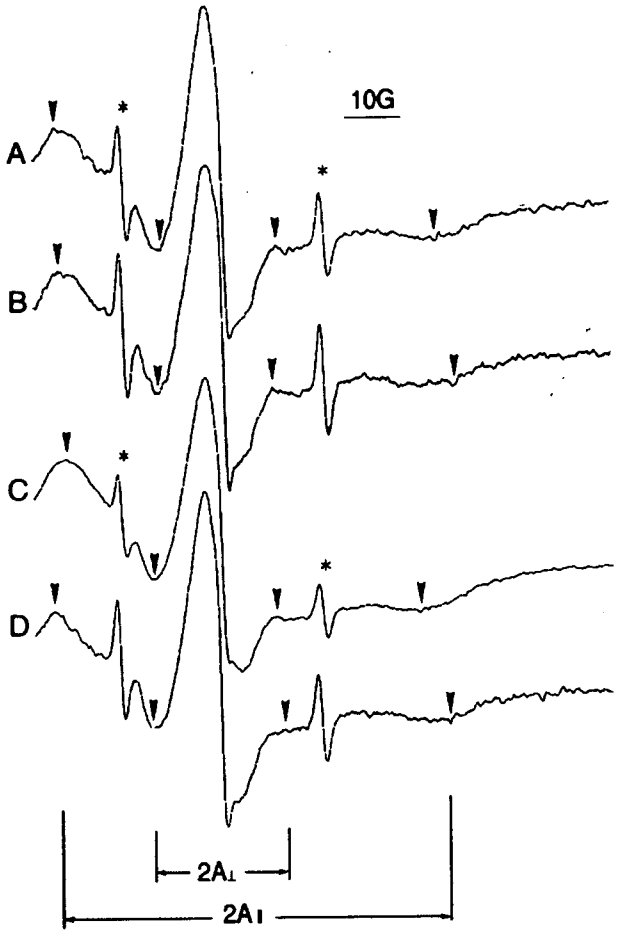

Fig. 2. ESR spectra of viral envelopes from intact C-2 (A), 0.5 -treated C-2 (B), C-2(MT-2) (C), and anti-HLA-II-treated C-2(MT-2) (D) viruses at 37 °C. Viruses (50 ml) were incubated in the presence or absence of 0.5 or anti-HLA-II antibodies at a final concentration of 1 g/ml at 37 °C for 40 min, and then labeled with 5-DSA for 20 min. Virus pellets were examined by ESR spectroscopy. The outer and inner hyperfine splittings, $2A_{//}$ and 2A , were measured as indicated by the arrowheads. Asterisks indicate the spectra of free 5-DSA. The order parameters (*S*-values) of (A), (B), (C) and (D) are 0.736, 0.787, 0.678, and 0.772, respectively. The scale (10G) of the horizontal axis is shown.

with its neutralization data. Another anti-V3 mAb, 694/98-D (1 g/ml) also suppressed the fluidity of the C-2 envelope (Fig. 3C). However, the non-neutralizing 670-30D (0.5 g/ml) and 246-D (1 g/ml) mAbs had no effects on the fluidization of the viral envelope (Fig. 3C). Similar effects on the plasma membrane fluidity were obtained when MOLT-4/C-2 cells were treated with 0.5 , 694/98-D, 670-30D and 246-D mAbs (data not shown). Thus, the 0.5 and 694/98-D mAbs suppressed the fluidity not only of the viral envelope but also of the plasma membrane.

HIV-1 neutralization by antibodies against non-HIV molecules

Adsorption-blocking, namely, the high-occupancy theory, cannot be completely excluded for the observed inhibition by the 0.5 and 694/98-D mAbs in the neutralization experiments. Therefore, we examined neutralization by non-HIV antibodies.

FACS analyses revealed that MOLT-4 cells did not express HLA-II molecules (Fig. 4A), whereas human T-cell leukemia virus type I (HTLV-I)-transformed MT-2 cells carried many of these molecules on their surface (Fig. 4B). MT-2 cells were also positive for HTLV-I gp46 detected by neutralizing LAT27 and non-neutralizing LAT12 antibodies (Fig. 4C). The binding affinity of LAT27 appeared to be stronger than that of LAT12 (Fig. 4C). HIV-$1_{C\text{-}2(MT\text{-}2)}$ viruses that budded from HIV-$1_{C\text{-}2}$-infected MT-2 cells were expected to have both HLA-II molecules and HTLV-I gp46 as phenotype-mixing progeny viruses. It has been reported that HIV-1 carries more HLA-II molecules than gp120 (Trubey et al., 2003). The anti-HLA-II

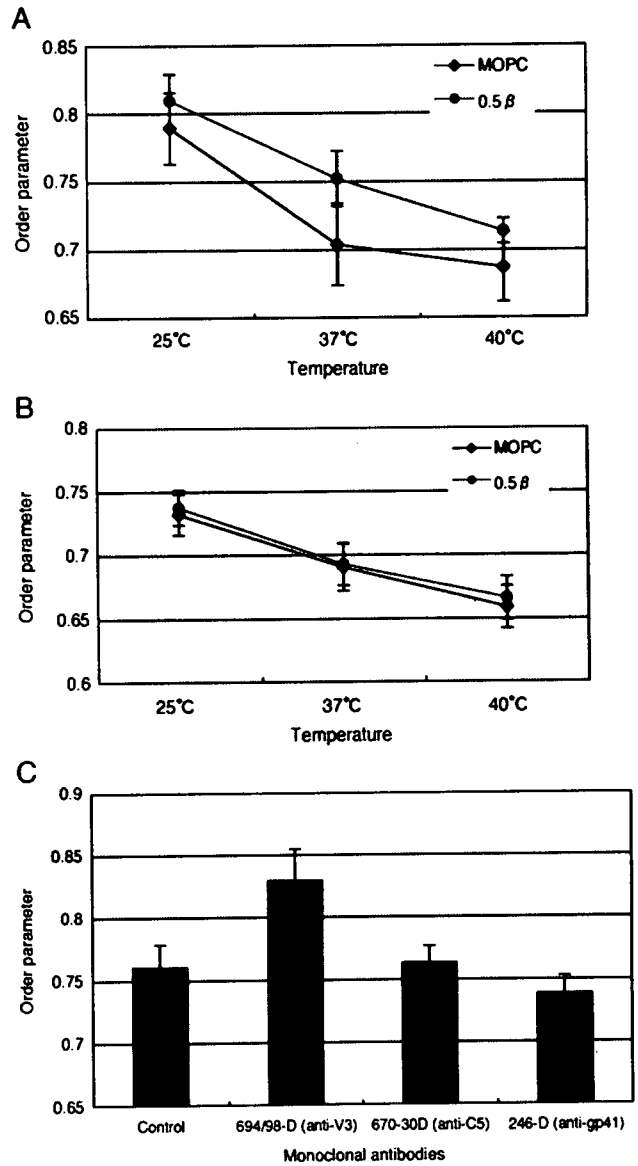

Fig. 3. Effects of 1 g/ml MOPC21 (diamonds) and 1 g/ml 0.5 (circles) on the envelope fluidity of C-2 (A) and esc.C-2 (B) viruses. Viruses (50 ml) were treated with each antibody at 37 °C for 40 min prior to labeling with 5-DSA for 20 min. All measurements were carried out in triplicate and the solid lines show the mean±SD. (C) Effects of 1 g/ml 694/98-D (n=8), 0.5 g/ml 670-30D (n=2), and 1 g/ml 246-D (n=5) on the envelope fluidity of C-2 viruses. As a control, PBS was used instead of an mAb (n=3). Bars show the mean±SD.

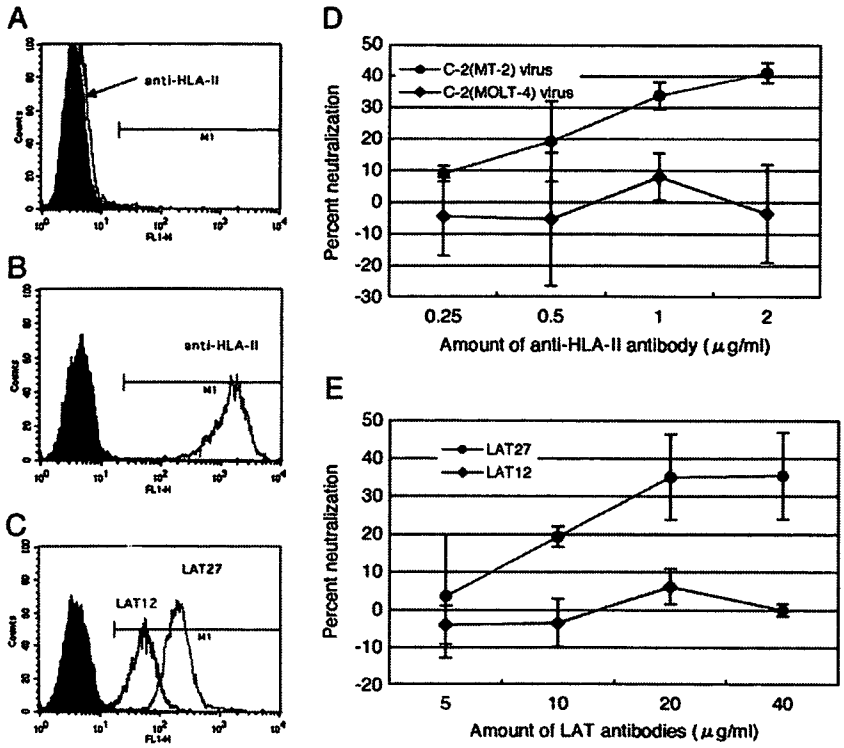

Fig. 4. Flow cytometric analysis of HLA-II expression in MOLT-4 (A) and MT-2 (B) cells, and HTLV-I gp46 expression in MT-2 cells (C). Neutralization of C-2(MT-2) (circles) and C-2(MOLT-4) (diamonds) viruses by anti-HLA-II (D) and neutralization of C-2(MT-2) viruses by LAT27 (circles) and LAT12 (diamonds) (E) are shown. The solid lines in panels D and E show the mean±SD of 6 independent experiments.

antibody at 2 g/ml neutralized 40% of the C-2(MT-2) viruses, whereas the C-2 viruses were not affected (Fig. 4D). C-2(MT-2) viruses were also neutralized by the neutralizing LAT27 antibody, but not by the non-neutralizing LAT12 antibody (Fig. 4E). However, 20–40 g/ml of the LAT27 antibody only neutralized 35% of the C-2(MT-2) viruses. In addition, the level of neutralization quickly reached a plateau with more than 20 g/ml of the antibody. Infection of C-2(MT-2) viruses in MAGI cells was almost completely blocked by 0.5 or T140, indicating that C-2(MT-2) viruses used HIV-1 gp120 and CXCR4 for the infection (data not shown). Nevertheless, HLA-II and HTLV-I gp46 on HIV-1 particles could be used as target molecules for neutralization of the virus by the respective antibodies.

One of the mechanisms proposed for HIV-1 neutralization by antibodies against non-HIV-1 molecules is the "coating theory," which suggests that a dense coat of antibodies over the virion surface could interfere with viral adsorption to cells by steric hindrance. However, treatment of C-2(MOLT-4) or C-2 (MT-2) viruses with the anti-HLA-II, LAT12 and LAT27 antibodies did not significantly inhibit the viral attachment to MOLT-4 cells (Fig. 5). As a positive control, 2 g/ml of the anti-V3 0.5 antibody suppressed C-2(MOLT-4) viral adsorption to the cells by only 50%, which could be partly explained by 0.5 mAb-mediated blocking of gp120 V3 binding to CXCR4. Other mechanisms, such as fluidity alterations in the viral envelope rather than steric hindrance, may be responsible for the HIV-1 neutralization by antibodies against non-HIV molecules.

Suppression of fluidization by neutralizing antibodies against non-HIV molecules

At 1 g/ml, the anti-HLA-II antibody strongly suppressed the envelope fluidity of C-2(MT-2) viruses (Figs. 2C and D; Fig. 6A), but did not suppress that of C-2 viruses (Fig. 6B). These findings were well correlated with the neutralization data (Fig. 4D). The anti-HLA-II suppressed the fluidity by 15% at 37 °C. Since the anti-HLA-II antibody (2 g/ml) was previously reported to suppress the membrane fluidity of MT-2 cells

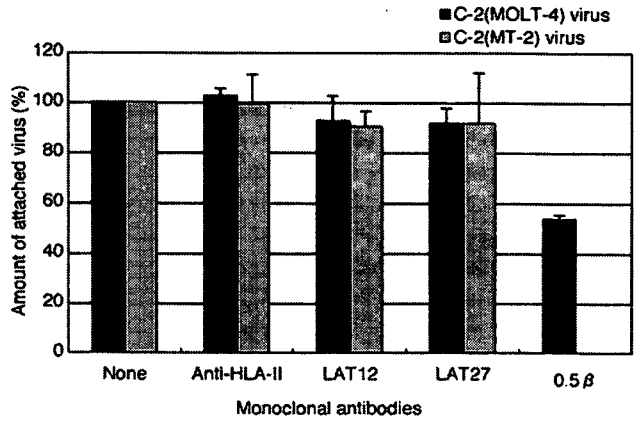

Fig. 5. Effects of MOPC21 (20 g/ml), anti-HLA-II (2 g/ml), LAT12 (20 g/ml), LAT27 (20 g/ml), and 0.5 (2 g/ml) on C-2(MOLT-4) or C-2(MT-2) virus adsorption on MOLT-4 cells. All experiments were carried out in triplicate, and the bars show the mean±SD.

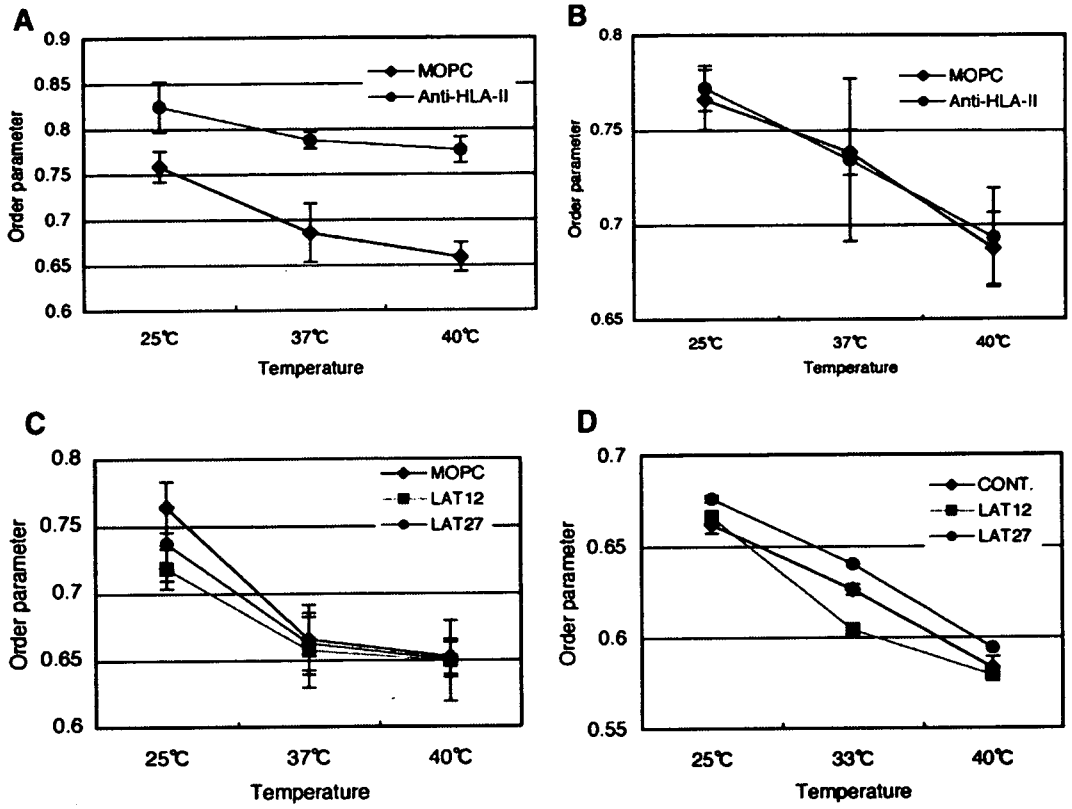

Fig. 6. Effects of 1 g/ml MOPC21 (diamonds) and 1 g/ml anti-HLA-II (circles) on envelope fluidity of C-2(MT-2) (A) and C-2 (B) viruses, and the effects of 10 g/ml LAT12 (squares) and 10 g/ml LAT27 (circles) on the fluidity of the viral envelope of C-2(MT-2) viruses (C) and plasma membrane of MT-2 cells (D). All measurements were carried out in triplicate and the solid lines show the mean±SD.

(Harada et al., 2004a), this antibody appears to universally lower the fluidity of lipid bilayers when it binds to membranes.

The LAT antibodies at 10 g/ml showed no significant suppression of fluidity in the C-2(MT-2) viruses (Fig. 6C). However, when MT-2 cells were treated with 10 g/ml of the LAT antibodies, the neutralizing LAT27 antibody suppressed the plasma membrane fluidity at all temperatures examined (Fig. 6D). In contrast, the non-neutralizing LAT12 antibody did not show any suppressive effects on MT-2 plasma membrane fluidity (Fig. 6D).

Discussion

The V3 loop of HIV-1 gp120 plays a critical role in determining the cell tropism of the virus (Clapham and McKnight, 2002). The loop is composed of approximately 35 amino acid residues with a global positive charge. An increase in the positive charge of the loop is thought to be associated with coreceptor usage (Hoffman and Doms, 1999; Moulard et al., 2000; Platt et al., 2001). A single amino acid change in V3 is able to switch viral coreceptor usage (CXCR4 or CCR5) (Hu et al., 2000; Shimizu et al., 1999). Furthermore, the V3 domain is considered to be the primary determinant of coreceptor usage (Cormier and Dragic, 2002) together with V1/V2 contributions (Dragic, 2001; Foda et al., 2001; Maeda et al., 2000). Anti-V3 antibodies strongly neutralize T-cell line-adapted strains of HIV-1 (Gorny et al., 2002; Zhang et al., 2002), and block the fusion induced by HIV-1 infection (Masuda et al., 1990) or gp120-expressing cells. The antibodies may neutralize viruses by interfering with several steps in the early phase of infection. It has been reported that anti-V3 mAbs interfere with the ability of gp120 and CD4 complexes to bind to CCR5 (Cormier et al., 2001). Thus, they act as receptor-blockers. However, HIV-1 neutralization by one or a few molecules of V3-targeted mAbs (Harada, 2002) and PAN (Armstrong et al., 1996) have been reported, suggesting the possibility of neutralizing effects after viral adsorption.

Engagement of the gp120 trimer with coreceptors induces gp41 rearrangement and exposure of the hydrophobic amino acid fusion domain, leading to fusion. The triggered fusion-activated gp41 structures are rod-like with a bundle of six -helices (Clapham and McKnight, 2002). It is conceivable that HIV-1 penetration into a cell requires a fusion-pore that is wide enough for the viral core to pass through. Accumulation of substantial numbers of fusion-activated gp41 structures with the help of fluidity of the lipid bilayer membrane must be necessary for this purpose. The formation of ring-like assemblies of fusion proteins has been reported in studies of rabies virus (Roche and Gaudin, 2002), baculovirus (Plonsky and Zimmerberg, 1996), influenza virus (Markovic et al., 2001) and Semliki Forest virus (Gibbons et al., 2003). Cell–cell fusion is temperature-dependent (Frey et al., 1995), and a time lag of 10–20 min between the six-helix bundle formation and fusion has been reported after transfer from 31.5 °C to 37 °C (Golding et al.,

2002). This indicates that the formation of a fusion pore requires a critical number of six-helix bundles.

The anti-HIV peptide T140 strongly inhibits X4 HIV-1 infection (Tamamura et al., 1998) through its specific binding to CXCR4, as shown by the finding that 1 M T140 could occupy most of the CXCR4 molecules on the cell surface. As a result, the binding of T140 to CXCR4 could prevent a loosely attached virus from forming additional multiple-site binding. In fact, the enhancement observed as PAE due to temperature shifts (25 °C to 37 °C and 25 °C to 40 °C) in the present study was strongly inhibited by T140, indicating that multiple-site binding was functioning and blocked by T140 occupation of CXCR4 molecules. We previously reported that PAE at 40 °C requires a time lag of 20–30 min before becoming apparent for blocking the enhancement by T140 after transfer from 25 °C to 40 °C (Harada et al., 2004a). This finding suggests that PAE requires the accumulation of gp120/receptor complexes through membrane fluidity, similar to the case for cell–cell fusion (Golding et al., 2002).

In the present study, V3-targeted 0.5 and 694/98-D mAbs inhibited the PAE of HIV-1 infection at 40 °C by 30–40% when the cells were pre-incubated with viruses at 25 °C. The PAN by anti-V3 antibodies was also observed when the shift was to 37 °C, but the infection was inhibited only by 10–20%. Thus, the antibodies mediate less potent PAN at 37 °C than at 40 °C, probably because the antibodies mainly suppress increased infectivity (PAE) due to increased temperature. Direct occlusion by the anti-V3 antibodies of open sites involved in the process of multiple-site binding to cells could be a way to prevent PAE at 40 °C. It is unknown whether these antibodies induce the detachment of adsorbed virions from cells. However, we found that PAE was only inhibited by 30–40% and a 10-fold higher dose (10 g/ml) of 0.5 showed no further inhibitory effect on the PAE, indicating that occupancy is not likely to happen in this case. Alternatively, the binding of the antibody to the viral surface could affect steric alterations to the viral envelope and restrain the envelope from enhancing the fluidity at 40 °C. In fact, we observed that binding of 0.5 and 694/98-D to C-2 viruses induced a more ordered or rigid envelope at 37 °C.

Neutralization of simian immunodeficiency virus (SIV) by anti-HLA and anti-hemagglutinin antibodies has previously been reported (Vzorov and Compans, 2000). In the present study, we also found that phenotype-mixed C-2(MT-2) viruses were neutralized by anti-HLA-II and LAT27 antibodies, although LAT27 exhibited a very weak neutralization activity. The reason why LAT27 is a neutralizing antibody, but LAT12 is not, remains unknown, although the binding affinity of LAT27 to MT-2 cells appeared to be stronger than that of LAT12. Two possible mechanisms are proposed for the neutralization of C-2 (MT-2) viruses by antibodies against non-HIV molecules. The first is the coating theory, which suggests that a dense coat of antibodies over the virion surface will interfere with viral adsorption to the cells, whereas the second is the steric alteration theory, which suggests that structural changes caused by binding of the antibodies will prevent penetration or uncoating of the virus. If the first mechanism is the case, adsorption of C-2 (MT-2) viruses, but not C-2(MOLT-4) viruses, should be blocked by antibodies against non-HIV molecules. However, we found that treatment of C-2(MOLT-4) and C-2(MT-2) viruses with anti-HLA-II, LAT12 and LAT27 antibodies did not significantly inhibit the viral adsorption. Instead, the anti-HLA-II antibody suppressed the envelope fluidity of C-2(MT-2) viruses, but not that of C-2 viruses. However, the neutralizing LAT27 antibody showed no suppressive effect on the fluidity of the C-2(MT-2) envelope, but did suppress the MT-2 plasma membrane fluidity, probably due to the increased HTLV-I gp46 expression on the MT-2 plasma membrane. Currently, the reason why the LAT27 neutralized C-2(MT-2) viruses is unknown. More sensitive techniques to measure minute changes of envelope fluidity might be required to explore the mechanism.

In the present study, the levels of neutralization (more than 90%) by 1 g/ml 0.5 and 694/98-D in conventional assays were much stronger than that of PAN (30–40% inhibition of PAE). PAN could be mainly exerted by suppression of the viral envelope fluidity. Apparently, anti-V3 antibodies have multifarious neutralization mechanisms, such as occupancy of V3 and stabilization of envelope fluidity. We found that the anti-HLA-II antibody showed 35% neutralization against C-2(MT-2) viruses at the concentration of 1 g/ml. This modest neutralization was only achieved by suppressing the fluidity. However, the significance or biological meaning of HIV-1 neutralization by anti-HLA-II antibodies or autoantibodies is the extraordinary protection revealed in studies on SIV-infected macaques vaccinated with cells expressing the same HLA molecules as those employed to culture the SIV used for the challenge (Arthur et al., 1995; Chan et al., 1992). Furthermore, antibodies against HLA are frequently present in HIV-exposed but uninfected individuals (Brown et al., 1997).

Suppressing the fluidity of the viral envelope could be one of the essential, but additional, mechanisms for virus neutralization because fluidity is indispensable for infectivity due to the required formation of multiple-site binding and a fusion pore (Harada, 2005; Harada et al., 2005, 2007). Antibodies may need substantial amounts of epitope molecules with high binding affinities on the plasma membrane or viral envelope to decrease its fluidity. The mechanism for fluidity suppression by antibodies is still unknown, but it is unlikely to be actin-dependent as the fluidity of the viral envelope was affected. The attachment of an antibody molecule, especially if it bridges adjacent subunits, is assumed to cause local structural alterations or topological changes of the integral protein, which spreads to the whole envelope or membrane, thereby stabilizing the fluidization for unknown reasons. Similarly, retrocyclin 2 was reported to immobilize surface proteins by cross-linking membrane glycoproteins (Leikina et al., 2005). Therefore, retrocyclin 2 exhibits inhibitory effects on viral fusion and entry. If the binding of the antibody itself is essential for lowering the fluidity, this cannot explain the presence of sensitizing (non-neutralizing) antibodies against viruses. Hence, a hypothesis has been proposed that viruses bear heterogeneous envelope molecules (Harada et al., 2004b), such as functional and nonfunctional gp120, and non-neutralizing antibodies only react with the nonfunctional gp120 (Poignard et al., 2003).

Furthermore, there must be specific molecules or epitopes that switch fluidization on or off after binding of the respective antibody. Further studies will be needed to clarify the mechanisms involved in modifying the fluidity of lipid bilayer membranes.

Materials and methods

Cells and culture

MOLT-4, MOLT-4/HIV-1_{C-2}, MOLT-4/HIV-$1_{esc.C-2}$ (Masuda et al., 1990) and MT-2 (Harada et al., 1985) cells were cultured in RPMI1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml of penicillin and 0.1 mg/ml streptomycin (complete medium). Multinuclear activation of a galactosidase indicator (MAGI) cells derived from HeLa-CD4/ long terminal repeat- -galactosidase cells (Kimpton and Emerman, 1992) were maintained in complete medium supplemented with 0.1 mg/ml G418 and 0.05 mg/ml hygromycin B. GHOST/CXCR4 cells derived from a human osteosarcoma cell line (HOS) were cultured in complete medium supplemented with 0.2 mg/ml G418, 0.05 mg/ml hygromycin B and 2 mg/ml puromycin as previously described (Cecilia et al., 1998).

Preparation of viruses

Plaque-cloned HIV-1_{C-2} viruses (III_B X4 strain) and their 0.5 -escaped HIV-$1_{esc.C-2}$ viruses (Harada et al., 1985; Masuda et al., 1990) were obtained from 3-day-old culture supernatants of persistently infected MOLT-4/HIV-1_{C-2} and MOLT-4/HIV-$1_{esc.C-2}$ cells, respectively. HTLV-I-transformed MT-2 cells were infected with C-2 viruses and cultured for 4 or 5 days. Progeny viruses in the supernatant were used as HIV-$1_{C-2(MT-2)}$. After filtration through a membrane (0.45 m pore size), the supernatants were stored at −80 °C until use. X4 envelope-pseudotyped viruses with a luciferase reporter gene (NL43-luc viruses) were produced by the calcium phosphate transfection method (Maeda et al., 2000). 293T cells were cotransfected with an envelope-deficient NL43 construct carrying the luciferase gene (pNL43-luc) and a pCXN2 vector expressing the envelope glycoprotein from pNL43.

Antibodies and reagents

A mouse IgG1 mAb, 0.5 , recognizing 24 amino acids in the V3 loop of gp120, was used (Matsushita et al., 1988). Three human anti-HIV mAbs were kindly provided by Dr. Susan Zolla-Pazner. Specifically, these mAbs were 694/98-D specific for the V3 region of gp120, 670-30D specific for the C5 region of gp120 and 246-D specific for the cluster I region of gp41 (Nyambi et al., 2000). An anti-HLA-II antibody (mouse anti-human HLA DR, DP, DQ) was purchased from Serotec (Oxford, UK) and stored at a concentration of 20 mg/ml in phosphate-buffered saline (PBS) at −80 °C. This antibody recognizes a monomorphic determinant common to each chain. Sodium azide showed no effects on the HIV-1 infectivity, adsorption, and membrane fluidity at 0.0002%, which was the maximum concentration used in this study. Mouse myeloma ascites IgG1 MOPC21 (Cappel, Cochranville, PA) was used as an IgG control. Rat LAT12 and LAT27 mAbs, which are non-neutralizing and neutralizing antibodies against HTLV-I, respectively, were also used (Tanaka et al., 1991). LAT27 recognizes amino acids 191–196 of HTLV-I gp46. A CXCR4 antagonist, T140, was kindly provided by Drs. H. Tamamura and N. Fujii (Tamamura et al., 1998). 5-Doxyl stearic acid (5-DSA) was purchased from Sigma-Aldrich (St. Louis, MO) and stored at a concentration of 20 mg/ml in ethanol at 4 °C until use.

Measurement of membrane fluidity in intact cells and viruses by electron spin resonance (ESR) spectroscopy

For ESR analyses, 1 ml of cells (7 10^6) or 50 ml of culture supernatant containing viruses (2–3 g of p24) were mixed with 1 ml of 60 g/ml 5-DSA or 25 ml of 90 g/ml 5-DSA in PBS, respectively, to make a final concentration of 30 g/ml, and incubated at 37 °C for 20 min (Sauerheber et al., 1980). The cells were washed thrice with PBS to remove the free spin label. The viruses were centrifuged at 114,000 *g* for 1 h at 4 °C in a 60 Ti Beckman rotor. The cell or virus pellets were resuspended in 40 l of PBS and drawn into capillary tubes. The ends of the tubes were sealed, before they were placed in quartz glass tubes and taken from the P3 facility. Spectra were recorded with a JES-RE1X ESR spectrometer (JEOL, Tokyo, Japan) equipped with a variable temperature control accessory. The instrument conditions were 2 min scan time, 5.0 mT sweep width, 0.1 mT field modulation width, 10 mW microwave power and 0.3 s time constant (Harada, 2005; Harada et al., 2005, 2007). Fig. 2 shows representative spectra, for which the outer hyperfine splitting indicates $2A_{//}$, and the inner one denotes 2A . The horizontal axis reflects the varying magnetic fields and the vertical axis represents the absorption of microwaves. The order parameter (*S*) was calculated as follows; $S=(A_{//}-A\ )/[A_{zz}-(A_{xx}+A_{yy})/2]=(A_{//}-A\ )/27.3G$.

Conventional neutralization and MAGI assay

Aliquots (200 l) of 2-fold-diluted antibodies (anti-HLA-II or LAT antibodies) were mixed with 200 l of C-2(MT-2) viruses, and incubated at 37 °C for 30 min. Next, aliquots (100 l/well) of the mixtures were seeded in triplicate on MAGI cells (8 10^4/well) that had been split into a flat-bottom 12-well plate on the previous day. The plate was incubated at 37 °C for 1 h with occasional shaking. After one wash with complete medium, 1 ml/well of complete medium was added, and the cells were cultured at 37 °C for 2 days before HIV-1-positive cells were stained by the MAGI assay. The infected cells were fixed with 1% formaldehyde and 2% glutaraldehyde in PBS for 5 min, washed twice with PBS, and incubated with staining solution (3 mM potassium ferrocyanide, 3 mM potassium ferricyanide, 1 mM $MgCl_2$ and 0.4 mg of X-Gal in PBS) at 37 °C for 40 min (Kimpton and Emerman, 1992). Doses of C-2

(MT-2) and C-2(MOLT-4) viruses were adjusted to make 100 to 200 positive cells per well without antibodies. The percent neutralization was calculated as follows: (1−positive cells in test wells/positive cells in control wells) 100.

Blocking of post-attachment enhancement (PAE) and infectivity assay with luciferase readout

GHOST/CXCR4 cells (2.5 10^4 cells/well) that had been seeded into a flat-bottom 48-well plate on the previous day were infected with 100 l/well of NL43-luc pseudoviruses (50 ng p24/ml). Viral adsorption was allowed to take place at 25 °C for 1 h. After one wash with complete medium, the cells were incubated in the presence or absence of T140 or mAbs against HIV-1 at 37 °C or 40 °C for 1 h and then washed once (Harada et al., 2004a, 2005). Subsequently, the plate was incubated at 37 °C for 2 days, and the infected cells were lysed with 100 l/well of luciferase assay buffer (Promega, Madison, WI). The luciferase activity was measured by adding 50 l of luciferase assay substrate (Promega) to 10 l of cell lysate and reading the light activity in a luminometer (Lumat LB 9501/16; EG&G Berthold, Bad Wildbad, Germany) (Song et al., 2001).

Viral adsorption experiment

C-2(MOLT-4) or C-2(MT-2) viruses (400 l each, 10 ng p24/ml) were mixed with MOPC21, anti-HLA-II, LAT12, LAT27 and 0.5 antibodies at final concentrations of 20, 2, 20, 20 and 2 g/ml, respectively, and incubated at 37 °C for 30 min. MOLT-4 cells (10^6 cells/tube) were treated with 100 l/tube of each mixture in triplicate, and incubated at 37 °C for 1 h. Next, the cells were subjected to two extensive washes with PBS and lysed with 50 l/tube of lysis buffer. The amount of p24 was assessed by ELISA (Cellular Products, Buffalo, NY).

Flow cytometric analysis

MOLT-4 or MT-2 cells (10^6 cells/tube) were treated with 50 l of anti-HLA-II (10 g/ml), LAT12 (10 g/ml) and LAT27 (10 g/ml) antibodies at 4 °C for 1 h. After two washes with PBS, the cells were incubated with 50 l of fluorescein isothiocyanate (FITC)-conjugated goat antiserum against mouse or rabbit antibodies (Organon Teknika, West Chester, PA) for 1 h at 4 °C. After two washes with PBS, the cells were resuspended with PBS for analysis using a FACScan system (Becton Dickinson, Mountain View, CA) (Song et al., 2001).

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Gp120 V3-dependent Impairment of R5 HIV-1 Infectivity Due to Virion-incorporated CCR5*

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Entry of R5 human immunodeficiency virus type 1 (HIV-1) into target cells requires sequential interactions of the envelope glycoprotein gp120 with the receptor CD4 and the coreceptor CCR5. We investigated replication of 45 R5 viral clones derived from the HIV-$1_{JR\text{-}FLan}$ library carrying 0–10 random amino acid substitutions in the gp120 V3 loop. It was found that 6.7% (3/45) of the viruses revealed ≥10-fold replication suppression in PM1/CCR5 cells expressing high levels of CCR5 compared with PM1 cells expressing low levels of CCR5. In HIV-$1_{V3L\#08}$, suppression of replication was not associated with entry events and viral production but with a marked decrease in infectivity of nascent progeny virus. HIV-$1_{V3L\#08}$, generated from infected PM1/CCR5 cells, was 98% immunoprecipitated by anti-CCR5 monoclonal antibody T21/8, whereas the other infectious viruses were only partially precipitated, suggesting that incorporation of larger amounts of CCR5 into the virions caused impairment of viral infectivity in HIV-$1_{V3L\#08}$. The results demonstrate the implications of an alternative influence of CCR5 on HIV-1 replication.

Entry of R5 human immunodeficiency virus type 1 (HIV-1)[2] into a target cell requires cooperative interactions of the viral envelope protein gp120 with the receptor CD4 and the coreceptor CCR5 (or CXCR4 for X4 HIV-1) (1–3). These interactions depend on the concentration and distribution of receptor and coreceptor molecules on the cell surface (4–7). Cells with a large amount of CD4 only require trace amounts of CCR5 for maximal susceptibility to infection by R5 HIV-1, whereas cells low in CD4 require larger amounts of CCR5 for maximal infection (6, 8). Sequential binding of the viral surface glycoprotein gp120 to CD4 and CCR5 initiates R5 HIV-1 infection; CD4 attachment induces a conformational change in gp120 that exposes a CCR5 binding domain (9–11). The coreceptor-binding site located in the bridging sheet and the V3 loop of gp120 also play a crucial role in interacting with the N-terminal domains of the CCR5 (12–16). Finally, direct interaction between CCR5 and the V3 loop (35–37 amino acid residues) in gp120 induces structural rearrangements of a fusion peptide of gp41, allowing fusion of viral and cellular membranes (14, 15, 17–19).

Envelope viruses are known to down-modulate the receptor expression on infected cells to prevent reinfection (20, 21). Post-entry, HIV infection leads to a rapid and potent down-modulation of CD4 molecules expressed at the cell surface. Three viral gene products, Nef, Env, and Vpu, are involved in trafficking and catabolism of down-modulating CD4. Nef enhances CD4 internalization and directs the receptor to lysosomes for degradation (22–27), whereas Env and Vpu interfere with the transport of newly synthesized CD4 to the cell surface (28, 29). Without strict CD4 down-modulation, CD4 induces trapping and aggregation of nascent progeny virions at the cell surface by the high affinity of gp120 for CD4 (30) and a dramatic reduction in the infectivity of released virions by recruitment and sequestration of gp120 molecules away from budding sites or recruitment of nonfunctional gp120-CD4 complexes at the virion surface (31, 32).

On the other hand, strict down-modulation of coreceptor CCR5 is not observed in HIV-infected cells, although CCR5 expression on the cell surface is partially reduced by Nef (33). Partial down-regulation of CCR5 and strict down-regulation of CD4 prevent the superinfection of cells in which viral replication is already progressing. CCR5 binding domains, V3 loop, and the bridging sheet domain of gp120 are not exposed until conformational changes in gp120 are induced by interaction with CD4 on the cell surface; therefore, CCR5 would not trap nascent progeny virions at the cell surface. This may be one reason why CCR5 does not need to be strictly down-regulated after viral entry. However, a presence of an unknown inhibitory effect of CCR5 on R5 HIV-1 replication is possible. This paper addresses whether the level of CCR5 in CD4 T-cell lines influences R5 HIV-1 replication, including late stages of the viral lifecycle. To evaluate the effect of V3 loop on viral replication with respect to CCR5 expression, 45 replication-competent mutant viruses carrying multiple amino acid substitutions in the gp120 V3 loop were used which were derived from an R5 HIV-1 V3 loop library using HIV-$1_{JR\text{-}FLan}$ as background (34). The library contained a set of random combinations of 0–10 polymorphic amino acid substitutions observed in 31 R5 clinical isolates. Replication of the viruses in a CD4 T-cell line PM1 expressing low levels of CCR5 and PM1/CCR5 cells expressing

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[2] The abbreviations used are: HIV, human immunodeficiency virus; mAb, monoclonal antibodies; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

high levels of CCR5 was examined. It was found that the viruses revealed different replication phenotypes with respect to CCR5 expression. The present study focused on a viral clone, HIV-$1_{V3L\#08}$, with a replication in PM1 cells comparable with wild type HIV-$1_{JR\text{-}FLan}$ but with dramatically suppressed replication in PM1/CCR5 cells. This is the first report that suppression of replication by high expression of CCR5 is V3 loop-dependent and associated with late stages of viral replication.

EXPERIMENTAL PROCEDURES

Cells and Viruses—The human CD4 T-cell line PM1 (35) was provided by the National Institutes of Health (NIH) AIDS Research and Preference Reagent Program, Division of AIDS NIAID, NIH, and maintained in RPMI1640 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Vitromex). PM1/CCR5 cells were generated by standard retrovirus-mediated transduction of PM1 cells by coculture with PA317 clone #8 cells transfected with pG1TKneo-CCR5 (36) without cloning. MAGIC5 (37) and 293T cells were maintained in Dulbecco's modified Eagle's medium (ICN Biomedicals) supplemented with 10% heat-inactivated fetal calf serum.

pJR-FL_{an} was created in our laboratory from pJR-FL (kindly provided by Dr. Y. Koyanagi, Kyoto University), incorporating AflII and NheI sites into Env at 6395 and 6562 nucleotides, respectively. R5 viruses, carrying a set of random amino acid substitutions in the gp120 V3 loop, were derived from the HIV-1 V3 loop library (34). For construction of V3 loop mutant viruses, amino acid substitutions were introduced into the gp120 V3 loop of pJR-FL_{an}, as described previously (34). For virus preparation, 293T cells (1 10^6) were transfected with 10 g of molecular clone DNA using the calcium phosphate ProFection Mammalian Transfection System (Promega). The supernatant was collected at 28 h post-transfection and filtered through a 0.22- m filter unit (Millipore) and stored at 80 °C until use.

HIV-1 single-cycle luciferase reporter viruses were produced by cotransfection of 293T cells with pNL-LucR E (38) and Env-expressing plasmids pCXN-$Env_{JR\text{-}FLan}$, pCXN-$Env_{JR\text{-}FLan\text{-}A69T}$, pCXN-$Env_{V3L\#08}$, or pCXN-$Env_{V3L\#08\text{-}A69T}$. Culture supernatant containing pseudoviruses at a final concentration of 8 ng/ml p24 was added to 1 10^4 cells/well PM1 or PM1/CCR5 cells in a 48-well plate. After 2 h, the cells were washed twice with phosphate-buffered saline (PBS), and firefly luciferase activity was measured 48 h post-infection according to the manufacturer's directions (Promega).

Viral Replication Assay—For determination of replication phenotype, 4 10^4 of PM1 or PM1/CCR5 cells were infected with 8 ng of p24 Gag for 2 h. After washing twice with PBS, the infected cells were incubated at 37 °C in a 5% CO_2 atmosphere. On day 6 post-infection, p24 Gag in the supernatant was measured using a p24 Gag ELISA (Zeptometrix).

Flow Cytometry—Cell surface expression of CD4 and CCR5 was analyzed by flow cytometry. Cells were incubated in the staining solution (3% fetal calf serum plus 0.05% sodium azide in PBS) with the mouse monoclonal antibodies (mAbs) anti-human CD4 (SK3, BD Biosciences Pharmingen) or anti-human CCR5 (2D7, BD Biosciences Pharmingen) at 4 °C for 30 min. The cells were washed with PBS, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody was used for antibody-staining. Flow cytometry was performed with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with BD Cell Quest Version 3.1 software (BD Biosciences Pharmingen).

Quantitative PCR—Virus (8 ng p24 Gag) was pretreated in culture fluids with 690 units of DNase I (Worthington Biochem) and added to PM1 or PM1/CCR5 cells (4 10^4) for 2 h at 37 °C. Cells were then washed and incubated at 37 °C for 8 h. Total DNA was purified using the QIAamp DNA blood kit (Qiagen) and eluted in a total volume of 200 l. Two l of DNA was analyzed by real-time quantitative PCR. Late reverse transcription products were detected using primers amplifying the region between nucleotides 685 and 789 of the provirus: forward primer (5 -ACATCAAGCAGCCATGCAAAT-3), reverse primer (5 -ATCTGGCCTGGTGCAATAGG-3), and probe (5 -FAM-CATCAATGAGGAAGCTGCAGAATGG-GATAGA-TAMRA-3). Reactions were performed in triplicate in TaqMan Universal PCR master mix using 0.9 pmol of each primer/ l and 0.25 pmol of probe/ l. After 10 min at 95 °C, reactions were cycled for 15 s at 95 °C followed by 1 min at 60 °C for 40 repeats on an ABI Prism model 7700 thermal cycler (Applied Biosystems).

Virus Infectivity Assay—For infectivity assay, 5 10^3 of MAGIC5 cells (37) were plated 1 day before infection into 48-well tissue culture plates. After absorption of virus for 2 h at 37 °C, cells were washed twice with PBS and further incubated at 37 °C in 5% CO_2. At 48 h post-infection, the cells were stained, and the number of blue foci in each well was counted (39).

Western Blot Analysis—Four days post-infection, viruses in the supernatant of HIV-1-infected cells were pelleted by centrifugation at 175,000 *g* for 60 min. Viral proteins (10 ng of p24 Gag) were separated by 4–20% SDS-PAGE, transferred to a polyvinylidene difluoride Immobilon P membrane (Millipore), and blocked with 5% milk in PBST (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, and 0.05% Tween 20) for 8 h at room temperature. Immunodetection was performed with anti-gp120 antibody (Aalto Bio Reagents) and anti-gp41 antibody (2F5; National Institutes of Health AIDS Research and Preference Regent Program) followed by a secondary antibody conjugated to horseradish peroxidase (Sigma) and Chemi-Lumi One (Nacalai Tesque).

Virus Precipitation Assay—Virus immunoprecipitation assay was performed as described previously (40). Anti-HLA-DR (L243), anti-CCR5 (3A9), and anti-CXCR4 (12G5) mAbs were purchased from BD Biosciences Pharmingen. Anti-CCR5 (T21/8) mAb was purchased from BioLegend. A rat immunoglobulin G1 (IgG1) mAb against hepatitis C virus, Mo-8 (41), was used as a rat isotype-matched negative control. Virus (5 ng of p24 Gag) in PBS containing 3% bovine serum albumin was mixed with the mAb at a concentration of 10 g/ml in a final volume of 100 l and incubated for 12 h at 4 °C. Then, 10 l of Pansorbin (Calbiochem), a suspension of heat-killed *Staphylococcus aureus* cells pretreated for 1 h with 3% bovine serum albumin, was added to the virus/mAb mixture. After incubation for 30 min at room temperature, captured viruses were

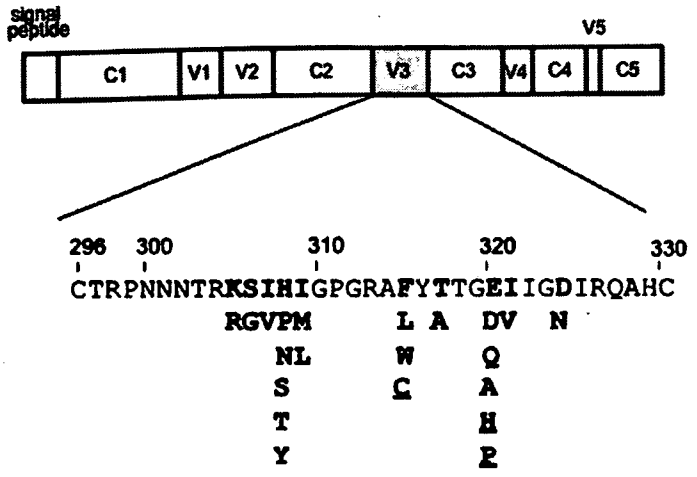

FIGURE 1. **Amino acid substitutions of the R5 HIV-1 V3 loop library.** Each viral clone contained 0–10 substitutions in the gp120 V3 loop. pJR-FL_{an} was used to construct the library as background. Theoretically, the number of possible combinations of 0–10 amino acid substitutions was calculated as 27,648. Residues in *boldface* indicate the substitutions that were randomly incorporated. *Underlined* residues indicate the substitutions that were not detected in the 31 R5 viruses, Phe^{315} to Cys or Glu^{320} to His or Pro but inevitably incorporated into the library due to combinations of nucleotide substitutions.

removed by centrifugation (350 *g* for 30 min). p24 Gag in the supernatant was determined by p24 Gag ELISA.

RESULTS

Replication Suppression of Viruses from the R5 HIV-1 V3 Loop Library in PM1/CCR5 Cells—An R5 HIV-1 V3 loop library was constructed carrying a set of random combinations of 0–10 amino acid substitutions in the V3 loop (34) (Fig. 1). Replication of 45 viruses randomly selected from the library was compared in PM1 and PM1/CCR5 cells (Table 1). PM1/CCR5 cells, generated by standard retrovirus transduction of PM1 cells with a CCR5 expression lentivector, regularly expressed high level of CCR5 and with similar levels of CD4 compared with PM1 cells (Fig. 2). A total of 36% of the viruses (16/45) failed to replicate in both cell types (1.0 ng/ml p24 Gag on day 6). Among replication competent viruses, ratios of p24 Gag ranged from 0.1 to 27, whereas the parental virus, HIV-$1_{JR\text{-}FLan}$, could replicate in both PM1 and PM1/CCR5 cells (ratio 1.9). Viruses were classified into the following three groups; 1) those that grew 10-fold more in PM1/CCR5 than PM1 cells (ratio 10), designated $R5^{H}$ phenotype, 2) those that revealed comparative replication kinetics in PM1 and PM1/CCR5 cells (0.1 ratio 10), designated $R5^{HL}$, and 3) viruses where replication was drastically suppressed in PM1/CCR5 cells (ratio 0.1), designated $R5^{L}$. Six of 45 viruses (13%), including HIV-$1_{V3L\#10}$, HIV-$1_{V3L\#16}$, HIV-$1_{V3L\#21}$, HIV-$1_{V3L\#23}$, HIV-$1_{V3L\#29}$, and HIV-$1_{V3L\#34}$, were classified as $R5^{H}$ phenotype, whereas 3 of the 45 (6.7%) viral clones, HIV-$1_{V3L\#08}$, HIV-$1_{V3L\#23}$, and HIV-$1_{V3L\#25}$, were $R5^{L}$ phenotype. These results indicated that the R5 HIV-1 V3 loop library contained unique replication phenotypes with respect to expression levels of CCR5 in the CD4 T-cell line. Note that these viruses carried amino acid substitutions in the V3 loop alone, indicating that the variety of replication phenotypes was dependent on their V3 loop structure.

Attention was then focused on the $R5^{L}$ phenotype, HIV-$1_{V3L\#08}$, to ascertain why high expression of CCR5 had a suppressive effect on viral replication. Replication of HIV-$1_{V3L\#08}$ was markedly suppressed in PM1/CCR5 cells, whereas the virus showed similar replication kinetics to HIV-$1_{JR\text{-}FLan}$ with 8 ng of p24 Gag in PM1 cells (Fig. 3, *A* and *B*). On day 6 post-infection, p24 Gag in the supernatant was 33-fold lower in PM1/CCR5 than PM1 cells. However, no replication suppression of HIV-$1_{V3L\#08}$ was observed in peripheral blood mononuclear cells or macrophages derived from three different donors compared with HIV-$1_{JR\text{-}FLan}$ (data not shown). HIV-$1_{V3L\#08}$ contained 8 amino acid substitutions: Ile^{307} to Val, His^{308} to Thr, Ile^{309} to Met, Phe^{315} to Leu, Thr^{317} to Ala, Glu^{320} to Asp, Ile^{321} to Val, and Asp^{324} to Asn in gp120 V3 loop alone (Fig. 3*C*).

A revertant of HIV-$1_{V3L\#08}$, designated HIV-$1_{V3L\#08\text{-}A69T}$, was isolated that restored replication ability in PM1/CCR5 cells with an additional substitution Ala^{69} to Thr in the C1 region of gp120 (Fig. 3*C*). HIV-$1_{JR\text{-}FLan\text{-}A69T}$ and HIV-$1_{V3L\#08\text{-}A69T}$ showed similar replication kinetics to HIV-$1_{JR\text{-}FLan}$ in PM1 cells (Fig. 3*A*). However, HIV-$1_{JR\text{-}FLan\text{-}A69T}$ and HIV-$1_{V3L\#08\text{-}A69T}$ showed a slightly higher replication profile in PM1/CCR5 cells on days 4 and 5 compared with HIV-$1_{JR\text{-}FLan}$ (Fig. 3*B*).

It is possible that suppression of HIV-$1_{V3L\#08}$ replication in PM1/CCR5 cells was due to high susceptibility of the virus to the chemokine(s) produced by the cells. To exclude this possibility, susceptibility of HIV-$1_{V3L\#08}$, HIV-$1_{V3L\#08\text{-}A69T}$, HIV-$1_{JR\text{-}FLan}$, and HIV-$1_{JR\text{-}FLan\text{-}A69T}$ to a -chemokine, RANTES (regulated on activation normal T cell expressed and secreted), was measured, but no differences were detected (data not shown).

Entry Efficiency of HIV-$1_{V3L\#08}$ into PM1/CCR5 Cells—To investigate whether the entry efficiency of HIV-$1_{V3L\#08}$ decreased, real-time PCR was utilized to analyze cellular accumulation of the late reverse transcriptase product, *gag*, synthesized shortly after virus entry into the cells (Fig. 4*A*). There was no decrease in *gag* DNA synthesis of HIV-$1_{V3L\#08}$ in PM1/CCR5 compared with PM1 cells. Rather, the DNA copies of HIV-$1_{V3L\#08}$ were 3.3-fold higher in PM1/CCR5 than in PM1 cells. Higher expression of CCR5 could promote more efficient viral entry when CD4 is not significantly expressed, consistent with previous reports for other viruses (1.9–2.5-fold) (6, 8). Moreover, there was no clear difference in DNA synthesis among the four viruses in PM1/CCR5 cells.

In addition, the virus pseudotyped with the envelope proteins of HIV-$1_{V3L\#08}$ revealed 2.0-fold more efficiency in PM1/CCR5 than in PM1 cells (Fig. 4*B*). Luciferase activity of other viruses in PM1/CCR5 cells also increased 1.7–2.2-fold compared with PM1 cells. Measured luciferase activity in cells infected with pseudotyped viruses serves as an indirect estimation of viral entry, integration, and transcriptional activity. The results demonstrate that suppression of HIV-$1_{V3L\#08}$ replication in PM1/CCR5 cells is not associated with early stages of the viral lifecycle.

Production of Virus from PM1/CCR5 Cells Infected with HIV-$1_{V3L\#08}$—A comparison was made of the number of virions generated from PM1/CCR5 and PM1 cells in the presence of a reverse transcriptase inhibitor (AZT) and a CCR5 inhibitor

TABLE 1
Replication of viruses from the HIV-1 V3 loop library in PM1 and PM1/CCR5 cells

Viral clone	V3 sequence	p24 Gag antigen (ng/ml)[a]		
		PM1	PM1/CCR5	Ratio[b]
$HIV\text{-}1_{JR\text{-}FLan}$	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC	140	270	1.9
#01	.....RG.PM.....HV.....	18	50	2.8
#02	.....L.....L.A..D...N.....	17	54	3.2
#03	.....RG.PL.....C.A..AV.....	<1.0	<1.0	–
#04	.....RGVYL.....PV.....	<1.0	<1.0	–
#05	.....R..NL.....L.....V..N.....	70	49	0.7
#06	.....R.VS.....L.A...V..N.....	46	17	0.4
#07	.....RG.PM.....W.A..H.....	23	20	0.9
#08	.....VTM.....L.A..DV..N.....	83	8.0	0.1
#09	.....GVNL.....L.A..DV.....	51	20	0.4
#10	.....S.....A..HV..N.....	21	220	10
#11	.....RGV.L.....PV.....	<1.0	<1.0	–
#12	.....VN.....A..PI..N.....	<1.0	<1.0	–
#13	.....R.VNL.....W....Q.....	4.0	5.0	1.3
#14	.....RGVPL.....A.....	<1.0	<1.0	–
#15	.....G..L.....C....H...N.....	<1.0	<1.0	–
#16	.....RG.YM.....L....QV.....	6.0	160	27
#17	.....RGVPL.....A..DV..N.....	3.0	10	3.3
#18	.....YL.....L....P...N.....	<1.0	<1.0	–
#19	.....GVT.....W.A.....	38	29	0.8
#20	.....RGVYM.....C....A...N.....	<1.0	<1.0	–
#21	.....NL.....Q...N.....	7.0	140	20
#22	.....VPM.....A.VAV..N.....	5.0	53	11
#23	.....VNM.....L....D.....	120	14	0.1
#24	.....R.VPL.....W.A.....N.....	3.0	3.0	1.0
#25	.....R.V.M.....V..N.....	70	6.0	0.1
#26	.....GVTL.....L.....V.....	<1.0	<1.0	–
#27	.....GVNL.....L.....V.....	13	8.0	0.6
#28	.....G.PL.....L.A..HV..N.....	40	47	1.2
#29	.....RG.PM.....PV..N.....	7.0	100	27
#30	.....SM.....W.A..P...N.....	2.0	2.0	1.0
#31	.....L.....C....H...N.....	<1.0	<1.0	–
#32	.....RG..L.....L.....N.....	3.0	3.0	1.0
#33	.....T.....W....PV..N.....	3.0	5.0	1.7
#34	.....GVYM.....L.S..D.....	7.0	170	24
#35	.....RGV.L.....W.A..H...N.....	2.0	2.0	1.0
#36	.....RG.....H.....	3.0	3.0	1.0
#37	.....TM.....L.A..P...N.....	<1.0	<1.0	–
#38	.....G.Y.....D.....	3.0	23	7.7
#39	.....Y.....P.....	5.0	37	7.4
#40	.....RG.TL.....W.A.....	<1.0	<1.0	–
#41	.....G..M.....C....PV..N.....	<1.0	<1.0	–
#42	.....RG.....L.A..PV..N.....	<1.0	<1.0	–
#43	.....L.....L.A..PV.....	60	24	0.4
#44	.....VP.....C.A..A...N.....	<1.0	<1.0	–
#45	.....RGET.....L....AV..N.....	<1.0	<1.0	–

[a] PM1 or PM1/CCR5 cells (4 10^4) were infected with each virus (8 ng of p24 Gag). On day 6 the extent of viral replication was measured by p24 Gag ELISA. Results represent the average of three independent experiments.
[b] Ratio, the concentration of p24 Gag in the supernatant of PM1/CCR5 cells was divided by that of PM1 cells.

(TAK-779) to block secondary infection by nascent progeny virus. The concentrations of AZT and TAK-779 used were 4 and 1 M, respectively, 78- and 32-fold higher than the respective IC_{50} levels (the concentration required to inhibit 50% of the blue foci formation in MAGIC5 cells) (data not shown). Note that on day 2 post-infection, the number of viruses generated from