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Enfuvirtide Resistance Mutations: Impact on Human Immunodeficiency Virus Envelope Function, Entry Inhibitor Sensitivity, and Virus Neutralization

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Enfuvirtide (ENF/T-20/Fuzeon), the first human immunodeficiency virus (HIV) entry inhibitor to be licensed, targets a structural intermediate of the entry process. ENF binds the HR1 domain in gp41 after Env has bound CD4, preventing conformational changes needed for membrane fusion. Mutations in HR1 that confer ENF resistance can arise following ENF therapy. ENF resistance mutations were introduced into an R5- and X4-tropic Env to examine their impact on fusion, infection, and sensitivity to different classes of entry inhibitors and neutralizing antibodies. HR1 mutations could reduce infection and fusion efficiency and also delay fusion kinetics, likely accounting for their negative impact on viral fitness. HR1 mutations had minimal effect on virus sensitivity to other classes of entry inhibitors, including those targeting CD4 binding (BMS-806 and a CD4-specific monoclonal antibody [MAb]), coreceptor binding (CXCR4 inhibitor AMD3100 and CCR5 inhibitor TAK-779), or fusion (T-1249), indicating that ENF-resistant viruses can remain sensitive to other entry inhibitors in vivo. Some HR1 mutations conferred increased sensitivity to a subset of neutralizing MAbs that likely target fusion intermediates or with epitopes preferentially exposed following receptor interactions (17b, 48D, 2F5, 4E10, and IgGb12), as well as sera from some HIV-positive individuals. Mechanistically, enhanced neutralization correlated with reduced fusion kinetics, indicating that, in addition to steric constraints, kinetics may also limit virus neutralization by some antibodies. Therefore, escape from ENF comes at a cost to viral fitness and may confer enhanced sensitivity to humoral immunity due to prolonged exposure of epitopes that are not readily accessible in the native Env trimer. Resistance to other entry inhibitors was not observed.

Highly active antiretroviral therapy employs combinations of protease and reverse transcriptase inhibitors to suppress human immunodeficiency virus (HIV) replication. However, viral rebound resulting from the acquisition of mutations in the reverse transcriptase and protease genes is not uncommon, and multidrug-resistant virus strains are responsible for an increasingly large fraction of new infections in North America (18, 24). New antiretroviral drugs that prevent virus entry and that are effective against these drug-resistant viruses are being developed, with one (enfuvirtide [ENF]/Fuzeon/T-20) having been licensed thus far (29). ENF is a subcutaneously injected 36-amino-acid peptide that is based on the amino acid sequence of the HR2 domain in the gp41 subunit of the HIV type 1 (HIV-1) Env protein. ENF binds to the HR1 domain in gp41, which becomes exposed after CD4 binding (16, 19). After coreceptor binding, the HR1 and HR2 domains interact with each other, forming a six-helix bundle in the context of a trimer, bringing the viral and cellular membranes into close proximity, subsequently resulting in membrane fusion and virus entry (13). ENF prevents this interaction, blocking membrane fusion and virus infection (10).

ENF and other classes of entry inhibitors target the highly variable HIV Env protein directly or, as with CD4 and coreceptor inhibitors, indirectly. Env variability likely accounts for the considerable baseline variability in sensitivity of HIV-1 strains to entry inhibitors (20). Factors that influence sensitivity to entry inhibitors include the affinity with which Env binds to its coreceptors as well as coreceptor expression levels (34, 35). Increased coreceptor affinity or expression levels are associated with accelerated fusion kinetics, reducing the period of time during which the ENF-binding site on HR1 is exposed (34, 35). Whether these factors influence clinical outcome is not yet known, but enhanced baseline resistance to ENF could increase the likelihood that full virologic resistance could emerge. Viruses resistant to ENF have been selected for in vitro and have been observed in patients (37, 49; reviewed in references 17 and 28). In these cases, mutations in the highly conserved HR1 domain have been documented (17, 28, 37, 49). With the use of ENF expanding, it is important to define virus resistance mechanisms as well as the consequences of ENF resistance for viral sensitivity to other classes of entry inhibitors and for viral fitness.

To evaluate the impact of ENF resistance mutations on Env function and sensitivity to other entry inhibitors, we introduced mutations into HR1 that have been shown to confer ENF

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resistance both in vitro and in vivo (37, 49). The mutations were introduced into two virus strains, one that uses the CXCR4 coreceptor and one that uses CCR5. We found that these HR1 mutations had minimal effect on viral sensitivity to small molecule or monoclonal antibody (MAb) inhibitors of CD4, CCR5, or CXCR4 binding. Additionally, these HR1 mutations did not confer resistance to T-1249, a peptide fusion inhibitor related to ENF. However, some HR1 mutations did result in reduced infection and fusion efficiency and slower fusion kinetics, prompting us to determine if ENF-resistant mutants were more sensitive to neutralizing MAbs. In fact, viruses with HR1 mutations were more sensitive to a subset of neutralizing MAbs and sera from HIV-positive individuals, including MAbs that bind to epitopes in gp41 and gp120, suggesting that there are kinetic as well as spatial constraints that limit the ability of some antibodies to neutralize HIV. Indeed modulation of CCR5 expression levels, which influences fusion kinetics (34, 35), can alter neutralization efficiency. Thus, high-level ENF resistance associated with changes in the HR1 domain of gp41 is not associated with resistance to other classes of entry inhibitors. Furthermore, resistance to ENF may be influenced in vivo by the humoral immune response.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells (PBMCs) were cultured and depleted of $CD8^+$ T cells as previously described (35). 293T, QT6, U87/CD4/CCR5, and U87/CD4/CXCR4 (5, 12) and HeLa/CD4/CCR5 (RC49) (33) cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DMEM/10). In addition, 0.3 mg of G418/ml plus 1 μg of puromycin/ml was used to maintain CD4 and coreceptor expression in U87/CD4/CCR5 and U87/CD4/CXCR4 cells. CD4/T-REx/CCR5 cells, which stably express CD4 and allow tetracycline-regulated expression of CCR5, were generated by transfecting T-REx/CCR5 cells (34) with a CD4 expression vector carrying a neomycin resistance gene followed by G418 selection of a stable CD4-expressing cell line (50). Cells were maintained in DMEM/10 supplemented with 200 μg of zeocin/ml, 1 mg of G418/ml, and 5 μg of blasticidin/ml to maintain *ccr5*, *cd4*, and *tet* repressor genes, respectively. Variable levels of CCR5 expression were induced by addition of different concentrations (0.1 to 10 ng/ml) of doxycycline to the culture medium. CCR5 expression levels were determined by flow cytometric analysis of cells immunostained with a phycoerythrin-conjugated CCR5-specific antibody (Pharmingen).

Plasmids. LAI and YU-2 Env proteins were cloned into the MluI and PspOM1 sites of a pCI expression construct modified to contain hepatitis B virus PRE to enable high-level, *rev*-independent Env expression (6, 34). G36D, V38M, and G36D/V38M amino acid changes (numbering according to HXB2 gp41 sequence) were introduced into the HR1 region of LAI and YU-2 Env by using specific oligonucleotides and the QuikChange site-directed mutagenesis kit (Stratagene).

Cell-cell fusion assay. QT6 cells, transfected with Env expression plasmids and infected with a T7 polymerase-encoding vaccinia virus (vTF1.1) (1), were added to QT6 cells cotransfected with CD4 and coreceptor expression plasmids and a luciferase reporter construct under the control of a T7 promoter. Cell-cell fusion of Env and receptor-expressing cells was detected by assaying for T7 polymerase-driven luciferase expression. This assay has previously been described in detail (39). The ability of sCD4 to induce fusion of CD4-negative, coreceptor-expressing cells was determined by adding sCD4 to coreceptor-expressing cells immediately prior to addition of Env-expressing cells.

Env fusion kinetics. The fusion kinetics of LAI, YU-2, and HR1 mutant Env proteins were determined in a β-lactamase reporter cell-cell fusion assay as previously described (23, 35). Briefly, QT6 cells cotransfected with Env and β-lactamase expression constructs and infected with vTF1.1 were added to HeLa/CD4/CCR5 cells labeled with CCF2-AM (an acetoxymethylester derivative of CCF2 which contains a donor fluorophore [coumarin] linked to an acceptor [fluorescein] via a β-lactam ring). Cell-cell fusion was detected by assaying for a shift from green to blue fluorescence indicating β-lactamase cleavage of CCF2. Fluorescence was quantitated in a fluorometer, and results were expressed as the ratio of blue/green fluorescence obtained with Env-transfected cells to background blue/green fluorescence obtained with empty-vector-transfected cells.

Virus infection assays. Luciferase reporter pseudotype viruses bearing LAI, YU-2, and HR1 mutant Env proteins were generated by cotransfection of 293T cells with gp160 and pNL-luc-E^- expression constructs as previously described (9, 11). Pseudotypes, normalized for p24 content, were used to infect U87/CD4/CXCR4 or U87/CD4/CCR5 cells, and infection was analyzed by assaying for luciferase expression 3 days postinfection.

LAI, YU-2, and HR1 mutant replication-competent viruses, generated by transfection of proviral constructs into 293T cells, were normalized for p24 content and used to infect PBMCs depleted of $CD8^+$ T cells. Input virus was removed by washing, and culture supernatants were harvested at various times postinfection for p24 analysis of viral replication.

RESULTS

ENF resistance mutations in the HR1 domain of gp41 and their impact on fusion, infection, and replication. Mutations in HR1 that confer ENF resistance can arise following ENF therapy or selection in vitro (17, 28, 37, 49). These mutations, which include G36D, V38M, and the double mutation G36D/V38M, can alter the affinity of ENF for HR1 (37) and are also likely to alter the affinity of HR2 for HR1 and may thus impact fusion efficiency. We introduced these mutations into both X4 (LAI) and R5 (YU-2) Env proteins to examine the impact of these mutations on fusion, infection, and sensitivity to other classes of entry inhibitors. Flow cytometry studies showed that the mutations did not affect Env expression, while Western blot analyses showed that the Env proteins were processed normally (data not shown). LAI mutations reduced fusion and pseudotype virus infection on cell lines by 1.5- to 3-fold, while mutations in YU-2 caused a minimal to at most a 2.5-fold reduction in fusion and pseudotype virus infection efficiency (Fig. 1A and B).

In both cell-cell fusion and pseudotype virus infection assays, the single amino acid mutations increased the amount of ENF required to inhibit fusion activity from 3.5- to 30-fold, while the double G36D/V38M mutation increased ENF resistance by 30- to 360-fold (Fig. 2). These effects are similar to those previously reported (37, 49). Sensitivity differences between cell-cell fusion and infection assays have been observed previously (34), likely due to factors including variation in levels of Env and receptor expression.

To examine the effects of the HR1 mutations on virus infection and replication in primary cells, we introduced the mutations into LAI and YU-2 proviruses (21, 32, 34). Equivalent amounts of each virus were then used to infect PBMCs, depleted of $CD8^+$ T cells, and the amount of viral p24 produced over time was measured. Relative to LAI, infection assessed at day 3 of the G36D mutant was reduced sixfold, that for the V38M mutant was reduced by threefold, and that for the LAI G36D/V38M mutant was reduced by 29-fold (Fig. 1C). In the context of YU-2, the G36D, V38M, and G36D/V38M mutations reduced infection efficiency at day 3 by 1.5-, 2-, and 5.5-fold, respectively (Fig. 1C). Additionally, the mutations generally reduced both the rate and/or extent of virus replication over time in PBMC cultures (Fig. 3). Thus, the HR1 mutations reduced infection efficiency of PBMCs to a greater extent than on cell lines, which may be reflective of differences in receptor expression levels and measurement of the effect of multiple infection cycles. These results are consistent with a

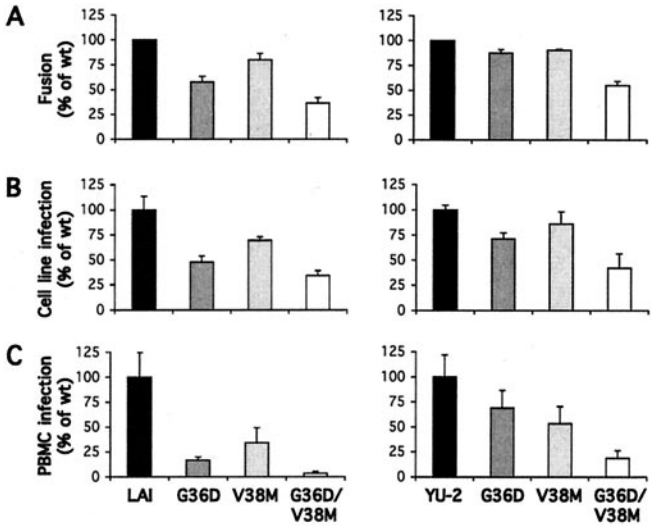

FIG. 1. Fusion and infection of LAI and YU-2 HR1 mutants. Relative fusion and infection levels mediated by HR1 mutant Env proteins are expressed as a percentage of wt Env-mediated fusion or infection. (A) Cell-cell fusion between Env-expressing cells and receptor-expressing cells (CD4/CXCR4 for LAI and CD4/CCR5 for YU-2). Results represent the average + standard error of the mean of at least three independent experiments. (B) Pseudotype virus infection of U87/CD4/CXCR4 (LAI) or U87/CD4/CCR5 (YU-2) cells. Results represent the average + standard deviation of triplicate wells representative of at least three independent experiments. (C) Replication-competent virus infection of PBMCs depleted of $CD8^+$ T cells, evaluated by analyzing culture supernatants for p24 content at 3 days postinfection. Results are from a representative experiment and correspond to average p24 values + standard deviation from infections performed in triplicate with virus generated from two independent clones of each provirus.

recent study showing that these mutations reduce viral fitness in vitro (25).

Mutations in gp120 or gp41 or truncations of the cytoplasmic tail of gp41 can affect the sensitivity of Env to CD4-induced structural rearrangements that result in membrane fusion (14, 35, 36). We examined the ability of a soluble form of CD4

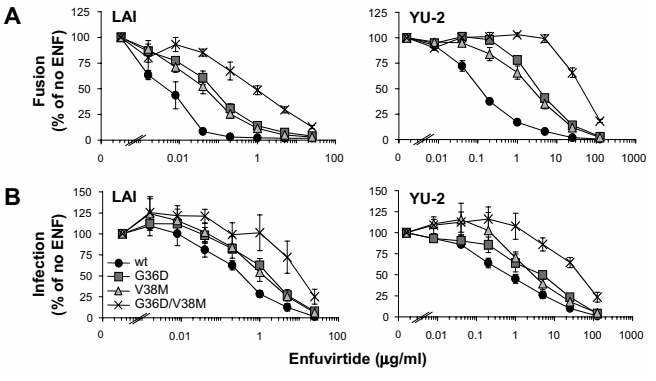

FIG. 2. ENF sensitivity of LAI and YU-2 HR1 mutants. ENF sensitivity of HR1 mutant Env proteins was determined in cell-cell fusion (A) and pseudotype virus infection inhibition (B) assays. Results are expressed as a percentage of wt Env-mediated fusion or infection in the absence of ENF and represent the average ± standard error of the mean of at least three independent experiments.

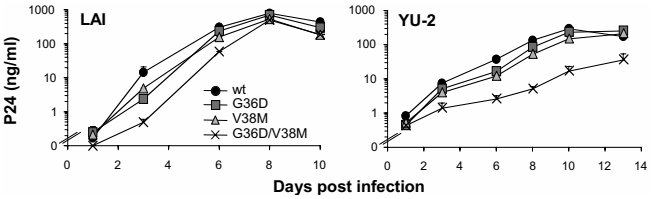

FIG. 3. Replication of LAI and YU-2 mutant viruses. Shown are growth curves of LAI and YU-2 viruses in PBMCs depleted of $CD8^+$ T cells. Input virus was removed by washing, and viral replication was evaluated by analyzing culture supernatants for p24 content at indicated days postinfection. Results are from a representative experiment and correspond to average p24 values + standard deviation from infections with triplicate wells of virus generated from two independent clones of each provirus.

(sCD4) to trigger fusion of cells expressing wild-type (wt) and HR1 mutant Env proteins with luciferase reporter $CD4^-$/$CXCR4^+$ (LAI) or $CD4^-$/$CCR5^+$ (YU-2) target cells compared to respective cellular $CD4^+$ target cells. Compared to wt Env proteins, V38M mutants were less susceptible to sCD4-induced fusion (Fig. 4). sCD4 triggered wt levels of fusion in the context of the YU-2 G36D/V38M mutant and either wt-level fusion (low concentrations) or reduced fusion (high concentrations) by LAI G36D/V38M (Fig. 4). Surprisingly, the G36D mutants were especially responsive to low concentrations of sCD4, being triggered up to 13-fold more efficiently than wt Env proteins (Fig. 4). Thus, a G36D mutation in the HR1 region of LAI and YU-2 lowers the threshold of sCD4 required to induce membrane fusion. This may indicate that certain ENF resistance mutations in HR1 may reduce the stability of gp41, resulting in an Env that becomes triggered to undergo structural rearrangements, resulting in membrane fusion, more readily following CD4 binding. This reduced threshold for sCD4-induced fusion did not correlate with an enhancement in the kinetics of fusion (see below). Thus, G36D is

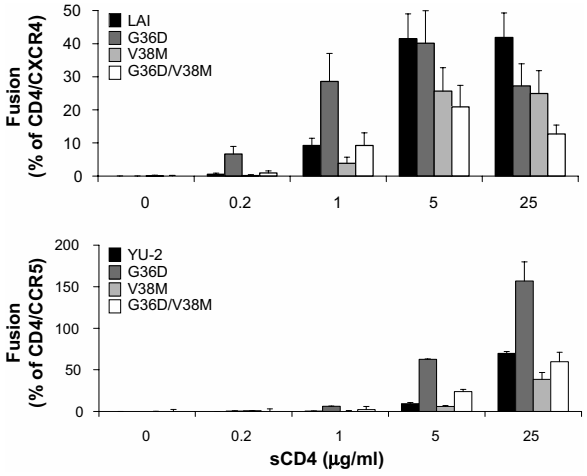

FIG. 4. sCD4-induced fusion of HR1 mutant Env proteins. The ability of sCD4 to trigger fusion between Env-expressing cells and CD4-negative coreceptor-expressing cells (CXCR4 for LAI and CCR5 for YU-2) is expressed as a percentage of fusion on $CD4^+$/coreceptor$^+$ cells (CD4/CXCR4 for LAI and CD4/CCR5 for YU-2). Results represent the average + standard error of the mean of three independent experiments, each performed in triplicate.

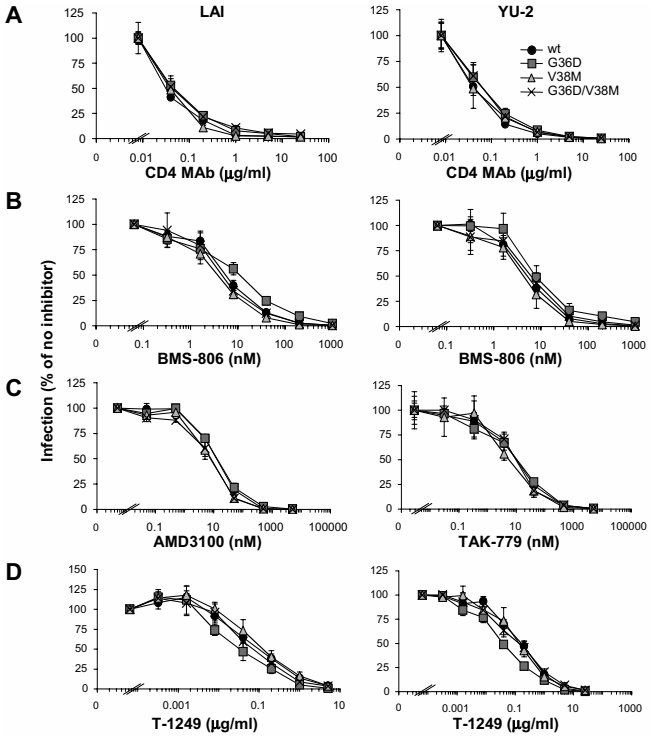

FIG. 5. Entry inhibitor sensitivities of HR1 mutant viruses. Shown are CD4 MAb19 (A), BMS-806 (B), AMD3100 and TAK-779 (C), and T-1249 (D) sensitivities of HR1 mutant pseudotype. Results are expressed as a percentage of infection in the absence of inhibitor and represent the average ± standard error of the mean of at least three independent experiments.

likely to remain in a CD4-triggered state for a longer period of time, which may result in enhanced sensitivity to inhibitors that bind to structural intermediates of the fusion process (supported by results of neutralization assays; see below).

Impact of ENF resistance mutations on sensitivity to different classes of entry inhibitors. With the use of ENF increasing, the emergence of drug-resistant viruses is of concern. Since ENF resistance mutations in HR1 can affect virus entry (Fig. 1), it is important to determine if sensitivities to other classes of entry inhibitors that are under clinical development are also affected. To address this, we infected cells with virus pseudotypes bearing either wt or ENF-resistant Env proteins in the presence of inhibitors that prevent CD4 binding, coreceptor binding, or fusion. We observed less-than-twofold differences in the sensitivities of LAI and YU-2 Env proteins to a MAb specific to domain 1 of CD4 (MAb 19), with similar 50% inhibitory concentrations (IC_{50}s) for the X4- and R5-tropic Env proteins (Fig. 5A). Likewise, the HR1 mutations had little effect on virus sensitivity to BMS-806, a small molecule inhibitor that binds to Env and prevents a functional CD4 interaction (22, 43) (Fig. 5B). Of note, LAI G36D exhibited the biggest differential sensitivity to BMS-806 from a wt Env, with a twofold-reduced sensitivity which may be reflective of the fact that this Env was more easily triggered to fuse by sCD4. Thus, these mutations in HR1 that confer increased ENF resistance have little or no effect on sensitivity to inhibitors targeting CD4 interactions.

We also assessed the impact of the HR1 mutations on sensitivity to the CXCR4-specific small molecule inhibitor AMD3100 (42) in the context of LAI and to the CCR5-specific small molecule inhibitor TAK-779 (2) in the context of YU-2. We observed less than a twofold difference in AMD3100 IC_{50}s between wt and ENF-resistant LAI viruses (Fig. 5C). Likewise, we observed less than a twofold difference in sensitivity to TAK-779 between YU-2 and YU-2 mutant viruses (Fig. 5C). Therefore, these mutations in HR1 that have been observed in patients who have failed ENF therapy have little or no impact on virus sensitivity to coreceptor inhibitors.

Finally, we examined the impact of the HR1 mutations on virus sensitivity to the fusion inhibitor T-1249. Like ENF, T-1249 is a peptide based on the amino acid sequence of HR2, but it inhibits fusion more potently than ENF and is active against many ENF-resistant viruses (15, 35). The V38M and G36D/V38M mutations had very little impact on the T-1249 sensitivity of LAI or YU-2 viruses, while G36D increased the T-1249 sensitivity of LAI and YU-2 approximately 3- and 4.5-fold, respectively (Fig. 5D). This may be reflective of enhanced CD4 triggering but slower fusion kinetics (see below) of the G36D mutants and would be supportive of G36D mutants remaining in structural intermediate conformations for a longer time. Thus, any potential reduced binding of T-1249 to a G36D mutant HR1 sequence may be compensated for by the slower fusion kinetics of G36D mutants, resulting in enhanced T-1249 sensitivity. Therefore, viruses that acquire significant resistance to ENF by these mutations in HR1 remain fully sensitive to the more potent fusion inhibitor T-1249.

Sensitivity of HR1 mutants to neutralizing antibodies. Changes in the HR1 domain, which plays a critical role in the entry process, impact entry efficiency. While we found little impact of HR1 mutations on sensitivity to other entry inhibitors, including those targeting the viral receptors, reduced entry efficiency has the potential to impact virus sensitivity to inhibitors directed against recessed or induced epitopes in Env, such as neutralizing antibodies. To examine the impact of HR1 mutations on neutralization sensitivity, we performed virus neutralization assays using both luciferase reporter and replication-competent viruses on U87 cell lines and human PBMCs.

We first tested MAbs 4E10 and 2F5, which bind to epitopes in gp41 between HR2 and the membrane-spanning region (31, 51). 4E10 neutralized wt LAI pseudotype virus with an IC_{50} of approximately 19 μg/ml (Fig. 6A). The LAI G36D and G36D/V38M mutant pseudotype viruses, which were 5.3- and 30-fold more resistant to ENF, were approximately 6- and 4.5-fold more sensitive to neutralization by 4E10, while the V38M mutant, which was 3.5-fold more resistant to ENF, was 1.6-fold more sensitive to neutralization. Similar results were obtained with MAb 2F5 (Fig. 6B). The same relative order of neutralization sensitivity was observed for 4E10 and 2F5 inhibition of replication-competent LAI and LAI HR1 mutant infection on PBMCs (data not shown). The YU-2 HR1 mutants were also more sensitive to neutralization by 4E10 and 2F5, with both G36D and V38M (4.5- and 7.5-fold more resistant to ENF) being two- to threefold more sensitive to neutralization by 4E10 and 2F5, and G36D/V38M (65-fold more resistant to ENF) being between five- and eightfold more sensitive to neutralization by these antibodies (Fig. 6A and B). Thus, the HR1 mutations studied here confer significant resistance to ENF

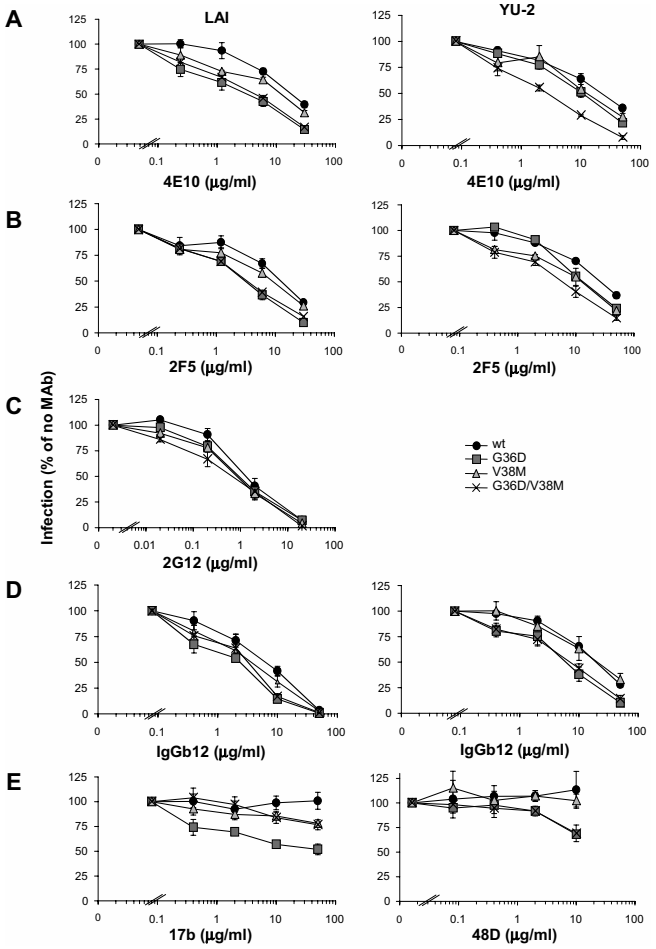

FIG. 6. Neutralizing antibody sensitivity of HR1 mutant viruses. Shown is neutralization of HR1 mutant pseudotype virus infection by 4E10 (A), 2F5 (B), 2G12 (C), IgGb12 (D), and 17b and 48D (E). Results are expressed as a percentage of Env-mediated infection in the absence of MAb and represent the average ± standard error of the mean of at least three independent experiments.

but enhance sensitivity to neutralization by MAbs directed against gp41 that target fusion intermediates or that bind to epitopes that are better exposed following receptor binding (4, 40, 48).

We also examined the impact of HR1 mutations on sensitivity to neutralization by gp120-specific MAbs that act to block infection by different mechanisms. 2G12 binds to a surface-exposed carbohydrate epitope in gp120 and is thought to block infection by interfering with viral attachment to cell surface receptors (47). None of the mutations had a significant effect on 2G12 neutralization of LAI (Fig. 6C). 2G12 was unable to neutralize YU-2 infection at concentrations up to 25 μg/ml (data not shown). A second broadly cross-reactive antibody directed against gp120, IgGb12, binds to a conformational epitope and blocks CD4 binding (8). IgGb12 binds monomeric gp120 better than trimeric Env (38), indicating that its epitope is likely less accessible on virions, where it is predicted to be somewhat recessed between gp120 subunits. The LAI HR1 mutants were slightly more sensitive to IgGb12 neutralization than was wt LAI, with G36D, V38M, and G36D/V38M exhibiting 2.9-, 1.7-, and 2-fold-reduced IC_{50}s (Fig. 6D). YU-2 and YU-2 V38M exhibited similar neutralization by IgGb12, while YU-2 G36D and G36D/V38M were approximately threefold more sensitive to IgGb12 neutralization (Fig. 6D). The fact that these HR1 mutations had more of an impact on IgGb12 compared to BMS-806 sensitivity may indicate that the small molecule inhibitor is less encumbered by structural constraints.

More notable differences were observed between wt and HR1 mutant pseudotype virus sensitivities to MAb 17b, which recognizes a CD4-induced epitope ($CD4^i$) (44). LAI infection was not neutralized by up to 50 μg of 17b/ml, whereas 50 μg of 17b/ml neutralized approximately 25% of V38M and G36D/V38M and almost 50% of G36D infection (Fig. 6E). 17b did not neutralize YU-2 infection (data not shown). YU-2 and YU-2 V38M infection was not neutralized by up to 10 μg of MAb 48D/ml, which also has a $CD4^i$ epitope (30, 44); however, 10 μg of 48D/ml neutralized approximately 35% of G36D and G36D/V38M infection (Fig. 6E).

In summary, HR1 mutations that confer relative ENF resistance can result in enhanced sensitivity to a subset of gp120- as well as gp41-specific MAbs that target fusion intermediates or whose epitopes are induced or exposed better following receptor binding, with fold enhancement of neutralization sensitivity from wt Env proteins sometimes exceeding fold resistance to ENF. Mutations that conferred the greatest reduction in relative fusion and infection efficiency and that exhibited enhanced sensitivity to sCD4 triggering, but reduced fusion rates (see below), conferred maximum increases in neutralization sensitivity.

Mechanism of enhanced neutralization sensitivity. How can mutations in HR1 enhance the ability of MAbs that bind between HR2 and the membrane-spanning region, or in gp120, to neutralize HIV? It is possible that the HR1 mutations increased exposure of the 2F5 and 4E10 epitopes in the native Env trimer or after Env binds to CD4 and perhaps coreceptor. To address the first possibility, we performed flow cytometry analyses using these antibodies on cells expressing wt or HR1 mutant Env proteins. No significant differences in antibody binding were observed (data not shown). An alternative hypothesis was that the HR1 mutations led to slower fusion kinetics, which could enhance antibody binding to structural intermediates of the fusion process and which could explain the increase in sensitivity of HR1 mutants to gp41 as well as $CD4^i$ MAbs. To examine this possibility, cells expressing CD4 and coreceptor were loaded with the fluorescent dye CCF2-AM, after which they were mixed with cells expressing Env proteins and β-lactamase. Upon cell-cell fusion, β-lactamase is introduced into the cytoplasm of the target cell, where it cleaves CCF2, resulting in a change in emission wavelength that can be measured using a fluorometer. Using this assay, we found that the HR1 mutations resulted in slower membrane fusion kinetics as judged either by initial fusion rates or by the time required to reach half-maximal fusion (Fig. 7; Table 1). The G36D and G36D/V38M mutants exhibited the lowest initial rates of fusion and were the most sensitive to MAb neutralization. Thus, reduced fusion kinetics correlated with enhanced sensitivity to neutralization by gp41-specific MAbs as well as by MAbs to $CD4^i$ epitopes and IgGb12.

If mutations in HR1 enhance sensitivity to neutralization by prolonging fusion kinetics, it should be possible to modulate

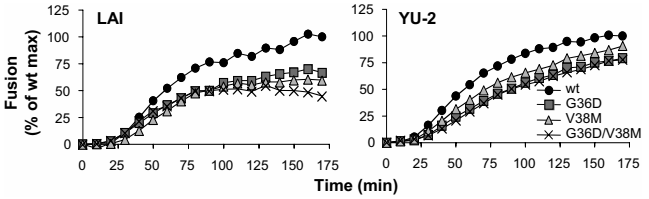

FIG. 7. Kinetics of HR1 mutant fusion. Shown are kinetics of HR1 mutant Env-mediated cell-cell fusion determined in a β-lactamase reporter assay. Fusion is expressed as a percentage of maximal wt Env-mediated fusion, and points represent the average of three experiments. Analyses of kinetic parameters are detailed in Table 1.

the neutralization sensitivity of wt virus by varying conditions in a way that alters membrane fusion rates, such as altering coreceptor expression levels (34). We therefore infected a $CD4^+$ cell line in which CCR5 expression can be modulated by an inducible promoter and found that at basal CCR5 expression levels the IC_{50} for neutralization of YU-2 by MAb 4E10 was 4.1 μg/ml, with progressively higher IC_{50}s of 11, 31, and 40 μg/ml needed to block infection on cells induced to express low, medium, or high levels of CCR5 (Fig. 8). Therefore, increasing CCR5 expression levels, which increases fusion rates (34), made HIV-1 YU-2 more resistant to neutralization by MAb 4E10. Therefore, we conclude that neutralization of HIV-1 by some antibodies can be affected by rates of membrane fusion and that, if fusion rates are prolonged either by reducing coreceptor expression levels or by mutations in Env, neutralization sensitivity is enhanced.

Sensitivity of HR1 mutants to sera from HIV-1-infected individuals. Since ENF resistance mutations in HR1 can confer enhanced sensitivity to neutralization by a subset of human-derived MAbs, it is possible that HR1 mutations that arise following ENF therapy in vivo may confer enhanced sensitivity to the humoral immune response. To address this, we compared LAI and LAI HR1 mutant sensitivities to neutralization by a panel of sera from HIV^+ individuals. Two sera potently neutralized LAI and HR1 mutants with similar efficiencies (serum 45 is shown as an example in Fig. 9). Four other sera exhibited limited neutralization activity against wt LAI and were unable to neutralize 50% of infection at a minimal dilution of 1 in 50. However, G36D/V38M and/or G36D mutants

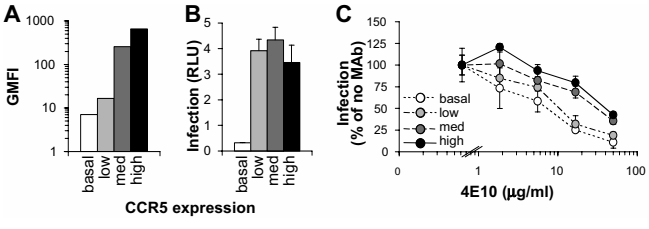

FIG. 8. Impact of CCR5 expression levels on YU-2 neutralization by MAb 4E10. Shown is 4E10 neutralization of YU-2 infection on $CD4^+$ cells induced to express different levels of CCR5. (A) CCR5 expression quantitated by flow cytometry as geometric mean fluorescence intensity (GMFI). (B) Pseudotype virus infection of cells induced to express different CCR5 levels, assessed as relative light units (RLU). (C) 4E10 neutralization expressed as a percentage of infection in the absence of MAb. Points represent the average ± standard deviation of triplicate wells from a representative experiment.

TABLE 1. Kinetic parameters of HR1 mutant cell-cell fusion[a]

Envelope	Y_{max} (% wt)[b]	IR (RFU/min)[c]	$t_{1/2}$ (min)[d]	b[e]
LAI wt	100.0	0.052 ± 0.010	61 ± 12	16.1 ± 4.8
LAI G36D	75.0 ± 8.4	0.037 ± 0.008	75 ± 19	24.6 ± 9.6
LAI V38M	69.4 ± 23.4	0.040 ± 0.010	70 ± 16	18.7 ± 6.9
LAI G36D/V38M	55.7 ± 20.8	0.031 ± 0.004	48 ± 1	12.6 ± 3.3
YU-2 wt	100.0	0.121 ± 0.034	64 ± 10	21.5 ± 4.4
YU-2 G36D	79.7 ± 9.2	0.078 ± 0.029	82 ± 14	25.8 ± 4.4
YU-2 V38M	93.0 ± 11.9	0.097 ± 0.029	80 ± 16	27.1 ± 5.7
YU-2 G36D/V38M	87.9 ± 8.8	0.079 ± 0.012	88 ± 8	32.1 ± 1.8

[a] Kinetic parameters of HR1 mutant Env-mediated cell-cell fusion were determined in a β-lactamase reporter assay (Fig 5). Fusion was assayed from 0 to 170 min in three independent experiments, and data were fitted to the equation $Y = Y_{max}/\{1 + \exp[-(t - t_{1/2})/b]\}$. The coefficients extracted from these curves ± standard errors of the means are presented.
[b] Fusion expressed as percentage of maximum fusion of wt Env.
[c] IR, initial rate of fusion (relative fluorescence units [RFU] per minute).
[d] Time to half-maximal fusion (minutes).
[e] Exponential rate constant.

were more sensitive to neutralization. For example, serum 7 blocked 50% of G36D and G36D/V38M infection at dilutions of approximately 1 in 210 and 1 in 79, respectively, representing >4.2- and >1.6-fold-enhanced neutralization sensitivity compared to wt LAI and V38M (Fig. 9). Thus, acquisition of HR1 mutations during ENF therapy that delay fusion kinetics may result in viruses with enhanced sensitivity to the immune response in vivo.

DISCUSSION

The entry of HIV into cells can be blocked by ENF, which binds to HR1 and prevents subsequent HR2 interactions that are needed for membrane fusion (10). In addition to being the first in the new class of antiretroviral drugs collectively referred to as entry inhibitors, ENF is unusual in that it targets a structural intermediate of the viral entry process (29). The ENF binding site is not exposed in the native Env trimer but rather becomes accessible as a consequence of receptor binding (16, 19). The transient exposure of the ENF binding site during the entry process makes possible a variety of mechanisms by which HIV could potentially acquire resistance to this novel drug. With ENF being administered to a growing number of patients, the mechanisms by which HIV can become resistant to ENF and the implications of ENF resistance for sensitivity to other classes of entry inhibitors, neutralizing antibodies, and viral fitness assume greater importance.

There are at least two general mechanisms by which changes

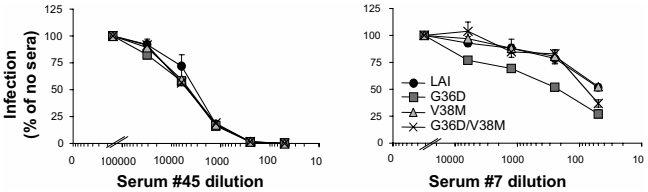

FIG. 9. Serum neutralization of LAI HR1 mutants. Shown is neutralization of LAI HR1 mutant pseudotype virus infection by sera from HIV-1-infected individuals. Results are expressed as a percentage of infection in the absence of sera and represent the average ± standard error of the mean of three independent experiments.

in Env could affect ENF sensitivity. Perhaps the most obvious is the classic mechanism in which changes in the drug binding site confer resistance and a selective growth advantage in the face of therapy. While limited work has been done thus far, it is known that selection for highly drug-resistant viruses in vitro can be associated with single amino or double acid changes in the ENF binding site in HR1 (37). Importantly, some of these changes have also been observed in patients who have experienced viral rebound while on ENF therapy (17, 28, 49), either alone or in combination with other antiretroviral drugs, and it is these mutations that we examined in this study. While changes in HR1 can directly affect ENF binding (10), they are also likely to impact binding of the viral HR2 region. If so, reduced affinity between HR1 and HR2 might be expected to negatively impact membrane fusion activity. Two of the HR1 changes that we examined in this study have recently been shown to reduce virus fitness in direct competition with wt virus when grown on cell lines in vitro (25). In this study, we find that changes in HR1, without compensatory mutations in HR2, make fusion less efficient and delay fusion kinetics, perhaps accounting for their negative impact on virus fitness in vitro.

A second general mechanism by which sensitivity to ENF can be altered is through changes in membrane fusion kinetics. Since ENF targets a structural intermediate of the membrane fusion process, altering the time during which the ENF binding site is exposed would logically be expected to modulate virus sensitivity to this entry inhibitor. All other things being equal, we have found that alterations in fusion kinetics can have a significant impact on ENF sensitivity (34). Fusion kinetics, in turn, can be increased by higher coreceptor expression levels and by higher-affinity interactions between Env and coreceptor (34, 35). Both of these serve to accelerate what appears to be the rate-limiting step in HIV entry—coreceptor binding—and minimize the period of time during which the ENF-binding site in HR1 is exposed. Our work here identifies changes in the HR1-HR2 binding site as being yet another factor that can affect the rate of HIV Env-induced membrane fusion to a significant degree.

An important finding in our study is that resistance to ENF resulting from changes in HR1 has little or no effect on virus sensitivity to a range of other entry inhibitors, including the fusion inhibitor T-1249, a peptide more potent than ENF that binds to a site on HR1 that overlaps that of ENF and which can block ENF-resistant viruses in vitro and in vivo (15, 35). The fact that Env proteins containing ENF resistance mutations remain sensitive to different classes of entry inhibitors is encouraging, since there is a good theoretical basis for employing entry inhibitors in combination (34). For example, coreceptor inhibitors reduce the rate of membrane fusion by reducing coreceptor availability, which in turn results in prolonged exposure of the ENF-binding site. The interplay between receptor binding and the exposure (from CD4 binding) and ultimate loss (after coreceptor binding) of the ENF binding site likely accounts for the ability of entry inhibitors to synergistically inhibit HIV infection in vitro (45, 46). Since we have found that resistance to ENF need not impact sensitivity to other entry inhibitors, the rationale for combination entry inhibitor therapy is strengthened.

Our results also help explain why mutations in HR1 that increase ENF resistance may be selected against, in vitro and in vivo, in the absence of ENF. The HR1 mutations examined here decreased membrane fusion activity as well as viral infectivity by various amounts depending on the mutation and whether cell lines or human PBMCs were used. Further, the HR1 mutations delayed membrane fusion kinetics by an appreciable amount. It is not clear if the slower fusion kinetics resulting from the HR1 mutations were the cause or the result of reduced membrane fusion activity. In the context of virus, slower membrane fusion kinetics could increase the probability that virus will be internalized and delivered to lysosomes prior to membrane fusion, resulting in less efficient virus infection (41). Alternatively, the change in free energy associated with the formation of the six-helix bundle is thought to provide the motive force needed to elicit membrane fusion (26), and a reduction in the strength of HR1-HR2 interactions could increase the likelihood that receptor-induced conformational changes fail to elicit membrane fusion. Given the effects of HR1 mutations on virus-membrane fusion and virus fitness (25), it will be important to determine if prolonged ENF therapy results not just in drug resistance but also in the selection of compensatory mutations that restore virus fitness. Interestingly, a virus dependent upon ENF for entry has recently been described (3).

Another, significant consequence of the delayed fusion kinetics resulting from HR1 mutations was that HIV became more sensitive to neutralization by some broadly cross-reactive MAbs as well as antibodies in the sera of some HIV-infected individuals. The Env protein of HIV is well adapted to function in the face of a robust and ever-changing humoral immune response (7). Conserved neutralizing epitopes may be inaccessible, sterically constrained, or shielded by carbohydrate and variable regions of the gp120 subunit (7). We found that the HR1 mutations enhanced sensitivity of virus to 2F5 and 4E10, two broadly cross-reactive neutralizing antibodies that bind to the base of the gp41 ectodomain, without obviously affecting their binding to native Env trimers. Thus, we favor the idea that the epitopes to which these antibodies bind either are more exposed as a consequence of receptor binding or become more accessible as a result of receptor-induced conformational changes (4, 40, 48). By lowering the rate of these conformational changes, antibody binding may be enhanced. Thus, in addition to spatial constraints that limit antibody binding to the native Env trimer, there are likely to be kinetic constraints as well. This clearly does not apply to all antibodies, as the sensitivity of virus to neutralization by 2G12, a MAb that binds to a surface-accessible epitope on gp120, was unaffected by the HR1 mutations. In contrast, MAbs to the coreceptor binding site that normally neutralize HIV weakly or not at all, due to the inaccessibility of their epitopes in the native trimer, exhibited enhanced neutralizing activity when certain mutations that reduced infectivity and delayed fusion kinetics were introduced into HR1. This was especially apparent with the G36D mutation, which fused at lower CD4 concentrations but exhibited the slowest fusion kinetics and may thus expose $CD4^i$ epitopes for the longest time. Thus, Env proteins that remain in a CD4-triggered state for a longer period of time due to reduced levels of coreceptor, reduced Env affinity for coreceptor, or mutations in the HR1-HR2 binding region that slow fusion kinetics may present epitopes that are present on structural

intermediates of the fusion process or that are sterically constrained in the native trimer for a longer period of time, enhancing antibody binding and virus neutralization as a result.

T-1249, like ENF, targets a structural intermediate of the fusion process. Therefore, T-1249 might be expected to exhibit more potent inhibitory activity against mutant viruses that fuse more slowly, although fusion kinetics has less of an impact on T-1249 than on ENF sensitivity, which may be due to the enhanced potency of T-1249 (35). On the other hand, ENF-resistant mutations in the HR1 binding site might be expected to confer some degree of cross-resistance to T-1249. Therefore, it is possible that the minimal impact of ENF resistance mutations on T-1249 sensitivity might reflect an interplay between increased exposure of the T-1249 binding site and reduced T-1249 binding efficiency due to some cross-resistance to ENF resistance mutations.

Resistance to antiretroviral drugs is typically associated with a reduction in virus fitness, though compensatory mutations may restore virus fitness to wt levels (27). Our work shows that resistance to ENF, and perhaps to other classes of entry inhibitors, may affect virus fitness in a way that is not readily apparent by standard in vitro replication assays. By prolonging the kinetic window during which structural intermediates of the membrane fusion process exist, novel epitopes or epitopes that are not readily accessible in the native Env trimer may become better exposed. Can this be exploited during antiretroviral therapy so as to limit the emergence of drug-resistant viruses? Some of the viruses studied here were more sensitive to neutralization by antibodies present in the sera of some but not all HIV-infected individuals tested. It is not known how commonly neutralizing antibodies such as 2F5, 4E10, and 17b are elicited in HIV-infected individuals. However, if immunization strategies can be identified that elicit such antibodies, then a mutually beneficial relationship between the humoral immune response and entry inhibitor therapy may arise. By slowing virus entry either by reducing available receptor levels or by selecting for mutations that prolong the entry process, entry inhibitors may make virus more sensitive to neutralization and may even increase the possibility that neutralizing antibodies may be elicited. The humoral immune response, in turn, may provide additional selective pressure against the emergence of virus strains that are resistant to ENF and perhaps other types of entry inhibitors as well. However, HIV can mutate to escape neutralizing antibody pressure as well as antiretroviral therapy, but escape may come at a cost to viral fitness.

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