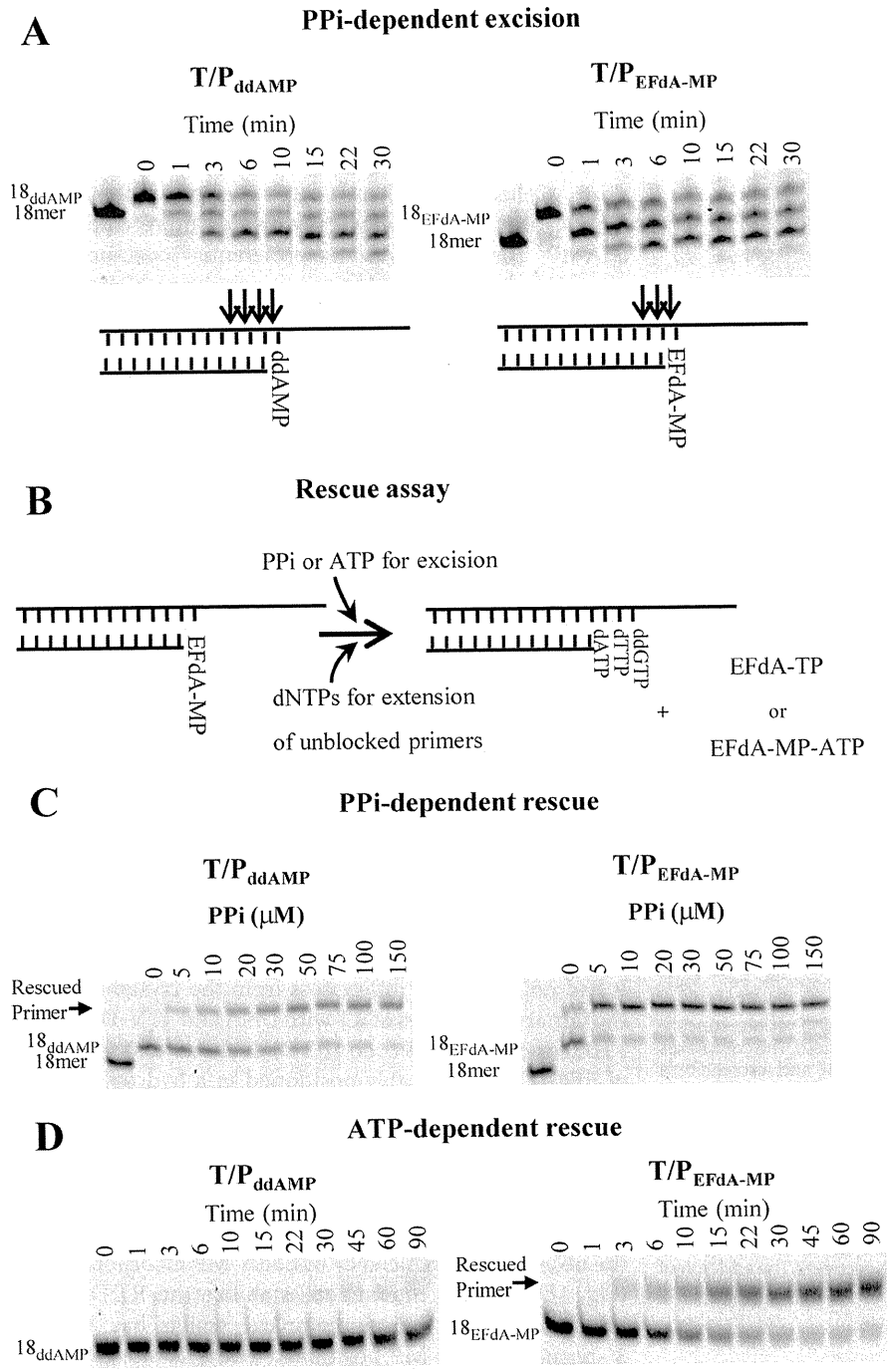

FIGURE 6. **PP_i and ATP-dependent unblocking of ddAMP and EFdA-MP terminated primers.** *A*, PP_i-dependent unblocking of T/P_{ddAMP} and $T/P_{EFdA\text{-}MP}$. Purified T/P_{ddAMP} or $T/P_{EFdA\text{-}MP}$ was incubated with HIV-1 RT in the presence of 6 mM $MgCl_2$ and 150 μM PP_i at 37 °C. Aliquots were removed and reactions stopped at the indicated time points (0–30 min). Cleavage sites are indicated with *arrows* in the schemes below the gels. *B*, schematic representation of PP_i- and ATP-dependent rescue assay. The excision products of PP_i- and ATP-dependent excision of EFdA-MP are EFdA-TP and the EFdA-MP-ATP dinucleoside tetraphosphate, respectively. *C*, PP_i-dependent rescue of T/P_{ddAMP} and $T/P_{EFdA\text{-}MP}$. Purified $T/P_{EFdA\text{-}MP}$ or T/P_{ddAMP} was incubated with HIV-1 RT in the presence of various amounts of PP_i (0–150 μM), dATP (100 μM), dTTP (0.5 μM), or ddGTP (10 μM) and 10 mM $MgCl_2$ at 37 °C. Aliquots of the reaction were stopped after 10 min. *D*, ATP-dependent rescue of T/P_{ddAMP} or $T/P_{EFdA\text{-}MP}$. Purified $T/P_{EFdA\text{-}MP}$ or T/P_{ddAMP} was incubated with HIV-1 RT in the presence of ATP (3.5 mM), dATP (100 μM), dTTP (0.5 μM), or ddGTP (10 μM) and 10 mM $MgCl_2$ at 37 °C. Aliquots of the reaction were stopped at the indicated time points (0–90 min).

clinically used NRTIs (Table 2 and Ref. 23), consistent with the inhibitory data obtained in transformed T-cell lines (13). The molecular basis for this exceptional antiviral activity of EFdA has to date been unclear. The detailed *in vitro* biochemical studies presented in this article show that EFdA inhibits HIV-1 reverse transcriptase mainly by blocking translocation after incorporation at the 3′-primer end and functioning as a TDRTI. The specificity of inhibition can vary depending on the type and sequence of the template (Fig. 2). Our studies also suggest that both the 3′-OH and the 4′-ethynyl groups of EFdA play important roles in the high antiviral potency exerted by this nucleoside analog.

The 4′-ethynyl group is essential for the mechanism of EFdA inhibition of RT-catalyzed DNA synthesis. The present work shows that EFdA-TP acts mainly as a potent terminator of RT-catalyzed DNA synthesis, despite having a 3′-OH group. It is possible that the presence of a 4′-ethynyl substitution on the ribose ring somehow affects the geometry and reactivity of its 3′-OH. However, in the presence of physiological concentrations of dNTPs (<50 μM) the chain terminating activity of EFdA appears to arise mainly from the difficulty of RT to translocate the 3′-EFdA-MP-terminated primer ($T/P_{EFdA\text{-}MP}$) following incorporation of the inhibitor. Under these circumstances the dNTP-binding site is not accessible, and incorporation of the next complementary nucleotide is prevented (Fig. 7*C*). Hence, EFdA appears to act as a translocation-defective RT inhibitor. The 4′-ethynyl moiety appears to be the critical factor in the difficulty presented by translocation of DNA primers with a 3′-terminal EFdA-MP residue. Our molecular model of the RT·DNA·EFdA-TP ternary complex suggests that the 4′-ethynyl moiety fits nicely into a hydrophobic pocket in the RT active site defined by residues Ala-114, Tyr-115, Phe-160, and Met-184 and the aliphatic portion of Asp-185 (Fig. 7*A*), similar to the proposed interactions of 4′-Ed4T at the same site (32). Once EFdA-MP has been added to the primer end to form the pre-translocation (or N-site) reaction product, these same RT residues serve to stabilize the terminal EFdA-MP in

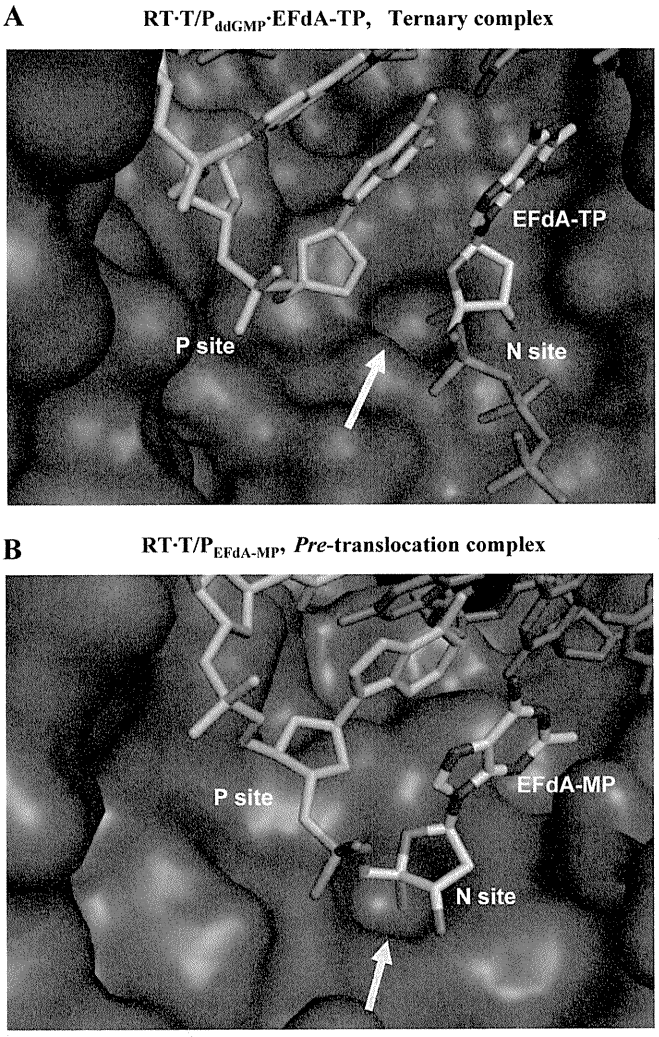

FIGURE 7. **Molecular models representing intermediates of the DNA polymerization reaction.** *A*, molecular model of a ternary complex among RT, DNA, and EFdA-TP. The primer is bound at the P-site, and the incoming EFdA-TP is bound at the N-site. The 4′-ethynyl group of EFdA-TP is bound at a hydrophobic pocket (shown by a *yellow arrow*) defined by residues Met-184, Ala-114, Tyr-115, and Phe-160 and the aliphatic chain of Asp-185. For purposes of clarity the p66 fingers subdomain is not shown. *B*, molecular model of RT bound to EFdA-MP-terminated T/P immediately after incorporation of the inhibitor at the primer terminus and before translocation. The EFdA-MP of the 3′-primer terminus is positioned at the N-site. *C*, schematic representation of RT inhibition by EFdA. After incorporation of EFdA-MP at the 3′-primer terminus RT remains bound to T/P$_{EFdA}$ mostly in a pre-translocation binding mode (*top*). In that binding mode the EFdA-MP at the 3′-primer terminus blocks binding of the incoming dNTP, thus inhibiting DNA polymerization.

the pre-translocation state (Fig. 7*B*). Hence, the stabilization of the primer 3′-terminal EFdA-MP in the pre-translocation state helps it to remain in a position antagonistic to further nucleotide addition and to inhibit DNA polymerization (Fig. 7*C*).

We have observed that in one instance RT stopped not only at the point of EFdA incorporation but also at the position following (Fig. 2*A*, positions *6* and *7*, respectively). Interestingly, we found that RT has enhanced translocation efficiency at this site, both on T/P$_{EFdA-MP}$ and on T/P$_{ddAMP}$ (data not shown). It appears that some translocated T/P$_{EFdA-MP}$ is also elongated by an additional nucleotide. Further polymerization may be inhibited by unfavorable interactions between the 4′-ethynyl group in the elongated template/primer (T/P$_{EFdA-MP-dNMP}$) with protein residues upstream in the DNA-binding cleft. The effect of template on the inhibition mechanism by EFdA is the subject of an ongoing investigation.

RT-catalyzed phosphorolytic excision of chain-terminating NRTIs is a major mechanism of HIV-1 resistance to the nucleoside analog class of therapeutics (26–28, 33). Our previous studies have shown that the NRTI phosphorolytic excision reaction is favored when the primer 3′-terminal nucleotide is in the pre-translocation or N-site (19, 34). The preference of the primer 3′-terminal EFdA-MP to remain in this site suggests that terminal EFdA-MP should undergo facile phosphorolytic removal. EFdA-MP was subject to excision by pyrophosphorolysis somehow faster than ddAMP, which tends to localize in the post-translocation site when at the 3′ terminus of the primer (see Fig. 5*A*). Although EFdA-MP can undergo excision, this process is not overly efficient, apparently because once the nucleotide is excised through pyrophosphorolysis to form EFdA-TP, the latter is rapidly reincorporated. These findings suggest that phosphorolysis may not play a significant role in HIV-1 resistance to EFdA, consistent with the relatively small loss of antiviral potency of EFdA against excision-enhanced HIV-1-containing mutations associated with resistance to AZT (13).

The importance of the 3′-OH in antiviral activity of EFdA is perhaps best highlighted by the observation that EFdA is 10,000-fold more potent at inhibiting HIV-1 replication in PBMCs than is the identical nucleoside lacking a 3′-OH, namely EFddA (Table 2). The 3′-OH on EFdA appears to play a number of roles in contributing to the exceptional antiviral potency of the compound. The 3′-OH on natural dNTP substrates contributes to the efficiency with which RT uses these substrates, and in general NRTIs that lack a 3′-OH are used less efficiently by RT than the base-analogous dNTP (35) (Table 3). Our *in vitro* biochemical data demonstrate that EFdA-TP is approximately 1 and 2 orders of magnitude more potent an inhibitor of RT-catalyzed DNA synthesis *in vitro* than are the

adenine-based NRTIs ddATP and TFV-DP, respectively (Fig. 1*D*), neither of which has a 3′-OH. Indeed, it appears that under identical conditions, RT is at least twice as likely to use EFdA-TP as a substrate over the natural nucleotide dATP (Table 3). This suggests that during HIV-1 reverse transcription, EFdA-TP might be preferentially incorporated, thereby leading to early and profound chain termination and contributing to the observed potent antiviral activity of this nucleoside analog.

NRTIs are administered therapeutically as prodrugs; these must undergo phosphorylation in target cells in order to exert their antiviral activity. The lack of a 3′-OH on current clinically used NRTIs can reduce recognition by cellular nucleoside/nucleotide kinases that have evolved to interact with the 3′-OH present in their natural nucleoside substrates (9). Preliminary studies suggest that EFdA appears to undergo rapid and facile intracellular conversion to the active antiviral EFdA-TP (36), showing that the 4′-ethynyl group does not interfere with recognition by cellular nucleoside/nucleotide kinases. Furthermore, the presence of fluorine at position 2 of the adenine base of EFdA helps to stabilize intracellular levels of EFdA and its phosphorylated products by hindering adenosine deaminase-catalyzed degradation of the molecule (13). Thus, both the 2-fluoro and the 3′-OH of EFdA may contribute to the intracellular accumulation of the antiviral EFdA-TP, thereby leading to its pronounced antiviral activity. We are presently carrying out detailed studies of the intracellular pharmacokinetics of EFdA in comparison with other NRTIs to better understand the dynamics of EFdA phosphorylation and turnover as contributors to the exceptional potency and persistence of its antiviral activity.

Other nucleoside analog inhibitors of HIV-1 RT that possess a 3′-OH have been described (21, 37–42), although these have mechanisms of action quite distinct from that of EFdA. North-methanocarba-2′-deoxyadenosine triphosphate and North-methanocarba-2′-thymidine triphosphate inhibit HIV-1 RT *in vitro* by a mechanism of delayed chain termination, where RT-catalyzed DNA synthesis pauses after the addition of several nucleotides following incorporation of the inhibitor (38, 39). Neither of these compounds has antiviral activity, presumably because of poor intracellular phosphorylation. Entecavir is a nucleoside analog with a 3′-OH that is approved for treatment of hepatitis B infection. Entecavir-TP also has been shown to inhibit HIV-1 RT-catalyzed DNA synthesis by a mechanism of delayed chain termination (40). Entecavir has only weak antiviral activity against HIV-1.

Additionally, nucleoside analogs substituted at the 4′-position have also been described previously (43–46). For example, 4′-azidothymidine and 4′-azidoadenosine both inhibit HIV-1 replication (46) although with potencies 200–2000-fold less than that of EFdA. Both 4′-azido nucleosides also have poor *in vitro* selectivity indices because of significant cytotoxicity. Azidothymidine-TP was shown to inhibit RT-catalyzed DNA synthesis by a type of delayed chain termination; incorporation of two sequential azidothymidine-MP molecules blocked DNA synthesis (44, 45). 4′-Methyl thymidine and 4′-ethyl thymidine both seem to cause pauses and stops in DNA synthesis at the point of incorporation (39). However, neither of these compounds has antiviral activity, because they cannot be phosphorylated by cellular nucleoside kinases. An analog of d4T that has a 4′-ethynyl substitution (Ed4T) is ~10 times more active than the parent compound (47, 48). Because Ed4T lacks a 3′-OH, it inhibits RT as a conventional chain terminator. Interestingly, Ed4T is a better substrate than d4T for phosphorylation by human thymidine kinase 1 (47–50), a property that leads to its increased antiviral potency compared with d4T. The antiviral activity of Ed4T is ~50-fold lower than that of EFdA.

In summary, EFdA is a TDRTI with two functionalities lacking in current therapeutic NRTIs, namely a 4′-ethynyl group and a 3′-OH. These additional properties impart superior antiviral activity to the compound and contribute to its mechanism of action, namely inhibition of primer translocation following EFdA-MP incorporation. This mechanism allows EFdA to act mainly as a *de facto* chain terminator of RT-catalyzed DNA synthesis, despite the presence of a 3′-OH. The present study validates RT nucleic acid translocation as a potential therapeutic target.

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Enhanced Exposure of Human Immunodeficiency Virus Type 1 Primary Isolate Neutralization Epitopes through Binding of CD4 Mimetic Compounds▽

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***N*-(4-Chlorophenyl)-*N'*-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamide (NBD-556) is a low-molecular-weight compound that reportedly blocks the interaction between human immunodeficiency virus type 1 (HIV-1) gp120 and its receptor CD4. We investigated whether the enhancement of binding of anti-gp120 monoclonal antibodies (MAbs) toward envelope (Env) protein with NBD-556 are similar to those of soluble CD4 (sCD4) by comparing the binding profiles of the individual MAbs to Env-expressing cell surfaces. In flow cytometric analyses, the binding profiles of anti-CD4-induced epitope (CD4i) MAbs toward NBD-556-pretreated Env-expressing cell surfaces were similar to the binding profiles toward sCD4-pretreated cell surfaces. To investigate the binding position of NBD-556 on gp120, we induced HIV-1 variants that were resistant to NBD-556 and sCD4 *in vitro*. At passage 21 in the presence of 50 μM NBD-556, two amino acid substitutions (S375N in C3 and A433T in C4) were identified. On the other hand, in the selection with sCD4, seven mutations (E211G, P212L, V255E, N280K, S375N, G380R, and G431E) appeared during the passages. The profiles of the mutations after the selections with NBD-556 and sCD4 were very similar in their three-dimensional positions. Moreover, combinations of NBD-556 with anti-gp120 MAbs showed highly synergistic interactions against HIV-1. We further found that after enhancing the neutralizing activity by adding NBD-556, the contemporaneous virus became highly sensitive to antibodies in the patient's plasma. These findings suggest that small compounds such as NBDs may enhance the neutralizing activities of CD4i and anti-V3 antibodies *in vivo*.**

Human immunodeficiency virus type 1 (HIV-1) replicates continuously in the face of a strong antibody (Ab) response, although Abs effectively control many viral infections (3). Neutralizing Abs (NAbs) are directed against the HIV-1 envelope (Env) protein, which is a heterodimer comprising an extensively glycosylated CD4-binding subunit (gp120) and an associated transmembrane protein (gp41). Env proteins are present on the virion surface as "spikes" composed of trimers of three gp120-gp41 complexes (20, 21, 29). These spikes resist neutralization through epitope occlusion within the oligomer, extensive glycosylation, extension of variable loops from the surface of the complex, and steric and conformational blocking of receptor binding sites (16, 18, 20).

Ab access to conserved regions is further limited because viral entry is a stepwise process involving conformational changes that lead to only transient exposure of conserved domains such as the coreceptor binding site (4, 5). However, some early strains of HIV-1 appear to be highly susceptible to neutralization by Abs (1, 10). For instance, subtype A HIV-1 envelopes from the early stage of infection exhibit a broad range of neutralization sensitivities to both autologous and heterologous plasma (1), suggesting that at least a subset of the envelopes have some preserved and/or exposed neutralization epitopes. It is well known that the potential for neutralizing properties of particular Abs is enhanced after binding of soluble CD4 (sCD4), especially NAbs against CD4-induced epitopes (CD4i Abs) (27) and some anti-V3 Abs (22). CD4i Abs are detected in plasma samples from many patients at an early stage of HIV-1 infection (9). Consequently, we hypothesize that small compounds such as sCD4 can enhance the neutralizing activities of CD4i Abs and some anti-V3 Abs not only *in vitro* but also *in vivo*.

In a previous report, two low-molecular-weight compounds that presumably interfere with viral entry of HIV-1 into cells were described (35). These two *N*-phenyl-*N'*-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamide analogs, NBD-556 and NBD-557, comprise a novel class of HIV-1 entry inhibitors that block the interaction between gp120 and CD4. These compounds were found to be equally potent inhibitors of both X4 and R5 viruses in CXCR4- and CCR5-expressing cell lines, respectively (35). Schön et al. (25) also reported that NBD-556 binds to gp120 in a process characterized by a large favorable change in enthalpy that is partially compensated for by a large unfavorable entropy change, representing a thermodynamic signature similar to that observed for binding of sCD4 to gp120. In a recent study, Madani et al. (23) reported the following findings: (i) NBD-556 binds within the Phe43 cavity, a highly conserved and functionally important pocket formed as gp120 assumes the CD4-bound conformation; (ii) the NBD-556 phenyl ring projects into the Phe43 cavity; (iii) the enhancement of CD4-independent infection by NBD-556 requires the induction of conformational changes in gp120; and (iv) increased

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affinities of NBD-556 analogs toward gp120 improve the antiviral potency during infection of CD4-expressing cells. The latter two studies demonstrated that low-molecular-weight compounds such as NBDs can induce conformational changes in the HIV-1 gp120 glycoprotein similar to those observed upon sCD4 binding (23, 25). The authors of these studies concluded that their data supported the importance of gp120 residues near the Phe43 cavity in binding to NBD-556 and lent credence to the docked binding mode.

In the present study, we investigated the binding position of NBD-556 on gp120 by inducing HIV-1 variants that were resistant to NBD-556 by exposing HIV-1_{IIIB} to increasing concentrations of the compound *in vitro*. We also induced sCD4-resistant HIV-1_{IIIB} variants and compared the profile of the sCD4-resistant mutations to that of the NBD-556-resistant mutations. We subsequently examined the virological properties of pseudotyped HIV-1 clones carrying the NBD-556 and sCD4 resistance-associated *env* gene mutations. Our findings provide a foundation for understanding the interaction of NBD-556 with the CD4-binding site of HIV-1 gp120. We also evaluated the anti-HIV-1 interactions between plasma NAbs and NBD-556 *in vitro* and considered the possibility of using the data as a key to opening the shield covering the conserved epitopes targeted by NAbs.

(This study was presented in part at the 15th Conference on Retroviruses and Opportunistic Infection, Boston, MA, 3 to 6 February 2008 [Abstract 736].)

MATERIALS AND METHODS

Cells, culture conditions, and reagents. The CD4-positive T-cell line PM1 was maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, UT), 50 U of penicillin/ml, and 50 μg of streptomycin/ml. PM1/CCR5 cells were generated by standard retrovirus-mediated transduction of PM1 cells with pBABE-CCR5 provided by the National Institutes of Health AIDS Research and Preference Reagent Program (NIH ARRRP) (24, 34). PM1/CCR5 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 U of penicillin/ml, 50 μg of streptomycin/ml, and 0.1 mg of G418 (Invitrogen, Carlsbad, CA)/ml. The TZM-bl cell line was obtained from the NIH ARRRP and maintained in Dulbecco modified Eagle medium (Sigma) supplemented with 10% FCS.

NBD-556 (molecular weight, 337.84) and YYA-004 (molecular weight, 303.4), which has the same structure as JRC-I-300 (23), were synthesized as previously described (23, 25, 30). KD-247 (12), 3E4, and 0.5γ (unpublished) are anti-gp120-V3 monoclonal Abs (MAbs). 17b (27), 4C11, and 4E9C (unpublished) are MAbs against CD4-induced epitopes (CD4i Abs). 17b, 2G12 (a MAb against the gp120 glycan), and b12 (a MAb against the CD4-binding site [CD4bs] epitope) were provided by the NIH ARRRP. The 0.5δ antibody established in our laboratory is an anti-CD4bs MAb (unpublished results). RPA-T4 (an anti-CD4 MAb) was purchased from BD Biosciences Pharmingen (San Jose, CA). Recombinant human sCD4 was purchased from R&D Systems, Inc. (Minneapolis, MN).

MAbs 3E4, 0.5γ, 0.5δ, 4C11, and 4E9C were human MAbs established from a patient with long-term nonprogressive illness. B cells from the patient's peripheral blood mononuclear cells (PBMC) were transformed by Epstein-Barr virus, followed by cloning. Culture supernatant from an individual clone was screened for reactivity to $gp120_{SF2}$ by enzyme-linked immunosorbent assay (ELISA). The specificity of the antibodies was determined by gp120 capture ELISA and fluorescence-activated cell sorting analysis of HIV-1_{JR-FL}-infected PM1 cells in the presence or absence of sCD4. The binding specificity was further assessed by an ELISA using peptides corresponding to the V3 sequence of various isolates. Based on these binding data, we classified them as follows: V3 MAbs, 3E4 and 0.5γ; CD4bs MAb, 0.5δ; and CD4i MAbs, 4C11 and 4E9C.

The laboratory-adapted HIV-1 strains HIV-$1_{89.6}$, HIV-1_{BaL}, HIV-1_{SF162}, HIV-1_{JR-FL}, and HIV-1_{YU2} were propagated in phytohemagglutinin-activated PBMC. These viruses were then passaged in PM1/CCR5 cells, and the culture supernatants were stored at −150°C prior to use. R5 primary HIV-1 isolates (HIV-$1_{Pt.1}$, HIV-$1_{Pt.2}$, HIV-$1_{Pt.3}$, and HIV-$1_{Pt.4}$) were isolated from four Japanese patients in our laboratory. All patients were at a stage of chronic infection. HIV-$1_{Pt.1}$, HIV-$1_{Pt.3}$, and HIV-$1_{Pt.4}$ were isolated from drug-naive patients, and HIV-$1_{Pt.2}$ was isolated from a drug-experienced patient and passaged in phytohemagglutinin-activated PBMC. Infected PBMC were cocultured with PM1/CCR5 cells for 4 to 5 days, and the culture supernatants were stored at −150°C until used. Nucleotide sequences of the gp120 from the four primary isolates were deposited in the DNA Data Bank of Japan under accession numbers AB553911 to AB553914.

Susceptibility assay. The sensitivities of six laboratory-adapted viruses, four primary isolates, and HIV-1_{IIIB} viruses passaged in the presence of sCD4 or NBD-556 were determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as previously described with minor modifications (31). Briefly, PM1/CCR5 cells (2×10^3 cells/well) were exposed to 100 times the 50% tissue culture infective dose ($TCID_{50}$) of the viruses in the presence of various concentrations of sCD4 or NBD-556 in 96-well round-bottom microculture plates, followed by incubation at 37°C for 7 days. After removal of 100 μl of the medium, 10 μl of MTT solution (7.5 mg/ml) in phosphate-buffered saline (PBS) was added to each well. The plate was then incubated at 37°C for 3 h. Subsequently, the produced formazan crystals were dissolved by adding 100 μl of acidified isopropanol containing 4% (vol/vol) Triton X-100 to each well. The optical densities at a wavelength of 570 nm were measured in a microplate reader. All assays were performed in duplicate or triplicate. We also determined the concentration for 50% cytotoxicity (CC_{50}) by using the MTT assay.

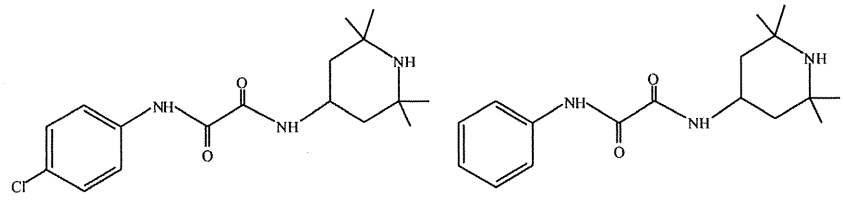
The sensitivities of the HIV-$1_{Pt.3}$ primary isolate to KD-247 (anti-V3 MAb), 4E9C (CD4i MAb), and autologous plasma IgG in the presence or absence of NBD-556 were also determined by using the MTT assay. To exclude any influence of plasma factors, such as antiviral drugs, cytokines, and chemokines, on the neutralization activities, we used IgG from the patient's plasma, which was purified using protein A-Sepharose (Affi-gel Protein A; Bio-Rad, Hercules, CA) (19).

Flow cytometric analysis. HIV-1_{JR-FL} chronically infected PM1 cells were preincubated with or without sCD4 (0.5 μg/ml) and NBD-556 (1, 3, 10, 30, 90, and 100 μM) for 15 min and then incubated with various anti-HIV-1 MAbs (17b, 4C11, KD-247, 3E4, and 0.5γ) at 4°C for 30 min. The cells were washed with PBS, and a fluorescein isothiocyanate-conjugated goat anti-human IgG Ab was used for Ab detection. Flow cytometry was performed with a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed by using the BD CellQuest version 3.1 software (BD Biosciences).

Data analysis and evaluation of synergy. Analyses of the synergistic, additive, and antagonistic effects of the antiviral agents were initially performed according to the median effect principle using the CalcuSyn version 2 computer program (6) to provide estimates of the 50% inhibitory concentration (IC_{50}) values of the antiviral agents in combination. Combination indices (CIs) were estimated from the data and reflected the nature of the interactions between KD-247 and sCD4 or NBD-556 and between NBD-556 and CD4i MAb 4C11 or anti-CD4bs MAb 0.5δ against HIV-1_{JR-FL} or HIV-1_{IIIB} on PM1/CCR5 cells as determined by the MTT assay. A CI of <0.9 indicated synergy, a CI between 0.9 and 1.1 indicated additivity, and a CI of >1.1 indicated antagonism. The CI value was directly proportional to the amount of synergy for the combination regimen. For example, values of <0.5 represented a high degree of synergy, while values of >1.5 represented significant antagonism. This approach has been widely used in analyses of antiviral interactions and was chosen to allow comparability with published literature.

Docking simulation. The structure for NBD-556 was built in SYBYL 7.1 (Tripos, St. Louis, MO) and minimized with the MMFF94 force field and partial charges (15). Using FlexSIS through its SYBYL module, docking of NBD-556 was performed into the crystal structure of gp120 obtained from the Protein Data Bank (PDB; entry 1RZJ). The binding site was defined as residues Val255, Asp368, Glu370, Ser375, Ile424, Trp427, Val430, and Val475, including residues located within a radius of 4.4 Å. The structure of the ligand was treated flexibly, and all other options were set to their default values. Figures were generated using SwissPdb Viewer version 3.9 (SPdbViewer) (13) and ViewerLite version 5.0 (Accelrys, Inc., San Diego, CA). We also generated a simian immunodeficiency virus (SIV) gp120 figure (PDB entry 2BF1) to compare the sites of the mutations in HIV-1 gp120 using the same software programs.

Isolation of NBD-556- and sCD4-resistant mutants from HIV-1_{IIIB} *in vitro*. To select NBD-556 and sCD4 escape viruses, HIV-1_{IIIB} was treated with various concentrations of NBD-556 or sCD4 and then infected into PM1/CCR5 cells as previously described with minor modifications (32). Viral replication was monitored by observation of any cytopathic effects in PM1/CCR5 cells. The culture supernatants were harvested on day 7 and used to infect fresh PM1/



NBD-556 YYA-004

FIG. 1. Structures of NBD-556 and YYA-004.

CCR5 cells for the next round of culture in the presence of increasing concentrations of NBD-556 or sCD4. When the viruses began to propagate in the presence of NBD-556 or sCD4, the concentration was further increased. After the viruses were passaged using up to 50 μM NBD-556 or 20 μg of sCD4/ml in PM1/CCR5 cells, the resulting viruses, designated NBD-556(20)14p, NBD-556(50)17p, and sCD4(20)5p, were recovered from the passaged cell culture supernatants.

Proviral DNA extracts from cells cultured with several concentrations of NBD-556 and sCD4 were subjected to PCR amplification using *Taq* polymerase (Takara, Shiga, Japan). The amplified products were cloned into pCR2.1 (Invitrogen), and the *env* regions in both the passaged and selected viruses were sequenced by using an ABI Prism 3110 automated DNA sequencer (ABI, Foster City, CA).

Construction of mutant Env expression vectors. Proviral DNA was extracted from the passaged HIV-1_{IIIB}-infected PM1/CCR5 cells by using a QIAamp DNA blood minikit (Qiagen, Valencia, CA). For the construction of Env expression vectors, we used pCXN2, which contains a chicken actin promoter. Briefly, we amplified the passaged HIV-1_{IIIB} gp160 regions using LA *Taq* (Takara) with the primers ENVA (5′-GGCTTAGGCATCTCCTATGGCAGGAAGAA-3′) and ENVN (5′-CTGCCAATCAGGGAAGTAGCCTTGTGT-3′). The PCR products were inserted into pCR-XL-TOPO (Invitrogen). The EcoRI fragment of pCR-XL-IIIB containing the entire *env* region was ligated into pCXN2 to give pCXN-IIIBwt. pCXN-IIIB(S375N), pCXN-IIIB(V255E), and pCXN-IIIB(A433T) were generated by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) in accordance with the manufacturer's instructions with the primer pairs S375Nfw (5′-AAATTGTAAC GCACAATTTTAATTGTGGAGG-3′) and S375Nrv (5′-CCTCCACAATTAA AATTGTGCGTTACAATTT-3′), V255Efw (5′-GAATTAGGCCAGTAGAAT CAACTCAACTGCT-3′) and V255Erv (5′-AGCAGTTGAGTTGATTCTACT GGCCTAATTC-3′), and A433Tfw (5′-CAGGAAGTAGGAAAAACAATGTA TGCCCCTC-3′) and A433Trv (5′-GAGGGGCATACATTGTTTTTCCTACTT CCTG-3′), respectively.

Pseudovirus preparation. Portions, 5 μg of pSG3ΔEnv and 0.5 μg of pRSV-Rev (17), supplied by the NIH ARRRP, and a 4.5-μg portion of HIV-1_{IIIB} Env-expressing pCXN2 were cotransfected into 293T cells using the Effectene transfection reagent (Qiagen). At 48 h after transfection, the pseudovirus-containing supernatants were harvested, filtered through a 0.2-μm-pore-size filter, and stored at −80°C. The pseudovirus activities were measured with a luminescence assay using TZM-bl cells as previously described (28).

Single-round virus infection assay. A single-cycle infectivity assay was used to measure the neutralization of HIV-1_{IIIB} pseudoviruses as described previously (26, 28). Briefly, NBD-556, YYA-004, sCD4, 2G12, b12, RPA-T4, or 4C11 at various concentrations and a pseudovirus suspension corresponding to 100 $TCID_{50}$ were preincubated in the absence or presence of 1 μM NBD-556 for 15 min on ice. The virus-compound mixtures were added to TZM-bl cells, which had been seeded in a 96-well plate (1.5×10^4 cells/well) on the previous day. The cultures were incubated for 2 days at 37°C, washed with PBS, and lysed with lysis solution (Galacto-Star mammalian reporter gene assay system; ABI). After transfer of the cell lysates to luminometer plates, the β-galactosidase activity (in relative light units) in each well was measured by using 50-fold-diluted Galacto-Star substrate in a reaction buffer diluent (100 μl/well; ABI) in a TR717 microplate luminometer (ABI). The reduction in infectivity was determined by comparing the relative light units in the presence or absence of each compound and expressed as the percentage of neutralization. Each assay was repeated two to three times.

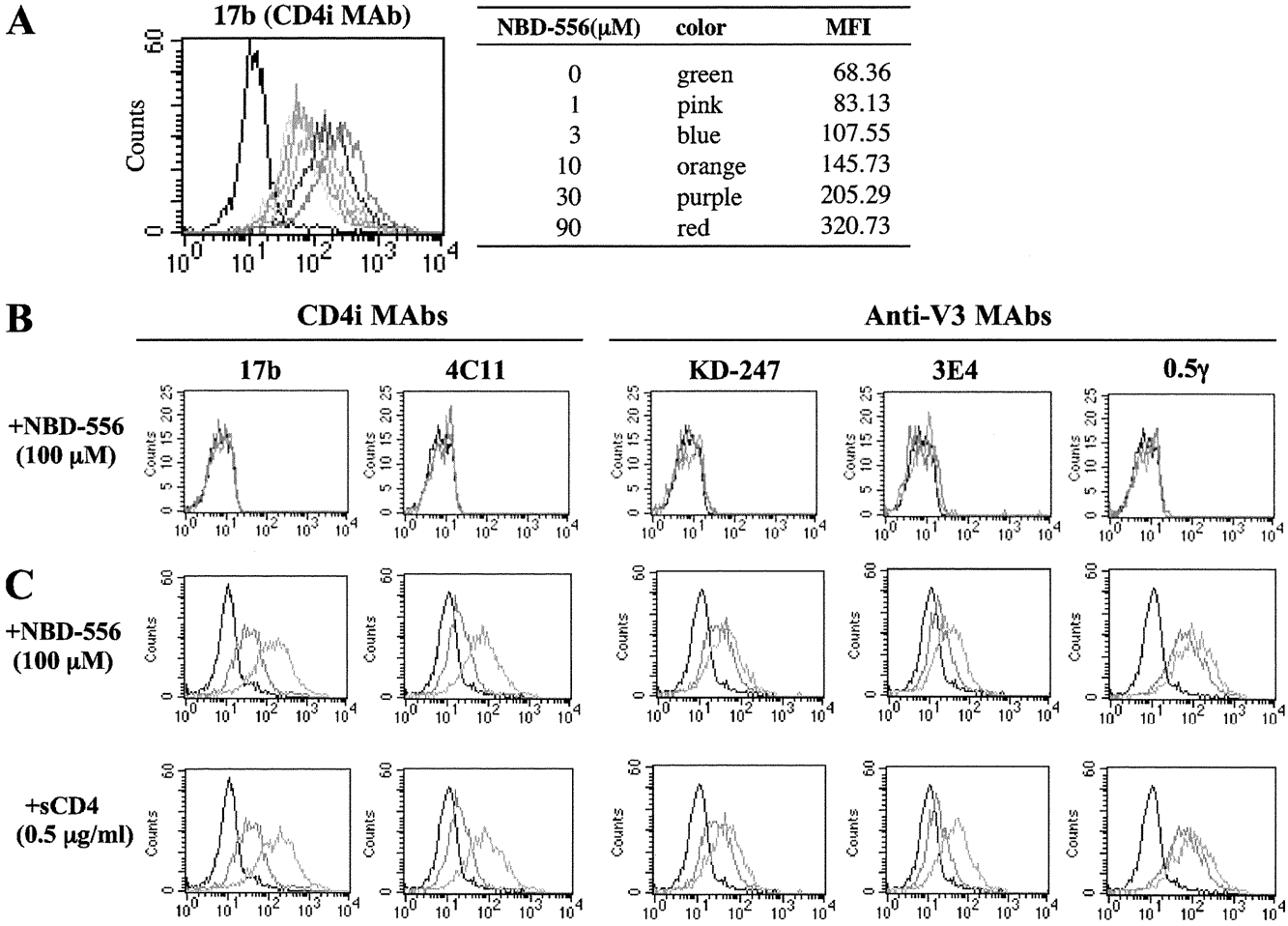
RESULTS

Anti-HIV-1 activities of sCD4, NBD-556, and YYA-004 for laboratory strains and primary HIV-1 isolates. Initially, we determined the inhibitory activities of sCD4, NBD-556, and YYA-004, which has a phenyl group instead of the *p*-chlorophenyl group of NBD-556 (Fig. 1), on the infection of PM1/CCR5 cells by different laboratory-adapted HIV-1 strains and different HIV-1 primary isolates of subtype B, including both X4 and R5 viruses, by using a previously reported method (33). sCD4 inhibited the laboratory-adapted HIV-1 strains HIV-1_{IIIB}, HIV-$1_{89.6}$, HIV-1_{BaL}, HIV-1_{SF162}, HIV-1_{JR-FL}, and HIV-1_{YU2} with IC_{50}s ranging from 0.26 to 6.1 μg/ml (Table 1). NBD-556 inhibited the X4 virus HIV-1_{IIIB} and dualtropic virus HIV-$1_{89.6}$ with IC_{50}s of 7.8 and 11.4 μM, respectively, but did not inhibit the R5 viruses HIV-1_{BaL}, HIV-1_{SF162}, HIV-1_{JR-FL}, and HIV-1_{YU2} with IC_{50}s of >30 μM. We also tested sCD4 and NBD-556 against the R5 primary isolates HIV-$1_{Pt.1}$, HIV-$1_{Pt.2}$, HIV-$1_{Pt.3}$, and HIV-$1_{Pt.4}$. sCD4 effectively inhibited all of the primary isolates at concentrations of 0.2 to 7.4 μg/ml. On the other hand, NBD-556 inhibited two of the four primary

TABLE 1. Inhibitory activities of sCD4 and NBD-556 toward infection by laboratory and primary strains of HIV-1

Virus	Subtype	Cell	Mean IC_{50}[a] ± SD		
			sCD4 (μg/ml)	NBD-556 (μM)	YYA-004 (μM)
Laboratory-adapted viruses					
X4					
HIV-1_{IIIB}	B	PM1/CCR5	0.26 ± 0.17	7.8 ± 2.6	>100
Dual					
HIV-$1_{89.6}$	B	PM1/CCR5	0.87 ± 0.09	11.4 ± 2.4	>100
R5					
HIV-1_{BaL}	B	PM1/CCR5	1.7 ± 0.28	>30	>100
HIV-1_{SF162}	B	PM1/CCR5	3.6 ± 0.64	>30	>100
HIV-1_{JR-FL}	B	PM1/CCR5	3.6 ± 0.71	>30	>100
HIV-1_{YU2}	B	PM1/CCR5	6.1 ± 2.00	>30	>100
Primary isolates					
R5					
HIV-$1_{Pt.1}$	B	PM1/CCR5	0.2 ± 0.04	3.6 ± 0.67	>100
HIV-$1_{Pt.2}$	B	PM1/CCR5	1.6 ± 0.21	>30	>100
HIV-$1_{Pt.3}$	B	PM1/CCR5	3.7 ± 0.42	11.8 ± 1.6	>100
HIV-$1_{Pt.4}$	B	PM1/CCR5	7.4 ± 1.30	>30	>100

[a] PM1/CCR5 cells (2×10^3) were exposed to 100 $TCID_{50}$ of each virus and then cultured in the presence of various concentrations of sCD4, NBD-556, or YYA-004 as indicated. The IC_{50}s were determined by using the MTT assay on day 7 of culture. All assays were conducted in duplicate, and the data shown represent the means derived from the results of two to three independent experiments. For NBD-556, CC_{50} = 140 μM; for YYA-004, CC_{50} = 350 μM. (The CC_{50} is the concentration for 50% cytotoxicity.)



NBD-556(μM)	color	MFI
0	green	68.36
1	pink	83.13
3	blue	107.55
10	orange	145.73
30	purple	205.29
90	red	320.73

FIG. 2. Comparisons of MAb binding to cell surface-expressed gp120 with sCD4 and NBD-556. HIV-1_{JR-FL} chronically infected PM1 cells were preincubated with or without NBD-556 (A, 1 to 90 μM; C, 100 μM) or sCD4 (C, 0.5 μg/ml), and uninfected PM1 cells were also preincubated with or without NBD-556 (B, 100 μM) for 15 min, and then incubated with various anti-HIV-1 MAbs (17b, 4C11, KD-247, 3E4, and 0.5γ) at 4°C for 30 min. The cells were washed, and a fluorescein isothiocyanate-conjugated anti-human IgG was used for detection. (A) Color lines show the concentrations of NBD-556: green, 0 μM; pink, 1 μM; blue, 3 μM; orange, 10 μM; purple, 30 μM; and red, 90 μM. (B and C) Red line shows no preincubated with NBD-556 or sCD4. Blue line shows preincubated with NBD-556 or sCD4. Black line shows using a control IgG MAb.

isolates, HIV-$1_{Pt.1}$ and HIV-$1_{Pt.3}$, with IC_{50}s of 3.6 and 11.8 μM, respectively (Table 1). YYA-004 did not show significant anti-HIV activity against any of the strains tested up to a concentration of 100 μM. The *in vitro* cytotoxicities of NBD-556 and YYA-004 toward PM1/CCR5 cells used for the anti-HIV-1 infectivity studies were determined by using the MTT assay. The CC_{50} values of NBD-556 and YYA-004 toward PM1/CCR5 cells were 140 and 350 μM, respectively (Table 1).

Comparison of Ab binding to cell surface-expressed HIV-1_{JR-FL} Env with NBD-556 and sCD4. To compare the effect of NBD-556 with that of sCD4 with respect to the induction of conformational change in the trimeric gp120, the binding of CD4i MAbs (17b and 4C11) and anti-V3 MAbs (KD-247, 3E4, and 0.5γ) to cell surface-expressed Env proteins on HIV-1_{JR-FL} chronically infected PM1 cells was analyzed by fluorescence-activated cell sorting. Comparisons of the binding profiles of the Abs to the cell surfaces were carried out using the mean fluorescence intensity (MFI). The binding of CD4i MAb 17b increased gradually as the amount of the CD4-mimicking small compound NBD-556 increased from 0 to 90 μM (Fig. 2A, the MFI increased from 68.36 to 320.73). As shown in Fig. 2C, the binding of both CD4i MAbs 17b and 4C11 increased remarkably after pretreatment with 100 μM NBD-556 (the MFIs increased from 43.3 to 201.57 and from 24.43 to 96.06, respectively). Moreover, the binding of all of the anti-V3 MAbs—KD-247, 3E4, and 0.5γ—was enhanced by pretreatment with NBD-556 (the MFIs increased from 34.59 to 51.9, from 22.97 to 39.07, and from 86.61 to 145.08, respectively). sCD4 pretreatment of the Env-expressing cell surface also caused remarkable enhancement of the binding for not only the CD4i MAbs but also the three anti-V3 MAbs, similar to pretreatment with NBD-556. These results indicate that the CD4-mimicking compound NBD-556 can induce the conformational changes in gp120 that are caused by binding of sCD4.

Highly synergistic interactions of KD-247 combined with NBD-556. Both neutralizing anti-V3 MAb KD-247 and NBD-556 block the viral entry process, especially at the stage of the interaction between CD4 and gp120 (CD4-binding site). Each of these agents binds to either the V3 loop or the CD4 cavity. Furthermore, our previous observations suggested that neu-

TABLE 2. Combination indices for KD-247, 4C11, or 0.5δ and for sCD4 or NBD-556 against HIV-1_{JR-FL} and HIV-1_{IIIB}

Combination	Virus	CI values at different ICs[a]		
		IC_{50}	IC_{75}	IC_{90}
KD-247+sCD4	HIV-1_{JR-FL}	0.313	0.266	0.277
KD-247+NBD-556	HIV-1_{JR-FL}	0.174	0.043	0.011
4C11+NBD-556	HIV-1_{IIIB}	0.473	0.445	0.860
0.5δ+NBD-556	HIV-1_{IIIB}	47.8	20.1	8.56

[a] The multiple-drug effect analysis of Chou et al. (6) was used to analyze the effects of the drugs in combination. IC, inhibitory concentration. CI < 0.9, synergy; CI = 0.9 to 1.1, additivity; CI > 1.1, antagonism. The data shown are representative of two or three separate experiments.

TABLE 3. Inhibitory activities of NBD-556 and sCD4 toward infection of HIV-1_{IIIB} escape variants from NBD-556 and sCD4

Virus	IC_{50}[a]	
	NBD-556 (μM)	sCD4 (μg/ml)
HIV-1_{IIIB}	12	0.52
HIV-$1_{NBD-R(20)14p}$	>30	5.7
HIV-$1_{NBD-R(50)17p}$	>30	>10
HIV-$1_{sCD4-R(20)5p}$	>30	>10

[a] PM1/CCR5 cells (2×10^3) were exposed to 100 $TCID_{50}$ of each passaged virus and then cultured in the presence of various concentrations of sCD4 or NBD-556. The IC_{50}s were determined by using the MTT assay on day 7 of culture. All assays were conducted in duplicate. The data shown are representative of two or three separate experiments.

tralizing MAb KD-247 selects escape variants with greater sensitivities to sCD4 (33). Based on this notion, we examined the synergy of this MAb with sCD4 or the CD4-mimicking compound NBD-556 against wild-type HIV-1_{JR-FL}. The multiple-drug effect analysis of Chou et al. (6) was used to analyze the effects of combining KD-247 with sCD4 or NBD-556. As shown in Table 2, all of the CI values for KD-247 with the two CD4-gp120 interaction inhibitors (sCD4 and NBD-556) were <0.5 against HIV-1_{JR-FL} at all of the inhibitory concentrations tested. In particular, the CI values for the combinations of KD-247 with NBD-556 were <0.1 for IC_{75} and IC_{90}. These results suggest that combinations of KD-247 with the CD4-gp120 binding inhibitors sCD4 and NBD-556 produce very highly synergistic effects. We further examined the synergy of CD4i MAb 4C11 or anti-CD4bs MAb 0.5δ with NBD-556 against wild-type HIV-1_{IIIB}. The combination of 4C11 and NBD-556 showed synergy against HIV-1_{IIIB} for IC_{50} and IC_{75}. As expected, the IC values for NBD-556 and anti-CD4 binding site MAb, 0.5δ, which may compete with the CD4 mimetic for the CD4-binding site, were >5 against HIV-1_{IIIB} at all of the inhibitory concentrations tested. However, at lower concentrations, additive effects were observed between NBD-556 and anti-CD4bs MAb 0.5δ (data not shown). These results indicate that NBD-556 may bind within or near the epitope of the anti-CD4bs MAb and then induce the conformational changes in Env.

Selection of NBD-556 and sCD4 escape variants. To select NBD-556- and sCD4-resistant HIV-1 variants *in vitro*, we exposed PM1/CCR5 cells to HIV-1_{IIIB} and serially passaged the viruses in the presence of increasing concentrations of NBD-556 or sCD4. As a control, HIV-1_{IIIB} was passaged under the same conditions without the antiviral agents to allow us to monitor the spontaneous changes that occurred in the virus during prolonged PM1/CCR5 cell passages (designated the passage control). The selected viruses were initially propagated in the presence of 1 μM NBD-556 or 0.5 μg of sCD4/ml and, during the course of the selection procedure, the concentrations of the NBD-556 and sCD4 were increased to 50 μM and 20 μg/ml, respectively. At passages 14 and 17 for NBD-556 and passage 5 for sCD4, the supernatants containing the viruses, which were designated HIV-$1_{NBD-R(20)14p}$, HIV-$1_{NBD-R(50)17p}$, and HIV-$1_{sCD4-R(20)5p}$, respectively, were harvested, and the sensitivities of the viruses to NBD-556 and sCD4 were determined by the MTT assay (Table 3). The IC_{50}s for NBD-556 against HIV-1_{IIIB}, HIV-$1_{NBD-R(20)14p}$, and HIV-$1_{NBD-R(50)17p}$ were 12, >30, and >30 μM, respectively. The IC_{50}s of sCD4 against HIV-1_{IIIB} and HIV-$1_{sCD4-R(20)5p}$ were 0.52 and >10 μg/ml, respectively. HIV-$1_{NBD-R(20)14p}$, HIV-$1_{NBD-R(50)17p}$, and HIV-$1_{sCD4-R(20)5p}$ were also examined for their cross-resistance with one another. Each resistant variant was found to be cross-resistant to NBD-556 and sCD4 (Table 3). These results indicate that the HIV-1_{IIIB} virus acquired resistant phenotypes against NBD-556 and sCD4 during the distinct *in vitro* selection processes.

Sequences of the envelope region of the NBD-556 and sCD4 mutants. To determine the genetic basis of the resistance in the variant HIV-1_{IIIB} strains, the C1 to C4 region of the *env* gene was amplified from genomic DNA extracted from the infected cells and cloned, and the PCR-amplified products were sequenced (Fig. 3). At passage 8 for 6 μM NBD-556, five mutations (A281D, E370A, S375N, A433T, and A436T) were observed. At passage 21 in the culture where HIV-1_{IIIB} was propagating in the presence of 50 μM NBD-556, four amino acid substitutions of Ser to Asn at position 375 (S375N, 11 of 11 clones) in C3, Ala to Lys at position 342 (A432K, 1 of 11 clones) in C4, Ala to Thr at position 433 (A433T, 4 of 11 clones) in C4, and Ala to Thr at position 436 (A436T, 1 of 11 clones) in C4 were observed (Fig. 3A). These results did not contradict a previous study in which gp120 mutants (S375W, I424A, W427A, and M475A) with changes in residues that contacted the Phe43 cavity did not detectably bind NBD-556 by isothermal titration calorimetry (23). On the other hand, in the selection with sCD4, seven mutations (E211G, P212L, V255E, N280K, S375N, G380R, and G431E) appeared during the passages. At passage 5 in the culture where HIV-1_{IIIB} was propagating in the presence of sCD4 (20 μg/ml), four substitutions of E211G (1 of 10 clones), V255E (5 of 10 clones), G380R (1 of 10 clones), and G431E (2 of 10 clones) were detected for sCD4 at 20 μg/ml (Fig. 3B).

To compare the two mutation profiles obtained during the *in vitro* selection with NBD-556 and sCD4, molecular modeling of NBD-556 docked into gp120 was performed by docking simulations using the FlexSIS module of SYBYL 7.1 (Fig. 4). The atomic coordinates of the crystal structure of gp120 with sCD4 were retrieved from the PDB (entry 1RZJ). As shown in Fig. 4, almost all of the mutations lay along the inside of the CD4 cavity in the selection of NBD-556, with similar three-dimensional positions to the mutations induced by sCD4. These findings demonstrate that NBD-556 binds to the CD4 cavity or in the vicinity of the CD4-binding site.

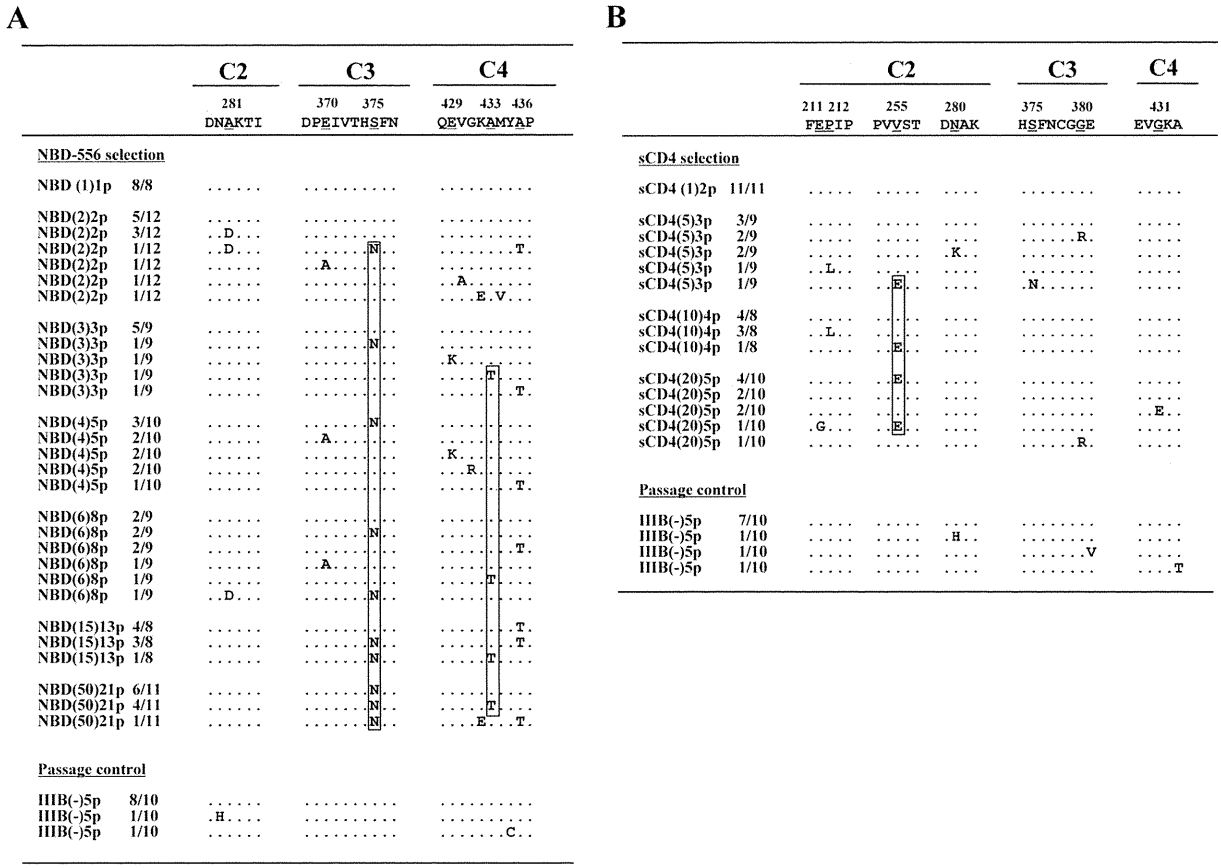


FIG. 3. Alignment of the gp120 amino acid sequences from the indicated passages in the NBD-556 and sCD4 escape processes. The amino acid sequences were deduced from the nucleotide sequences of the *env*-encoding regions of proviral DNA isolated from cells infected with the HIV-1_{IIIB} variants selected in the presence of NBD-556 (A) or sCD4 (B) and the passage control. The amino acid sequences of the envelope proteins of the baseline HIV-1_{IIIB} are shown at the top as a reference. The identity of the sequences at the individual amino acid positions is indicated by dots. The numbers of clones with the given amino acid substitutions among a total of 8 to 12 clones are listed. The number in parentheses denotes the concentrations of NBD-556 or sCD4. The major mutations of NBD-556 and sCD4-resistant variants at final passage are boxed.

Sensitivities of β-galactosidase reporter HIV strains pseudotyped with the sCD4- and NBD-556-resistant envelope mutations to NBD-556, sCD4, and MAbs. To confirm whether the mutations were responsible for the reduced sensitivities to NBD-556 and sCD4, a single-round replication assay was performed. The β-galactosidase reporter viruses were pseudotyped with wild-type Env (HIV-1_{WT}) or Env singly mutated with V255E in C2 (HIV-1_{V255E}), S375N in C3 (HIV-1_{S375N}), and A433T in C4 (HIV-1_{A433T}). The mutations that arose in the absence of NBD-556 (the passage control) are not related to resistance because the control passage did not show any significant increase in IC_{50} (data not shown). With respect to the mutations in the presence of NBD-556 three mutations, S375N, V255E, and A433T were consistently and increasingly observed during the process of selection. Additional mutations in "escape variants" other than S375N, V255E, and A433T were observed; however, these mutations were not consistently detected in passages and did not accumulate during selection. Thus, we considered the three mutations—S375N, V255E, and A433T—related to the development of resistance to both NBD-556 and sCD4, although some involvement of additional mutations in the development of a resistant phenotype is undeniable. As shown in Fig. 5A, all of the mutant clones were completely resistant to NBD-556 at concentrations of up to 20 μM. YYA-004 without the *p*-chlorophenyl group was unable to inhibit infection of all of the clones tested (Fig. 5B). The clone with V255E, which was induced by *in vitro* selection with sCD4, was highly resistant to sCD4 compared to the wild-type virus (114-fold-higher IC_{50}) (Fig. 5C). However, the other pseudotyped viruses, HIV-1_{S375N} and HIV-1_{A433T}, were slightly resistant compared to HIV-1_{WT} (4- and 2-fold-higher IC_{50}s, respectively). We also examined the sensitivities of the pseudotyped clones containing Env mutations to anti-gp120 glycan MAb 2G12, anti-CD4bs MAb b12, and anti-CD4 MAb RPA-T4 by a single-round replication assay (Fig. 5D to F). All of the mutant viruses showed almost the same neutralization sensitivities as the wild-type virus to the 2G12, b12, and RPA-T4 MAbs. These results indicate that the three mutations induced by *in vitro* selection with NBD-556 and sCD4 were responsible for the resistance to NBD-556, whereas the NBD-selected variants containing S375N in C3 and A433T in C4 of gp120 had moderately resistant phenotypes against sCD4, as shown by the sensitivities of the NBD-556-passaged viruses to sCD4 determined by the multiround assay (Table 3).

To examine whether the resistance mutations affected the sensitivity of a CD4i MAb against HIV-1, we determined the

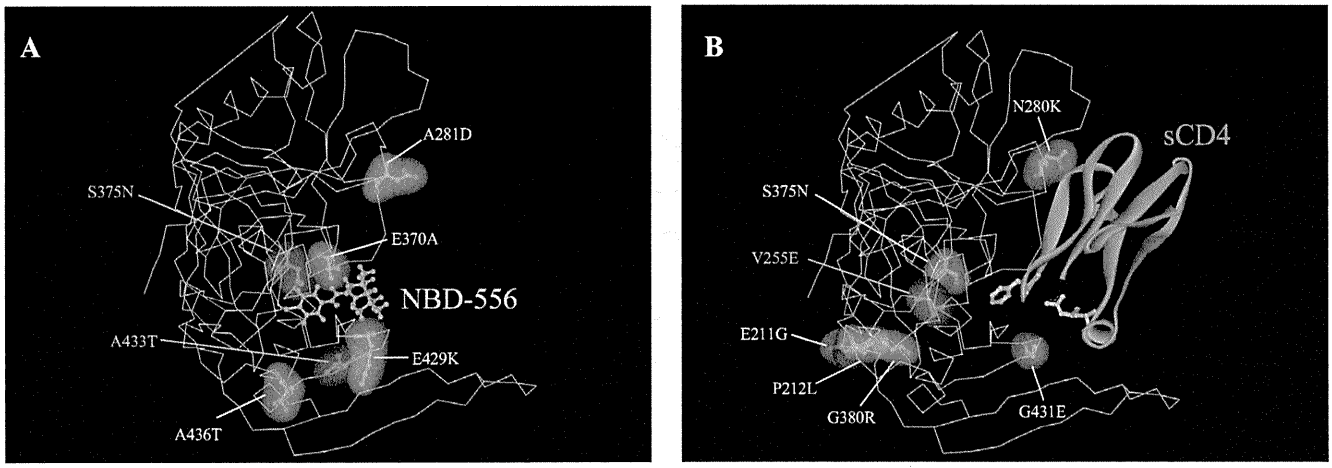


FIG. 4. Locations of substitutions in HIV-1_{IIIB} gp120 induced by *in vitro* selection with NBD-556 or sCD4. The side chains of the mutated residues that appeared during the *in vitro* selection with NBD-556 (A) or sCD4 (B) are shown in yellow and purple. The amino acid substitutions that confer resistance in HIV-1 are indicated in purple. The crystal structure of gp120 with sCD4 was retrieved from the PDB (entry 1RZJ). The structure of compound NBD-556 docked into gp120 was created by using the FlexSIS module of SYBYL 7.1.

sensitivities of HIV strains pseudotyped with the sCD4- and NBD-556-resistant envelope mutations to CD4i MAb 4C11 with or without the CD4-mimicking compound. As expected, NBD-556-pretreated HIV-1_{WT} was more sensitive to 4C11 than the untreated virus (IC_{50}s, 0.12 versus 0.72 μg/ml) (Fig. 6). On the other hand, all of the mutant viruses were completely resistant to 4C11 with or without NBD-556 pretreatment. These results suggest that the CD4 and NBD-556 resistance mutations in gp120 hide the epitope for a particular Ab against a CD4-induced epitope, similar to primary R5 viruses.

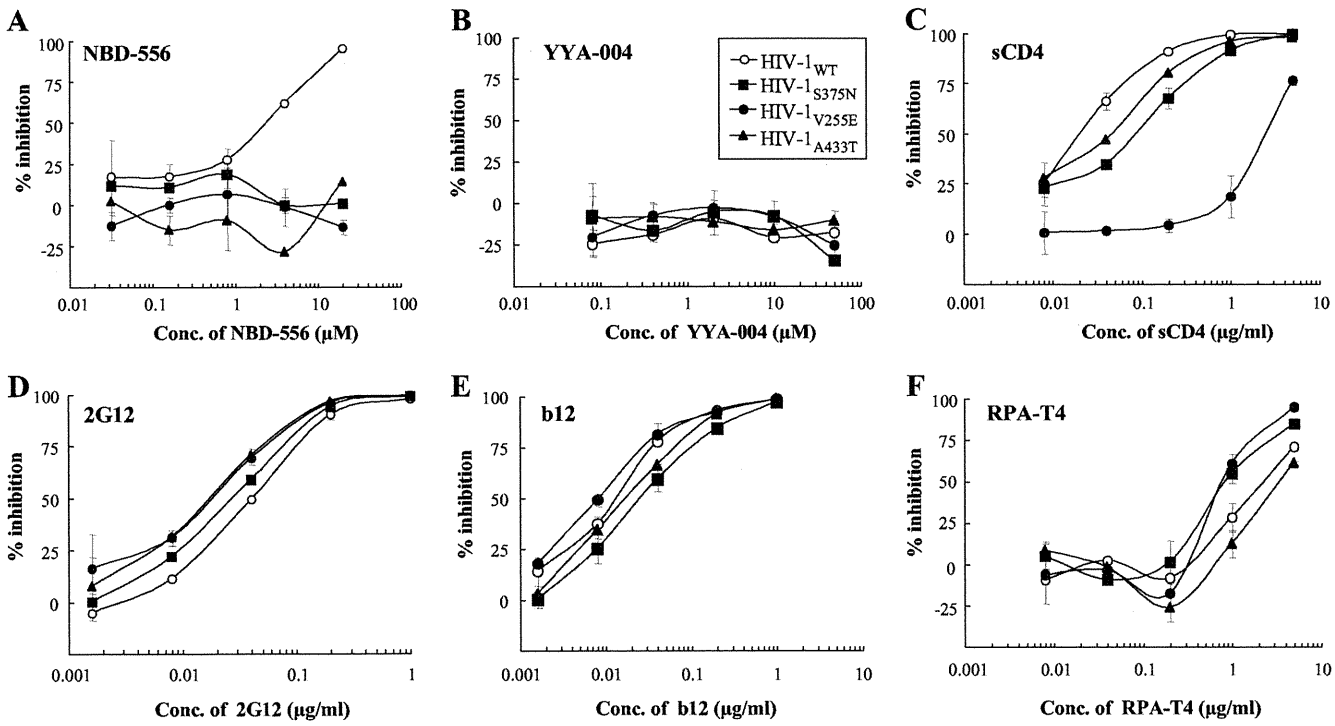


FIG. 5. Sensitivities of β-galactosidase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistance envelope mutations to NBD-556, YYA-004, sCD4, and MAbs. The sensitivities of β-galactosidase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistance envelope mutations to NBD-556 (A), YYA-004 (B), sCD4 (C), 2G12 (D), b12 (E), and RPA-T4 (F) are shown. NBD-556, YYA-004, sCD4, and MAbs at various concentrations and a pseudovirus suspension corresponding to 100 $TCID_{50}$ were preincubated for 15 min on ice and then added to the target cells (TZM-bl). The inhibitory effects were determined by measuring the β-galactosidase activities on day 2 of culture. All assays were conducted in triplicate, and the data shown represent the means ± the standard deviations (SD) derived from the results of two to three independent experiments.

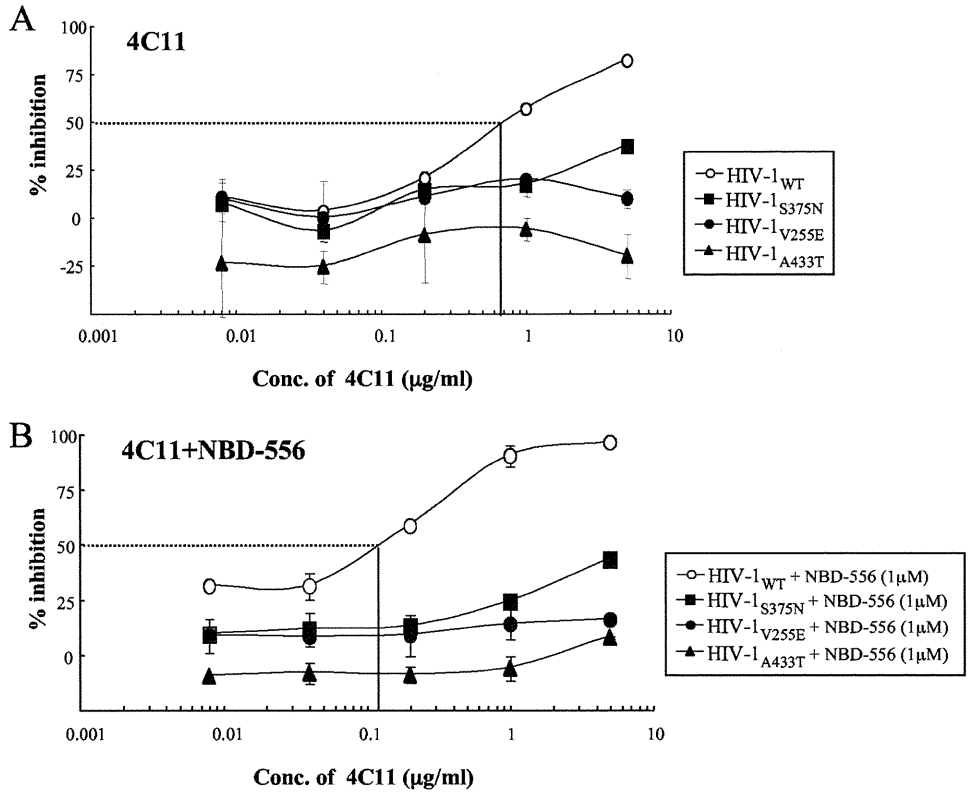


FIG. 6. Sensitivities of β-galactosidase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistance envelope mutations to CD4i MAb 4C11 with or without NBD-556. The sensitivities of β-galactosidase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistance envelope mutations to CD4i MAb 4C11 in the absence (A) or presence (B) of NBD-556 are shown. 4C11 at various concentrations and a pseudovirus suspension corresponding to 100 $TCID_{50}$ were preincubated with or without NBD-556 (1 μM) for 15 min on ice and then added to the target cells (TZM-bl). The inhibitory effects were determined by measuring the β-galactosidase activities on day 2 of culture. All assays were conducted in triplicate, and the data shown represent the means ± the SD derived from the results of two to three independent experiments.

NBD-556-mediated enhancement of the neutralization activities of plasma Abs against an autologous isolate. Neutralization escape has been documented in HIV-1 subtype B viruses, with contemporaneous viruses showing less sensitivity to autologous neutralization than earlier viruses (2). For one patient (patient 3 [Pt.3]) infected with a subtype B virus, the autologous neutralizing activities in plasma IgG obtained close to the time of the virus isolation were measured in the presence or absence of NBD-556 (0, 1, 2, 4, and 8 μM) by the MTT assay. As shown in Fig. 7A, the plasma IgG neutralizing activity was much less potent against the variant ($HIV\text{-}1_{Pt.3}$) from the same time point (IC_{50} of >200 μg/ml for IgG). However, $HIV\text{-}1_{Pt.3}$ pretreated with at least 1 μM NBD-556 became sensitive to the contemporaneous plasma IgG compared to the untreated virus. To examine which kinds of NAbs are enhanced by NBD-556, we determined the susceptibilities of $HIV\text{-}1_{Pt.3}$ to anti-V3 MAb KD-247 and CD4i MAb 4E9C with or without NBD-556. The virus was completely resistant to both MAbs (IC_{50}s of >100 μg/ml) in the absence of NBD-556, while NBD-556-pretreated $HIV\text{-}1_{Pt.3}$ became sensitive to KD-247 and 4E9C (IC_{50}s of 10.0 and 20.8 μg/ml, respectively) (Fig. 7B). These results indicate that CD4-mimicking small compounds such as NBDs have potent NAb-enhancing activities toward plasma Abs that cannot access the neutralizing epitopes hidden within the trimeric Env, such as CD4i and anti-V3 Abs.

DISCUSSION

In this study, we observed that NBD-556 could bind to a CD4-binding site, followed by the induction of conformational changes in gp120 similar to those observed upon sCD4 binding. Although we used a limited number of viruses and plasma IgG preparations obtained from an HIV-1-positive patient for testing the synergistic effects between NBD-556 and neutralizing antibody, we also found highly synergistic interactions between NBD-556 and not only CD4i MAbs but also anti-V3 MAbs. Moreover, our data indicated that small compounds such as NBDs can enhance the potency of NAbs in HIV-1-infected patients against the contemporaneous viruses, which are resistant to neutralization by Abs in the plasma.

We illustrated the sites of the mutations induced by NBD-556 on the structure of unliganded gp120 of SIV obtained from the PDB (entry 2BF1) to compare the sites before and after binding of the CD4-mimicking compound. As shown in Fig. 8, the mutations lay in front of the outer domain in gp120, which was near to or within the CD4-binding site. These findings indicate that NBD-556 attaches to the CD4-binding site or the

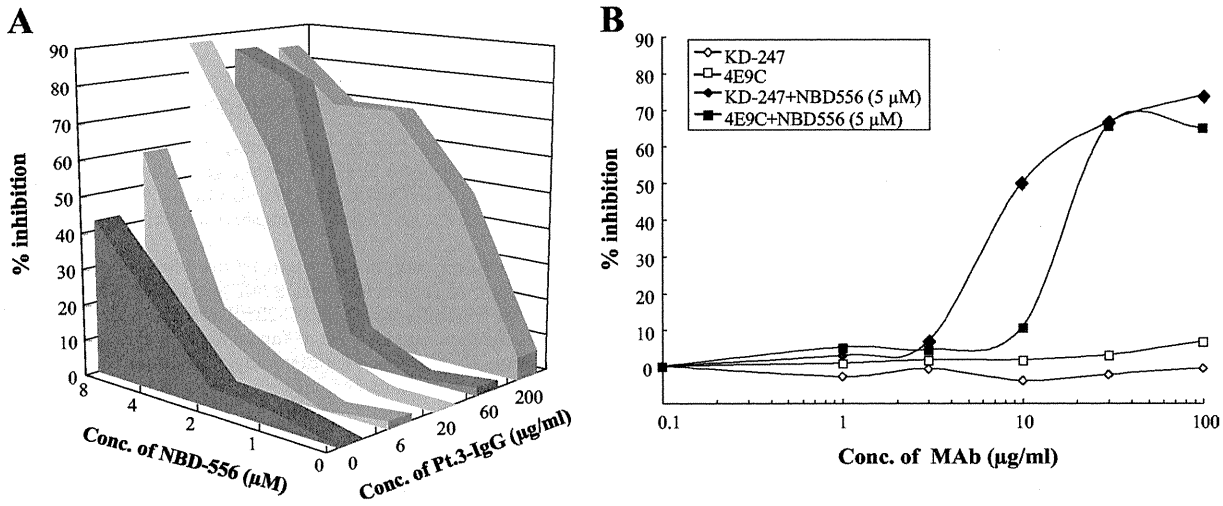


FIG. 7. NBD-556-mediated enhancement of the neutralization activity of plasma IgG against the autologous isolate. (A) The sensitivities of the HIV-$1_{Pt.3}$ primary isolate to the autologous plasma IgG (Pt.3-IgG) in the absence or presence of NBD-556 (1, 2, 4, and 8 μM) were determined by the MTT assay. (B) The sensitivities of the HIV-$1_{Pt.3}$ primary isolate to KD-247 (anti-V3 MAb; diamonds) and 4E9C (anti-CD4i MAb; squares) in the absence (open symbols) or presence (filled symbols) of 5 μM NBD-556 were determined by the same assay. The data shown are representative of two or three separate experiments.

surrounding residues in the unliganded form of gp120 and that, after the conformational changes of the envelope glycoproteins, probably the CD4-liganded form induced by the attack by NBD-556, the compound could penetrate and be held for a while in the CD4 cavity. In a recent study, Haim et al. (14) showed that sCD4-mimicking compounds have the ability to inactivate HIV-1 by prematurely triggering active but transient intermediate states of the envelope glycoproteins. In the transient intermediate states, several neutralizing epitopes in gp120 may be accessible to the neutralizing Abs. These data and our present results suggest that some NBD analogs, which bind to the cavity tightly and for a longer time, as well as cell surface CD4 inducing a more stable envelope glycoprotein intermediate state, show highly potent NAb-enhancing activities.

Madani et al. (23) reported that replacement of gp120 Ser375 with a glycine residue dramatically reduced the HIV-1 sensitivity to enhancement by any of the NBD-556 analogs, suggesting that a certain element of the Ser375 side chain contributes to the NBD-556 efficacy. They also reported that viruses bearing envelope glycoproteins with Ser375 mutated to alanine exhibited greater enhancement by NBD-556 and some NBD-556 analogs than the viruses with wild-type envelope glycoproteins, suggesting that the hydroxyl group of Ser375 is detrimental to the binding and/or activity of some NBD-556 analogs that contain large para-phenyl substituents. Mutations

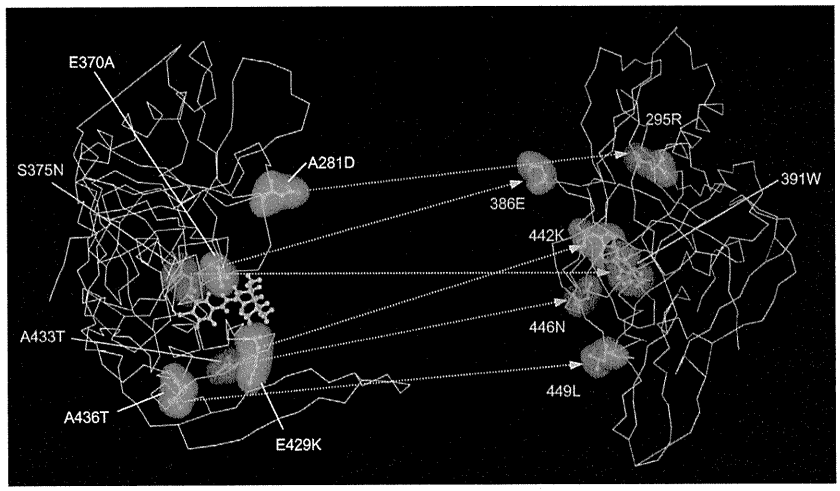


FIG. 8. Comparisons of the locations of the mutations induced by NBD-556 between the structures of unliganded and liganded gp120. The side chains of the mutated residues that appeared during *in vitro* selection with NBD-556 are shown in yellow, green and purple in the liganded (left) or unliganded (right) structures. The amino acid substitutions that confer resistance in HIV-1 are indicated in purple (S375N and A433T). The crystal structures of liganded and unliganded gp120 were retrieved from the PDB (entries 1RZJ and 2BF1, respectively). The corresponding sites of the NBD-resistant mutations are also shown on the unliganded gp120.

of other gp120 residues lining the Phe43 cavity or vestibule (Val255, Thr257, Glu429, and Val430) significantly decreased the enhancement of virus infection by the NBD-556 analogs. Our *in vitro* selection study showed that the key mutations for NBD-556 resistance were S375N and A433T and that minor mutations related to NBD-556 resistance were A281D, E370A, E429K, and A436T (Fig. 4). Thus, alterations to several gp120 residues, namely, S375N, A433T, and V255E, that line the Phe43 pocket or reside around and inside the cavity can negatively affect the entry inhibitory effect of NBD-556 on HIV-1 infection (Fig. 5).

Decker et al. (9) reported that the chemokine coreceptor binding sites of HIV-1 from subtypes A, B, C, D, F, G, and H and circulating recombinant form (CRF) 01, CRF02, and CRF11 elicit high titers of CD4i Abs during natural human infection and that these Abs bind and neutralize viruses as divergent as HIV-2 in the presence of sCD4. Recently, Davis et al. (7) showed that transplantation of HIV-1 V3 epitopes into an HIV-2 envelope scaffold provides a sensitive and specific means to detect and quantify HIV-1 V3 epitope-specific NAbs in human sera. They used this HIV-2/HIV-1 V3 scaffolding strategy to study the kinetics of the development and breadth of V3-specific NAbs in longitudinal sera from individuals acutely infected with subtype C or subtype B HIV-1. Their results indicated that high-titer broadly reactive V3-specific Abs are among the first to be elicited during acute and early HIV-1 infection, although these Abs lack neutralizing potency against primary HIV-1 viruses, which effectively shield V3 from Ab binding to the functional Env trimer (8). These observations strongly support the idea that the major problem facing the development of CD4i-based or V3-based immunogens is not sequence variation within the epitopes, but rather that access of most CD4i and anti-V3 Abs to their epitopes in functional Env complexes is blocked. As shown in Fig. 7A, plasma IgG from a seropositive patient exhibited strongly enhanced neutralizing activity against the contemporaneous virus after treatment with NBD-556. Therefore, we consider that small compounds such as NBDs can enhance the neutralizing activities of CD4i and certain anti-V3 Abs *in vivo* at the acute stage of HIV-1 infection or in combination with anti-V3 NAbs as a passive immunization.

In general, small molecules have certain advantages from a therapeutic standpoint because of their low propensity for immunogenicity, high metabolic stability, easy large-scale production, and relatively low cost. Small molecule Ab-enhancing therapeutics such as NBD compounds would have additional benefits over available treatment approaches to HIV. Since CD4i and anti-cryptic V3 Abs are already present in a large number of HIV-1-infected patients, no prevaccination would be necessary for the induction of NAbs. Moreover, the use of bifunctional small molecules, such as an entry inhibitor and a NAb enhancer, should be effective for passive immunization of the anti-HIV NAbs enhanced by the accessibility of epitopes after binding of sCD4, such as 17b (27) and KD-247 (11, 12). Elucidation of the molecular details governing the interactions between gp120 and NBD compounds will assist in optimization efforts, as well as in the evaluation of this strategy in more complex biological models for HIV infection. Consequently, we will continue to synthesize such NBD analogs to search for drugs with more potent power to change the tertiary structure of the envelope glycoproteins and lower toxicity toward the host cells.

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Long-Term Control of HIV-1 in Hemophiliacs Carrying Slow-Progressing Allele HLA-B*5101▽†

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HLA-B*51 alleles are reported to be associated with slow disease progression to AIDS, but the mechanism underlying this association is still unclear. In the present study, we analyzed the effect of HLA-B*5101 on clinical outcome for Japanese hemophiliacs who had been infected with HIV-1 before 1985 and had been recruited in 1998 for this study. HLA-B*5101$^+$ hemophiliacs exhibited significantly slow progression. The analysis of HLA-B*5101-restricted HIV-1-specific cytotoxic T-lymphocyte (CTL) responses to 4 HLA-B*-restricted epitopes in 10 antiretroviral-therapy (ART)-free HLA-B*5101$^+$ hemophiliacs showed that the frequency of Pol283-8-specific CD8$^+$ T cells was inversely correlated with the viral load, whereas the frequencies of CD8$^+$ T cells specific for 3 other epitopes were positively correlated with the viral load. The HLA-B*5101$^+$ hemophiliacs whose HIV-1 replication had been controlled for approximately 25 years had HIV-1 possessing the wild-type Pol283-8 sequence or the Pol283-8V mutant, which does not critically affect T-cell recognition, whereas other HLA-B*5101$^+$ hemophiliacs had HIV-1 with escape mutations in this epitope. The results suggest that the control of HIV-1 over approximately 25 years in HLA-B*5101-positive hemophiliacs is associated with a Pol283-8-specific CD8$^+$ T-cell response and that lack of control of HIV-1 is associated with the appearance of Pol283-8-specific escape mutants.

Human immunodeficiency virus type 1 (HIV-1)-specific CD8$^+$ T cells play a critical role in the control of HIV-1 infections (26, 5), but HIV-1 escape occurs during acute and chronic phases of an HIV-1 infection (6, 14). There are several mechanisms affording HIV-1 escape from the host immune system. They include the appearance of mutants that escape from HIV-1-specific cytotoxic T lymphocytes (CTLs) (6, 14) and neutralizing antibodies (27, 47, 48), impaired recognition of HIV-1-infected cells by HIV-1-specific CTLs due to Nef-mediated downregulation of HLA class I molecules (8, 42), and impaired function of HIV-1-specific T cells (3).

It is well known that long-term nonprogressors (LTNPs), who remain disease free and have very low or undetectable viral loads (VLs) in the absence of antiretroviral therapy (ART), exist as a very small population of HIV-1-infected individuals (7, 21, 38). A small minority of these LTNPs were infected by HIV-1 containing deletions in viral accessory molecules (10, 17, 24). HLA alleles such as HLA-B*57/5801, HLA-B*27, and HLA-B*51 are associated with slow progression to AIDS (19, 22, 37). Indeed, it is reported that many LTNPs carry these HLA alleles (31, 36). These findings imply that HIV-1-specific CTLs restricted by these alleles may play an important role in the control of HIV-1 replication in LTNPs. The mechanism of control of HIV-1 replication has been analyzed in LTNPs and slow progressors carrying HLA-B*57/5801, HLA-B*27, or HLA-B*13, and has been related to the Gag-specific CD8$^+$ T-cell epitopes presented by these alleles (9, 11, 14, 16, 34). On the other hand, the mechanism underlying the association between HLA-B*5101 and slow progression remains unclear. To date, no study of the mechanism of control of HIV-1 in HLA-B*5101$^+$ LTNPs has been reported.

Since the data indicate that HIV-1 replication can be controlled for more than 20 years in LTNP hemophiliacs, analysis of HIV-1-specific immune responses and HIV-1 in these patients is useful for investigating the immunological control of HIV-1. In Japan, HLA-B*57/58 and HLA-B*27 are very rare alleles (18). Therefore, it was speculated that only HLA-B*51 would play an important role in the control of HIV-1 replication in HIV-1-infected Japanese donors.

We showed previously that 2 Pol peptides and 1 Gag peptide were HLA-B*5101-restricted immunodominant CTL epitopes (45). Two Pol-specific CTLs are known to have strong abilities to suppress HIV-1 replication *in vitro* (43). Our recent study using 9 cohorts showed that of these T cells, Pol283-specific CTLs select mutations at position 8 (position 135 of reverse transcriptase [RT]) in the epitope (20). A Thr mutation at position 8 (8T) was found predominantly in HIV-1-infected HLA-B*5101$^+$ donors, whereas the 8R, 8L, and 8V mutations were also found in these donors. The 8T, 8L, and 8R mutants had fitness similar to

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that of the wild-type virus, whereas the 8V mutation had a higher fitness cost than the others.

In the present study, we analyzed the effect of HLA-B*5101 on clinical outcome in Japanese hemophiliacs infected with HIV-1. In addition, we investigated the role of HLA-B*5101-restricted HIV-1-specific CTLs *in vivo* in HLA-B*5101$^+$ LTNP and slow-progressing Japanese hemophiliacs who had not been treated with antiretroviral therapy for approximately 25 years. Our results revealed a role for Pol283-8-specific HLA-B*5101-restricted HIV-1-specific CTLs in the long-lasting (approximately 25 years) control of HIV-1 replication.

MATERIALS AND METHODS

Patients. One hundred eight Japanese hemophiliacs who had been infected with HIV-1 before 1985, mostly around 1983, were recruited for the present study, which was approved by the ethics committees of Kumamoto University and the National Center for Global Health and Medicine. Written informed consent was obtained from all subjects according to the Declaration of Helsinki. Patient HLA type was determined by standard sequence-based genotyping. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood.

Cells. C1R and 721.221 cells expressing HLA-B*5101 (C1R-B*5101 and 721.221-B5101, respectively) were generated previously (15, 33, 44). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.15 mg/ml hygromycin B.

HIV-1 clones. An infectious proviral clone of HIV-1, pNL-432, and its mutant, pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), were reported previously (1). Pol283-8 and Pol743-9 mutant (Pol283-8L, -8T, -8V, and -8R; Pol743-1I, -5I, and -4I5I) viruses were generated based on pNL-432 by using the GeneTailor site-directed mutagenesis system (Invitrogen).

HLA class I tetramers. HLA class I-peptide tetrameric complexes (tetramers) were synthesized as described previously (2). Four HIV-1 specific epitopes (Pol283-8, Pol743-9, Gag327-8, and Rev71-11) (45) were used for the refolding of HLA-B*5101 molecules. Phycoerythrin (PE)-labeled streptavidin (Molecular Probes) was used for the generation of the tetramers.

Flow cytometric analysis using tetramers. PBMCs were incubated with the tetramers at 37°C for 30 min. The cells were subsequently washed twice with RPMI–10% newborn calf serum (NCS) and were then stained with an anti-CD8 monoclonal antibody (MAb). Next, they were incubated at 4°C for 30 min and were then washed twice with RPMI–10% NCS. The cells were finally resuspended in phosphate-buffered saline (PBS) containing 2% paraformaldehyde, and then the percentage of tetramer-positive cells among the CD8$^+$ population was determined by using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA).

Generation of CTL clones. Pol283-8-specific CTL clones and Pol743-9-specific CTL clones were generated from HIV-1-specific bulk-cultured T cells by limiting dilution in U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) containing 200 μl of cloning mixture (about 1×10^6 irradiated allogeneic PBMCs from healthy donors and 1×10^5 irradiated C1R-B*5101 cells prepulsed with the corresponding peptide at 1 μM in RPMI 1640 supplemented with 10% human plasma and 200 U/ml human recombinant interleukin-2 [rIL-2]) (43).

CTL assay for target cells infected with HIV-1. The cytotoxicity of CTL clones for 721.221-B5101 cells infected with HIV-1 (>30% p24 antigen [Ag]-positive cells) was determined by the standard ^{51}Cr release assay as described previously (42). The infected cells were incubated with 150 μCi $Na_2{}^{51}CrO_4$ in saline for 60 min, and then the infected cells were washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well) were added to each well of a U-bottom 96-well microtiter plate (Nunc, Roskilde, Denmark) with effector cells at an effector-to-target cell (E:T) ratio of 2:1. The cells were then incubated for 6 h at 37°C. The supernatants were collected and analyzed with a gamma counter.

Assay for 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 (42). CD4$^+$ T cells isolated from PBMCs were derived from an HIV-1-seronegative individual with HLA-B*5101. After the CD4$^+$ T cells had been incubated with the desired HIV-1 clones for 4 h at 37°C, they were washed three times with R10 medium. The HIV-1-infected CD4$^+$ T cells were then cocultured with HIV-1-specific CTL clones. From day 3 to day 7 postinfection, culture supernatants were collected, and the concentration of p24 Ag in the supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) (HIV-1 p24 Ag ELISA kit; ZeptoMetrix).

Sequencing of proviral DNA or plasma RNA. Genomic DNA was extracted from PBMCs by using a QIAamp DNA blood minikit (Qiagen). Viral RNA was extracted from the plasma of HIV-1-infected individuals by using a QIAamp Mini Elute virus spin kit (Qiagen). cDNA was synthesized from the RNA with SuperScript II and random primers (Invitrogen). We amplified HIV RT and integrase sequences by nested PCR using RT-specific primers 5′-CCAAAAGT TAAGCAATGGCC-3′ and 5′-CCCATCCAAAGGAATGGAGG-3′ or 5′-CC TTGCCCCTGCTTCTGTAT-3′ for the first round of PCR and 5′-AGTTAGG AATACCACACCCC-3′ and 5′-GTAAATCCCCACCTCAACAG-3′ or 5′-AA TCCCCACCTCAACAGAAG-3′ for the second round and integrase-specific primers 5′-ATCTAGCTTTGCAGGATTCGGG-3′ and 5′-CCTTAACCGTAG TACTGGTG-3′ or 5′-CCTGATCTCTTACCTGTCC-3′ for the first round of PCR and 5′-AAAGGTCTACCTGGCATGGG-3′ or 5′-TTGGAGAGCAATG GCTAGTG-3′ and 5′-AGTCTACTTGTCCATGCATGGC-3′ for the second round. PCR products were either sequenced directly or cloned by using a TOPO TA cloning kit (Invitrogen) and then sequenced. Sequencing was done with a BigDye Terminator cycle sequencing kit (version 1.1; Applied Biosystems), and sequences were analyzed by use of an ABI PRISM 310 genetic analyzer.

Cell surface staining and intracellular cytokine staining (ICC assay). PBMCs from HIV-1-infected individuals were stimulated with the desired peptide (1 μM) and cultured for 12 to 14 days. These cultured PBMCs were assessed for gamma interferon (IFN-γ)-producing activity as previously described (42). After C1R-B*5101 cells had been incubated for 60 min with epitope peptides (1 μM), they were washed twice with RPMI 1640 containing 10% FCS. These C1R cells and the cultured PBMCs were incubated at 37°C for 6 h at an effector-to-stimulator ratio of 2:1 or 4:1 after the addition of brefeldin A (10 μg/ml). Next, the cells were stained with an anti-CD8 MAb (Dako Corporation, Glostrup, Denmark), fixed with 4% paraformaldehyde at 4°C for 20 min, and then permeabilized at 4°C for 10 min with PBS supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer). The cells were resuspended in the permeabilizing buffer and were then stained with an anti-IFN-γ MAb (BD Bioscience Pharmingen, San Diego, CA). Finally, they were resuspended in PBS containing 2% paraformaldehyde, and then the percentage of CD8$^+$ cells positive for intracellular IFN-γ was determined by using a FACSCalibur flow cytometer.

RESULTS

Association of HLA-B*5101 with long-term control of HIV-1 in HIV-1-infected Japanese hemophiliacs. We recruited 108 Japanese hemophiliacs who had been infected with HIV-1 before 1985. Eighteen of the patients had not been treated with any antiretroviral therapy (ART) and had CD4 counts of >350 (very-slow-progressor [VSP] group) by 1998, whereas the other 90 patients had been treated with ART and/or had a CD4 count of <350 (slow-progressor [SP] group). The frequency of HLA-B*5101 in the VSP group (9 of 18 donors [50.0%]) was higher than that in the SP group (15 of 90 donors [16.7%]), and the difference between these 2 groups was significant (P, 0.01). We analyzed the association of HLA class I alleles with disease progression during the years 1998 to 2007 in the VSP group. The 9 HLA-B*5101$^+$ VSP hemophiliacs exhibited significantly slower progression of the disease over this period than the 9 HLA-B*5101$^-$ subjects (Fig. 1), and no other HLA-B alleles or HLA-A/DR alleles showed any significant influence on the progression of the disease in this group (not shown). One HLA-B*3501$^+$ VSP hemophiliac was found in the HLA-B*5101$^+$ group, but none were found in the HLA-B*5101$^-$ group, indicating that HLA-B*3501, which is associated with rapid progression to AIDS, did not affect the results for the 2 VSP groups. Other HLA-A/B/DR alleles were not associated with the HLA-B*5101$^+$ or the HLA-B*5101$^-$ group (see Table S1 in the supplemental material). These results, taken together, show that the HLA-B*5101 allele was

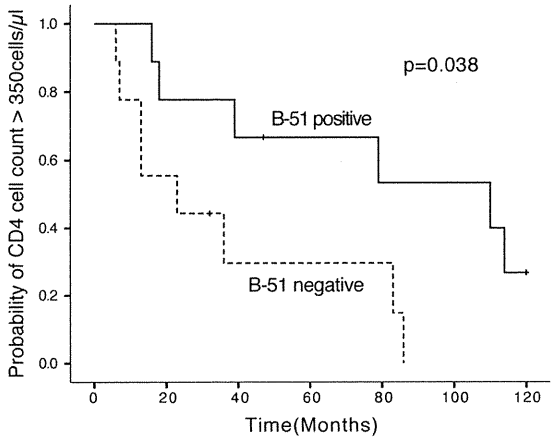

FIG. 1. Association of HLA-B*5101 with slow progression to AIDS. Kaplan-Meier survival analysis was used to estimate the time to the first CD4 cell count (24-week time-weighted average levels of CD4 cells) of $<350/\mu l^3$ for 9 HLA-B*5101-positive (solid line) and 9 HLA-B*5101-negative (dashed line) hemophiliacs who had not been treated with antiretroviral therapy (ART) and who had a CD4 count of $>350/\mu l$ in 1998.

still associated with slow progression of the disease more than 20 years postinfection.

Control of HIV-1 replication by HLA-B*5101-restricted CD8$^+$ T cells. A previous study demonstrated that 2 types of HLA-B*5101-restricted CTLs, Pol283-8 (TAFTIPSI)-specific and Pol743-9 (LPPVVAKEI)-specific CTLs, suppressed HIV-1 replication *in vitro* much more strongly than did other HLA-B*5101-restricted CTLs (43), suggesting that these CTLs may play a key role in the control of HIV-1 in the HLA-B*5101$^+$ SP group. To investigate the control of HIV-1 by these CTLs, we selected 10 HLA-B*5101-positive donors (8 VSPs and 2 SPs) who had not been treated with ART by 1998 and whose PBMC samples were available for analysis of HLA-B*5101-restricted CTLs (see Fig. S1 and Table S2 in the supplemental material). Three of the 8 VSP patients had VLs below 1,000 copies at all time points tested and were classified as LTNPs. We found that only 3 of the 108 HIV-1-infected hemophiliacs (KI-021, KI-051, and KI-124) were LTNPs for approximately 25 years and that all 3 of these LTNPs carried HLA-B*5101. We generated 4 HLA-B*5101 tetramers carrying Pol283-8, Pol743-9, Gag327-9, or Rev71-11, and we used them to determine the frequencies of HIV-1-specific CD8$^+$ T cells among PBMCs from these 3 LTNPs (Table 1 and Fig. 2). KI-021 had both Pol283-8- and Pol743-9-specific CD8$^+$ T cells but neither Gag327-9- nor Rev71-11-specific CD8$^+$ T cells during the years 1997 to 2005 (Fig. 2A). KI-051 also had both Pol283-8- and Pol743-9-specific CD8$^+$ T cells, whereas this patient had no Rev71-11-specific CD8$^+$ T cells and a low number of Gag327-9-specific CD8$^+$ T cells during the years 1999 to 2005 (Fig. 2B). KI-124 had Pol283-8-, Pol743-9-, and Gag327-9-specific CD8$^+$ T cells (Table 1). These results suggest that the 2 Pol-specific CD8$^+$ T cells may play an important role in the control of HIV-1 in these LTNPs carrying HLA-B*5101.

Selection of escape mutations of the Pol283-8 epitope in very slow progressors. Of the 8 HLA-B*5101$^+$ VSP hemophiliacs, KI-127 had Pol283-8-specific CD8$^+$ T cells at a low frequency in 1998, when the plasma viral load (pVL) was very low, whereas later this patient lost the response, and the pVL increased from an undetectable level to more than 10^3 copies (Fig. 2C). The other 4 VSPs, excluding 3 LTNBs, either had a low number of Pol283-8-specific CD8$^+$ T cells or did not have any of these cells at any time points studied. These results suggest that Pol283-8-specific CD8$^+$ T cells rather than Pol743-9-specific CD8$^+$ T cells may control HIV-1 *in vivo*.

To clarify the role of these HLA-B*5101-restricted CD8$^+$ T cells in the control of HIV-1 *in vivo*, we analyzed the correlation between the frequency of the HLA-B*5101-restricted CD8$^+$ T cells and the pVL in 10 HLA-B*5101$^+$ hemophiliacs. The frequency of Pol283-8-specific CD8$^+$ T cells was negatively correlated with the pVL (P, 5.6×10^{-8}), whereas the frequency of the other T cells was positively correlated with the pVL (Fig. 3). These results support the idea that Pol283-8-specific CD8$^+$ T cells drive the suppression of HIV-1 replication *in vivo*.

We speculated, therefore, that escape mutants within Pol283-8 epitopes were selected in slow progressors over a 25-year period, because these epitope-specific CTLs are thought to provide strong immune pressure on HIV-1. Two of the LTNPs had the Pol283-8V mutant, whereas the third had wild-type Pol283 in July 2002 but the 8V mutant in October

TABLE 1. Numbers of 4 types of HLA-B*5101-restricted CD8$^+$ T cells among HLA-B*5101$^+$ HIV-1-infected hemophiliacs

Patient	Median VL (copies/ml)[a]	Median no. of CD4 cells/μl[a]	Median no. (frequency) of HLA-B*5101-restricted CD8$^+$ T cells[b]				No. of times PBMCs were tested (dates)[c]
			Pol743	Pol283	Gag327	Rev71	
KI-021	50	618	1,910 (0.39)	1,900 (0.40)	<100 (0)	<100 (0)	10 (8/1997–11/2005)
KI-051	50	737	3,222 (0.53)	5,186 (0.87)	1,082 (0.16)	<100 (0)	5 (10/1999–9/2005)
KI-124	570	850	3,126 (0.43)	1,745 (0.24)	1,381 (0.19)	<100 (0)	8/2001
KI-386	360	459	3,164 (0.40)	554 (0.07)	5,774 (0.73)	396 (0.05)	8/2006
KI-363	1,700	676	6,696 (0.54)	1,488 (0.12)	496 (0.04)	1,116 (0.09)	11/1998
KI-127	5,500	597	8,100 (0.79)	257 (0.02)	23,411 (2.33)	<100 (0.01)	9 (2/1998–4/2006)
KI-121	16,650	327	4,853 (0.59)	134 (0.02)	<100 (0)	395 (0.04)	2 (12/1999, 8/2001)
KI-032	25,500	226	9,153 (1.80)	<100 (0)	344 (0.09)	<100 (0)	2 (10/2002, 9/2005)
KI-007	39,500	387	1,084 (0.12)	394 (0.05)	6,278 (0.68)	1,029 (0.12)	2 (6/2001, 4/2002)
KI-026	40,000	526	10,705 (1.32)	<100 (0)	6,164 (0.76)	568 (0.07)	7/2005

[a] At the time of tetramer analysis.
[b] Median number of HLA-B*5101-restricted CD8$^+$ T cells/μl among PBMCs (median frequency of HLA-B*5101-restricted T cells among CD8$^+$ T cells [expressed as a percentage]).
[c] If PBMCs were tested only once, only the date (month/year) is given.

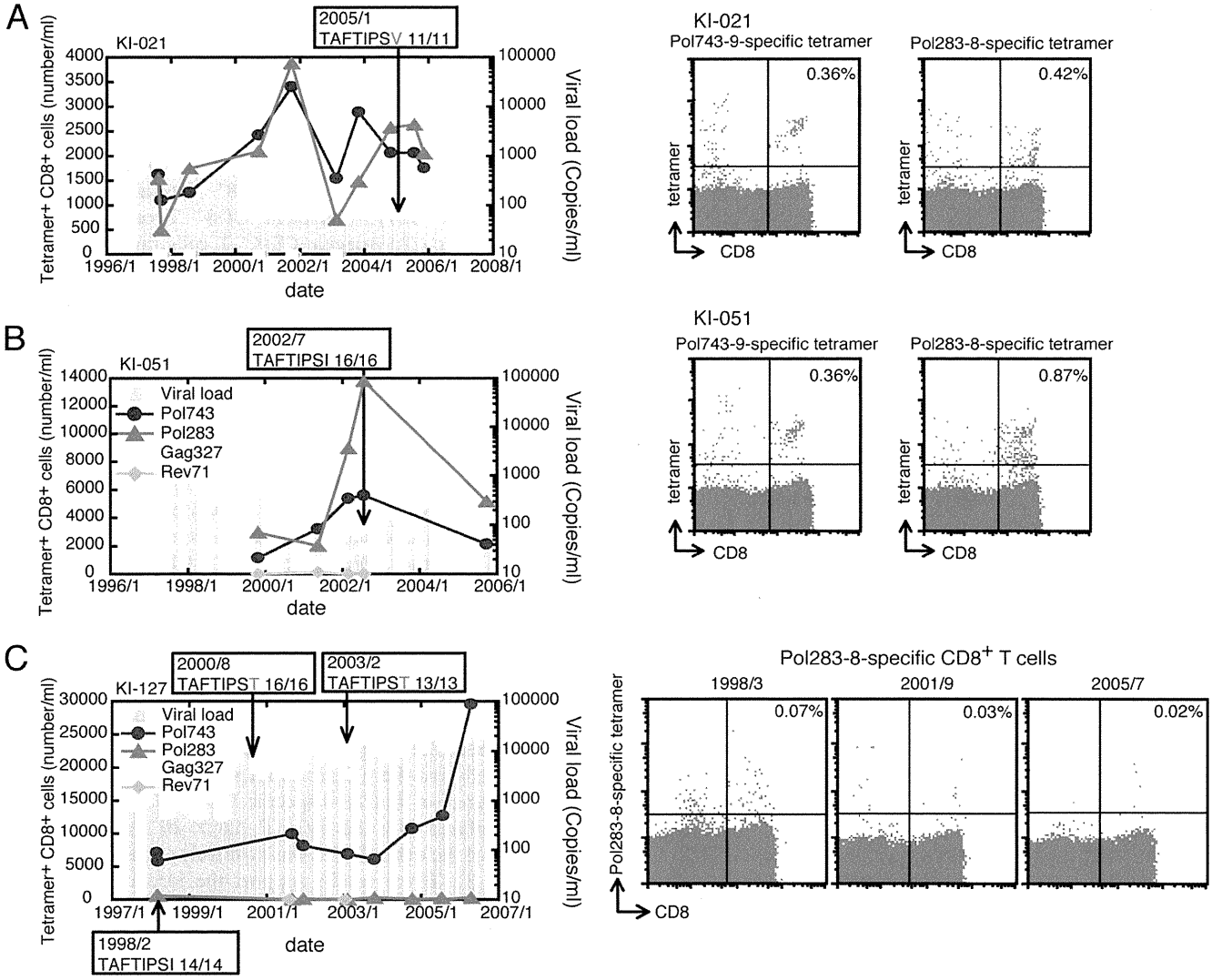

FIG. 2. Longitudinal analysis of HLA-B*5101-restricted $CD8^+$ T cells and Pol283 epitope sequences in 3 slow-progressing hemophiliacs. Four types of HIV-1-specific $CD8^+$ T cells were detected by use of specific tetramers. PBMCs from KI-021 (A), KI-051 (B), and KI-127 (C) were analyzed by using Pol743-9-specific and Pol283-8-specific tetramers. The percentage of tetramer-positive cells among the $CD8^+$ T-cell population is given in the upper right quadrant of each histogram. The sequence of the Pol283-8 epitope from each patient is shown. The detection limit of pVL was 400 copies/ml until 2000 and 50 copies/ml after 2000.

2006 (Table 2). As previously noted (34), Pol283-8-specific CTL clones showed the same killing activity toward target cells prepulsed with the Pol283-8V peptide as toward those prepulsed with the wild-type peptide. These T cells revealed similar killing activity toward 721.221-B*5101 cells infected with NL-432 carrying Pol283-8V (NL-Pol283-8V) as toward those infected with NL-432 (see Fig. S2A in the supplemental material) and only a marginally weaker ability to suppress the replication of NL-Pol283-8V (see Fig. S2B in the supplemental material). In contrast, the 5 VSPs and 2 SPs had Pol283-8T or Pol283-8R mutants (Table 2). Three Pol283-8-specific CTL clones failed to kill target cells infected with NL-432 carrying these mutants (NL-Pol283-8T and NL-Pol283-8R [see Fig. S2A in the supplemental material]) or to suppress the replication of these mutants (see Fig. S2B in the supplemental material), indicating that these were escape mutants.

Longitudinal analysis of KI-127 showed that the 8T mutant appeared in August 2000, when the VL had increased approximately 10-fold, whereas wild-type Pol283 was found in February 1998, when the VL was very low or undetectable (Fig. 2C). Previous population analysis using 9 cohorts showed strong association between HLA-B*51 and Pol283-8T (20). These observations together suggest that the 8T mutant is an escape mutant selected by Pol283-specific CTLs and implies that escape from this epitope reduces immune control of HIV-1.

***In vitro* selection of Pol283 escape mutants by Pol283-specific CTLs.** The results shown in Fig. 4 suggested that Pol283-specific CTLs selected 8T, 8R, and 8L escape mutants. To further confirm the selection of these mutants by Pol283-specific CTLs, we investigated whether Pol283-specific CTLs selected these mutant viruses *in vitro* when the CTLs were cul-

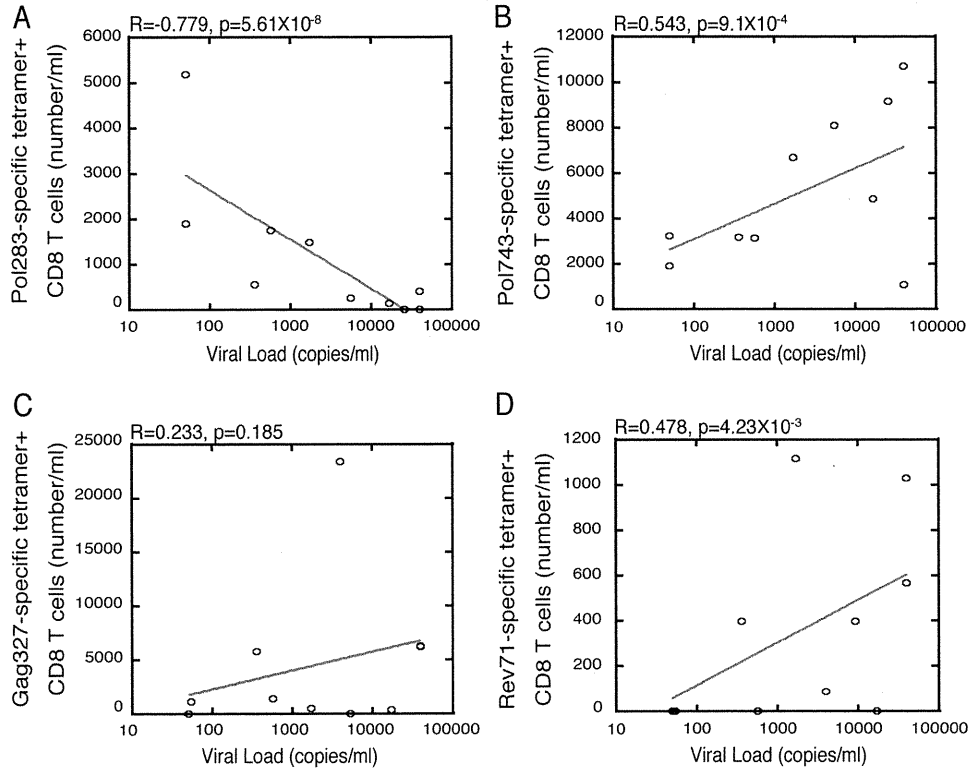

FIG. 3. Correlation of the number of HLA-B*5101-restricted CD8+ T cells with the viral load. The number of Pol283-8-specific (A), Pol743-9-specific (B), Gag327-specific (C), or Rev71-specific (D) CD8+ T cells among PBMCs from 10 HLA-B*5101+ hemophiliacs was measured at 1 time point or at 2 to 10 different time points (see Table 1) by using specific tetramers. The correlation of the median number of tetramer-positive cells with the median viral load was analyzed.

tured with HLA-B*5101-positive CD4+ T cells infected with NL-432 and the mutant virus together. Pol283-specific CTL clones selected these 3 mutant (8T, 8R, and 8L) viruses rapidly in this assay (Fig. 4A to C), supporting the notion that these mutants were selected as escape mutants by Pol283-specific CTLs.

Long-term maintenance of Pol283-8-specific memory CD8+ T cells and failure of induction of escape mutant-specific CD8+ T cells. If the Pol283-8T mutant was selected by Pol283-8-specific CTLs in donors first infected with HIV-1 carrying the Pol283-8 wild-type epitope, we can speculate that the donors had Pol283-8-specific memory CD8+ T cells but failed to elicit

TABLE 2. Sequences of Pol283-8 and Pol743-9 epitopes in HLA-B*5101+ HIV-1-infected hemophiliacs

Patient	Epitope				VL (copies/ml)	Date (mo/yr) of PBMC testing[b]
	Pol283-8		Pol743-9			
	Sequence	Clonal frequency[a]	Sequence	Clonal frequency		
NA[c] (wild-type sequence)	TAFTIPSI		LPPVVAKEI			
KI-021	-------V	11/11	---------	10/12	<50	1/2005
KI-051	---------	16/16	---------	15/15	63	7/2002
	-------V	DS	ND[d]	ND	<50	10/2006
KI-124	-------V	14/14	---------	14/15	600	8/2001
KI-386	-------T	DS	---------	DS	1,200	10/2006
KI-363	-------T	DS	---------	DS	1,700	11/1998
KI-127	-------T	13/13	---------	17/17	5,300	2/2003
KI-121	-------T	16/16	I--------	12/13	9,300	12/1999
KI-032	-------T	13/13	---------	15/15	17,000	10/2002
KI-007	-------R	15/16	---II----	18/18	33,000	6/2001
KI-026	-------T	DS	I--------	DS	28,000	1/2004

[a] Expressed as (number of clones carrying the indicated sequence)/(number of clones tested). DS, direct sequence.
[b] The sequence for patient KI-021 is from proviral DNA; those for all other patients are from plasma RNA.
[c] NA, not applicable.
[d] ND, not determined.