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Recombination leads to the rapid emergence of HIV-1 dually resistant mutants under selective drug pressure

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ABSTRACT **The potential contribution of recombination to the development of HIV-1 resistance to multiple drugs was investigated. Two distinct viruses, one highly resistant to a protease inhibitor (SC-52151) and the other highly resistant to zidovudine, were used to coinfect T lymphoblastoid cells in culture. The viral genotypes could be distinguished by four mutations conferring drug resistance to each drug and by other sequence differences specific for each parental virus. Progeny virions recovered from mixed infection were passaged in the presence and absence of both zidovudine and SC-52151. Dually resistant mutants emerged rapidly under selective conditions, and these viruses were genetic recombinants. These results emphasize that genetic recombination could contribute to high-level multiple-drug resistance and that this process must be considered in chemotherapeutic strategies for HIV infection.**

The extensive genetic diversity of HIV is well documented (1–4). This diversity results from the contribution of high levels of virus replication (5), coupled to a high mutation rate of HIV-1 per replication cycle (3×10^{-5}/bp) (6) and probably to genetic recombination (7–11). Retroviral recombination occurs frequently during reverse transcription, a consequence of having two RNA genomes packaged per virion (12, 13). Two models have been proposed for homologous recombination: the forced copy choice model (14) and the strand displacement–assimilation model (15). Both require the formation of heterozygous progeny virions in dually infected cells followed by genetic recombination during reverse transcription in newly infected cells by these progeny virions (16, 17). Recombination has been documented between distinct clades of HIV (18, 19) and recently in several cases of HIV-1 coinfection (20–23).

Antiviral therapy applies selective pressure on virus populations, leading to the disappearance of susceptible wild-type viruses and the emergence of resistant mutants (5, 24–26). The emergence of drug-resistant variants of HIV-1, first documented with HIV-infected individuals receiving zidovudine therapy (27), has been reported for most reverse transcriptase (RT) and protease (PR) inhibitors (28). These resistant variants are selected during antiretroviral therapy and probably preexist in the viral quasispecies (29, 30). The potential contribution of recombination to the development of HIV-1 resistance *in vitro* has been reported for zidovudine resistance (31) and for dual resistance to zidovudine and 3TC (32). However, in the first study, the selection conditions were insufficient to inhibit the replication of viruses with single mutations. The dual-resistance study did not represent a true infection model because polyethylene glycol was used to fuse infected cells and no selective drug pressure was applied. To assess the contribution of genetic recombination to the rapid emergence of multiple-drug-resistant variants, we infected T lymphoblastoid cells with a mixture of two viruses, one highly resistant to an RT inhibitor and one highly resistant to a PR inhibitor. Each virus had four mutations conferring drug resistance as well as underlying sequence differences to permit the discrimination between recombination and selection for new mutations. Serial passages were performed with and without drug selective pressure. We observed that dually resistant viruses were able to grow under selective pressure and that these viruses were genetic recombinants. These resistant mutants appeared quickly within one or two passages under selective conditions.

MATERIALS AND METHODS

Virus and Cells. A derivative of HIV-1_{LAI} resistant to the PR inhibitor SC-52151 (Searle) (33), with mutations 48V/71V/75I/81T in the PR gene[§¶], and HIV-1_{RTMC}, a zidovudine-resistant derivative of HXB2d with mutations 67N/70R/215F/219Q in the RT gene (34), were used (for the drug susceptibilities of these viruses, see Fig. 1 *A* and *B*). MT-2 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin and streptomycin (35).

Drug Susceptibility Assay. MT-2 cells (1×10^6/ml) were infected with the appropriate viruses at a multiplicity of infection (moi) of 0.01. After a 2-h inoculation period, the cells were washed and dispensed (6×10^4 per well) in triplicate into 96-well plates containing various concentrations of zidovudine or SC-52151. The production of p24 antigen (enzyme immunoassay; Abbott) was measured in the culture supernatant 3 and 7 days after infection, and the percentage of p24 antigen reduction was calculated based upon the control value without drugs. The concentrations of zidovudine or SC-52151 required to inhibit p24 production by 50% (IC_{50}) were determined.

Isolation of HIV-1 Dually Resistant Variants. MT-2 cells (5×10^5/ml) were infected with both HIV LAI resistant to SC-52151 and HXB2d resistant to zidovudine at an moi 1. After a 2-h inoculation period, the cells were washed three times in PBS to remove inoculum virus and resuspended in RPMI 1640 medium. The viruses were allowed to replicate for 2 days. The cell-free culture supernatant was harvested and used to infect fresh MT-2 cells (5×10^5/ml) in the presence or absence of both zidovudine (1 μM) and SC-52151 (1 μM) (passage 2, P2). The cells had been preincubated for 1 h with both drugs before infection. For subsequent passages, the supernatants were harvested when cultures exhibited both significant cytopathic effects and production of viral p24 antigen. One milliliter was used to infect fresh MT-2 cells as

Abbreviations: PR, protease; RT, reverse transcriptase; moi, multiplicity of infection; P1, passage 1; R1, recombinant 1.

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described. For experiment 1, individual virus progeny were obtained by endpoint dilution cloning of virus recovered after P2. PCR was then performed on these biologic clones to ensure that the predominant population would yield amplified product.

RNA Extraction and Amplification of the Viral *gag* and *pol* Gene Segments. RNA was extracted from the cell-free culture supernatant with phenol-chloroform. Two distinct regions of the viral RNA were amplified, (*i*) the PR and RT genes (1187-bp product) with primer 5′-taatacgactcactatagggagaccactaacttctgtatgt-3′ (LAI positions 1729–1748) and primer 5′-aattaaccctcactaaagggagacagagccaacagccccacca-3′ (LAI positions 2898–2916) and (*ii*) part of the p17/p24 genes (310-bp product) with primer 5′-cagacaggatcagaagaacttag-3′ (LAI positions 540–562) and primer 5′-cagcccagaagtaataccatgt-3′ (LAI positions 827–849). Reverse transcription and PCR amplification of RNA were performed with 0.2 mM of each deoxynucleoside triphosphate, PCR buffer (50 mM KCl/10 mM Tris, pH 8.3/2.5 mM $MgCl_2$/0.01% gelatin), and 0.25 μg of each primer. The samples were incubated for 90 s at 65°C and incubated for 60 s at 42°C. Moloney murine leukemia virus RT (100 units; GIBCO/BRL) was added and incubated at 42°C for 30 min. After reverse transcription, 2.5 units of native Taq DNA polymerase (Perkin–Elmer) was added, and amplification was carried out in a thermal cycler (Perkin–Elmer) for 2 min at 94°C and for 35 cycles (*pol* gene) or 30 cycles (*gag* gene) as follows: for *pol*, 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C; for *gag*, 20 s at 94°C, 20 s at 55°C, and 1 min at 72°C; and a final elongation cycle of 7 min at 72°C for both genes.

DNA Sequencing of the PR and Reverse Transcriptase Genes. The *pol* RNA PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining. The 1187-bp band was excised and purified (Qiaquick gel extraction kit; Qiagen, Chatsworth, CA). Four primers with overlaps in sequence data were used: 5′-aagagagcttcaggtctggggtag-3′ (+), 5′-gaactgtatcctttaactt-3′ (+), 5′-ggaagaaatctgttgactcagattgg-3′ (+), and 5′-aaagaacctccattcctttggatgggt-3′ (−) with LAI positions 1750–1773, 1814–1833, 2090–2115, and 2798–2824, respectively. Cycle sequencing was performed with use of the Applied Biosystems prism ready reaction dyedeoxy terminator cycle sequencing kit with AmpliTaq DNA polymerase, FS (Perkin–Elmer); excess dye-labeled terminators were removed from the extension products by spin column purification (CentriSep spin columns; Princeton Separations, Adelphia, NJ), and samples were loaded onto a sequencer (Perkin–Elmer/Applied Biosystems; model 373A). All these procedures were performed according to the manufacturer's instructions.

Restriction Enzyme Analysis of RNA PCR Product of the *gag* and *pol* Genes. The amplified products were digested with *Scr*FI for the *gag* gene or with *Ssp*I and *Acs*I alone or in combination for the *pol* gene, separated on a 1% or 2% agarose gel, respectively, and visualized by ethidium bromide staining.

RESULTS

Experimental Design to Detect Dually Resistant Recombinant Mutants. Two distinct viruses, HIV LAI resistant to SC-52151 PR inhibitor and HXB2d resistant to zidovudine (Fig. 1 *A* and *B*), were used to permit the discrimination between parental viruses and recombinant mutants selected under dual zidovudine and SC-52151 drug pressure (Fig. 2*A*).

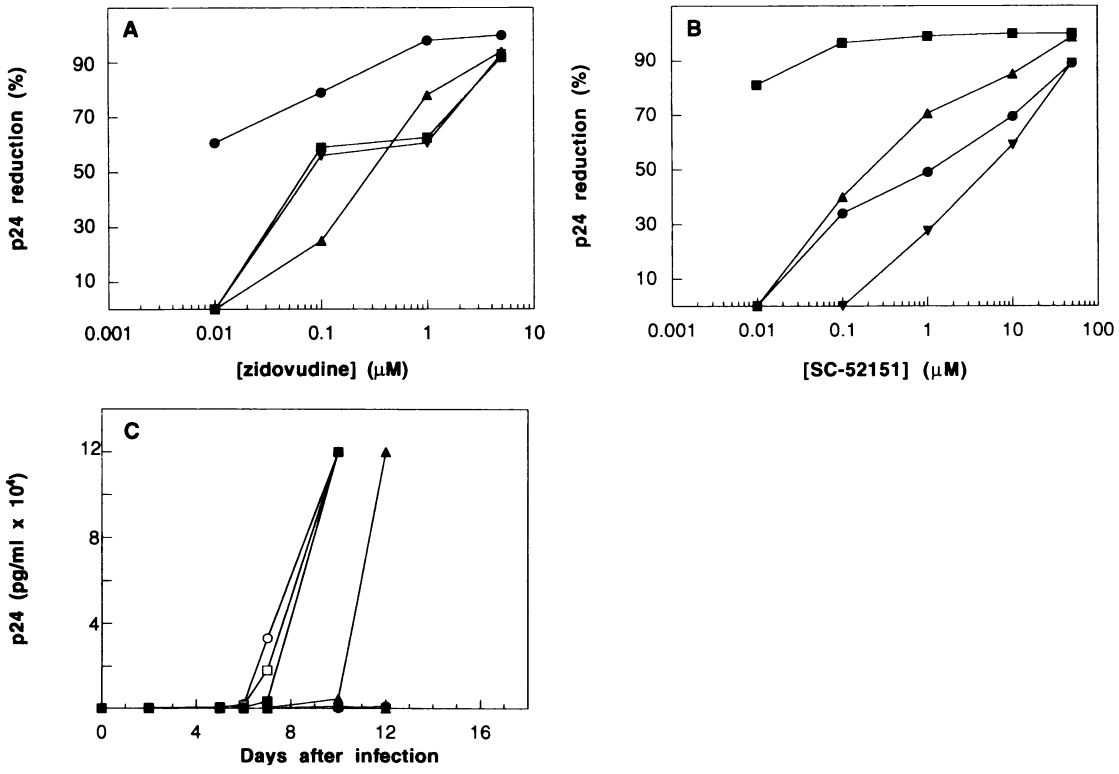

FIG. 1. Susceptibility of the parental and recombinant viruses to zidovudine (*A*) and to SC-52151 PR inhibitor (*B*). ○, LAI parental (mutations 48V/71V/75I/81T in the PR gene); ■, HXB2d parental (mutations 67N/70R/215F/219Q in the RT gene); ▲, LAI/HXB2d recombinant 1 P3 (mutations 48V in the PR gene and 67N/70R/215F/219Q in the RT gene); ▼, LAI/HXB2d recombinant 3 P4 (mutations 48V/71V/75I/81T in the PR gene and 67N/70R/215F/219Q in the RT gene). (*C*) Virus production assessed by p24 antigen level in MT-2 cells infected with LAI and HXB2d parental viruses in the presence of 1 μM zidovudine and 1 μM SC-52151 alone or in combination. The supernatant of MT-2 cells infected with LAI and HXB2d at an moi of 1 was harvested at day 2 and used in subsequent passage to infect fresh MT-2 cells. Virus production as assessed by p24 antigen in the presence of 1 μM zidovudine (●, LAI; ○, HXB2d), 1 μM SC-52151 (▲, LAI; △, HXB2d), or both 1 μM zidovudine and 1 μM SC-52151 (▼, LAI; ▽, HXB2d), or in the absence of drugs (■, LAI; □, HXB2d). No growth of the LAI in the presence of zidovudine, of the HXB2d in the presence of SC-52151, or of either virus in the presence of both drugs was detected.

The susceptibility of the parental viruses to zidovudine and SC-52151 was tested. The optimal drug concentration that inhibited the growth of sensitive viruses and allowed the growth of resistant viruses was 1 μM for both drugs. The growth curves, as measured by p24 antigen expression, of LAI resistant to SC-52151 and HXB2d resistant to zidovudine in the presence of either 1 μM zidovudine, 1 μM SC-52151, both, or neither are shown in Fig. 1*C*. Zidovudine or SC-52151 at 1 μM completely blocked virus production of the sensitive viruses, and no virus grew when both drugs (1 μM) were used. The growth curve of HXB2d resistant to zidovudine was similar with or without the presence of zidovudine (1 μM). LAI resistant to SC-52151, however, grew slower in the presence SC-52151 (1 μM), delaying viral production by about 3 days.

Serial Passage of HIV-1 Resistant Strains With and Without Selective Drug Pressure. MT-2 cells were inoculated with LAI resistant to SC-52151 and HXB2d resistant to zidovudine, each at an moi of 1. Culture supernatants were harvested at day 2 for the purpose of generating heterozygous virions before a second round of replication occurred. HIV p24 antigen levels of these supernatants were $\approx$1000 pg/ml for the first two experiments and $\approx$18000 pg/ml for the third experiment. Drug selection, 1 μM zidovudine and 1 μM SC-52151, was applied during P2 and maintained for subsequent passages. Viral production in MT-2 cells coinfected with resistant LAI and resistant HXB2d is shown at P2, P3, and P4 (Fig. 3 *A–C*). Despite the presence of both drugs, ongoing viral production was detected and sustained. Although virus production was delayed in comparison with virus production without drug selective pressure. Viruses derived from serial passages under selective pressure were dually resistant (Fig. 1 *A* and *B*).

Characterization of Dually Resistant Viruses. The genotype of dually resistant progeny viruses was characterized by sequencing. At least two separate RNA PCR amplifications were performed on each sample. Sequence analysis of the *pol* gene of the virus population recovered from P2 (experiment 1), and P3 and P4 (experiments 1, 2, and 3) revealed that the 5′ end corresponded to the LAI parental sequence and that the 3′ end corresponded to the HXB2d parental sequence (Fig. 2*A*). The LAI/HXB2d recombinants generated in those three separate experiments were designated recombinant 1 (R1), recombinant 2 (R2), and recombinant 3 (R3), respectively. The recombination region was mapped in two experiments (R1 and R2) between the regions of *pol* encoding amino acid residues 48 and 70 of the PR. In the third experiment (R3), the recombination region was mapped between the regions of *pol* encoding amino acid residues 6 and 40 of the RT.

To confirm that these recombinants represented the major population present, we sequenced the five isolates obtained by endpoint dilution of P2 culture supernatant of experiment 1. The sequences were identical, indicating that these double mutants represented the predominant population. To ensure that true recombination events had been generated, PCR with equal ratios and high template imput of LAI and HXB2d parental viruses were performed. These conditions greatly favor background PCR recombination (36). However, by direct sequencing, we always recovered mixtures of viruses at signature nucleotides without recombinants. Thus, this methodology would not uncover in a population viruses that have minor representation. Moreover, we amplified a region in *gag* that discriminated the two parental resistant viruses. The products were digested with *Scr*FI, which distinguishes the sequences of the parental viruses LAI and HXB2d at amino acid 126 of the p17 gene. RNA PCR-*Scr*FI digestion of virus populations recovered from experiment 1 (P2 and P3), experiment 2 (P3; Fig. 2*B*), and experiment 3 (P3; data not shown) corresponded to LAI in 5′, confirming that the dually resistant mutants R1, R2, and R3 were generated by genetic recombination. Sequencing data of the *pol* gene and digestion with *Scr*FI of the *gag* gene showed that in experiments 2 and 3, the virus population recovered from P2 corresponded to a mixture of LAI and HXB2d parental viruses with predominance of the LAI population.

The *pol* gene sequences of the virus passaged in the absence of drug selective pressure corresponded to HXB2d parental (mutations 67N/70R/215F/219Q in the RT gene) in experiment 1 (P2, P3, and P4), to LAI parental (mutations 48V/71V/75I/81T in the PR gene) in experiment 2 (P2, P3, and P4), and to a mixture of LAI and HXB2d parental in experiment 3 (P2, P3, and P4). These mutations were thus relatively stable in culture, and the emergence of dually resistant recombinant mutant was not seen without selective drug pressure.

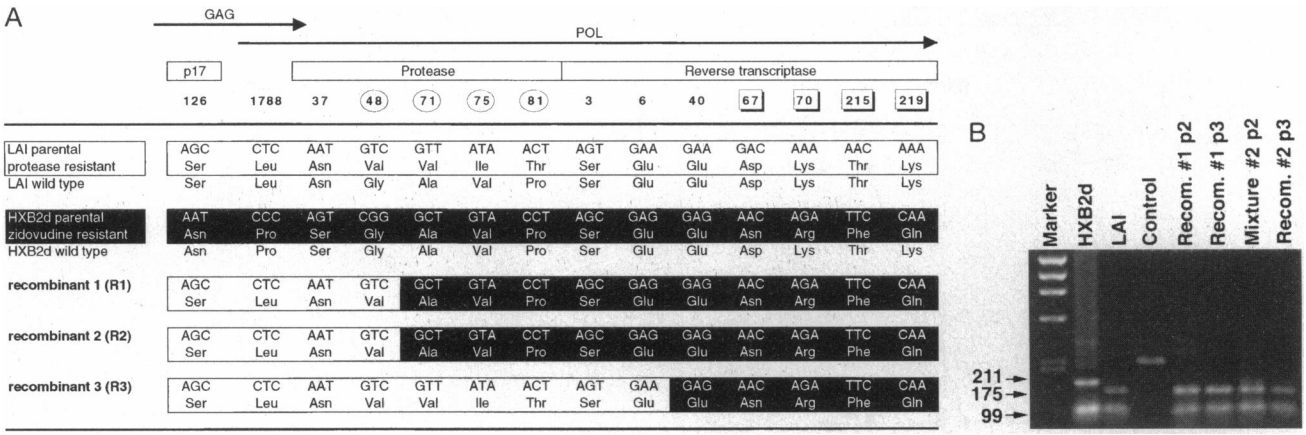

FIG. 2. (*A*) Schematic representation of the *gag-pol* region [matrix (p17), PR, and RT genes] of LAI and HXB2d parental viruses, and their recombinant progeny viruses. Amino acid residues that differentiate the viruses are indicated. The exception is 1788, which is a nucleotide number (HIV_{LAI} position) in *gag-pol* that differs in the two parental viruses. Amino acid changes conferring drug resistance to SC-52151 PR inhibitor (LAI mutations 48V/71V/75I/81T in the PR gene) or to zidovudine (HXB2d mutations 67N/70R/215F/219Q in the RT gene) and genetic sequences characteristic of LAI and HXB2d parental viruses are in circles and squares, respectively. Recombinants 1, 2, and 3 emerged in experiments 1, 2, and 3, respectively, when dual infection with LAI and HXB2d occurred in the presence of both 1 μM zidovudine and 1 μM SC-52151. (*B*) RNA PCR-*Scr*FI digestion of the *gag* gene to detect the p17 amino acid residue 126 difference between LAI and HXB2d. *Scr*FI cleaves specifically the LAI 310-bp RNA PCR product coding for Ala at p17 amino acid 126 to produce three bands of 38, 99, and 175 bp (lane 2) instead of two of 99 and 211 bp for HXB2d (lane 1); lane 3, uncut control. RNA from passages with selective drug pressure, P2 (lane 4, experiment 1; lane 6, experiment 2) and P3 (lane 5, experiment 1; lane 7, experiment 2), of MT-2 cells initially coinfected with LAI and HXB2d (P1) were subjected to PCR and digested with *Scr*FI. Lane M, ϕ174 *Hae*III marker.

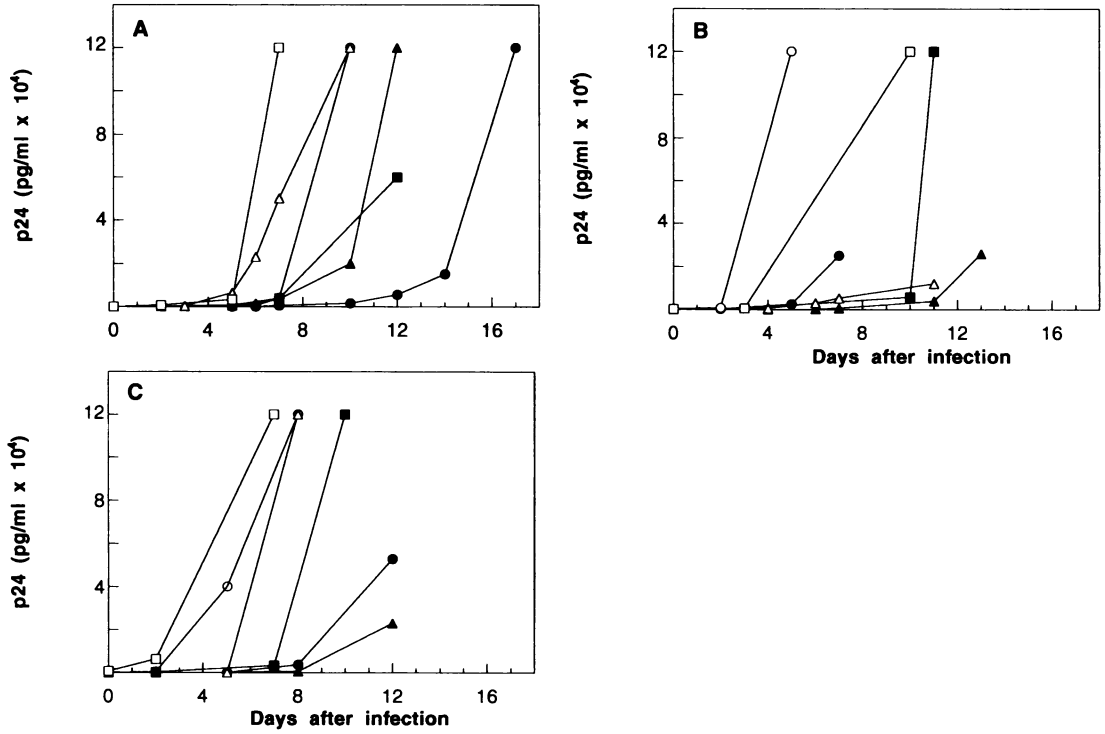

FIG. 3. Virus production assessed by p24 antigen in MT-2 cells coinfected with LAI and HXB2d parental viruses. MT-2 cells were coinfected by LAI and HXB2d at an moi of 1, and serial passages of viruses were performed in the presence of both 1 μM zidovudine and 1 μM SC-52151, or in the absence of drug. The results of three separate experiments are represented at P2 (*A*), P3 (*B*), and P4 (*C*). Experiment 1: ●, zidovudine + SC-52151; ○, without drug. Experiment 2: ▲, zidovudine + SC-52151; △, without drug. Experiment 3: ■, zidovudine + SC-52151; □, without drug.

Detection of Viral Mixtures by RNA PCR Digestion of the *pol* Gene Recovered from Serially Passaged Virus. Direct sequencing of PCR products using *Taq*-dye terminator may not detect genetic mixtures of viruses, especially if one population is poorly represented. To distinguish and determine the proportion of LAI, HXB2d, and LAI/HXB2d recombination mutants, we analyzed the RNA PCR products of the *pol* gene by restriction digestion with *Ssp*I and *Acs*I alone or in combination (Fig. 4). *Ssp*I cleaves specifically the PR gene with mutation 75I independently of the LAI or HXB2d genetic background. *Acs*I cleaves specifically the LAI parental virus coding for Asn at amino acid residue 37 in the PR gene. Thus, the LAI/HXB2d recombinant progeny could be distinguished from LAI and HXB2d parental viruses by *Ssp*I and *Acs*I digestion when recombination mapped between the region of *pol* encoding amino acid residues 48 and 70 of the PR (experiments 1 and 2). The recombinant mutants R1 and R2 yielded a fragment of 183 bp, implying that recombinants were present (Fig. 4*B*). Under selective drug pressure, the population was composed predominantly of LAI/HXB2d recombinants in experiments 1 (P2, P3, and P4) and 2 (P3 and P4). In experiment 2, the appearance of the dually resistant recombinant mutants was preceded by the production of a mixture of the original LAI and HXB2d populations with a predominance of LAI (P2). Without selective drug pressure, the virus population was purely HXB2d in experiment 1 (P2, P3, and P4) and LAI in experiment 2 (P2, P3, and P4). Although recombinant mutants could not be detected by restriction enzyme digestion when recombination mapped between the regions of *pol* encoding amino acid residues 6 and 40 of the RT (experiment 3), we could distinguish mixtures of LAI and HXB2d viruses or recombinant R3 and HXB2d viruses (Fig. 4*C*). Under selective pressure, the population initially was composed of a mixture of LAI and HXB2d, with a predominance of LAI (P2). The dually resistant recombinant mutants then appeared at P3 with the progressive disappearance of the mixed parental population, which was absent at P4 (data not shown). In the three experiments, viruses passaged without drug pressure (P2, P3, and P4) remained stable, corresponding to HXB2d, LAI, and a mixture of LAI and HXB2d parental viruses (data not shown for P4).

DISCUSSION

Under selective drug pressure *in vitro*, recombination has been demonstrated between viruses highly resistant to a PR inhibitor and highly resistant to zidovudine. This recombination resulted in the rapid emergence of dually resistant mutants. Genetic recombination was documented both with mutations conferring drug resistance and with genetic sequences characteristic of each of the two parents. PCR-mediated recombination could be ruled out (36) since the LAI/HXB2d recombinants conserved their genotype through successive passages and dominated the population. To ensure that true recombination events had been generated, PCR with equal ratios of LAI and HXB2d parental viruses were performed and resulted in mixtures of the sequences at signature nucleotides without recombinants. In addition, no dually resistant recombinant mutants were detected by population sequencing when mixed infections with LAI and HXB2d parents were passaged without the selective pressure of both drugs. Furthermore, the same LAI/HXB2d recombinant genotype was recovered in repeated assays by direct population sequencing that is representative of the dominant population. The sequence of the five biologic clones obtained by endpoint dilution of the virus population from experiment 1 (P2) also confirmed the predominance of the recombinant genotype present in the population.

Two previous reports have suggested that recombination events may be involved in the appearance of increased zidovudine resistance (31) and of double zidovudine and 3TC resistance (32). However, in the second study, the dually resistant

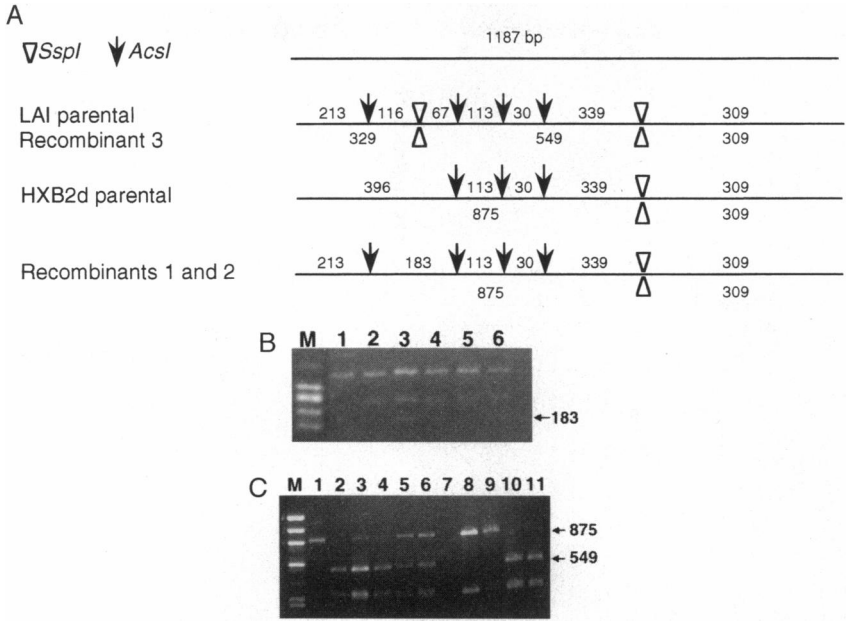

FIG. 4. (*A*) Detection of viral mixtures by RNA PCR digestion of the *pol* gene (1187-bp product) from virus released into the supernatant of MT-2 infected cells. (*B*) In experiments 1 and 2, RNA PCR products from passages with selective drug pressure were digested with *Ssp*I + *Acs*I. The 339- and 309-bp fragments comigrated, and only fragments between 183 and 396 bp are pictured (396-, 339- tot 309-, 213-, and 183-bp fragments). Lane 1, HXB2d parental; lane 2, LAI parental; lane 3, experiment 1, P2 (R1); lane 4, experiment 1, P3 (R1); lane 5, experiment 2, P2 (mixture of parental viruses); lane 6, experiment 2, P3 (R2); lane M, ϕ174 *Hae*III marker (fragments 310–118 bp). (*C*) RNA PCR product digested with *Ssp*I alone. Experiment 1, P2 (lane 8) and P3 (lane 9) without selective drug pressure. Experiment 2, P2 (lane 10) and P3 (lane 11) without selective drug pressure. Experiment 3, P2 with (lane 3) and without (lane 5) selective drug pressure and P3 with (lane 4) and without (lane 6) selective drug pressure. HXB2d parental (lane 1); LAI parental (lane 2). Lane M, ϕ174 *Hae*III marker. Lane 7, empty.

recombinants were recovered without selective pressure and with a procedure of cell fusion, which is not required for retroviral recombination. We have used a mixed infection system with selective conditions, 1 μM zidovudine and 1 μM SC-52151, that completely inhibited the growth of both LAI (48V/71V/75I/81T) and HXB2d (67N/70R/215F/219Q) parents that were highly resistant to only one of the drugs. The appearance and spread of the LAI/HXB2d recombinant mutants established that the mutants were stable. This occurred after one passage under selective drug pressure (P2) for the first experiment. For the second and third experiments, the dually resistant recombinant mutants emerged after two passages under selective drug pressure (P3), implying that a mixed virus population had been produced in the previous passage (P2).

Of possible interest, the genotype of the LAI/HXB2 recombinant mutants suggested that there had been a recombination event that mapped between the regions of *pol* encoding amino acid residues 48 and 70 of the PR in two of the three experiments. Recombination during reverse transcription occurs principally during minus-strand DNA synthesis (14, 17, 37) and studies have shown that HIV-1 RT is able to promote internal strand transfer from heteropolymeric and homopolymeric templates (38–40). Therefore, a specific site of RT pausing may promote the template switches and lead to recombination in the same region. Recombination theoretically should occur at similar frequencies in all regions of the viral genome (41, 42), and the efficiency of recombination should also depend on the length of the sequence identity (11). Thus, in our experiments, recombinants with crossover points in the same region could have emerged due to chance and to their general fitness combined with their growth advantage under selective drug pressure (5, 19, 24–26).

HIV-1 is present as a complex genetic mixture (quasispecies) in infected individuals. Recombination, which has been documented in HIV patients coinfected by divergent HIV strains (18–23), could be a significant source of genetic diversity (1, 2, 41). HIV infection is characterized by a rapid clearance of virions (5, 25, 26), the generation of $\approx 10^{10}$ new HIV virions daily, and a mutation rate of 3×10^{-5}/bp (6). Every single mutation and perhaps most double mutations are thus produced daily. The emergence of highly resistant virus with four or more mutations requires 6 months to 1 year or more for zidovudine (27) and PR inhibitors (43). The time to develop high-level resistance to multiple drugs directed to different targets such as PR and RT should take even longer. However, genetic recombination, as documented in this study, can accelerate this process and lead to the rapid emergence of multiply resistant mutants. Although the correlation between drug resistance and clinical disease progression in HIV infection has not been clearly defined (44), the accelerated selection of multiply and highly resistant variants by the generation of genetic recombinants must be considered.

To delay the emergence of drug resistance, combination regimens are likely to become the chemotherapeutic strategy to treat HIV infection. The present study indicates that incomplete suppression of virus replication can permit the accelerated generation of multiply resistant viruses by genetic recombination. Because recombination requires dual infection of cells, optimal suppression of HIV replication will diminish the opportunity for the selection of both new mutations and recombination.

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from the University of California, San Diego Center for AIDS Research (J.C.).

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