Emergence of Resistant Human Immunodeficiency Virus Type 1 in Patients Receiving Fusion Inhibitor (T-20) Monotherapy

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The synthetic peptide T-20 (enfuvirtide) represents the first of a new class of antiretroviral compounds to demonstrate in vivo potency by targeting a step in viral entry. T-20 inhibits a conformational change in the human immunodeficiency virus type 1 (HIV-1) transmembrane glycoprotein (gp41) that is required for fusion between HIV-1 and target cell membranes. The initial phase I clinical trial of T-20 treatment for HIV-infected patients thus provided a unique opportunity to evaluate the emergence of resistant virus in vivo to this novel class of antiretroviral agents. All four patients who received an intermediate dose of T-20 (30 mg twice daily) had an initial decline in plasma viral load over the first 10 days but a rising trend by day 14, suggestive of selection for resistant virus. Plasma virus derived from patients enrolled in all dosage groups of the phase I T-20 trial was analyzed by population sequencing before and after treatment. While no mutations were found within a highly conserved 3-amino-acid sequence (GIV) known to be critical for fusion at baseline, after 14 days of therapy, virus from one patient in the 30-mg dose group (30-1) developed a mutation in this motif, specifically an aspartic acid (D) substitution for glycine (G) at position 36. Multiple env clones were derived from the plasma virus of all four patients in the 30-mg dosage group. Sequence analysis of 49 clones derived from the plasma of patient 30-1 on day 14 revealed that 25 clones contained the G36D mutation, while 8 contained the V38A mutation. Dual mutations involving G36D and other residues within the HR1 domain were also identified. In 5 of the 49 env clones, other mutations involving residues 32 (Q32R or Q32H) and 39 (Q39R) were found in combination with G36D. Cloned env sequences derived from the plasma virus of subject 30-3 also had single mutations in the GIV sequence (V38M and I37V) detectable following therapy with T-20. The plasma virus from subjects 30-2 and 30-4 did not contain changes within the GIV sequence. To analyze the biological resistance properties of these mutations, we developed a novel single-cycle HIV-1 entry assay using JC53BL cells which express β-galactosidase and luciferase under control of the HIV-1 long terminal repeat. Full-length env clones were derived from the plasma virus of patients 30-1 and 30-3 and used to generate pseudotyped virus stocks. The mean 50% inhibition concentrations (IC₅₀s) for mutants G36D and V38A (patient 30-1) were 2.3 μg/ml and 11.2 μg/ml, respectively, statistically significant increases of 9.1- and 45-fold, respectively, compared with those of wild-type Env. The IC₅₀ for the V38 M mutation (patient 30-3) was 7.6 μg/ml, an 8-fold increase compared with that of the wild type. The I37V mutation resulted in an IC₅₀ 3.2-fold greater than that of the wild type. Ears with double mutations (Q32R plus G36D and Q32H plus G36D) exhibited a level of resistance similar to that of G36D alone. These findings provide the first evidence for the rapid emergence of clinical resistance to a novel class of HIV-1 entry inhibitors and may be relevant to future treatment strategies involving these agents.

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein complex controls key processes of viral entry. This complex determines viral tropism and mediates membrane fusion and thus enables virus entry and infection of the host cell. The HIV-1 envelope protein is initially synthesized as a 160-kDa polyprotein precursor (gp160) that is extensively glycosylated. Proteolytic cleavage of gp160 produces the gp120 surface subunit and the gp41 transmembrane subunit, which form oligomers and associate with each other through noncovalent interactions on the surface of the virion. On the surface of target cells, the gp120 surface subunit binds to its receptor and coreceptors and the gp41 transmembrane subunit mediates the fusion of the viral and cellular membranes (for a review, see reference 8). Subsequent to receptor binding, conformational changes occur in gp41 that lead to membrane fusion (7, 13, 18, 19, 21). To attain a fusion-active conformation, specific regions of the gp41 ectodomain must interact. The ectodomain contains a hydrophobic fusion peptide (FP) sequence at the amino terminus, followed by two leucine zipper-like motifs (heptad repeat 1 [HR1] and HR2). HR1 and HR2 consist of a 4,3 hydrophobic repeat that is predictive of an alpha-helical secondary structure and characteristic of coiled coils. Ultimately, through coiled coil interactions a trimer of antiparallel dimers of HR1 and HR2 is predicted to form (12). The formation of this six-stranded helical bundle induces a hairpin structure that brings the viral and cell membranes into proximity for fusion (2, 23).

Previous studies reported that synthetic peptides based on the HR2 sequence effectively block HIV-induced membrane...
fusión y porción celular (9, 12, 24–26). T-20, previamente conocida como DP178 y ahora como enfuvitrino, es una 36-
ácido aminolítico sintético homólogo a la última 36 amino ácidos del HR2 (3, 24). Mediante la bondanza de HR1, T-20
bloquea la formación del dominio natural necesario para fusión (3, 26). Estudios in vitro han mostrado que T-20 inhibe células
HF-1 infectadas y virus-mediadas célula-célula fusión (24, 26).

En los estudios in vitro se demostró que T-20 inhibe células HF-1 y virus-mediados célula-célula fusión (24, 26). Los estudios in vitro para 6 semanas en el caso de las concentraciones
de T-20, resistente variantes de HF-1 evolucionaron (16). Los estudios in vitro demostraron que el virus infectado contenía
mutaciones en el número de 3 aminoácidos residuos (GIV, posiciones 36 a 38) que son altamente conservadas en el HR1
dominio (16). Mutaciones en ambas posiciones 36 (G a D o S) y 38 (V a M) causaron un marcado descenso en la susceptibilidad a T-20
inhibición (16). Un nivel intermedio de sensibilidad fue observado con las mutaciones a posición 36 (G36S).

Kilby et al. reportaron resultados desde el primer estudio clínico de T-20, que proporcionó prueba de concepto para potentes,
dosis-dependiente de infección promovida por la inhibición de un paso en el viral endocito y/o fusión (10). A todo el grupo a quienes recibieron un mg-vido doce veces diariamente experimentaron un marcado descenso (mean, –1.96 log_{10}) en la infección de HF-1 RNA (vRNA), mientras que en los 3- y 10-mg BID dosis grupos mínimos o no variaciones en el viral laudo después del 14-día curso de la terapia. Los	

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Materials and Methods

Population nucleotide sequencing. Viral RNA was prepared from the plasma of 12 of the 16 patients enrolled in a phase I clinical trial of T-20 (10) by using the QIAamp viral RNA purification protocol (Qiagen). A 692-bp fragment of gp41 that includes the FP, HR1, and HR2 was amplified by PCR as described earlier (22) by using the following primers: outer sense primer 5'-GA GGGACAATGGAAAGAAGTAAT-3' (nt 7638 a 7686), outer antisense primer 5'-GGTGAATCCCTGCTAACTCTATGAT-3' (nt 8355 a 8378), inner sense primer 5'-GGAGAATTATATATATGAAAG-3' (nt 7674 a 7700), and inner antisense primer 5'-GCACGCGTCCGTCCGATACGTGC TCCACCC-3' (nt 8986 a 8918). Nucleotide numbers indicate the primer

Cloning and subcloning env. Full-length env genes were amplified from plasma RNA by nested PCR using the following primers: outer sense primer 5'-TAG AGGCCTGGAGACATGAGGAGAAG-3' (nt 5852 a 5876), outer antisense primer 5'-TTGCTACTTGTTGATGTCCTATG-3' (nt 8972 a 8950), inner sense primer 5'-GATCAGCTTTAAGGATCATCTTATGAAAGAAG-3' (nt 5957 a 5982), and inner antisense primer 5'-AGCTGCTGATCCGCCTGCTCT GAGATGACTGCTCACC-3' (nt 8986 a 8918). The PCR products of the full-length env genes were cloned into pCR-XL-TOPO (Invitrogen) and the individual clones were sequenced and analyzed as described above. Some of clones were chosen for phenotypic analysis and were subcloned into pCDNA3.1

Site-directed mutagenesis. Site-directed mutagenesis was done using the

Statistical analysis. To determine the T-20 dose corresponding to la 50%
inhibition concentration (IC_{50}) with a 95% confidence interval, a piecewise linear dose-response curve was calculated for each of the nucleotide of the template pCDNA3.1-env used for PCR amplification. PCR conditions were as follows: 95°C for 30 s, 58°C for 1 min, and 68°C for 18 min. After 20 cycles, the PCR product was digested with 10 U of DpnI to cleave template DNA at 37°C for 1 h. Mutants were identified by nucleotide sequencing.

VOL. 46, 2002 CLINICAL RESISTANCE OF HIV-1 TO T-20 1897

CLINICAL RESISTANCE OF HIV-1 TO T-20 1897
dependency. Furthermore, under the linearity assumption the standard error of $IC_{50}$ can be estimated.

RESULTS

Analysis of plasma vRNA load. The median viral load at baseline (day 0) for the four individuals treated with 30-mg doses of T-20 was $4.76 \log_{10}$, and after 14 days of treatment the median change was $-0.62 \log_{10}$ (10). To gain a better understanding of the clinical and virologic responses to T-20, the vRNA load of each patient treated with the 30-mg dose (patients 30-1, 30-2, 30-3, and 30-4) was evaluated separately (Table 2). Prior to treatment (day 0), the vRNA loads of patients 30-1, 30-2, 30-3, and 30-4 were 511,800, 84,610, and 31,600 copies/ml, respectively. The greatest decline in plasma vRNA among these subjects generally occurred 7 to 10 days after T-20 therapy was initiated. A plateau or increase in vRNA was measured after 14 days of treatment (Table 2). Thus, in contrast to the potent response in the 100-mg group (10), subjects in the 30-mg dose group had suboptimal responses.

Genetic analysis of plasma HIV-1 env. From the plasma virus of subjects enrolled in each of the four T-20 dosage groups, a 692-bp fragment of DNA containing the FP, HR1, and HR2 domains of gp41 was amplified by reverse transcriptase PCR (RT-PCR) directly from plasma and analyzed by population sequencing. Figure 2A compares the amino acid sequences of the FP, HR1, and HR2 regions prior to (day 0) and after (day 14) treatment with T-20. No differences in the viral population sequence were detected pre- and posttherapy for any of the patients in the 3-, 10-, or 100-mg arms of the study, although each patient’s virus, as expected, was unique. However, in the 30-mg arm, day 14 plasma virus from three of four patients contained a mutation within the HR1 domain. The DNA sequence chromatograms illustrated that approximately one-half of the plasma virus from subject 30-1 contained an aspartic acid residue in place of glycine at position 36 (G36D). Similarly, approximately one-half of the plasma virus from patient 30-2 contained a methionine in place of arginine at position 46 and virus from subject 30-3 contained an isoleucine in place of valine at position 69 (Fig. 2B). While the G36D
Clinical Resistance of HIV-1 to T-20

36 clones contained a single mutation at either position 36 or 38 (Fig. 3A). Twenty-five of the clones (51%) contained the G36D mutation, three (6%) contained the G36S mutation, and eight (16%) contained the V38A mutation. Interestingly, none of the sequences contained a double mutation at residues 36 and 38. The only other mutation detected in more than one of the env clones was located at either position 32 or 39, and these always occurred together with the G36D mutation. Of the 58 env clones derived from the day 14 plasma virus of patient 30-3 that were analyzed, 7 (12%) contained mutations within the GIV sequence. These included one I37V and six V38M mutants (Fig. 3B) which were not apparent from population sequencing. Thirteen of 33 clones (39%) derived from the day 14 plasma virus of patient 30-3 and 30-4 contained an arginine-to-methionine substitution at position 69 (R46M). Two of 12 (17%) of the day 14 plasma of patient 30-2 contained an arginine-to-methionine substitution at position 69. Two of 12 (17%) of the day 7 clones also contained the G36D mutation (Fig. 2A).

Phenotypic analysis of plasma virus susceptibility to T-20. Of four patients treated with the 30-mg dose of T-20, our genetic analysis identified mutant plasma virus for two whose virus load was incompletely suppressed, which is highly suggestive of in vivo selection. To analyze the biologic effect of specific mutations on HIV-1 replication, we analyzed the susceptibility of various env clones to inhibition by T-20 by using a broadly sensitive HIV entry assay capable of quantifying infection within a single cycle of replication. This assay is based on the JC53BL indicator cell line, which is highly sensitive to infection with both R5 and X4 primary viruses. DNA fragments containing the entire env gene were subcloned from the pCR-XL-TOPO plasmid into the pCDNA3.1 plasmid under.

TABLE 1. Comparison of sensitivities of JC53BL cells and PBMC to HIV-1 infection

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<th>p24:TCID\textsubscript{50}</th>
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\[\textsuperscript{a}\text{ Primary virus isolates were derived by coculture (10 days) of HIV-1-infected patient PBMC with PHA-stimulated normal donor PBMC. Viruses were cultured in the pCDNA3.1 plasmid. Multiple clones from each patient were amplified from plasma virus using RT-PCR and cloned in the pCR-XL-TOPO plasmid into the pCDNA3.1 plasmid.}

\[\textsuperscript{b}\text{ Phenotypic analysis of plasma virus susceptibility to T-20 by using a broadly sensitive HIV entry assay capable of quantifying infection within a single cycle of replication. This assay is based on the JC53BL indicator cell line, which is highly sensitive to infection with both R5 and X4 primary viruses. DNA fragments containing the entire env gene were subcloned from the pCR-XL-TOPO plasmid into the pCDNA3.1 plasmid under.}
control of the cytomegalovirus promoter. The pCDNA3.1-env expression plasmids were cotransfected into 293T cells with the HIV-1 env \textsuperscript{11002} pSG3\textsuperscript{9004} env proviral clone. The titers of the transfection-derived pseudotyped virions were determined and then analyzed for sensitivity to T-20 by using the J53BL indicator cell line. An overview of our approach for analyzing phenotypic resistance is illustrated in Fig. 1C.

Viral envelopes from patient 30-1 containing the G36D or V38A mutation were analyzed. Two different clones of each mutant were included in the analysis. Similarly, two wild-type (wt) env clones were analyzed, one derived from day 0 and the other from day 14 plasma virus. The mean IC\textsubscript{50} for the two V38A Env mutants was 11.2 \mu g/ml compared with 0.25 \mu g/ml for the wt Envs (P < 0.001), an increase of 45-fold (Fig. 4A). The G36D mutants also exhibited reduced levels of sensitivity to T-20. The IC\textsubscript{50} for G36D mutant no. 57 was 3.4 \mu g/ml, a statistically significant difference of 13.6-fold (P < 0.001). In repeated experiments the IC\textsubscript{50} of G36D mutant no. 2 was 1.13, an increase of 4.5-fold compared to that of the wt. It may be noteworthy that env no. 2 but not env no. 57 contained an

FIG. 2. Population sequence analysis of plasma HIV-1 env. (A) Comparison of the FP, HR1, and HR2 amino acid sequences pre- and posttreatment with T-20. The plasma virus from one subject (3-1) in the 3-mg dose group, two subjects (10-2 and 10-3) in the 10-mg dose group, and four subjects each in the 30- and 100-mg groups were analyzed by population sequencing from before day 0 (d 0) and after 14 days of treatment (d14) with T-20. The sequences were analyzed using Sequencher software and aligned against the LAI consensus sequence. The FP, HR1, and HR2 regions are depicted. The region within HR2 to which T-20 binds is underlined. (B) Quantitative detection of mutant plasma virus by automated population DNA sequencing. DNA sequence chromatograms of specified regions within HR1 are shown for patients 30-1 (residues 36 to 38), 30-2 (residues 45 to 47), and 30-3 (residues 68 to 69). Chromatograms are shown for each subject from before day 0 and after 14 days (Day 14) of T-20 treatment. The sequences shown were obtained from, and therefore are presented as, the minus (noncoding) DNA strand. The minus-strand sequence corresponds to the plus strand (coding), which is indicated on each chromatogram. Codon changes are indicated as plus-strand substitutions.
arginine-to-glycine (R122G) substitution within the HR2 domain (Fig. 4B). Since determinants of coreceptor specificity contained within the gp120 V3 loop can modulate sensitivity to T-20 (6), the V3 amino acid sequences of Envs 2 and 57 were compared. While some amino acid differences were noted in V3 (Fig. 4B), based on the study by Derdeyn et al. (6), these differences appear not to affect susceptibility to T-20. Three of the 30-1 env clones contained a double mutation involving residues 32 and 36: two contained Q32H plus G36D (Q32H/G36D) and one contained Q32R/G36D. Our phenotypic analysis of these double mutations indicated a loss in sensitivity similar to that of the single G36D mutation (Fig. 4C). This result suggested that neither the Q32R nor the Q32H mutation causes a further loss in viral susceptibility to T-20. A more comprehensive analysis that includes longer treatment trials and greater numbers of subjects will be necessary to delineate how mutations outside of the GIV sequence contribute to the emergence of T-20-resistant virus.

Although it was not apparent from population sequencing of plasma virus (Fig. 2), Env mutations, including V38M and I37V, were detected by clonal analysis in the day 14 plasma virus of patient 30-3 (Fig. 3B). The IC50 of T-20 for the V38M Env mutant was 7.6 μg/ml compared with a mean IC50 of 0.99 μg/ml for the wt Envs, a statistically significant increase of approximately 7.7-fold (P < 0.001) (Fig. 5). The IC50 of the I37V mutant was 3.0, which is threefold greater than the mean of those of the wt Envs. None of the other mutations that were detected in the HR1 domain, including R46M and V69I, were found to affect sensitivity to T-20 (data not shown).

Site-directed mutagenesis was used to confirm that single
FIG. 4. Sensitivity of GIV mutant Env to inhibition by T-20. One thousand infectious units of HIV-1 pseudotyped with mutant or wt Envs derived from patient 30-1 was used to infect cultures of JC53BL cells containing 0, 0.04, 0.2, 1, 5, and 25 µg of T-20 per ml. Two days later, virus entry was measured by analyzing the indicator cells for lucifer activity. Virus infectivity was calculated by dividing the mean lucifer activity value at each of the drug concentrations by the mean value of the control (0 µg). Resistance to T-20 is depicted by plotting relative infectivity on the y axis against T-20 concentration on the x axis. (A) HIV-1/SG3 env was pseudotyped with the G36D mutant Envs 57 and 2, the V38A mutant Envs 18 and 21, and wt Envs (wt-d0 and wt-d14). (B) Sequence analysis of the HR2 and V3 Env domains for the wt (d0-wt), no. 2 (d14-02), and no. 57 (d14-57) env clones. (C) HIV-1/SG3 env stocks were pseudotyped with G36D mutant Env 57, the 32H/36D double mutant Envs (32H, 36D), the 32R/36D double mutant Env (32R, 36D), and the wt Env (wt-d0). The results depicted in panels A and B were highly reproducible and are representative of three independent experiments.
mutations in the GIV sequence were sufficient to confer resistance to T-20. A wt, day 0 env clone derived from patient 30-1 was mutated using the Quik-Change site-directed mutagenesis kit (Stratagene Inc.) and degenerative primers. Virus pseudotyped with the wt Env had an IC\textsubscript{50} of 0.24, while that pseudotyped with the G36D site-directed mutant Env had an IC\textsubscript{50} of 1.4, a 5.8-fold increase (Table 3). The occurrence of Q32R in combination with G36D (G36D/Q32R) did not increase the IC\textsubscript{50} beyond that of G36D alone. Interestingly, the V38A/Q32R mutant Env exhibited an IC\textsubscript{50} of 1.5 µg/ml, supporting the earlier data for a role of the V38A mutation in resistance to T-20. These results indicated that single mutations within the GIV sequence were sufficient to confer HIV-1 resistance to T-20 in vivo.

**Detection of T-20-resistant HIV-1 cultured from plasma.**

Both our genetic and phenotypic analyses indicated rapid in vivo selection of HIV-1 with reduced sensitivity to T-20. Since the plasma HIV-1 load of patient 30-1 remained relatively high during therapy and approximately 50% of the plasma virus contained a mutation in the GIV sequence, it seemed likely that resistant virus could be selected directly from the plasma. Plasma taken from patient 30-1 prior to T-20 administration and after 14 days of treatment was cocultured with PHA-stimulated PBMC. After 18 days of culture, the supernatants were collected and the infectious virus titers were determined using JC53BL cells. The titers of both virus stocks were greater than 10\textsuperscript{5} per ml. The baseline IC\textsubscript{50} (day 0 virus) was 0.13 µg/ml. The day 14 virus had an IC\textsubscript{50} of 0.79 µg/ml, a statistically significant increase of 6.1-fold (Fig. 6). Population sequence analysis confirmed that approximately 50% of the virus derived by coculture contained the G36D env mutation (see insert). This result confirmed the presence of T-20-resistant, replication-competent virus. It also suggested that the in vivo susceptibility of HIV-1 to T-20 can be analyzed from isolates of primary virus.

**DISCUSSION**

The phase I T-20 clinical trial consisted of four dose groups: 3, 10, 30 and 100 mg administered twice daily. Those enrolled in the 3- and 10-mg dose groups had no significant changes in viral load, suggesting the lack of a pharmacological effect. On the other hand, the plasma vRNA loads of all subjects in the 100-mg group declined to less than 500 copies/ml (median change, \(-1.96 \log\) by ultrasensitive assays) (10). Subjects in the 30-mg arm experienced partial declines in viral load, but the median change on day 14 was only \(-0.62 \log\). One individual in the 30-mg arm did not have sustained suppression below baseline. The vRNA load of each of the 30-mg subjects remained well above (at least 10-fold) the limits of detection during T-20 treatment. It was notable that the greatest decline in viral load in three of the four subjects occurred on day 10, with a modest increase on day 14 (Table 1). Sequence analysis provided evidence for the selection of resistant virus. Of multiple env clones that were derived from subjects in all dose groups at baseline, none contained mutations in the GIV motif. However, after 14 days of treatment a significant proportion of env clones derived from two of the four subjects that

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**TABLE 3. Site-directed mutagenesis**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>IC\textsubscript{50}</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>G36D</td>
<td>1.4</td>
<td>5.8</td>
</tr>
<tr>
<td>G36D/Q32R</td>
<td>1.3</td>
<td>5.4</td>
</tr>
<tr>
<td>V38A/Q32R</td>
<td>1.5</td>
<td>6.3</td>
</tr>
</tbody>
</table>

\*Mutations were introduced into a wt, day 0 env clone (pcDNA3.1-env) derived from patient 30-1 by using the Quik-Change kit (Stratagene Inc.) and degenerate oligonucleotide primers. Mutations were identified by nucleotide sequence analysis.
received the 30-mg dose of T-20 contained mutations in the GIV sequence. These same two subjects had the highest viral load at baseline. Our analyses demonstrated that a population of resistant virus had emerged in 2 of 16 HIV-1 infected adults enrolled in the phase I T-20 clinical trial during 14 days of treatment.

It has recently been shown that coreceptor specificity contained within the gp120 V3 loop can modulate sensitivity to T-20 (6). Viruses dependent on CCR5 coreceptor usage are less sensitive to inhibition by T-20 compared with those that utilize CXCR4 (6). To understand whether coreceptor specificity may have affected the emergence of resistant virus in subjects 30-1 and 30-3, we analyzed coreceptor usage by using GHOST indicator cells and found that the viral Envs from all four subjects in the 30-mg dose group, including 30-1 and 30-3, used CCR5 exclusively (data not shown). Therefore, in this study it is unclear what, if any, contribution virus coreceptor usage had on the emergence of T-20-resistant virus.

Despite a relatively conserved target in the gp41 sequence, our findings demonstrate that in certain clinical settings, HIV-1 membrane fusion inhibitors may select for resistance-conferring mutations. This determination is similar to those for the presently available classes of antiretroviral therapies: a single mutation in the RT sequence that conveys resistance to the nucleoside analog lamivudine (3TC) is detectable within weeks in some individuals (20), monotherapy with nonnucleoside RT inhibitors may select for one or two critical resistance-conferring mutations in less than 6 weeks (17), and single-agent therapy with protease inhibitors may select for a series of stepwise mutations over months that convey cross-resistance to multiple agents within this class (4). The present phase I trial of T-20 was short (14 days), yet clear-cut virologic resistance developed; it seems likely that with longer periods of treatment, the proportion of patients with virologic resistance would rise (10a). The GIV sequence appears to be of central importance, but our analysis also suggested that mutations in adjacent residues (such as residues 39 and 42) also contribute to viral resistance.

Finally, in this report we present data showing that the sensitivity of the JC53BL-13 cell line to infection by R5 and X4 viruses is equal to, or in some cases greater than, that of normal PHA-stimulated human lymphocytes. This observation, together with the viral envelope glycoprotein pseudotype experiments showing entry inhibition by and escape from T-20, suggests that the entry assay described herein may be useful for detecting and characterizing, at the biological and molecular levels, naturally occurring neutralizing antibodies to HIV-1.

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REFERENCES


