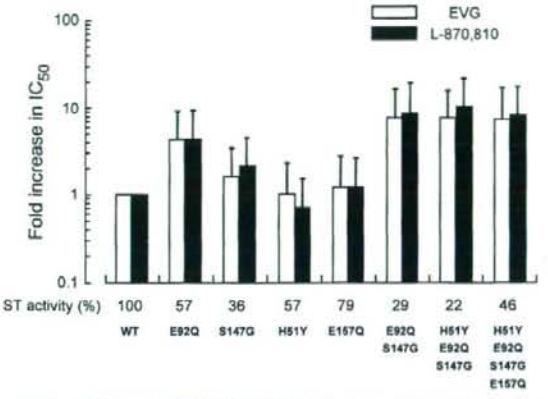

FIG. 4. Effect of EVG-selected mutations on IN strand transfer activity and on the inhibition of strand transfer by IN inhibitors. The strand transfer activities of recombinant IN enzymes carrying EVG-selected mutations were determined using an oligonucleotide-based strand transfer assay. Strand transfer (ST) activity of IN mutants was compared to that of the wild type (WT); results are shown as percentages of wild-type activity. The effect of IN inhibitors on strand transfer was also determined for wild-type and mutant IN enzymes; results are expressed as the increase (n-fold) in IC_{50} values of inhibitors relative to those of the wild type.

bility to EVG at the level of inhibition of strand transfer, consistent with its identification as a primary EVG resistance mutation in the virological analyses.

Replication kinetics of IN inhibitor-resistant variants. The effects of IN mutations on the replication kinetics of HIV-1 variants were assessed by comparing their levels of p24 production in culture supernatants to that of wild-type virus (Fig. 5). At day 5 postinfection, levels of p24 production by the HIV-1_{E92Q} and HIV-1_{Q146P} variants were 86% and 82% of HIV-1_{WT} levels, respectively. These variants showed high-level (36-fold) or moderate (11-fold) resistance to EVG (Table 3), whereas the replication levels of both were similar to those of the wild type. However, the introduction of additional EVG resistance mutations further decreased p24 production, which is indicative of a decline in the levels of viral replication. In particular, HIV-$1_{T66I/Q146P/S147G}$, HIV-$1_{T66I/Q95K/Q146P/S147G}$, HIV-$1_{T66I/Q95K/E138K/Q146P/S147G}$, HIV-$1_{H51Y/E92Q/S147G}$, and HIV-$1_{H51Y/E92Q/S147G/E157Q}$ all showed significantly reduced levels of p24 production (less than 20% of wild-type levels by day 5 in all cases). Thus, there was an inverse correlation between the levels of EVG resistance and the viral replication capacity; that is, as resistance to EVG increased, viral replication decreased. Interestingly, viral variants carrying L-870,810-selected mutations had more moderate reductions in replication capacity, even in the case of the HIV-$1_{V72I/F121Y/T125K/V151I}$ variant that had high-level resistance to both L-870,810 and EVG (68% of wild-type levels). These results indicate that mutations associated with resistance to IN inhibitors can have various effects on viral replication capacity. The reduced replication capacity of EVG-resistant variants was not rescued in the presence of the inhibitor (data not shown), as was observed previously for NFV-resistant variants in the presence of NFV (35). Thus, the reduced replication capacity of IN inhibitor-resistant variants may present a barrier to their emergence in vivo.

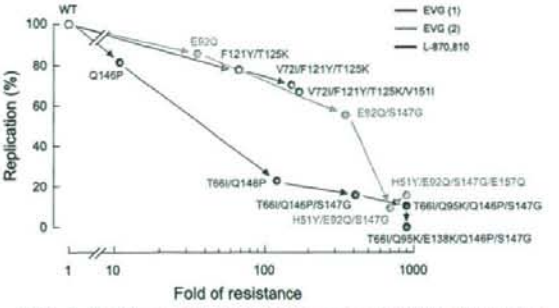

FIG. 5. Replication kinetics of EVG- and L-870,810-resistant viral variants. The replication kinetics of wild-type and IN inhibitor-resistant viral variants were determined by p24 ELISA. The relationship of replication capacity and change (n-fold) in susceptibility (shown in Table 3) is depicted. Variants are plotted according to the observed order of their emergence during selection experiments in vitro. Replication kinetics of EVG-selected mutants derived from the two independent selection experiments (shown in Fig. 3) are plotted in different colors. WT, wild type.

Antiviral effect of IN inhibitors on retroviruses. The antiviral activity of EVG against other retroviruses, including MLV and SIV, was assessed. EVG and L-870,810 inhibited the integration of the HIV-based vector used as a positive control for the luciferase assay (EC_{50} values of 0.8 and 5.0 nM, respectively), as observed in the MAGI assay with HIV-1_{IIIB} (Fig. 6). EVG and L-870,810 suppressed the replication of MLV infection (EC_{50} values of 5.8 and 22 nM, respectively) as well as that of the primate retrovirus SIV (0.5 and 3.2 nM, respectively), indicating that IN inhibitors have antiviral activity against a broad range of retroviruses.

DISCUSSION

The data described here show that EVG inhibits HIV replication by specifically blocking the strand transfer reaction mediated by IN, as demonstrated by the intracellular accumulation of 2-LTR DNA products, a signature of nonproductive integration. Furthermore, EVG directly blocked the production of strand transfer products in an in vitro strand transfer assay. Confirming that EVG is a bona fide IN inhibitor, we selected EVG-resistant viral variants in vitro and demonstrated that the resulting viral variants had acquired multiple mutations in the IN coding region and had simultaneously acquired reduced phenotypic susceptibility to EVG. HIV-1 molecular clones carrying the EVG-selected IN mutations had an EVG-resistant phenotype and in many cases also had reduced susceptibility to another IN inhibitor, L-870,810. These data provide formal proof that the observed IN mutations are indeed EVG resistance mutations and that EVG is an IN inhibitor.

Among the IN mutations observed to be selected by EVG, two mutations, T66I and E92Q, appeared to provide the major contribution to EVG resistance. Both of these individual mutations resulted in >30-fold reduced susceptibility to EVG. The T66I mutation conferred cross-resistance to S-1360 and

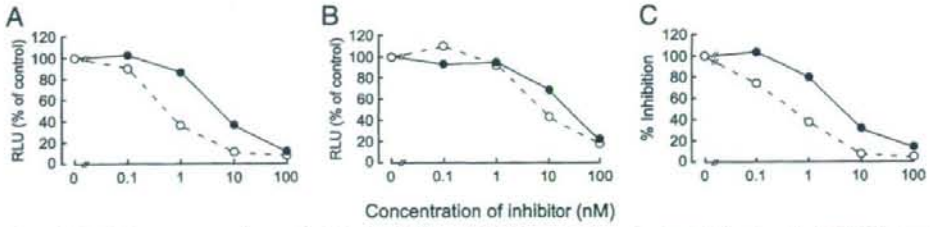

FIG. 6. Effect of IN inhibitors on retroviruses. Antiviral activities of EVG (open circles with dashed lines) and L-870,810 (closed circles with solid lines) against HIV-based (A) or MLV-based (B) vectors harboring the luciferase gene were determined by measuring luciferase activity at 48 h posttransduction. Results are expressed as percentages of relative luciferase units (RLU) compared to those of the no-inhibitor control. (C) Anti-SIV activity was determined using the MAGI assay. These results shown are one representative assay from three independent experiments.

L-731,988 (Table 3) and was also previously observed in an independent EVG selection by Jones et al. (26). The E92Q mutation, when introduced into a recombinant IN enzyme, also reduced the susceptibility of the resulting mutant IN enzyme to EVG, as measured by the reduced EVG inhibition of the in vitro strand transfer assay (Fig. 4). The other IN mutations identified, including H51Y, Q95K, E138K, Q146P, S147G, and E157Q, individually resulted in lower changes (*n*-fold) in EVG susceptibility (1.0- to 11.0-fold) but, when added to either the T66I or the E92Q mutation, further increased resistance to EVG to various degrees relative to either mutation alone. Interestingly, the accumulation of these EVG-selected IN mutations resulted in a significant attenuation of viral replication kinetics. Thus, the emergence of resistance to IN inhibitors may be associated with reductions in viral fitness, which may provide a barrier to the emergence of these mutations in vivo or be associated with lower viral loads if they do emerge.

Of the three HIV enzymes PR, RT, and IN, the structure and mechanism of IN are the least well understood, and despite extensive efforts, the structure of the complete IN enzyme remains to be determined. Only partial two-domain crystal structures of the IN apoenzyme are available, and no structure showing full-length IN bound to its viral cDNA substrate has been published. During integration in vivo, IN functions in the preintegration complex, which also includes RT and the viral DNA (2, 3). Some limited evidence suggests that RT interacts with the active site of IN (39). IN has also been proposed to function with several cellular factors including IN interactor 1 (Ini1) (27) and lens-epithelium-derived growth factor (LEDGF/p75) (7). In the context of these associated cellular factors, IN may retain a different conformation compared to that of the recombinant enzyme alone. This may be one of the reasons that only moderate EVG resistance was observed in the oligonucleotide-based strand transfer assay compared to a cell-based antiviral assay.

Alignment of several IN CCD structures deposited in the Protein Data Bank indicates that there are two regions with poorly defined or disordered structures, including residues 47 to 56 and 140 to 152 (Fig. 7; see Fig. S1 in the supplemental material). Of these two disordered regions, residues 140 to 152 have been implicated as a flexible loop involved in viral cDNA binding (20, 21, 53). Although the precise structural details are unknown, the flexible loop has been proposed to adopt different conformations in the presence or absence of the viral cDNA (12). Notably, several of the EVG-selected mutations that we observed are located on or adjacent to this proposed flexible loop, including E138K, Q146P, and S147G. The flexible loop is important for the catalytic activity of IN (21, 32), and as shown in Fig. 4, the introduction of mutations in these residues, especially S147G, drastically reduced the catalytic activity of IN. Previously published data also demonstrated that another mutation at codon 147 (S147I) resulted in HIV-1 that was highly replication defective, including effects on viral DNA synthesis (47). Indeed, S147 is highly conserved among various retroviruses (see Fig. S2 in the supplemental material), highlighting the importance of the loop for IN function. It is possible that IN inhibitor resistance mutants may have additional pleiotropic effects on processes in viral replication other

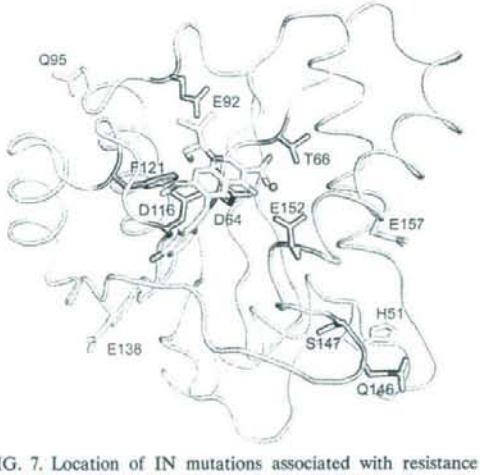

FIG. 7. Location of IN mutations associated with resistance to EVG. EVG in complex with the HIV-1 IN CCD is shown along with the catalytic triad residues (D64, D116, and E152) (green) and a magnesium ion (magenta). Amino acid residues conferring resistance to EVG as primary mutations (T66, E92, F121, Q146, and S147) or as secondary mutations (H51, Q95, E138, and E157) are shown in red and cyan, respectively. The flexible loop (residues 140 to 152) is shown in pink.

than integration; in particular, RT and IN were previously suggested to interact functionally (25).

Recently, an in silico docking simulation of HIV IN with several IN inhibitors including EVG was reported (44). Notably, that author showed that in the best-fit model for EVG docked to IN, the isobutyl substituent on the quionolone moiety of EVG orients directly towards IN residue E92. Interestingly, the hydroxyl component of the isobutyl on the quinolone replaces a water molecule that is coordinated by residue E92 between the two catalytic residues D64 and E152. This docking structure may provide insight into the mechanism of IN inhibition by EVG and provides a starting point for understanding the mechanism of EVG resistance mediated by the E92Q substitution. However, it is uncertain whether this docking simulation represents the precise binding mode of EVG with IN in vivo. Therefore, to accurately assess the binding mode of IN inhibitors with IN, available structural data need to be supplemented by a variety of other approaches. In this study, a virological approach and an enzymatic approach were integrated to characterize the mechanism of action, antiviral activity, and resistance profile of EVG in vitro.

As shown in Fig. 7, primary EVG resistance mutations are located around the catalytic triad of the CCD of IN and are surrounded by the secondary mutations. Among the residues affected by primary mutations, E92 and F121 are located close to EVG on the model and might interact with the IN inhibitor. However, the mechanism by which these mutations interact with the IN inhibitor or with the viral cDNA to mediate resistance is currently unclear. Recently, clinical isolate data from patients experiencing virologic failure in ongoing phase III studies of another IN inhibitor, raltegravir, were reported; E92Q was among the mutations noted to develop in these raltegravir failure patients, usually in combination with another IN mutation, N155H (11, 48). These preliminary clinical data and the data presented here with L-870,810, indicate that the E92Q mutation may be able to mediate resistance and potential cross-resistance to multiple IN inhibitors including EVG and raltegravir. Consistent with the data described here, site-directed mutant HIV carrying the E92Q mutation has been confirmed to show resistance to EVG and to have low-level (approximately sixfold) reduced susceptibility to raltegravir (26).

Several of the IN residues affected by primary mutations observed in EVG-selected variants including T66, E92, and S147 are absolutely conserved among the retroviruses tested (HIV-1, HIV-2, SIV, and MLV) and in retroviruses from multiple mammalian species (see Fig. S2 in the supplemental material). The significant conservation of mammalian retroviral IN CCDs at both the level of sequence homology and structure of the active site was demonstrated by the ability of EVG to inhibit HIV, SIV, and MLV IN activity. This suggests that EVG, and probably other IN inhibitors, binds to a conformationally conserved region of all retroviral INs; the binding of EVG and other IN inhibitors to IN is also likely to involve the catalytic magnesium ion. Taken together, these results suggest that several distinct mechanisms may contribute to IN inhibitor resistance, including conformational changes in the structure of IN that affect the binding of the IN inhibitor, charge effects, steric hindrance, loss of stabilizing binding interactions, or, possibly, alterations in magnesium binding.

A similar reduction in viral replication capacity as a result of drug resistance mutations was previously reported for NRTI resistance mutations (K65R, L74V, and M184V) (45, 55) and for PI resistance mutations (D30N) (49). Mutations that act to compensate for some of the loss of viral replication resulting from drug resistance, for example, GAG processing mutants, have also been described (18, 36, 52). At least one of the EVG secondary mutations, E157Q, may have an analogous role, as it partially restored strand transfer activity that was attenuated by other EVG-selected mutations and also further enhanced resistance to EVG (Fig. 4). Some secondary IN mutations might act to compensate for the altered conformation of IN resulting from the structural effects of primary resistance mutations. The E138K mutation may be such an example, as on its own, it showed no effect on susceptibility to either EVG or L-870,810. The clinical implications of the reduction in fitness resulting from the selection of EVG-resistant mutations are not yet understood.

In conclusion, EVG is a potent inhibitor of the HIV IN enzyme that acts by blocking the strand transfer reaction and is effective not only against HIV but also against other retroviruses. Moreover, the emergence of viral variants that were highly resistant to EVG was associated with significant reductions in viral replication in vitro. These results indicate that EVG should be highly effective for the treatment of HIV-1-infected patients, including those who have had virologic failure of their highly active antiretroviral therapy due to the emergence of HIV-1 drug resistance to approved antiretroviral drugs.

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HIV-1–specific CTLs effectively suppress replication of HIV-1 in HIV-1–infected macrophages

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Both CD4+ T cells and macrophages are major reservoirs of HIV-1. Previous study showed that HIV-1–specific cytolytic T lymphocytes (CTLs) hardly recognize HIV-1–infected CD4+ T cells because of Nef-mediated HLA class I down-regulation, suggesting that HIV-1 escapes from HIV-1–specific CTLs and continues to replicate in HIV-1–infected donors. On the other hand, the CTL recognition of HIV-1–infected macrophages and the effect of Nef-mediated HLA class I down-regulation on this recognition still remain unclear. We show a strong HIV-1 antigen presentation by HIV-1–infected macrophages. HIV-1–specific CTLs had strong abilities to suppress HIV-1R5 virus replication in HIV-1–infected macrophages and to kill HIV-1R5–infected macrophages. Nef-mediated HLA class I down-regulation minimally influenced the recognition of HIV-1–infected macrophages by HIV-1–specific CTLs. In addition, HIV-1–infected macrophages had a stronger ability to stimulate the proliferation of HIV-1–specific CTLs than HIV-1–infected CD4+ T cells. Thus, the effect of Nef-mediated HLA class I down-regulation was less critical with respect to the recognition by HIV-1–specific CTLs of HIV-infected macrophages than that of HIV-1–infected CD4+ T cells. These findings support the idea that the strong HIV-1 antigen presentation by HIV-1–infected macrophages is one of the mechanisms mediating effective induction of HIV-1–specific CTLs in the acute and early chronic phases of HIV-1 infection. (Blood. 2007;109:4832-4838)

Introduction

HIV-1–specific CD8+ T cells play a critical role in the control of HIV-1 infections.[1,2] However, HIV-1–infected individuals develop AIDS if they are not treated with antiretroviral therapy. HIV-1 escape occurs during acute and chronic phases of an HIV-1 infection.[3] Several hypotheses concerning HIV-1 mechanisms affording escape from the host immune system have been proposed.[4-6] One of these is impaired activity of HIV-1–specific cytotoxic T lymphocytes (CTLs) to kill HIV-1–infected CD4+ T cells and to suppress HIV-1 replication by Nef-mediated down-regulation of HLA class I molecules. Previous studies reported that HIV-1–specific CTL clones failed to kill CD4+ T cells infected with Nef+ HIV-1.[7,8] Our previous studies using NL-432 X4 clone and NL-M20A lacking Nef function for HLA class I molecules showed that most HIV-1–infected CTLs failed to kill NL-432–infected CD4+ T cells and partially suppressed NL-432 replication but that they could effectively kill NL-M20A–infected CD4+ T cells and completely suppress NL-M20A replication,[8,9] indicating that Nef-mediated HLA class I down-regulation critically affects recognition of HIV-1–infected CD4+ T cells by HIV-1–specific CTLs. The effects of Nef-mediated HLA class I down-regulation on these antiviral activities of HIV-1–specific CTLs varied among CTLs specific for various HIV-1 epitopes.[9]

CD4+ T cells and macrophages are major targets of HIV-1.[10,11] Macrophages are persistently infected with HIV-1 and serve as a reservoir of the M-tropic/R5 strain of HIV-1.[11,12] HIV-1–infected macrophages are detected in the various tissues of individuals infected with HIV-1, disseminating HIV-1 throughout the body.[13] Therefore, the ability of CTLs to suppress HIV-1 replication in macrophages may be an important factor in the control of HIV-1 infections. Previous studies showed that HIV-1–specific CTLs can kill HIV-1–infected alveolar macrophages derived from HIV-1–infected individuals.[14,15] However, it still remains unclear whether such CTLs effectively suppress HIV-1 replication in macrophages and whether Nef-mediated HLA class I down-regulation critically affects HIV-1–specific CTL recognition of HIV-1–infected macrophages as it does that of HIV-1–infected CD4+ T cells.

The X4 virus infects CD4+ T cells and weakly infects macrophages, whereas the R5 virus infects both macrophages and CD4+ T cells. The X4 virus dominantly appears in late phase of HIV-1 infection, whereas the R5 virus involves in the transmission and replicates in the early phase. Analysis of CTL responses to X4 virus-infected CD4+ T cells, R5 virus-infected CD4+ T cells, and R5 virus-infected macrophages is important to understand CTL-mediated immune responses in both early and late phases of HIV-1 infection.

In this study, we tested the ability of HIV-1–specific CD8+ T cells to kill HIV-1R5 virus-infected macrophages and to suppress the replication of HIV-1R5 virus in macrophages, and we also investigated the effect of Nef-mediated HLA class I down-regulation on the recognition by HIV-1–specific CD8+ T cells of HIV-1R5 virus-infected macrophages. In addition, we compared the antiviral activities of these cells against HIV-1R5 virus-infected macrophages with those against HIV-1-R5 virus-infected or X4 virus-infected CD4+ T cells. Finally, we investigated the mechanisms underlying the effective recognition of HIV-1–infected macrophages by HIV-1–specific CTLs.

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Materials and methods

This study was approved by Kumamoto University Ethics Committee. Informed consent was obtained from all subjects, in accordance the Declaration of Helsinki.

Isolation and culture of macrophages and CD4+ T cells

Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of HLA-B*5101+ or HLA-B*3501+ healthy donors by an adherence method as previously described.[16] CD4+ T cells were purified from nonadherent cells by means of anti–human CD4 monoclonal antibody (mAb)–coated magnetic beads (magnetic-activated cell sorting [MACS] beads; Miltenyi Biotec, Bergisch Galdbach, Germany). These cultured macrophages and CD4+ T cells were infected with HIV-1 clones as previously described.[8]

HIV-1–specific CTL clones

HIV-1–specific CTL clones (HLA-B*5101-restricted CTL clones: Pol743-8-40, Pol283-8-237, -240, -320, and -340; Gag327-9-131, -142, -148, and -287; Rev71-11-8, -17, and -55; and HLA-B*3501-restricted CTL clones: Env77-9-110, Pol273-9-2; and an HLA-A*1101-restricted CTL clone: Gag349-11-18 and -22 as mismatched CTL clone) were generated as previously described.[17-19] These CTL clones predominantly showed $CD27^-CD28^-CD45RA^-$ phenotype (data not shown).

HIV-1 clones

An infectious proviral clone of HIV-1, pNL-432, and its Nef mutant, pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), were reported previously.[20] pJRFL and its Nef-defective mutant, pJR-Xh, which has a frame shift at a *Xho*I site (44th amino acid of Nef protein), were kindly donated by Dr Koyanagi (Kyoto University, Kyoto, Japan). $pJRFL_{NL\text{-}432\ Nef}$ and $JRFL_{NL\text{-}M20A\ Nef}$ were constructed by exchanging the Nef region of JRFL for that of NL-432 or NL-M20A.

CTL assay

The cytotoxicity of CTL clones against HIV-1–infected macrophages or CD4+ T cells (40-50% p24 antigen-positive cells) was determined by a standard ^{51}Cr-release assay, as previously described.[8]

Flow cytometric analysis

Cells infected with HIV-1 clone were stained to assess the expression of HLA class I in HIV-1–infected macrophages or CD4+ T cells, as previously described.[9] For detection of intracellular cytokines, HIV-1–specific CTL clones were cocultured with HIV-1–infected cells for 6 hours at an effector-stimulator (E/S) ratio of 1:4. Then, brefeldin A was added (10 μg/mL). After a 6-hour incubation, the cells were stained with FITC-labeled anti–human IFN-γ, PE-labeled anti–human MIP-1β, PerCp-labeled anti–human CD8, or APC-labeled anti–human TNF-α mAbs (BD Biosciences, San Jose, CA), as previously described.[21]

Suppression of HIV-1 replication by HIV-1–specific CTLs

The ability of HIV-1–specific CTLs to suppress HIV-1 replication was examined as previously described.[8] Briefly, CD4+ T cells or macrophages were incubated with a given HIV-1 clone for 12 hours at 37°C. After several washes with R10 medium, the cells were cocultured with HIV-1–specific CTL clones. From day 3 to 12 after infection, 10 μL culture supernatant was collected, and the concentration of p24 antigen was measured by use of an enzyme immunoassay (HIV-1 p24 antigen enzyme-linked immunosorbent assay [ELISA] kit; ZeptMetrix, Buffalo, NY). The percentage of suppression of HIV-1 replication was calculated as follows: % suppression = (1 − concentration of p24 antigen in the supernatant of HIV-1–infected CD4+ T cells cultured with HIV-1–specific CTLs/concentration of p24 antigen in the supernatant of HIV-1–infected CD4+ T cells culture without the CTLs) × 100.

Western blot analysis

Cells were lysed in lysis buffer (1% Triton X-100, 50 mM Tris HCl, pH 7.6, 1 mM $MgCl_2$, 150 mM NaCl, and 0.1 mM EDTA) containing a mixture of protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Samples were boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-10% polyacrylamide gel electrophoresis (PAGE), and transferred to an Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA). Protein detection was performed after incubation with appropriate first and secondary antibodies by using a Chromogenic Western Blot Immunodetection Kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The first antibodies for p24 and Nef were purchased from ZeptoMetrix and Advanced Biotechnologies, respectively. Quantification was performed by using National Institutes of Health Image.

Proliferation assay

HIV-1–infected CD4+ T cells or macrophages were irradiated and cocultured with thawed HIV-1–specific CTL clones (5×10^3 cells/well) for 3 days in triplicate in 96-well plates at an E/S ratio of 1:4. Then 0.5 μCi/well (0.0185 MBq) of ^3H [thymidine] was added, and the cells were subsequently incubated for an additional 16 hours. The incorporation was measured by a scintillation counter.

Results

Strong abilities of HIV-1–specific CTL clones to suppress HIV-1 replication in macrophages and to kill HIV-1–infected macrophages

To investigate CTL recognition of HIV-1–infected macrophages, we measured the ability of HIV-1–specific CTLs to suppress the replication of HIV-1 R5 strain JRFL and X4 strain NL-432 in HIV-1–infected macrophages and CD4+ T cells, respectively. We used CTL clones specific for 4 HLA-B*5101-restricted epitopes (Pol743-9, Pol283-8, Gag327-9, and Rev71-11) and 2 HLA-B*3501-restricted epitopes (Env77-9 and Pol273-9). Previous studies using these epitope-specific CTL clones demonstrated that the 2 B*5101-restricted Pol-specific CTL clones completely suppressed the replication of HIV-1 X4 strain NL-432 but that other CTL clones only partially suppressed it.[8,9] We measured the ability of these 6 CTL clones to suppress the replication of JRFL and its Nef-defective mutant JR-Xh in HIV-1–infected macrophages. The surface expression of HLA-B*5101 molecules was down-regulated in JRFL-infected macrophages and NL-432–infected CD4+ T cells but not in JR-Xh–infected macrophages and NL-M20A–infected CD4+ T cells (Figure 1). The down-regulation of HLA-B*3501 molecules was also found in only JRFL-infected macrophages and NL-432–infected CD4+ T cells (data not shown). SF2-Rev71-11-8 partially suppressed the replication of JRFL, whereas the other 5 clones completely suppressed the replication of both JRFL and JR-Xh (Figure 2A). On the other hand, only Pol743-9– and Pol283-8–specific CTLs completely suppressed the replication of both NL-432 and NL-M20A in HIV-1–infected CD4+ T cells (Figure 2B). These CTL clones showed similar effects in terms of their cytolytic activity toward HIV-1–infected macrophages and CD4+ T cells (Figure 2C-D). These results of the CTL clones for JRFL-infected macrophages contrast with those for NL-432–infected CD4+ T cells.[8,9]

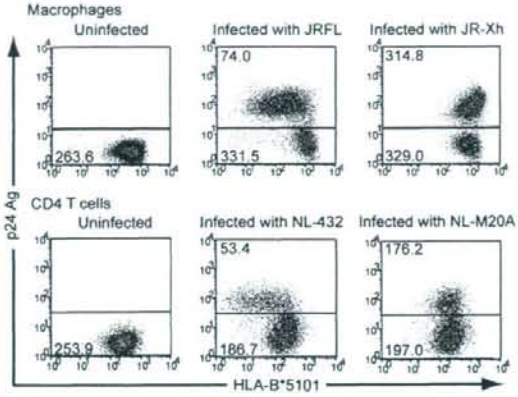

Figure 1. Expression of HLA class I molecules on macrophages or CD4⁺ T cells infected with Nef⁺ or Nef⁻ HIV-1. Macrophages established from monocytes and CD4⁺ T cells of an HLA-B*5101⁺ donor were infected with HIV-1 JRFL or JR-Xh and NL-432 or NL-M20A, respectively, and then cultured for 6 days. The cultured macrophages and CD4⁺ T cells were stained with anti-p24 and 4D12 anti–HLA-B5 mAbs. The surface expression of HLA-B*5101 on p24⁺ or p24⁻ cells is shown as the mean fluorescence intensity (MFI) in each figure.

Comparison between abilities of HIV-1–specific CTLs to suppress R5 virus replication in CD4⁺ T cells and macrophages

It remains possible that the strong ability to suppress the replication of JRFL and the effect of Nef-mediated HLA class I down-regulation were a strain-dependent effect. To exclude this possibility, we generated 2 R5 chimera viruses, specifically, $JRFL_{NL\text{-}432Nef}$ (JRFL carrying NL432-derived Nef) and $JRFL_{NL\text{-}M20ANef}$ (JRFL carrying NL-M20A–derived Nef), and then investigated the ability of 4 HLA-B*5101-restricted CTL clones to suppress the replication of these chimera viruses in HIV-1–infected CD4⁺ T cells and macrophages. The down-regulation of HLA class I molecules was found to occur in $JRFL_{NL\text{-}432\ Nef}$-infected cells, but not in $JRFL_{NL\text{-}M20A\ Nef}$-infected cells (data not shown). The Rev71-11-8 CTL clone suppressed the replication of $JRFL_{NL\text{-}M20A\ Nef}$ in $JRFL_{NL\text{-}M20A\ Nef}$-infected macrophages more strongly than that of $JRFL_{NL\text{-}432\ Nef}$ in $JRFL_{NL\text{-}432\ Nef}$-infected macrophages, whereas other CTL clones showed the same ability to suppress the replication of the 2 JRFL chimera viruses (Figure 3A).

These CTL clones revealed more than 10- to 100-fold stronger ability to suppress the replication of the chimera viruses in HIV-1–infected macrophages than in HIV-1–infected CD4⁺ T cells (Figure 3A). The replication kinetics of $JRFL_{NL\text{-}432\ Nef}$ and $JRFL_{NL\text{-}M20A\ Nef}$ between these 2 cell types were similar, as shown in Figure 3B, thus indicating that the difference in the ability of the specific CTL clones to suppress the chimera virus replication was unrelated to replication kinetics. Thus, these results indicate that HIV-1–specific CTLs could recognize HIV-1–infected macrophages more effectively than HIV-1–infected CD4⁺ T cells.

Ability of HIV-1–infected macrophages to stimulate HIV-1–specific CTLs

We further analyzed the cytokine production from the HLA-B*5101-restricted CTL clones after having stimulated them with either HIV-1–infected CD4⁺ T cells or HIV-1–infected macrophages. IFN-γ–, MIP-1β–, or TNF-α–producing CTL clones were much more detectable after the clones had been stimulated with HIV-1 chimera virus-infected macrophages than after stimulation with HIV-1 chimera virus-infected CD4⁺ T cells (Figure 4A-B). We considered p24⁻ cells to be HIV-1–uninfected cells because only p24⁺ cells showed down-regulation of CD4 (data not shown). HIV-1–infected cells might exist in p24⁻ cells, but they should express very low level of HIV-1 proteins and can hardly stimulate HIV-1–specific CTLs. Therefore we counted p24⁺ cells as HIV-1–infected cells. Frequencies of Pol283-8-340 CTL clones producing

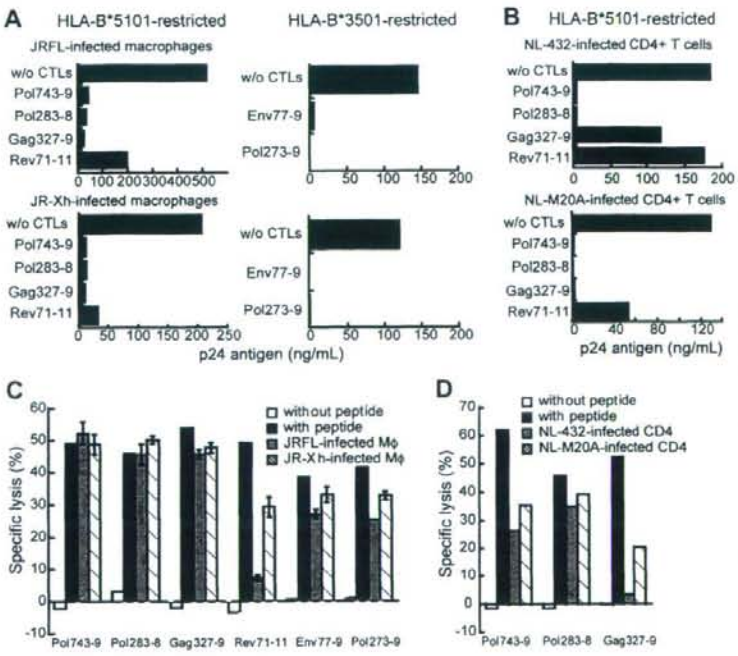

Figure 2. Strong abilities of HIV-1–specific CTLs to suppress HIV-1 replication in HIV-1–infected macrophages and to kill them. (A-B) Ability of HIV-1–specific CTL clones to suppress HIV-1 replication in HIV-1–infected macrophages and in HIV-1–infected CD4⁺ T cells. Macrophages and CD4⁺ T cells from an HLA-B*5101⁺ donor and an HLA-B*3501⁺ donor were infected with JRFL or JR-Xh and NL-432 or NL-M20A, respectively, and then cocultured with each HIV-1–specific CTL clone at an E/T ratio of 1:1. HIV-1 p24 antigens in the supernatant were measured on day 9 after infection by use of an enzyme immunoassay. Data shown in the figure are averages of triplicate assays for each HIV-1–specific CTL clone. (C) Cytotoxic activity against HIV-1–infected macrophages. Macrophages from an HLA-B*5101⁺ donor and an HLA-B*3501⁺ donor were infected with JRFL or JR-Xh. JRFL-infected (56% of total cells were p24 antigen-positive), JR-Xh–infected (48% of total cells were p24 antigen-positive) macrophages were used as target cells at an E/T ratio of 2:1. Data shown in the figure are averages ± SD of triplicate assays for each HIV-1–specific CTL clones. (D) Cytotoxic activity against HIV-1–infected CD4⁺ T cells. CD4⁺ T cells from an HLA-B*5101⁺ donor were infected with NL-432 or NL-M20A. NL-432–infected (81.6% of total cells were p24 antigen-positive) and NL-M20A–infected (79.1% of total cells were p24 antigen-positive) were used as target cells at an E/T ratio of 2:1.

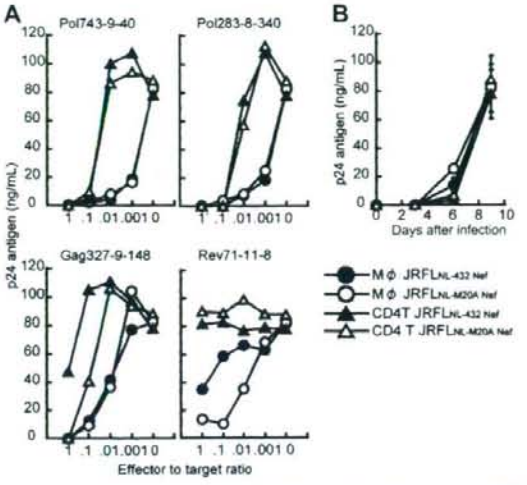

Figure 3. Comparison between abilities of HIV-1–specific CTLs to suppress HIV-1 replication in CD4⁺ T cells and macrophages infected with HIV-1 R5 strain. (A) The ability of HIV-1–specific CTL clones to suppress $JRFL_{NL\text{-}432\ Nef}$ and $JRFL_{NL\text{-}M20A\ Nef}$ replication in CD4⁺ T cells and macrophages infected with $JRFL_{NL\text{-}432\ Nef}$ or $JRFL_{NL\text{-}M20A\ Nef}$. CD4⁺ T cells and macrophages from HLA-B*5101⁺ donor were infected with $JRFL_{NL\text{-}432\ Nef}$ or $JRFL_{NL\text{-}M20A\ Nef}$ and then cocultured with HLA-B*5101-restricted HIV-1–specific CTL clones at various E/T ratios. The amount of HIV-1 p24 antigen in the supernatant on day 9 after infection was measured by using an enzyme immunoassay. (B) Kinetics of $JRFL_{NL\text{-}432\ Nef}$ and $JRFL_{NL\text{-}M20A\ Nef}$ replication in CD4⁺ T cells and macrophages infected with $JRFL_{NL\text{-}432\ Nef}$ or $JRFL_{NL\text{-}M20A\ Nef}$. The amount of HIV-1 p24 antigen in the supernatant on days 3 to 9 after infection was measured by the enzyme immunoassay. Data shown in the figure are averages of triplicate assays for each time point. The experiments shown in panels A and B were performed simultaneously.

at least one cytokine were 69.6% and 72.5%, after the clones had been stimulated with $JRFL_{NL\text{-}432\ Nef}$-infected and $JRFL_{NL\text{-}M20A\ Nef}$-infected macrophages, respectively, whereas they were 29.7% and 43.8% after stimulation with $JRFL_{NL\text{-}432\ Nef}$-infected and $JRFL_{NL\text{-}M20A\ Nef}$-infected CD4⁺ T cells, respectively. HIV-1–specific CTL clones stimulated with HIV-1–infected CD4⁺ T cells predominantly produced a single or 2 cytokines, whereas cells producing 3 cytokines were more frequently found among those stimulated with HIV-1–infected macrophages (Figure 4B). Similar results were found for other CTL clones tested (Figure 4A). These results suggest that HIV-1–infected macrophages can stimulate HIV-1–specific CD8⁺ T cells more strongly than HIV-1–infected CD4⁺ T cells in vivo.

HIV-1–specific CTLs are frequently found in HIV-1–infected individuals, although their number decreases in the late chronic phase of an HIV-1 infection.[22] Since we found the ability of HIV-1 antigen presentation by HIV-1–infected CD4⁺ T cells to be much weaker than that by HIV-1–infected macrophages and macrophages are well known to be professional antigen-presenting cells, we speculated that HIV-1–infected macrophages would induce the proliferation of HIV-1–specific CD8⁺ T cells more effectively than HIV-1–infected CD4⁺ T cells. To test this possibility, we analyzed the capacities of HIV-1–infected macrophages and HIV-1–infected CD4⁺ T cells to induce the proliferation of HLA-B*5101-restricted HIV-1–specific CTL clones (Pol283-8-340, Gag327-9-148, Rev71-11-17, and HLA-mismatched CTL clones; Figure 5A). All 3 HLA-B*5101-restricted CTL clones cocultured with $JRFL_{NL\text{-}M20A\ Nef}$-infected or $JRFL_{NL\text{-}432\ Nef}$-infected macrophages or $JRFL_{NL\text{-}M20A\ Nef}$-infected CD4⁺ T cells showed significantly higher proliferation than those cocultured with uninfected macrophages or uninfected CD4⁺ T cells, respectively, whereas when the clones were cocultured with $JRFL_{NL\text{-}432}$–infected CD4⁺ T cells, only Pol283-8-340 showed significantly higher proliferation compared with that obtained with uninfected CD4⁺ T cells. The proliferation abilities of these CTL clones stimulated with $JRFL_{NL\text{-}M20A\ Nef}$-infected macrophages were significantly higher than those of the clones stimulated with $JRFL_{NL\text{-}M20A\ Nef}$-infected CD4⁺ T cells. Similar results were found for the proliferation of all 3 HLA-B*5101-restricted HIV-1–specific CTL clones stimulated with $JRFL_{NL\text{-}432\ Nef}$-infected macrophages or $JRFL_{NL\text{-}432\ Nef}$-infected CD4⁺ T cells. To confirm these

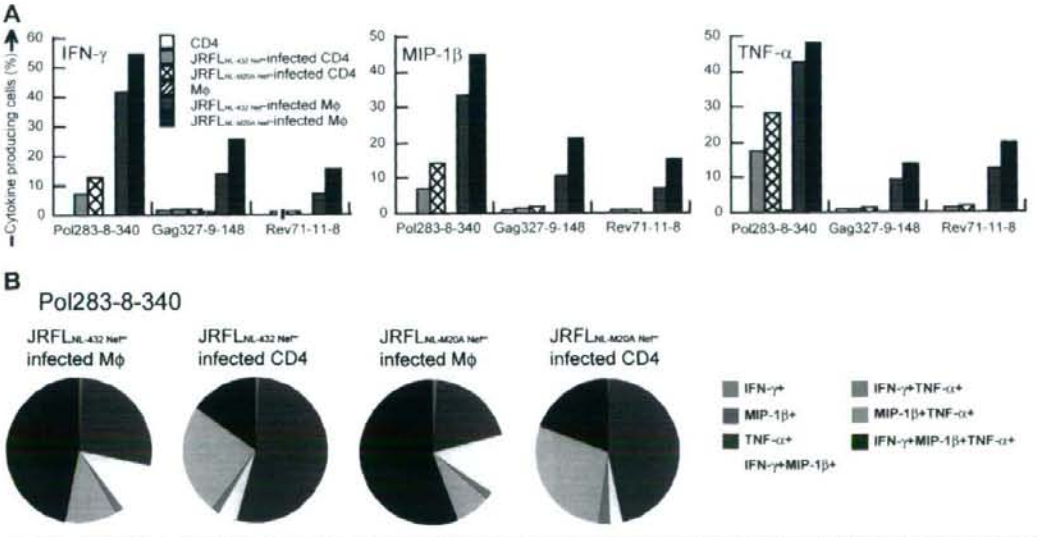

Figure 4. Ability of HIV-1–infected CD4⁺ T cells and HIV-1–infected macrophages to stimulate cytokine production by HIV-1–specific CTLs. Cultured CD4⁺ T cells and macrophages were infected with $JRFL_{NL\text{-}432\ Nef}$ or $JRFL_{NL\text{-}M20A\ Nef}$. $JRFL_{NL\text{-}432\ Nef}$-infected macrophages (22.7% p24 antigen-positive) and CD4⁺ T cells (19.9% p24 antigen-positive), as well as $JRFL_{NL\text{-}M20A\ Nef}$-infected macrophages (21.5% p24 antigen-positive) and CD4⁺ T cells (22.4% p24 antigen-positive) were used to stimulate 3 HLA-B*5101-restricted CTL clones at an effector-stimulator (E/S) ratio of 1:4. (A) The frequency of cells expressing each cytokine is shown as a percentage of the total number of CD8⁺ cells. (B) The frequency of cells expressing these cytokines among total cytokine-producing cells is also shown in this pie chart.

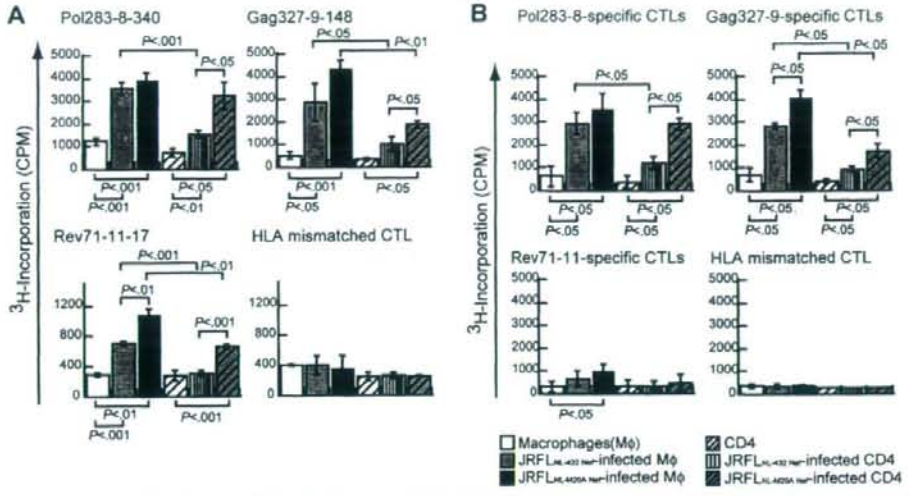

Figure 5. Ability of HIV-1–infected CD4$^+$ T cells and HIV-1–infected macrophages to induce proliferation of HIV-1–specific CTLs. Eleven HLA-B*5101-restricted CTL clones (Pol283-8-340, -320, -237, and -240; Gag327-9-148, -142, -267, and -131; Rev71-11-8, -55, and -17) were cocultured for 96 hours with uninfected macrophages, irradiated JRFL$_{NL\text{-}432\ Nef}$-infected macrophages (17.8% p24 antigen-positive), JRFL$_{NL\text{-}M20A\ Nef}$-infected macrophages (23.2% p24 antigen-positive), uninfected CD4$^+$ T cells, JRFL$_{NL\text{-}432\ Nef}$-infected CD4$^+$ T cells (20.8% p24 antigen-positive), or JRFL$_{NL\text{-}M20A\ Nef}$-infected CD4$^+$ T cells (24.8% p24 antigen-positive) at an E/S ratio of 1:4. The incorporation was measured after an additional 16-hour incubation. (A) Typical example of ^{3}H-incorporation in HLA-B*5101-restricted CTL clones (Pol283-8-340, Gag327-9-148, and Rev71-11-17), and HLA-mismatched CTL clone. Data shown in this figure are averages ± SD of triplicate assays. (B) Average ± SD of proliferation in triplicate assays for 4 Pol283-8–, 4 Gag327-9–, or 3 Rev71-11–specific CTL clones.

results, we measured the proliferation of other CTL clones with the same specificity (Figure 5B). A higher proliferation of the CTL clones stimulated with HIV-1–infected macrophages than with HIV-1–infected CD4$^+$ T cells was confirmed for Pol283-8– and Gag327-9–specific CTL clones but not for the Rev71-11–specific CTL clones. Furthermore, they showed a higher proliferation when they were stimulated with JRFL$_{NL\text{-}M20A\ Nef}$-infected cells than with JRFL$_{NL\text{-}432\ Nef}$-infected cells, but the influence of Nef-mediated down-regulation of HLA class I molecules was less crucial for the stimulation with HIV-1–infected macrophages than for that with HIV-1–infected CD4$^+$ T cells. These results strongly suggest that HIV-1–infected macrophages can much more effectively induce proliferation of HIV-1–specific CTLs than can HIV-1–infected CD4$^+$ T cells in vivo and support our idea that HIV-1–specific CTLs are strongly induced by HIV-1–infected macrophages in the acute and early chronic phases but that they are weakly induced in the late chronic phase, since the X4 virus predominantly replicates in this phase.

High expression of HIV-1 proteins in HIV-1–infected macrophages

We speculated that the difference in the suppressive effect of HIV-1–specific CTLs on JRFL replication between macrophages and CD4$^+$ T cells may have resulted from a difference in the amount of surface expression of HLA class I molecules between these HIV-1–infected cells. Flow cytometric analysis using mAb specific for HLA-B*5101 revealed that the surface expression of HLA-B*5101 on CD4$^+$ T cells was approximately 2-fold lower than that on macrophages (data not shown). To investigate the effect of this difference in surface expression of HLA-B*5101 on the recognition by HIV-1–specific CTLs, we measured the killing activity of 3 HLA-B*5101–restricted CTL clones toward HLA-B*5101$^+$CD4$^+$ T cells or macrophages prepulsed with the appropriate epitope peptides (Figure 6A). The ability of CTLs to kill the

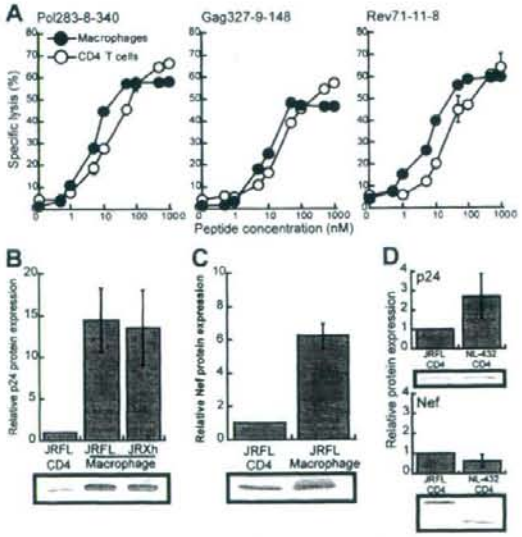

Figure 6. Different expression of HLA class I molecules and HIV-1 proteins between CD4$^+$ T cells and macrophages infected with HIV-1. (A) Comparison of the susceptibility between CD4$^+$ T cells and macrophages for cytotoxic activity of HIV-1–specific CTL clones. Cytotoxic activity of HLA-B*5101-restricted CTL clones was examined for CD4$^+$ T cells and macrophages prepulsed with each epitope peptide at an E/T ratio of 2:1. Data shown in the figure are averages of triplicate assays for each CTL clone. (B-C) The expression of p24 and Nef proteins in JRFL-infected CD4$^+$ T cells and macrophages. After p24$^+$ cells had become 20% to 30% of the total cell population, these cells were lysed. The cell lysates (6 μg) were analyzed by Western blotting with anti-p24 or anti-Nef mAb. Relative protein expression indicates the ratio of the amount of the p24 and Nef proteins in JRFL or JR-Xh–infected macrophages to that in JRFL-infected CD4$^+$ T cells per equal cell number. Data are shown as the average for 3 independent experiments. (D) The expression of p24 and Nef proteins in CD4$^+$ T cells infected with either NL-432 or JRFL. Data are shown as the average ± SD for 3 independent experiments.

peptide-pulsed macrophages (LL_{50}, peptide concentration providing a half of maximum percent specific lysis) was approximately 3-fold lower (2.67 ± 0.53) than that to kill the peptide-pulsed CD4+ T cells. These results suggest that the difference in surface expression of HLA-B*5101 molecules between macrophages and CD4+ T cells may partially influence the recognition of these cells by HIV-1–specific CD8+ T cells. Another possibility is that HIV-1 antigens are much more expressed in HIV-1–infected macrophages than in HIV-1–infected CD4+ T cells. To examine this possibility, we measured the amount of p24 and Nef proteins in HIV-1–infected macrophages and HIV-1–infected CD4+ T cells. The amount of p24 was approximately 13-fold larger in either JRFL- or JR-Xh–infected macrophages than in JRFL-infected CD4+ T cell (Figure 6B), and that of Nef protein was more than 7-fold larger in JRFL-infected macrophages than in JRFL-infected CD4+ T cells (Figure 6C). There was no difference in the amount of p24 or Nef protein between NL-432–infected CD4+ T cell and JRFL-infected CD4+ T cells (Figure 6D). Such results indicate that HIV-1–infected macrophages can synthesize much more HIV-1 protein than HIV-1–infected CD4+ T cells. Thus, it is likely that the difference in HIV-1 antigen presentation between the 2 cells resulted from the difference in the production of HIV-1 epitope peptide, because the difference in HIV-1 protein expression was much larger than that in HLA class I expression. These results suggest that HIV-1–infected macrophages can present a sufficient amount of peptide-MHC class I complexes for CTL recognition in spite of Nef-mediated down-regulation of HLA class I molecules.

Discussion

Previous studies showed that HIV-1–specific CTLs can kill HIV-1–infected alveolar macrophages derived from HIV-1–infected individuals but that they failed to kill HIV-1–infected CD4+ T cells.[7,14,15] These results imply that HIV-1–infected macrophages can present HIV-1 antigens more effectively than HIV-1–infected CD4+ T cells. Our previous studies using NL-432 X4 clone and NL-M20A lacking Nef function for HLA class I molecules showed that most HIV-1–infected CTLs failed to kill NL-432–infected CD4+ T cells and partially suppressed NL-432 replication but that they could effectively kill NL-M20A–infected CD4+ T cells and completely suppress NL-M20A replication,[8,9] indicating that Nef-mediated HLA class I down-regulation critically affects recognition of HIV-1–infected CD4+ T cells by HIV-1–specific CTLs. These studies together suggest that the assay measuring the ability of HIV-1–specific CTLs to suppress HIV-1 replication is more sensitive than the cytotoxic assay and imply that the effect of Nef-mediated HLA class I down-regulation is much stronger on the recognition by HIV-1–specific CTLs of HIV-1–infected CD4+ T cells than that of HIV-1–infected macrophages. In fact, we here demonstrated that HIV-1–specific CTLs much more strongly suppressed JRFL replication in the culture of HIV-1–specific CTLs with JRFL-infected macrophages than that in those of HIV-1–specific CTLs with JRFL-infected CD4+ T cells. Thus, the present study indicates that Nef-mediated HLA class I down-regulation only partially affected recognition of HIV-1–infected macrophages by HIV-1–specific CTLs.

The difference in the suppressive effect of HIV-1–specific CTLs on HIV-1 replication between macrophages and CD4+ T cells may be explained by several mechanisms such as differences of HLA class I surface expression and HIV-1 protein expression between macrophages and CD4+ T cells. The present study demonstrated that the surface expression of HLA class I molecules on macrophages was approximately 2-fold higher than that on CD4+ T cells and that this difference weakly influenced ability of HIV-1–specific CTL clones to kill these cells prepulsed with the epitope peptides. These results suggest that the difference in HLA class I surface expression between these 2 cells only partially influenced that in the suppressive effect of HIV-1–specific CTLs on HIV-1 replication. On the other hand, we demonstrated that HIV-1 antigens were much more expressed in HIV-1–infected macrophages than in HIV-1–infected CD4+ T cells. Thus, it is likely that the difference in HIV-1 protein expression between the 2 cells resulted in the difference in HIV-1 antigen presentation since the difference in HIV-1 protein expression was much larger than that in HLA class I expression. Because macrophages are also known to carry costimulatory molecules and function as professional antigen-presenting cells, HIV-1–specific CTLs can effectively proliferate when stimulated by HIV-1–infected macrophages.

A previous study showed that most HIV-1–specific CTLs partially suppress NL-432 replication in NL-432–infected CD4+ T cells,[8,9] whereas the present study exhibited that they also had similar ability to suppress JRFL replication in JRFL-infected CD4+ T cells, indicating that HIV-1–specific CTLs fail to suppress HIV-1 replication in CD4+ T cells in early and late phases of HIV-1 infection. In contrast, HIV-1–specific CTLs strongly suppressed HIV-1 replication in macrophages. These observations imply that HIV-1 replication is more controlled by the CTLs in the early phase than in the late stage.

It is well known that dendritic cells (DCs) play an important role in the transmission of HIV-1 to CD4+ T cells and in antigen presentation.[23] DCs can present antigens to naïve T cells, whereas macrophages present antigens only to memory and effector T cells.[24-27] These findings suggest that DCs present HIV-1 antigens to naïve T cells, so that HIV-1–specific effector and memory T cells are induced in the early stage of an HIV-1 infection. On the other hand, HIV-1–infected macrophages may play a role in maintenance of HIV-1–specific memory and effector T cells, because macrophages can stimulate memory and effector T cells but not naïve T cells. In fact, the present study demonstrated that HIV-1–infected macrophages stimulated HIV-1–specific CTL clones much more strongly than did HIV-1–infected CD4+ T cells, indicating that HIV-1–specific CD8+ T cells are maintained in HIV-1–infected donors due to stimulation by HIV-1–infected macrophages but not due to that by HIV-1–infected CD4+ T cells. In HIV-1–infected individuals, the number of DCs is decreased and their functional impairment is observed,[28,29] suggesting that HIV-1–specific memory and effector T cells may be maintained by antigen presentation by HIV-1–infected macrophages rather than by DCs. Since the X4 virus, which infects only CD4+ T cells, dominantly appears in the late phase, the antigen presentation by HIV-1–infected macrophages would not be expected in this phase. In addition to the loss of HIV-1–specific helper T cells and DCs,[28-32] this may be one of the mechanisms that mediates the reduction in the number of HIV-1–specific T cells and failure of suppression of HIV-1 replication in the late phase.

In the present study, we demonstrated a strong HIV-1 antigen presentation by HIV-1–infected macrophages and less effect of Nef-mediated HLA class I down-regulation on the recognition of HIV-1–infected macrophages by HIV-1–specific CD8+ T cells. HIV-1 R5 virus-infected macrophages could induce higher proliferation of HIV-1–specific CTLs. Antigen presentation by HIV-1–infected macrophages and DCs are major pathways for the induction of HIV-1–specific T cells in HIV-1–infected donors.

Because HIV-1–infected macrophages are frequently detected in various tissues,[33-36] they may be considered to be involved in the maintenance of HIV-1–specific acquired immunity in acute and early chronic phases of an HIV-1 infection. However, because HIV-1 expression depends on the activation statuses of the cells, it still remains unclear that HIV-1–infected macrophages can strongly express HIV-1 proteins and can strongly stimulate HIV-1–specific CTLs in vivo. Further studies of HIV-1–infected macrophages in vivo are necessary to clarify whether HIV-1–infected macrophages are strong professional antigen-presenting cells in vivo.

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Authorship

Contribution: M.F. performed experiments, analyzed data, and helped to write the manuscript; M.T. designed experiments and helped to write the manuscript.

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Isolation and Characterization of Human Immunodeficiency Virus Type 1 Resistant to the Small-Molecule CCR5 Antagonist TAK-652[▽]

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TAK-652, a novel small-molecule chemokine receptor antagonist, is a highly potent and selective inhibitor of CCR5-using (R5) human immunodeficiency virus type 1 (HIV-1) replication in vitro. Since TAK-652 is orally bioavailable and has favorable pharmacokinetic profiles in humans, it is considered a promising candidate for an entry inhibitor of HIV-1. To investigate the resistance to TAK-652, peripheral blood mononuclear cells were infected with the R5 HIV-1 primary isolate KK and passaged in the presence of escalating concentrations of the compound for more than 1 year. After 67 weeks of cultivation, the escape virus emerged even in the presence of a high concentration of TAK-652. This virus displayed more than 200,000-fold resistance to TAK-652 compared with the wild type. The escape virus appeared to have cross-resistance to the structurally related compound TAK-779 but retained full susceptibility to TAK-220, which is from a different class of CCR5 antagonists. Furthermore, the escape virus was unable to use CXCR4 as a coreceptor. Analysis for Env amino acid sequences of escape viruses at certain points of passage revealed that amino acid changes accumulated with an increasing number of passages. Several amino acid changes not only in the V3 region but also in other Env regions seemed to be required for R5 HIV-1 to acquire complete resistance to TAK-652.

The introduction of highly active antiretroviral therapy with reverse transcriptase inhibitors and protease inhibitors has achieved significant progress in the treatment of human immunodeficiency virus type 1 (HIV-1) infection (31). In addition, novel inhibitors targeting other essential molecules for viral replication, such as CCR5 and integrase, are now in human clinical trials (8, 22, 25). The chemokine receptors CCR5 and CXCR4 act as major coreceptors of HIV-1 in consort with the primary receptor CD4 (4, 16, 17). It has been reported that HIV-1 using CCR5 as a coreceptor (R5 HIV-1) is isolated predominantly during the asymptomatic stage (5). R5 HIV-1 is also responsible for virus transmission between individuals. On the other hand, HIV-1 using CXCR4 as a coreceptor (X4 HIV-1) generally emerges at the advanced stage of the disease and is related to acceleration of its progression (5, 20). However, several lines of evidence suggest that R5 HIV-1 still plays a major role even in the advanced stage (11, 30). Therefore, suppression of R5 HIV-1 in infected individuals may be more important than that of X4 HIV-1 in terms of blocking viral transmission and delaying disease progression. This hypothesis has been supported by the finding that individuals having homozygous CCR5-Δ32, a truncated and nonfunctional form of CCR5, display profound resistance to HIV-1 infection without obvious health problems (6, 12, 21). These findings have given us the idea that CCR5 antagonists may be effective as anti-HIV-1 agents without serious side effects, even though CCR5 is a host cellular factor.

The first small-molecule CCR5 antagonist, TAK-779, has been reported to be a potent and selective inhibitor of HIV-1 replication by our group (3). This compound inhibits R5 HIV-1 replication at nanomolar concentrations in cell cultures. However, TAK-779 is an anilide derivative with a quaternary ammonium moiety and could not be further developed as an antiretroviral agent because of its poor oral bioavailability. In the meantime, several groups have identified different classes of small-molecule and orally bioavailable CCR5 antagonists, most of which appeared to be promising candidates for further development (8, 13, 25). TAK-220 and TAK-652, novel orally bioavailable CCR5 antagonists, are successors of TAK-779. TAK-220 is one of a novel series of compounds with chemical structures totally different from that of TAK-779 (27). TAK-220 is orally bioavailable and highly inhibitory to HIV-1 replication in vitro. The other compound, TAK-652, is a derivative of TAK-779 with high oral bioavailability and favorable pharmacokinetic profiles in humans (2). This compound is also a highly potent inhibitor of R5 HIV-1 replication in vitro. Thus, both compounds are considered promising candidates for clinical development.

There may be no exceptions that drug-resistant HIV-1 will emerge under the selective pressure of any single antiretroviral agent. In the case of CCR5 antagonists, there is a serious concern that their long-term use could induce the evolution of X4 HIV-1 in patients (17, 19). In fact, drug-resistant viruses were isolated in long-term cultures of R5 HIV-1-infected cells by the selection

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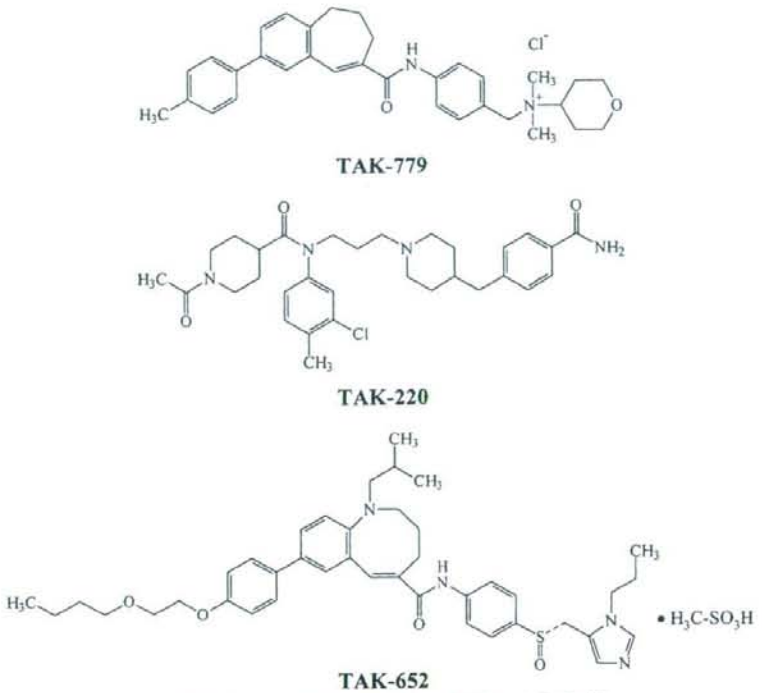

FIG. 1. Structures of TAK-779, TAK-220, and TAK-652.

pressure of some CCR5 antagonists, such as AD101 and vicriviroc (10, 15, 29). However, the escape viruses were found to retain the R5 phenotype. Therefore, in vitro isolation and analyses of drug-resistant viruses may be able to provide useful information for future clinical development of CCR5 antagonists. In this study, we conducted a long-term culture experiment with R5 HIV-1-infected peripheral blood mononuclear cells (PBMCs) with escalating concentrations of TAK-652. After serial passages of the infected cells for more than 1 year, an escape virus was obtained which displayed complete resistance to TAK-652 but retained full susceptibility to TAK-220.

MATERIALS AND METHODS

Compounds. The small-molecule CCR5 antagonists TAK-779 (3), TAK-220 (27), and TAK-652 (2) and the CXCR4 antagonist AMD3100 (23) were synthesized by Takeda Pharmaceutical Company, Osaka, Japan. The chemical structures of the CCR5 antagonists are shown in Fig. 1.

Cells and virus. PBMCs were obtained from healthy volunteers after obtaining their informed consent. The cells were isolated with Ficoll-Hypaque gradient density centrifugation and stimulated with 5 μg/ml phytohemagglutinin (PHA) in RPMI 1640 medium supplemented with 20% fetal bovine serum, 100 U/ml recombinant human interleukin-2 (Takeda Pharmaceutical Company), 100 U/ml penicillin G, and 100 μg/ml streptomycin for 3 days. The above medium without PHA was used in viral replication assays. U87 astroglioma cells expressing human CD4 and either CCR5 or CXCR4 (U87.CD4.CCR5 cells or U87.CD4.CXCR4 cells, respectively) were obtained from D. Littman (New York University School of Medicine, New York, NY) and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 300 μg/ml Geneticin, 1 μg/ml puromycin, and antibiotics. The above medium without Geneticin and puromycin was used in viral replication assays. The KK strain of R5 HIV-1 was used for infection of PBMCs. This strain was isolated from a patient in Kagoshima University Hospital who had no treatment history with any antiretrovirals until virus isolation. Its coreceptor usage was previously determined by a replication assay in U87.CD4.CCR5 cells and U87.CD4.CXCR4 cells, as described below.

Long-term culture of infected PBMCs with TAK-652. The KK strain of HIV-1 (100 ng of p24) was added to 6 ml of PHA-stimulated PBMCs (5×10^6 cells) and incubated at 37°C. To achieve sufficient infection of PBMCs with the clinical isolate, the cells were incubated for 2 days in the absence of compounds. After virus adsorption, the cells were washed three times with culture medium to remove unadsorbed virus particles. The cells were resuspended with culture medium (10 ml) in the presence of 0.2 nM TAK-652. On day 4 after virus infection, the infected cells were subcultured at a ratio of 1:4 with fresh culture medium containing the same concentration of the compound. On day 7, the number of viable cells was counted and adjusted to 1×10^6 cells/ml. Then, 1 ml of the infected cells and 4 ml of freshly prepared and uninfected PBMCs (4×10^6 cells) were suspended with culture medium (10 ml) in the presence of an appropriate concentration of TAK-652 and incubated at 37°C. As control cultures, exactly identical passages of the infected PBMCs in the absence of the compound were carried out in parallel with the cultures exposed to TAK-652. At each passage, p24 antigen levels of culture supernatants were monitored by using an enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix Corp., Buffalo, NY) to confirm virus replication. The concentration of TAK-652 was escalated when the p24 level in the TAK-652-treated culture exceeded 50% of that in the control culture at two consecutive passages. To exclude the effect of different PBMC donors, PBMCs from the same (one) donor were used for passages throughout the experiment. The escape viruses as well as the control viruses were propagated once in PBMCs to remove the compound, their infectivity was determined, and they were used for further experiments.

Susceptibility assay of escape viruses to CCR5 antagonists. PHA-stimulated PBMCs (4×10^6 cells) were infected with 1,400 50% cell culture infective doses of the virus and incubated at 37°C. After a 4-h incubation, the cells were washed with culture medium to remove unadsorbed viral particles and seeded into a 96-well plate (2×10^5 cells/well) with culture medium containing various concentrations (0.1 to 10,000 nM) of test compounds. On day 4 after virus infection,

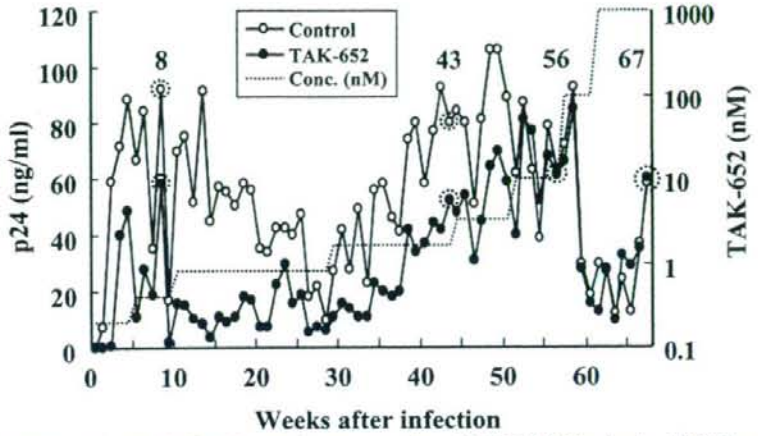

FIG. 2. Long-term culture of infected PBMCs with escalating concentrations of TAK-652. PHA-stimulated PBMCs were infected with an R5 HIV-1 clinical isolate (KK strain) and were passaged weekly by replenishing with fresh PBMCs in the presence or absence of TAK-652 at the indicated concentrations. The solid and dotted lines indicate the p24 levels and the concentrations of TAK-652 in culture supernatants, respectively. PBMCs obtained from one healthy volunteer were used throughout the experiment. At passages 8, 43, 56, and 67, viruses were isolated from the supernatants and subjected to phenotypic and genotypic analyses.

the cells were subcultured at a ratio of 1:2 with fresh culture medium containing the same concentration of the test compounds. On day 7 after infection, the culture supernatants were collected and p24 antigen levels were determined by using an ELISA kit (ZeptoMetrix Corp.).

Determination of coreceptor usage. U87.CD4.CCR5 or U87.CD4.CXCR4 cells were seeded into a 48-well plate (1×10^4 cells/well) and incubated overnight at 37°C. The culture supernatants were removed, and the cells were inoculated with the culture supernatant of each passage in a total volume of 400 μl. After an overnight incubation, the cells were washed thoroughly with culture medium to remove unadsorbed viral particles and further incubated. On day 4 after infection, the culture supernatants were removed and incubation continued with fresh culture medium. On day 6, the culture supernatants were collected and p24 antigen levels were determined by using an ELISA kit (ZeptoMetrix Corp.).

Sequence analysis of *env* genes. Genomic DNA was extracted from the infected PBMCs with a DNA extraction kit (Wako, Tokyo, Japan). The extracted DNA was subjected to PCR. The PCR consisted of 30 cycles (95°C for 15 s, 55°C for 30 s, and 68°C for 150 s) with the forward and reverse primers EnvS (5′-GAGCAGAAGACAGTGGCAATGAGAGTGAAG-3′) and EnvA (5′-TTTTGACCACTTGCCACCCATCTTATAGCA-3′), respectively, which generated a fragment including nucleotides −18 through 2566 of the *env* gene corresponding to the JR-FL strain of HIV-1 (GenBank accession number U63632). The amplified products were isolated by gel electrophoresis and purified with a PCR DNA and gel band purification kit (Amersham Pharmacia Biotech, Piscataway, NJ). The purified DNA was sequenced directly with a cycle sequence kit (BigDye Terminator version 3.1; Applied Biosystems Inc., Foster City, CA), using both forward and reverse primers on an automated DNA analyzer (model 3730; Applied Biosystems Inc.). Depending on the sequence result obtained by the analysis, the primer for the next sequence analysis was designed.

Data analysis. The 50% inhibitory concentrations (IC_{50}) of test compounds were calculated using the SAS system procedure NLIN, which produces least-squares estimates of the parameters of a nonlinear model (logistic model).

RESULTS

Isolation of escape viruses. To successfully isolate TAK-652-resistant viruses, it seemed important to start an experiment with a genetically heterogeneous primary R5 HIV-1 isolate. In addition, an isolate from a treatment-naïve patient would be preferable. Therefore, we chose the KK strain as the source of R5 HIV-1. In our previous study, TAK-652 was found to inhibit replication of the KK strain with an IC_{50} and IC_{90} of 0.043 nM and 0.19 nM, respectively (2). Therefore, PBMC cultures were started in the absence or presence of TAK-652 at a concentration of 0.2 nM (almost identical to its IC_{90}). After three passages, the p24 level of the TAK-652-treated culture rapidly increased and reached more than 50% of the control culture level (Fig. 2). Therefore, the compound concentration was elevated to 0.4 nM at passage 5. At passage 8, viruses were isolated from the TAK-652-treated and control cultures and designated as $KK_{652\text{-}8}$ and $KK_{C\text{-}8}$, respectively (Fig. 2).

Further passages of the infected PBMCs were carried out weekly with an increasing concentration of TAK-652 from 0.8 to 1.6 nM. During this period, HIV-1 replication in the TAK-652-treated culture appeared to be suppressed well, compared to that in the control culture. However, the p24 levels gradually increased after 37 passages, and viruses from the TAK-652-treated and control cultures were isolated at passage 43 ($KK_{652\text{-}43}$ and $KK_{C\text{-}43}$, respectively) (Fig. 2). At passage 52, the p24 levels of the TAK-652-treated and control cultures were comparable. After this point, suppression of HIV-1 replication was not observed for the TAK-652-treated culture, even when its concentration was elevated to 100 nM. Viruses were obtained from the TAK-652-treated and control cultures at passage 56 ($KK_{652\text{-}56}$ and $KK_{C\text{-}56}$, respectively) and passage 67 ($KK_{652\text{-}67}$ and $KK_{C\text{-}67}$, respectively) (Fig. 2). The concentrations of TAK-652 were 10 and 1,000 nM at passages 56 and 67, respectively.

Susceptibility of escape viruses to CCR5 antagonists. When TAK-652 was examined for its inhibitory effect on $KK_{652\text{-}67}$ replication in PBMCs obtained from two different donors, it did not show any significant inhibition at concentrations up to 10,000 nM (Fig. 3). TAK-779 exhibited a dose-dependent but only partial antiviral activity against $KK_{652\text{-}67}$. Interestingly, TAK-220, from a different class of CCR5 antagonists, was highly inhibitory to the replication of $KK_{652\text{-}67}$, irrespective of

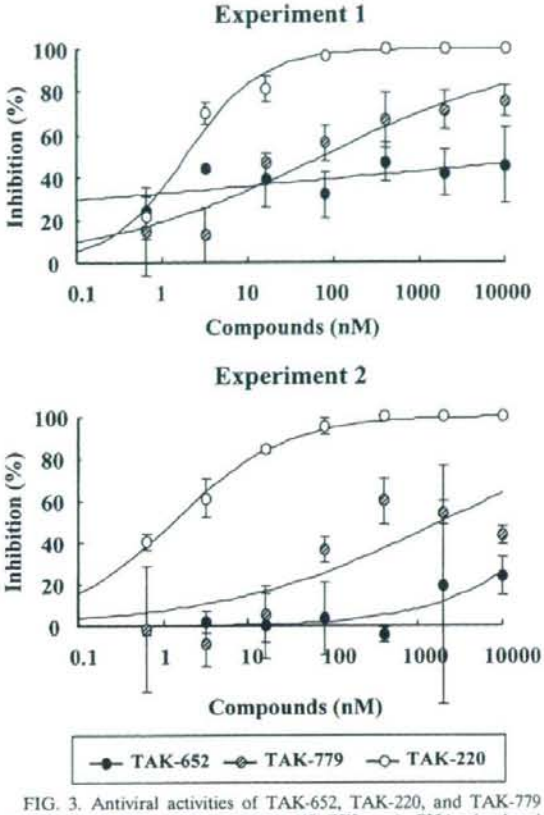

FIG. 3. Antiviral activities of TAK-652, TAK-220, and TAK-779 against the escape virus at passage 67 (KK_{652-67}). PHA-stimulated PBMCs were infected with the virus and incubated for 4 h. The cells were washed to remove unadsorbed viral particles and seeded into a 96-well plate with culture medium containing various concentrations of test compounds. On day 4 after virus infection, the cells were subcultured at a ratio of 1:2 with fresh culture medium containing the same concentration of the test compounds. On day 7 after infection, the culture supernatants were collected and p24 antigen levels were determined by ELISA. The results of two different experiments are shown. In each experiment, different donors were used for the antiviral assays of the compounds.

PBMC donor. As shown in Table 1, the IC_{50} of TAK-652 for KK_{652-67} was more than 10,000 nM. Considering that the IC_{50} of TAK-652 for the wild-type virus (KK_{WT}) was 0.043 nM in PBMCs, KK_{652-67} had strong (more than 200,000-fold) resistance to this compound. The IC_{50}s of TAK-779 for KK_{652-67} were 77 and 2,000 nM in experiments 1 and 2, respectively. In another experiment under identical assay conditions, an IC_{50} of 2.1 nM was obtained with TAK-779 for the wild type (Table 1), suggesting that KK_{652-67} displayed cross-resistance to TAK-779. In contrast, KK_{652-67} appeared to retain complete susceptibility to TAK-220 because little, if any, increase in its IC_{50} was observed in comparison with those for KK_{WT} (Table 1). Surprisingly, TAK-652 did not inhibit the replication of the control virus isolated at passage 67 (KK_{C-67}) at concentrations up to 10,000 nM (data not shown), suggesting that the virus is an X4

TABLE 1. Anti-HIV-1 activities of TAK-652, -220, and -779 against KK_{652-67} in PBMCs[a]

Compound	KK_{WT}[b]	IC_{50} (nM) (fold increase)	
		KK_{652-67}	
		Expt 1	Expt 2
TAK-652	0.043	>10,000 (>230,000)	>10,000 (>230,000)
TAK-220	1.2	1.9 (1.6)	1.3 (1.1)
TAK-779	2.1	77 (37)	2,000 (940)

[a] Cells from different donors were used in each experiment. The IC_{50} is the 50% inhibitory concentration based on the reduction of p24 antigen levels in culture supernatants on day 7 after virus infection. Assays were performed in triplicate wells. Values in parentheses represent the fold increase (ratio of the IC_{50} for the wild type to the IC_{50} for KK_{652-67}).

[b] The IC_{50}s of TAK-652 and TAK-220 for the wild type were taken from references 2 and 27, respectively. The IC_{50} of TAK-779 for the wild type is based on an experiment performed under identical assay conditions.

HIV-1, a dual-tropic (R5X4) HIV-1, or a mixture of R5 HIV-1 and X4 HIV-1.

It was important to examine the susceptibility of the viruses isolated at certain points during the long-term passage of infected PBMCs. For accurate evaluation and comparison of these viruses, all escape and control viruses as well as the wild type needed to be examined simultaneously. In the first experiment, the IC_{50} of TAK-652 for the wild type was 0.14 nM (Table 2). This value was 3.3-fold higher than that obtained previously (2). When the IC_{50} for the escape virus at passage 8 (KK_{652-8}) was compared to that for its control virus (KK_{C-8}), no reduction in its susceptibility to TAK-652 was observed in either experiment 1 or 2. However, slight (2.0- to 3.3-fold) and considerable (110- to 380-fold) increases in the IC_{50}s were identified for KK_{652-43} and KK_{652-56}, respectively, compared with those of the corresponding control viruses. Thus, viruses with a different degree (low, middle, or high) of resistance to TAK-652, namely, KK_{652-43}, KK_{652-56}, and KK_{652-67}, were obtained in the long-term culture experiment.

Coreceptor usage of escape viruses. To determine whether the escape viruses and their control viruses acquired the ability to use CXCR4 as an alternative coreceptor, the replication of these viruses were examined in U87.CD4.CCR5 or U87.CD4.CXCR4 cells. All viruses except for KK_{C-67} replicated well in

TABLE 2. Anti-HIV-1 activity of TAK-652 against escape and control viruses obtained at passages 8, 43, and 56 in PBMCs[a]

Virus	IC_{50} (nM) (fold increase)	
	Expt 1	Expt 2
KK_{WT}	0.14	ND[b]
KK_{C-8}	0.24 (1)	0.27 (1)
KK_{652-8}	0.13 (0.55)	0.26 (0.97)
KK_{C-43}	0.20 (1)	0.34 (1)
KK_{652-43}	0.66 (3.3)	0.69 (2.0)
KK_{C-56}	0.63 (1)	0.64 (1)
KK_{652-56}	240 (380)	67 (110)

[a] Cells from different donors were used in each experiment. The IC_{50} is the 50% inhibitory concentration based on the reduction of p24 antigen levels in culture supernatants on day 7 after virus infection. Assays were performed in triplicate wells. Values in parentheses represent the fold increase (ratio of the IC_{50} for virus from the control passage to the IC_{50} for virus from the corresponding TAK-652-treated passage).

[b] ND, not determined.

TABLE 3. Replication of escape and control viruses obtained at passages 8, 43, 56, and 67 in U87.CD4.CCR5 and U87.CD4.CXCR4 cells[a]

Virus	p24 (ng/ml)[b]	
	U87.CD4.CCR5	U87.CD4.CXCR4
KK_{C-8}	+	−
KK_{C-43}	+	−
KK_{C-56}	+	−
KK_{C-67}	+	+
KK_{652-8}	+	−
KK_{652-43}	+	−
KK_{652-56}	+	−
KK_{652-67}	+	−

[a] Assay procedures are described in Materials and Methods.
[b] +, the p24 level in the culture supernatant was above 1 ng/ml or the virus replicated in the indicated cells; −, the p24 level was below 0.1 ng/ml or the virus did not replicate in the indicated cells.

U87.CD4.CCR5 cells but not in U87.CD4.CXCR4 cells (Table 3), indicating that they could not use CXCR4 as a coreceptor for infection. Although we did not examine their replication in U87 cells expressing other chemokine receptors, it would be unlikely that these viruses could use a chemokine receptor other than CCR5 and CXCR4. KK_{652-67} was found to be highly susceptible to TAK-220 (Fig. 2 and Table 1), which is an antagonist highly specific to CCR5 (27).

KK_{C-67} could replicate in both U87.CD4.CCR5 and U87.CD4.CXCR4 cells (Table 3). Therefore, to determine whether the virus is a dual-tropic (R5X4) HIV-1 or a heterogeneous mixture of R5 HIV-1 and X4 HIV-1, PBMCs were infected with KK_{C-67} and cultured in the presence of either 5 μM TAK-652, 5 μM AMD3100 (a specific inhibitor of CXCR4), or both. These concentrations of TAK-652 and AMD3100 are enough to suppress the replication of R5 HIV-1 and X4 HIV-1, respectively. After a 4-day incubation, the cells were washed thoroughly and further incubated for 3 days in the absence of any compounds. Active replication of KK_{C-67} was observed for the cells initially exposed to TAK-652 alone or AMD3100 alone. However, no replication was observed for the cells initially exposed to both compounds (data not shown). Then, PBMCs were infected with the virus derived from the TAK-652-exposed culture and incubated in the presence of 5 μM AMD3100, and no virus replication was identified (data not shown). An identical result was obtained when PBMCs were infected with the virus derived from the AMD3100-exposed culture and incubated in the presence of 5 μM TAK-652 (data not shown). These results indicate that KK_{C-67} is likely to be a heterogeneous mixture of R5 HIV-1 and X4 HIV-1 rather than R5X4 HIV-1.

Amino acid changes of escape viruses. To determine what amino acid changes are associated with resistance to TAK-652, full-length *env* genes of four escape and four control viruses as well as the wild type were sequenced. Several amino acid changes were observed not only in the gp120 subunit but also in the gp41 subunit (Fig. 4). Each amino acid change could be classified into one of three categories. The first category includes changes that were identified for both escape and control viruses. The second one is changes that were identified only for the control viruses. The last one, which is the most important, includes the changes that were identified only for the escape viruses under the selection pressure of TAK-652. The amino acid changes in the last category have been summarized in Table 4. KK_{652-8} had no amino acid changes in this category, which corresponds to the observation that the susceptibility of KK_{652-8} to TAK-652 was comparable that of KK_{C-8} and KK_{WT} (Table 2). Six amino acid changes, including one heterogeneous change, were observed for the modestly resistant virus KK_{652-43}. The number of amino acid changes increased with increasing passage number and resistance. The highly resistant virus KK_{652-67} had 12 amino acid changes: one in C2, two in V3, two in V4, two in C4, and five in gp41 (Table 4).

DISCUSSION

In this study, we isolated an R5 HIV-1 virus highly resistant to the novel CCR5 antagonist TAK-652 through long-term culture of infected PBMCs. The phenotypic analysis revealed that the virus was highly resistant to TAK-652 and had partial cross-resistance to TAK-779, probably due to their structural similarities (Fig. 1 and Table 1). In contrast to TAK-779, TAK-220, a structurally different CCR5 antagonist, was equally inhibitory to the replication of the TAK-652-resistant virus and the wild type. A similar finding has been reported for SCH-C and vicriviroc (SCH-D) (25, 26). Although both compounds are structurally related, the subtype G Russian clinical isolate RU570, which was weakly susceptible to inhibition by SCH-C (IC_{50} > 1 μM), retained high susceptibility to vicriviroc. Furthermore, four amino acid changes in the V3 region of gp120 were necessary and sufficient to confer resistance to SCH-C (10), whereas vicriviroc-resistant viruses had no amino acid changes in the V3 region (1). Thus, Env amino acid changes responsible for resistance to CCR5 antagonists differ from one compound to another. It would be of special interest to determine whether TAK-652 has sufficient antiviral activity against TAK-220-resistant R5 HIV-1.

We have also conducted a long-term culture experiment with R5 HIV-1-infected PBMCs under the selection pressure of TAK-220. However, no escape virus could be obtained with escalating concentrations of TAK-220, even after 132 passages (data not shown). At present, the reason for such difficulty in inducing TAK-220-resistant viruses is unclear. Nishikawa and colleagues recently analyzed the binding sites for TAK-220 on human CCR5 and found that TAK-220 shares some interacting amino acid residues with TAK-779 but also requires distinct amino acid residues for its inhibitory effect on HIV-1 (18). It is possible that the conformation of CCR5 might be more extensively altered by binding of TAK-220 to CCR5 than by binding of TAK-652. Nevertheless, TAK-652 has unique properties with which TAK-220 and other CCR5 antagonists are not endowed. TAK-652 has good oral bioavailability and a long plasma half-life in humans (2). Therefore, it is assumed that TAK-652 is able to retain a plasma concentration sufficiently higher than that required for virus inhibition by once-daily administration at a reasonable dose. TAK-652 is a potent inhibitor of ligand binding not only to CCR5 but also to CCR2b, which has been observed for neither TAK-220, maraviroc, vicriviroc, nor aplaviroc (8, 13, 25, 27).

Amino acid changes in the Env region accumulated with an increasing period of cultivation (Fig. 4). Among the amino acid

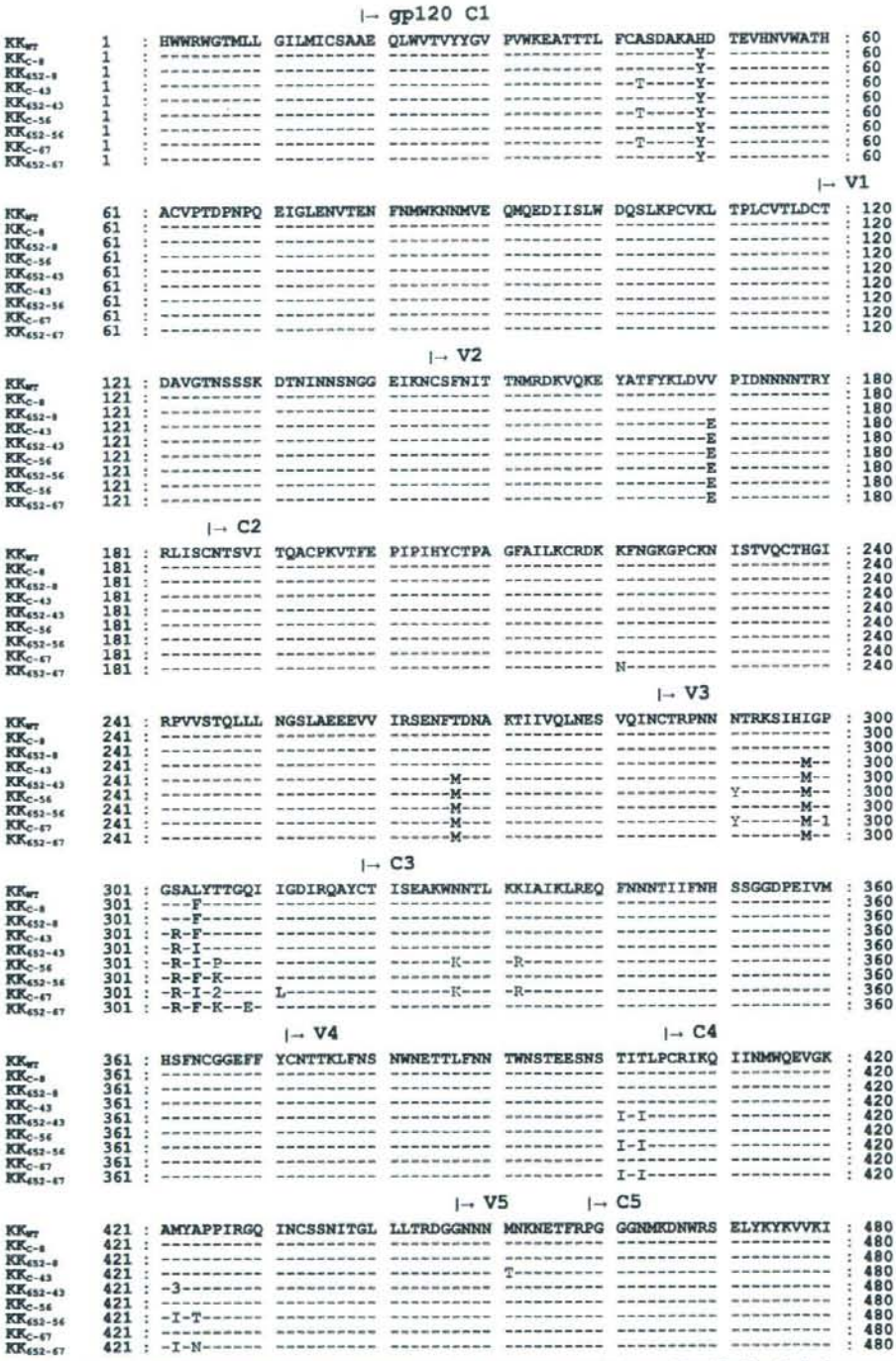

FIG. 4. Env amino acid sequences of isolated viruses. Blue letters indicate the amino acid changes identified for both drug escape and control viruses. Green letters indicate the amino acid changes identified only for the control viruses but not for the escape viruses. Red letters indicate the amino acid changes identified only for the escape viruses but not for the control viruses. There was heterogeneity for four amino acids, which are indicated numerically (1, P or A; 2, P or T; 3, M or I; 4, S or L).

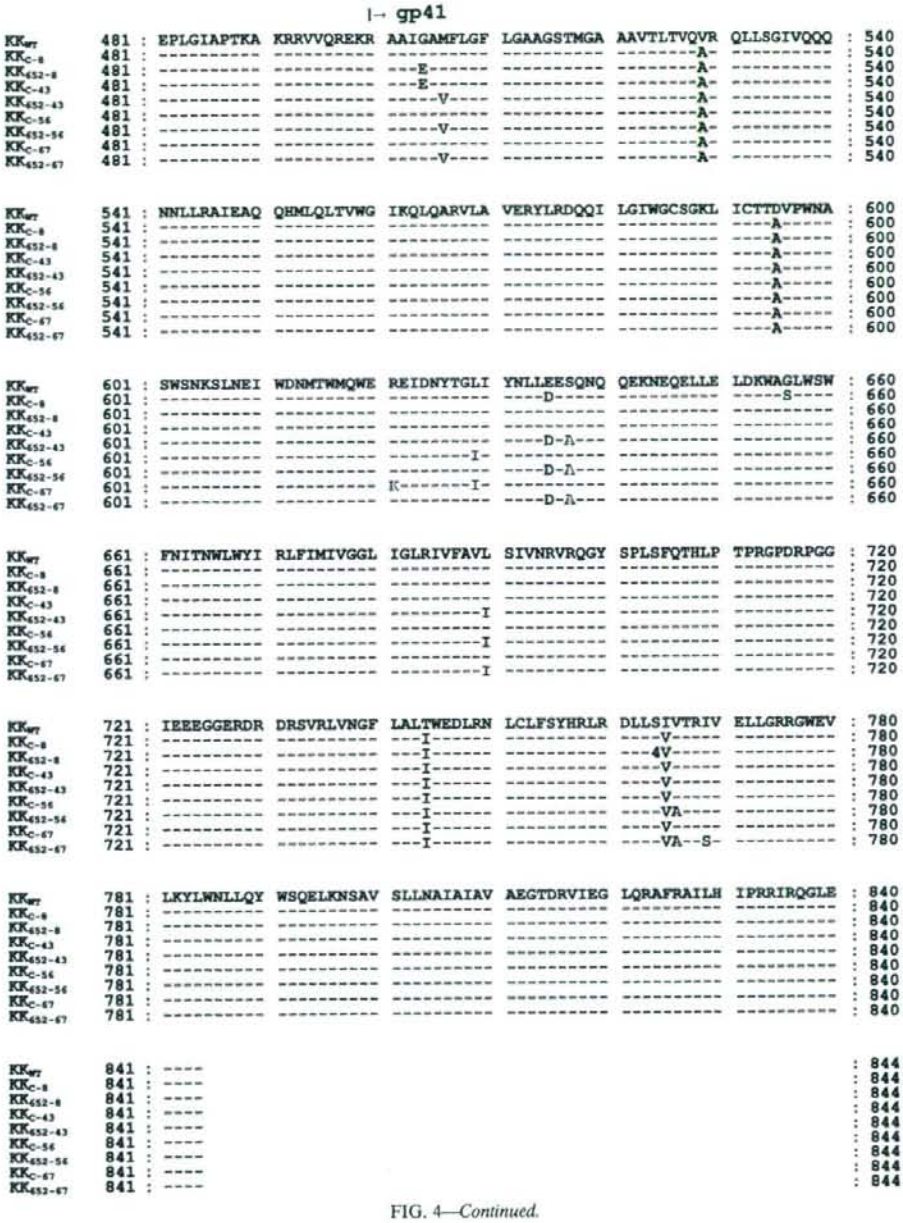

FIG. 4—*Continued.*

changes, several changes did not seem to be attributable to the selection pressure by TAK-652 but were the consequences of in vitro passage of infected cells, since these changes could be identified not only for the escape viruses but also for the corresponding control viruses. In addition, there were some amino acid changes that were found only for the control viruses. Selection of viruses with certain amino acid changes despite the absence of compounds has been commonly observed after in vitro passages of primary isolates and may be attributed to better replication fitness of these viruses. Apart from the amino acid changes unrelated to TAK-652 resistance, there were amino acid changes identified only for the escape viruses, and they accumulated with an increased period of cultivation (Table 4). In particular, a considerable gap in drug

TABLE 4. Env amino acid changes considered to be involved in TAK-652 resistance[a]

Virus	Amino acid at position:											
	221 (C2)	306 (V3)	309 (V3)	401 (V4)	403 (V4)	422 (C4)	424 (C4)	506 (gp41)	637 (gp41)	690 (gp41)	766 (gp41)	769 (gp41)
KK_{WT}	K	T	Q	T	T	M	A	M	S	L	V	I
KK_{652-8}	—[b]	—	—	—	—	—	—	—	—	—	—	—
KK_{652-43}	—	—	—	I	I	M/I	—	V	A	I	—	—
KK_{652-56}	—	K	—	I	I	I	T	V	A	I	A	—
KK_{652-67}	N	K	E	I	I	I	N	V	A	I	A	S

[a] Based on the amino acid sequence data shown in Fig. 4.
[b] —, identical to the amino acid of the wild type.

susceptibility was found between KK_{652-43} and KK_{652-56} (Table 2). In addition to the amino acid changes observed for KK_{652-43}, the three changes T306K (V3), M424T (C4), and V766A (gp41) were identified for KK_{652-56}. Furthermore, three amino acid changes, K221N (C2), Q309E (V3), and I769S (gp41), occurred in the Env region of the highly resistant virus KK_{652-67}. It has been reported that, unlike the resistance to reverse transcriptase and protease inhibitors, one amino acid change of the Env region does not bring about the resistance to CCR5 antagonists (10, 14). Trkola and colleagues have proposed two possible mechanisms that confer resistance to CCR5 antagonists on HIV-1 (29). The first one is the increase of gp120 binding affinity to CCR5. In this case, the virus can compete more strongly with a CCR5 antagonist and infect target cells. The second one is the creation of a substantially different binding site on gp120 for CCR5. In this case, the virus is still able to infect the target cells, even when the binding site of gp120 is already occupied with a CCR5 antagonist. Since the two mechanisms may not be mutually exclusive and can act sequentially, further studies, including the introduction of site-directed mutations, are required to elucidate the amino acids responsible for the resistance to TAK-652.

In accordance with previous experiments by others (29), no HIV-1 coreceptor switch from CCR5 occurred in the escape viruses in this study (Table 3). Instead, the control virus, KK_{C-67}, could use both CCR5 and CXCR4 for infection. It is known that only a few amino acid changes, especially in the V3 region of gp120, can convert R5 HIV-1 to X4 HIV-1 (7, 9, 24). Indeed, seven amino acid changes, including two heterogeneous changes, were found in the V3 region of KK_{C-67}, suggesting that the virus might be a heterogeneous mixture of R5 HIV-1 and X4 HIV-1 rather than a dual-tropic (R5X4) virus. This assumption was confirmed by the drug-swapping experiment with TAK-652 and AMD3100 (see Results for details). Since the original strain, KK_{WT}, is a clinical isolate from a treatment-naïve patient, it is not surprising that a small population of X4 HIV-1 existed in the infected cells and expanded during their long-term culture.

In conclusion, this study provides important information on TAK-652-resistant viruses, such as no cross-resistance to TAK-220 and no coreceptor switch to X4 HIV-1. While our experiments using a clinical isolate and a single PBMC donor may reflect the in vivo scenario of drug resistance better than those using a laboratory strain and multiple donors, it is possible that different mutants will be selected in individual experiments. Furthermore, in a clinical setting, CCR5 antagonists must be used in combination with existing antiretrovirals (28), which may alter the pattern for TAK-652 resistance. Thus, the emergence of drug resistance should be further investigated and confirmed in clinical trials.

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