Brief Report

# SEXUAL TRANSMISSION OF AN HIV-1 VARIANT RESISTANT TO MULTIPLE REVERSE-TRANSCRIPTASE AND PROTEASE INHIBITORS

FREDERICK M. HECHT, M.D.,
ROBERT M. GRANT, M.D., M.P.H.,
CHRISTOS J. PETROPOULOS, PH.D.,
BETH DILLON, M.S.W., M.P.H.,
MARGARET A. CHESNEY, PH.D., HUAN TIAN, PH.D.,
NICHOLAS S. HELLMANN, M.D.,
NIRMALA I. BANDRAPALLI, M.S., LAURA DIGILIO, M.D.,
BERNARD BRANSON, M.D., AND JAMES O. KAHN, M.D.

COMBINATION treatments with agents that inhibit protease and reverse transcriptase of human immunodeficiency virus type 1 (HIV-1) decrease mortality and slow disease progression.[1] The development of resistance to these drugs, however, limits the benefit of such treatments.[2,3] There have been reports of the transmission of HIV-1 variants that are resistant to nucleoside and non-nucleoside inhibitors of reverse transcriptase.[4-9] The transmission of HIV-1 variants that are resistant to protease inhibitors could represent an important emerging clinical and public health problem. We report a case of transmission of an HIV-1 variant with multiple mutations that conferred resistance to both protease inhibitors and reverse-transcriptase inhibitors.

## CASE REPORT

The index patient was a middle-aged homosexual man who had tested negative for HIV-1 antibodies six months before an episode of fever (temperature, up to 39°C), night sweats, severe fatigue, and malaise. On the fifth day of symptoms, the patient was evaluated by his primary care physician, who considered a diagnosis of acute HIV-1 infection. An enzyme immunosorbent assay for HIV-1 antibody (Organon Teknika, Durham, N.C.) was nonreactive, but a quantitative HIV-1 p24 antigen test (Abbott Laboratories, Abbott Park, Ill.) was positive, detecting levels of 165 pg of p24 antigen per milliliter of plasma. A rapid plasma reagin test was nonreactive. Seventeen days after the onset of symptoms, treatment with zidovudine, lamivudine, and nelfinavir was begun. Two days later the patient enrolled in the Options Project, a study of primary HIV-1 infection at the University of California, San Francisco, at which time the results of Western blotting for HIV-1 antibodies were indeterminate. Physical examination revealed no abnormalities. Two weeks after treatment was begun, indinavir was substituted for nelfinavir.

The patient reported one recent risk factor for exposure to HIV-1: an episode of receptive anal intercourse without a condom four days before the onset of symptoms. According to the patient (and as subsequently corroborated by his partner), intercourse was discontinued before ejaculation. During three detailed interviews, the patient recalled only one other possible exposure to HIV, during an episode of protected receptive anal intercourse with a different partner three months earlier.

After obtaining the consent of the index patient, we contacted the suspected source patient. The source patient consented to a review of his medical records and agreed to provide blood for testing. He had been given a diagnosis of HIV-1 infection in 1990. Before the index patient became infected, the source patient had received multiple antiretroviral agents, including zidovudine, zalcitabine, lamivudine, stavudine, saquinavir, ritonavir, and indinavir (Fig. 1), and had admittedly been poorly compliant with treatment. On the day the index patient was infected the source patient underwent branched-chain DNA testing (bDNA 2.0, Chiron, Emeryville, Calif.), which showed a viral load of 28,670 copies of HIV-1 RNA per milliliter. One week later, treatment was changed to nelfinavir, lamivudine, zidovudine, and delavirdine in an effort to reduce the viral burden. The new regimen was not successful, and the patient discontinued all antiretroviral medications eight weeks later and remained untreated until he was tested in connection with the index case.

## METHODS

HIV-1 RNA levels were measured with the Chiron bDNA 2.0 assay. Results that were below the limits of quantification of the assay were tested with the Roche Ultrasensitive HIV-1 RNA polymerase-chain-reaction (PCR) test (lower limit of quantification, 50 copies per milliliter; Roche Diagnostics, Nutley, N.J.). Phenotypic and genotypic resistance assays were performed on the first plasma specimen obtained from each patient. The first specimen was obtained from the index patient 23 days after the suspected date of exposure, 19 days after the onset of primary HIV-1 symptoms, and 2 days after antiretroviral therapy was begun. The specimen from the source patient was obtained 22 weeks after the date of exposure, 13 weeks after he had stopped antiretroviral therapy.

Phenotypic analysis of drug susceptibility was performed with a newly developed method (unpublished data). The assay uses HIV-1 vectors that are assembled by inserting amplified reverse-transcriptase and protease viral gene segments derived from the patient into a modified HIV-1 genome that carries a luciferase indicator gene and is restricted to a single round of viral replication. A control vector was constructed with pNL4-3, an infectious molecular clone of HIV-1.[10] The assay consists of transfecting cells with HIV-1 vector DNA, collecting viral particles 48 hours after transfection, and then infecting fresh cells. Cells are lysed 48 hours after infection (one round of viral replication), and luciferase activity is measured. Protease inhibitors are added to cells shortly after transfection, and reverse-transcriptase inhibitors are added to cells before infection. Drug susceptibility is measured by comparing luciferase activity in control cultures and cultures of HIV-1 derived from the patient in the presence and absence of antiretroviral drugs. Drugs that inhibit HIV-1 reverse transcriptase or protease reduce the amount of luciferase activity in the target cell. The concentration of drug that inhibited luciferase activity by 50 percent ($IC_{50}$) was used as a measure of drug susceptibility. In this assay, in more than 95 percent of replicate studies, results varied by less than a factor of 2, suggesting that larger reductions in the $IC_{50}$ indicate reduced drug susceptibility. The drug susceptibility of HIV-1 from the index patient was measured in duplicate with separate test vector preparations derived from separate amplification reactions for each sample. The results of drug-susceptibility measurements of the duplicate vector preparations varied by a factor of less than 1.4.

From the AIDS Program, San Francisco General Hospital and University of California, San Francisco, San Francisco (F.M.H., R.M.G., J.O.K.); the Gladstone Institute of Immunology and Virology, San Francisco (R.M.G., N.I.B., L.D.); ViroLogic, South San Francisco, Calif. (C.J.P., H.T., N.S.H.); the Centers for Disease Control and Prevention, Atlanta (B.D., B.B.); and the Center for AIDS Prevention Studies, University of California, San Francisco, San Francisco (M.A.C.). Address reprint requests to Dr. Hecht at the UCSF AIDS Program, 995 Potrero Ave., Ward 84, San Francisco, CA 94110.

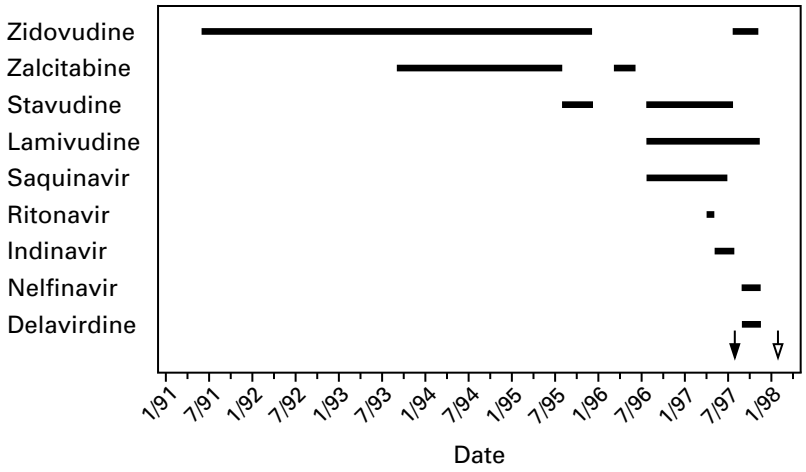


**Figure 1.** History of Antiretroviral Therapy in the Source Patient.
The solid arrow indicates the date of transmission of the virus to the index patient, and the open arrow the date on which blood samples were obtained from the source patient.

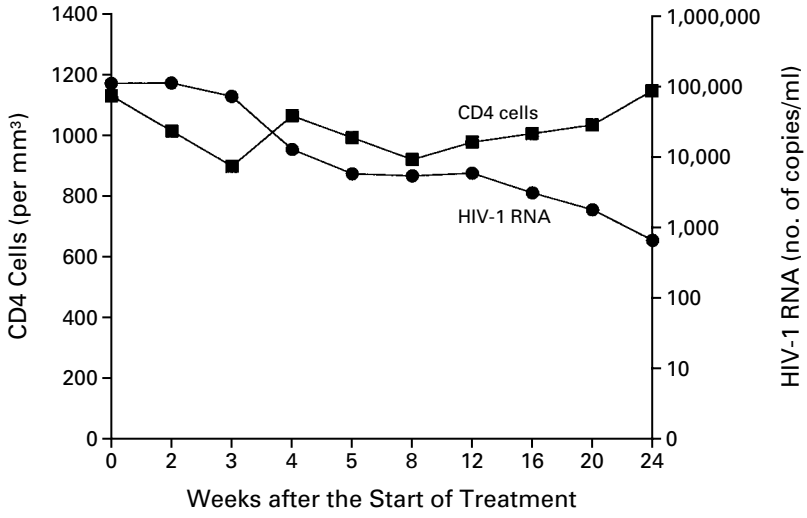


**Figure 2.** Plasma HIV-1 RNA Levels and CD4 T-Lymphocyte Counts in the Index Patient after the Initiation of Antiviral Treatment.

We sought genotypic evidence of antiviral-drug resistance by amplifying the entire HIV-1 protease gene and the first 250 codons of the reverse-transcriptase gene from plasma and directly sequencing the amplification product using arrays of HIV-1 nucleic acid probes.[11-13] We used an automated procedure involving fluorescence detection of the chain-terminator cycle sequence[14] to confirm protease and reverse-transcriptase sequences in samples from the patients in the two separate HIV-1 test vector preparations used to evaluate drug susceptibility. For each test vector preparation, DNA sequences were determined for the population as a whole and for approximately 10 clones isolated from each population.

The *env* V3 region of HIV-1 from the index patient, the source patient, and six other patients with primary HIV-1 infection who were enrolled in the Options Project was amplified by nested PCR and directly sequenced with standard fluorescence cycle sequencing to determine whether HIV-1 gene sequences were similar enough to be used as evidence to support the suspected mode of transmission in the index patient. Sequence alignment was determined with the Clustal W 1.7 computer program[15] and manually reviewed before analysis. Phylogenetic relations were inferred by the neighbor-joining method after the elimination of sites with gaps and compensation for multiple substitutions. Precautions to prevent contamination of sequence data were implemented, as described previously.[16] Bootstrap analysis was performed to evaluate the level of confidence in the observed phylogenetic clusters of *env* gene sequences among the eight tested specimens.[17,18] The value obtained by bootstrap analysis represents the proportion of 1000 trials in which random sampling with replacement of nucleotide sites in the sequence alignment indicated that the two sequences came from the same branch of the phylogenetic tree.

## RESULTS

HIV-1 RNA levels and CD4 T-lymphocyte counts in the index patient are shown in Figure 2. Phenotypic drug-resistance studies demonstrated that the $IC_{50}$ of samples from the index patient was at least twice that

of control samples for zidovudine, lamivudine, saquinavir, ritonavir, indinavir, and nelfinavir (Fig. 3). Before the index patient became infected, the source patient had used all these medications except nelfinavir.

Genotypic analyses showed multiple codons in the protease and reverse-transcriptase genes in both the index and the source patients that differed from those of the wild-type virus and that have been associated with drug resistance (Table 1). The Asp67Asn, Thr215Tyr, and Lys219Gln substitutions found in the reverse-transcriptase gene amplified from HIV-1 from the index patient have been associated with resistance to zidovudine, whereas the Met184Val substitution predicts resistance to lamivudine.[19] Each of these substitutions, mixed with wild-type variants, was also identified in virus amplified from the source patient. Seven amino acid substitutions associated with resistance to protease inhibitors were identified in HIV-1 from the index patient; four of these substitutions were found as either the dominant variant or in mixture with the wild type in the source patient. Although some of the observed substitutions in the protease gene have been reported to be naturally occurring polymorphisms (Met36Ile, Leu63Pro, and Ala71Thr),[20,21] the Met46Ile and Leu90Met substitutions have only been observed in association with treatment with protease inhibitors.[22,23] The Leu90Met substitution is associated with resistance to all four protease inhibitors now in clinical use, and the Met46Ile substitution is associated with resistance to ritonavir, indinavir, and nelfinavir. Codon 82 is an important site for mutations leading to resistance to indinavir and ritonavir. The substitution of isoleucine for methionine at codon 82, however, is not typically associated with resistance to currently licensed protease inhibitors, but it has been reported in association with an experimental protease inhibitor, A-77003.[24]

Bootstrap analysis of the *env* V3 sequences from the index patient and the source patient revealed values of 100 percent in 1000 trials, indicating that the sequence clustering was unlikely to have occurred by chance alone. The degree of divergence of the *env* V3 sequences between the index patient and the source patient was 1.3 percent, whereas the degree of divergence between any other pairs of *env* V3 sequences in the analysis was more than 7 percent. The high degree of similarity between viral envelope sequences from the two patients indicates a recent ancestral relationship, as would occur after viral transmission from one patient to another. Phylogenetic analysis of the reverse-transcriptase and protease gene segments also indicated that the viral sequences from the source patient and index patient clustered together, with bootstrap values of 100 percent.

## DISCUSSION

Phenotypic and genotypic studies of the drug susceptibility of HIV-1 obtained from the index patient at the onset of treatment suggest that he was infected with a strain that was resistant to multiple protease and reverse-transcriptase inhibitors. Analysis of his history suggested that the source of HIV-1 infection was a partner with an extensive history of treatment with antiretroviral agents, including protease inhibitors. This epidemiologic link was supported by the high degree of similarity in HIV-1 *env* gene sequences from the two patients. The finding of fewer genotypic substitutions coding for drug resistance in the source patient may be the result of a reversion to wild-type virus in the absence of treatment with antiretroviral drugs, a founder effect during transmission, or the presence of different dominant viruses in the semen and plasma in the source patient.

The multiple mutations required to induce resistance to protease inhibitors can reduce the reproductive efficiency of HIV-1, although this is not true in all cases.[25,26] Theoretically, a reduction in the reproductive efficiency of HIV-1 could impair its ability to establish infection in a new host, supporting speculation that protease-inhibitor–resistant HIV-1 might not be readily transmitted. Some HIV-1 variants that are resistant to zidovudine may be at a selective disadvantage during transmission as compared with wild-type virus.[27] Although the findings in our case do not rule out the possibility that multidrug-resistant HIV-1 is less readily transmitted, they confirm that an HIV-1 variant with multiple mutations coding for resistance to protease and reverse-transcriptase inhibitors can be transmitted.

The slow decrease in the number of copies of HIV-1 RNA in the index patient contrasts with the course of infection in 36 other patients whom we have treated with similar drug combinations in the early stage of disease; all had undetectable levels of HIV-1 RNA on the branched-chain DNA assay within 12 weeks after treatment was begun, as compared with 20 weeks for the index patient.[28] The observed decrease was most likely due in part to naturally occurring declines in HIV-1 RNA that occur after primary HIV-1 infection.[29]

Protease inhibitors have been widely used for only two years. The index patient is the only patient with genotypic evidence of the transmission of protease-resistant HIV-1 among 37 patients with early or primary HIV-1 infection who have been tested by the Options Project. The frequency of infection with protease-inhibitor–resistant virus may increase dramatically, especially in communities in which the use of these treatments is widespread.

From a public health perspective, this case has several important implications. First, if the history of risk factors given by both partners is accurate, it confirms that the practice of withdrawal before ejaculation is dangerous because pre-ejaculate secretions can contain infectious HIV-1,[30] a risk that the patients in this case did not understand. Second, it points out

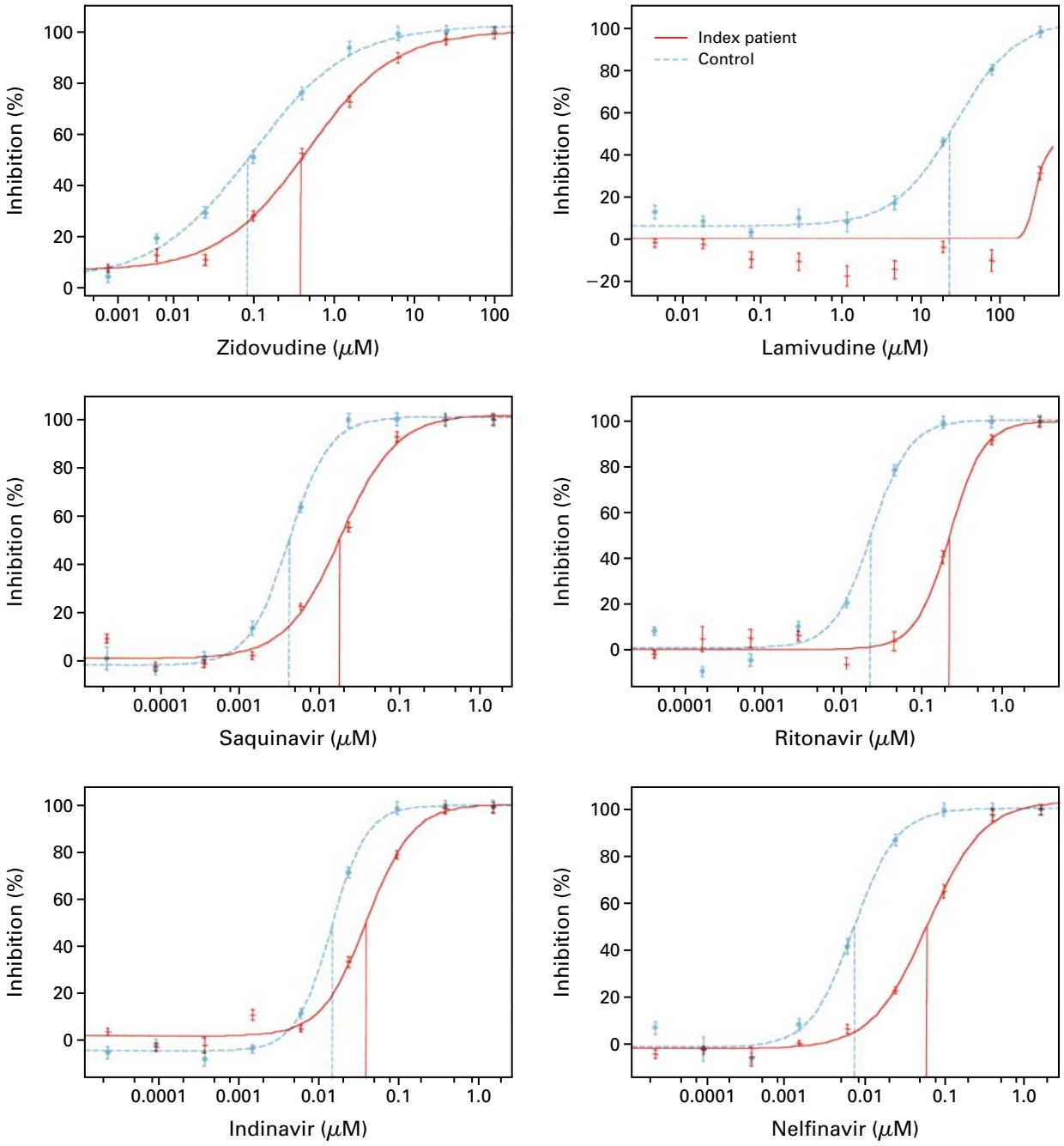


**Figure 3.** Results of Phenotypic Assay of the Drug Susceptibility of HIV-1 from the Index Patient.

The curves show the drug concentrations required to achieve the designated levels of inhibition for the index patient and control HIV-1. The I bars indicate means ±SE. The vertical lines indicate the concentrations of drug that inhibited luciferase activity by 50 percent ($IC_{50}$). The respective mean $IC_{50}$ values for control HIV-1 and HIV-1 from the index patient (with the factor by which resistance was greater in the patient given in parentheses) were as follows: 0.103 and 0.457 μM for zidovudine (4.4), 25.4 and >300 μM (higher concentrations of lamivudine could not be tested owing to cell toxicity) for lamivudine (>12), 0.0042 and 0.0207 μM for saquinavir (4.9), 0.0196 and 0.247 μM for ritonavir (12.6), 0.0141 and 0.0383 μM for indinavir (2.7), and 0.0081 and 0.0633 μM for nelfinavir (7.8).

**TABLE 1.** GENOTYPIC ANALYSIS OF THE DRUG-RESISTANCE MUTATIONS OF HIV-1 FROM THE INDEX AND SOURCE PATIENTS.

| ENZYME | AMINO ACID SEQUENCE (NO. OF CLONES WITH GENOTYPE/NO. OF CLONES TESTED)* | |
|---|---|---|
| | INDEX PATIENT | SOURCE PATIENT |
| Reverse transcriptase | Asp67Asn (20/20) | WT (18/19)/Asp67Asn (1/19) |
| | Met184Val (20/20) | WT (16/19)/Met184Val (3/19) |
| | Thr215Tyr (20/20) | WT (15/19)/Thr215Tyr (4/19) |
| | Lys219Gln (20/20) | WT (15/19)/Lys219Gln (4/19) |
| Protease | Met36Ile (20/20) | Met36Ile (19/19) |
| | Met46Ile (20/20) | WT (19/19) |
| | Leu63Pro (20/20) | Leu63Pro (13/19)/WT (6/19) |
| | Ala71Thr (20/20) | WT (19/19) |
| | Asn88Asp (20/20) | WT (19/19) |
| | Val82Ile (20/20) | Val82Ile (12/19)/WT (7/19) |
| | Leu90Met (20/20) | WT (18/19)/Leu90Met† |

*The results indicate the amino acid sequences identified by direct sequencing of the amplification product and confirmed by fluorescence cycle sequencing. In addition, multiple clones were amplified and sequenced with fluorescence cycle sequencing to determine the proportion of clones with the indicated sequences. The proportion of clones with the indicated amino acid sequence is noted in parentheses. WT denotes wild-type virus.

†Although direct sequencing and fluorescence cycle sequencing indicated the presence of the Leu90Met substitution, none of the 19 clones sequenced had this substitution.

an additional hazard for the uninfected: the acquisition of multidrug-resistant HIV-1. This emphasizes the importance of careful maintenance of HIV risk-reduction efforts, even with the availability of more effective treatments. Third, it underlines the need to develop systems to monitor the prevalence of the transmission of antiretroviral-drug–resistant strains in order to guide the implementation of appropriate clinical and public health responses.

Supported by grants from the Centers for Disease Control and Prevention (U64/CCU913941, to Drs. Hecht and Chesney), the Center for AIDS Research (P30 AI 27763, to Drs. Grant, Digilio, and Kahn), the AIDS Clinical Research Center, San Francisco (CC96-SF-176, to Drs. Kahn and Grant), and the National Institutes of Health (UO1 41531, to Drs. Kahn and Hecht).

Dr. Kahn is a paid advisor to ViroLogic, Inc.

*We are indebted to Options Project staff members John Melichar, Nelson Murcar, R.N., and Sara S. Batya, who helped make this report possible; to ViroLogic employees Neil T. Parkin, Yolanda S. Lie, and Jeannette M. Whitcomb for technical assistance and data analysis; and to the patients and the referring physician for their cooperation.*

## REFERENCES

**1.** Hammer SM, Squires KE, Hughes MD, et al. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. N Engl J Med 1997;337:725-33.
**2.** McDonald CK, Kuritzkes DR. Human immunodeficiency virus type 1 protease inhibitors. Arch Intern Med 1997;157:951-9.
**3.** Deeks SG, Smith M, Holodniy M, Kahn JO. HIV-1 protease inhibitors: a review for clinicians. JAMA 1997;277:145-53.
**4.** Imrie A, Beveridge A, Genn W, Vizzard J, Cooper DA. Transmission of human immunodeficiency virus type 1 resistant to nevirapine and zidovudine: Sydney Primary HIV Infection Study Group. J Infect Dis 1997; 175:1502-6.
**5.** Veenstra J, Schuurman R, Cornelissen M, et al. Transmission of zidovudine-resistant human immunodeficiency virus type 1 variants following deliberate injection of blood from a patient with AIDS: characteristics and natural history of the virus. Clin Infect Dis 1995;21:556-60.
**6.** Ippolito G, Del Poggio P, Arici C, et al. Transmission of zidovudine-resistant HIV during a bloody fight. JAMA 1994;272:433-4.
**7.** Angarano G, Monno L, Appice A, et al. Transmission of zidovudine-resistant HIV-1 through heterosexual contacts. AIDS 1994;8:1013-4.
**8.** Conlon CP, Klenerman P, Edwards A, Larder BA, Phillips RE. Heterosexual transmission of human immunodeficiency virus type 1 variants associated with zidovudine resistance. J Infect Dis 1994;169:411-5.
**9.** Erice A, Mayers DL, Strike DG, et al. Primary infection with zidovudine-resistant human immunodeficiency virus type 1. N Engl J Med 1993; 328:1163-5.
**10.** Adachi A, Gendelman HE, Koenig S, et al. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 1986;59:284-91.
**11.** Kozal M, Shah N, Shen N, et al. Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. Nat Med 1996;2:753-9.
**12.** Lipshutz RJ, Morris D, Chee M, et al. Using oligonucleotide probe arrays to access genetic diversity. Biotechniques 1995;19:442-7.
**13.** Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP, Fodor SPA. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. Proc Natl Acad Sci U S A 1994;91:5022-6.
**14.** Wei X, Ghosh SK, Taylor ME, et al. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 1995;373:117-22.
**15.** Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673-80.
**16.** Learn GH Jr, Korber BTM, Foley B, Hahn BH, Wolinsky SM, Mullins JI. Maintaining the integrity of human immunodeficiency virus sequence databases. J Virol 1996;70:5720-30.
**17.** Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985;39:783-91.
**18.** Sanderson M. Confidence limits on phylogenies: the bootstrap revisited. Cladistics 1989;5:113-29.
**19.** Schinazi RF, Lloyd RM Jr, Nguyen M-H, et al. Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. Antimicrob Agents Chemother 1993;37:875-81.
**20.** Yamaguchi K, Byrn RA. Clinical isolates of HIV-1 contain few pre-existing proteinase inhibitor resistance-conferring mutations. Biochim Biophys Acta 1995;1253:136-40.
**21.** Barrie KA, Perez EE, Lamers SL, et al. Natural variation in HIV-1 protease, Gag p7 and p6, and protease cleavage sites within gag/pol polyproteins: amino acid substitutions in the absence of protease inhibitors in mothers and children infected by human immunodeficiency virus type 1. Virology 1996;219:407-16.
**22.** Ives KJ, Jacobsen H, Galpin SA, et al. Emergence of resistant variants of HIV in vivo during monotherapy with the proteinase inhibitor saquinavir. J Antimicrob Chemother 1997;39:771-9.
**23.** Zhang YM, Imamichi H, Imamichi T, et al. Drug resistance during indinavir therapy is caused by mutations in the protease gene and in its Gag substrate cleavage sites. J Virol 1997;71:6662-70.
**24.** Kaplan AH, Michael SF, Wehbie RS, et al. Selection of multiple human immunodeficiency virus type 1 variants that encode viral proteases with decreased sensitivity to an inhibitor of the viral protease. Proc Natl Acad Sci U S A 1994;91:5597-601.
**25.** Gulnik SV, Suvorov LI, Liu B, et al. Kinetic characterization and cross-resistance patterns of HIV-1 protease mutants selected under drug pressure. Biochemistry 1995;34:9282-7.
**26.** Rayner MM, Cordova B, Jackson DA. Population dynamics studies of wild-type and drug-resistant mutant HIV in mixed infections. Virology 1997;236:85-94.
**27.** Wahlberg J, Fiore J, Angarano G, Uhlen M, Albert J. Apparent selection against transmission of zidovudine-resistant human immunodeficiency virus type 1 variants. J Infect Dis 1994;169:611-4.
**28.** Hecht F, Chesney M, Busch MP, et al. Treatment of primary HIV with AZT, 3TC, and indinavir. Presented at the Fifth Conference on Retroviruses and Opportunistic Infections, Chicago, February 1–5, 1998. abstract.
**29.** Kahn JO, Walker BD. Acute human immunodeficiency virus type 1 infection. N Engl J Med 1998;339:33-9.
**30.** Pudney J, Oneta M, Mayer K, Seage G III, Anderson D. Pre-ejaculatory fluid as potential vector for sexual transmission of HIV-1. Lancet 1992;340:1470.