ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1992, p. 153–157
0066-4804/92/010153-05$02.00/0

Vol. 36, No. 1

Human Immunodeficiency Virus Type 1 *pol* Gene Mutations Which Cause Decreased Susceptibility to 2′,3′-Dideoxycytidine

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Received 3 September 1991/Accepted 28 October 1991

To investigate whether human immunodeficiency virus type 1 *pol* gene mutations are selected during prolonged 2′,3′-dideoxycytidine (ddC) therapy, we used the polymerase chain reaction to amplify a portion of the reverse transcriptase segment of the *pol* gene from the peripheral blood mononuclear cell DNA of a patient with AIDS before and after an 80-week course of ddC therapy. The consensus sequence from the second sample contained a unique double mutation (ACT to GAT) in the codon for reverse transcriptase amino acid 69, causing substitution of aspartic acid (Asp) for the wild-type threonine (Thr). A mutation (ACA to ATA) also occurred in the codon for position 165, causing substitution of isoleucine (Ile) for Thr. The GAT (Asp) codon was introduced into the *pol* gene of a molecular clone of human immunodeficiency virus via site-directed mutagenesis. Following transfection, mutant and wild-type viruses were tested for susceptibility to ddC by a plaque reduction assay. The mutant virus was fivefold less susceptible to ddC than the wild type; cross-resistance to 3′-azido-3′-deoxythymidine or 2′3′-dideoxyinosine was not found. The Ile-165 mutation did not confer additional ddC resistance. The Asp-69 substitution may have contributed to the generation of resistant virus in this patient.

Antiretroviral therapy for the treatment of human immunodeficiency virus type 1 (HIV-1) infection has proven effective in increasing the length and quality of life of infected individuals (6, 29, 30). However, after approximately 2 years many patients become refractory to therapy and their symptoms return (5, 20, 21). Larder et al. (14) found that 3′-azido-3′-deoxythymidine (AZT)-resistant viruses could be isolated from patients treated for 6 months or more. These viruses were found to contain up to five mutations (four amino acid substitutions) in the *pol* gene of HIV (16). Other studies have now confirmed that HIV regularly acquires resistance during AZT therapy (3, 12, 24) and that this resistance is associated with the presence of one or more of these mutations (3, 15, 17).

The antiretroviral agents 2′,3′-dideoxycytidine (ddC) and 2′,3′-dideoxyinosine (ddI), now undergoing extensive clinical trials, are used in attempts to salvage patients who have become unresponsive to AZT and possibly (in combination with AZT) to delay the emergence of resistant virus (4). In this report we describe mutations occurring in the HIV *pol* gene in a patient with AIDS during prolonged ddC therapy and the effect of these mutations on the susceptibility of HIV-1 to ddC in vitro.

MATERIALS AND METHODS

Patient history. The patient was a 24-year-old man who presented with *Pneumocystis carinii* pneumonia in October 1988 and was found to be seropositive for HIV-1 at that time. He was enrolled in a treatment protocol (ACTG 047) and received ddC (0.03 mg/kg every 4 h on alternating weeks) from December 1988 through June 1990 (80 weeks). During the first year of ddC therapy, aside from intermittent episodes of thrush, the patient remained well. His CD4 cell counts remained low (<100 cells per mm^3) throughout the treatment period, but plasma p24 levels dropped from approximately 1,000 pg/ml prior to treatment to between 132 and 190 pg/ml after 4 months. Thereafter, his p24 levels began to rise again and were in the range of 12,000 pg/ml after 1 year of therapy. After 18 months, the patient's clinical status declined rapidly, with marked neurological deterioration ascribed clinically to progressive HIV infection. One month later ddC therapy was stopped and he was started on AZT (100 mg every 4 h). Neurological deterioration continued, and the patient died in March 1991.

Oligonucleotides. Oligonucleotide primers P5 and P7, used for amplification of a 654-bp *pol* gene fragment (corresponding to reverse transcriptase amino acids 63 to 279, not including primers) from peripheral blood mononuclear cell DNA, have been described previously (7). This region was chosen for amplification because it had previously been shown to be involved in nucleotide binding and polymerase function (11), as well as to harbor AZT resistance mutations (16). All other oligonucleotides used in this study are listed in Table 1, along with the corresponding HIV-1 NL4-3 coordinates (22).

Identification of *pol* gene mutations. Amplification of *pol* gene fragments from the patient's peripheral blood mononuclear cells by the polymerase chain reaction (PCR) (25) was performed as previously described (7, 10); we used 40 cycles of amplification with a final Mg^{2+} concentration of 3 mM. Under these conditions the *Taq* polymerase error rate was found to be 0.5% or 2.4×10^{-4} per nucleotide per amplification cycle (10). *pol* clones were identified and analyzed as previously described (7), except that oligonucleotide probes P69D1 and P165I1 (Table 1), which hybridize to sequences

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence	NL4-3 coordinates
P69D1	5′-AAAAGACAGTGATAAATGGAGAA-3′	2744–2766
P69D2	5′-TTCTCCATTTATCACTGTCTTTT-3′	2766–2744
P69N	5′-AGACAGTAATAAATGGA-3′	2747–2763
P8	5′-ATACTAGGTATGGTAAATGC-3′	2953–2934
P165I1	5′-AAGATTTTTATCATGCTA-3′	3052–3035
P165I2	5′-TAGCATGATAAAAATCTT-3′	3035–3052
P30	5′-CCATTAGTCCTATTGAGAC-3′	2551–2569
P31	5′-GCCTTAACTGTAGTACTG-3′	4612–4595

carrying the Asp-69 and Ile-165 mutations, respectively, were used in screening. Filters for Southern hybridizations were prepared by the method of Maniatis et al. (19) and probed by the method of Berent et al. (2).

Site-directed mutagenesis. Construction of HIV-1 clones carrying the Asp-69 and Ile-165 mutations is diagrammed in Fig. 1. Overlap extension mutagenesis was performed by the method of Ho et al. (9). PCRs were run on 0.5 μg of template at a Mg^{2+} concentration of 1.5 mM. Reactions were run for 25 cycles (*Pst*I fragment [Fig. 1B]) or 20 cycles (*Bal*I fragment [Fig. 1E]) as follows: 1 min at 94°C, 2 min at 45°C, and 2 min at 70°C. Under these conditions the error rate was reduced to approximately 0.02% (data not shown), presumably owing to the high level of template, the low Mg^{2+} concentration, and fewer cycles; this is in agreement with results of earlier work (9).

Orientation of cloned fragments was determined by restriction analysis and confirmed by sequencing. The nucleotide sequence of pJF3 was determined from the 3′ *Pst*I junction to the upstream *Bal*I site; it was identical to the pNL4-3 sequence (22) except for the Asp-69 double mutation. The 5′ *Bal*I junction of pJF4A was verified by sequencing. The nucleotide sequence of the entire 1.9-kbp *Bal*I fragment of pJF6A was determined. In addition to the Asp-69 and Ile-165 mutations, the sequence differed from the published pNL4-3 sequence (22) by the presence of a G at position 3608 and A's at positions 4197, 4198, 4199. However, sequencing of our preparation of pNL4-3 revealed these latter residues, suggesting that the published sequence is in error and demonstrating that these are not PCR-induced mutations.

Preparation of virus stocks. Plasmid DNA (20 μg) was used to transfect $T4^+$ HeLa cells (18) via the modified calcium phosphate technique described by Sambrook et al. (26). At 3 days after transfection, numerous syncytia were observed in the experimental cultures but not in the mock-transfected control. At this time supernatants were removed and used to infect CEM.SS cells (23) by addition of 10^6 cells, in 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum plus antibiotics, to 1 ml of the $T4^+$ HeLa supernatants. The mixtures were incubated at 37°C for 2 h; then 4 ml of fresh Dulbecco's modified Eagle's medium containing 10% fetal bovine serum plus antibiotics was added and the cultures were incubated further. To generate virus stocks, we added 10^6 uninfected CEM.SS cells to approximately 1.5×10^6 cells from the infected cultures in a total volume of 6 ml of fresh medium. These cultures were incubated for 4 to 6 days, and then supernatants were harvested and titers were determined by plaque assay (generally 1×10^4 to 2×10^4 PFU/ml). Virus stocks were stored at −70°C prior to use.

Plaque assays. Stocks of pNL4-3, pJF4A, and pJF6A were used in a quantitative plaque reduction assay to assess viral susceptibility to ddC (Hoffmann-LaRoche Inc., Nutley, N.J.), ddI (Sigma Chemical Co., St. Louis, Mo.), and AZT (Burroughs Wellcome Co., Research Triangle Park, N.C.). Plaque assays were performed as described by Larder et al. (13) with $T4^+$ HeLa cells (18) at an initial cell density of 1.5×10^4 cells per well and a virus input of 100 to 300 PFU per well.

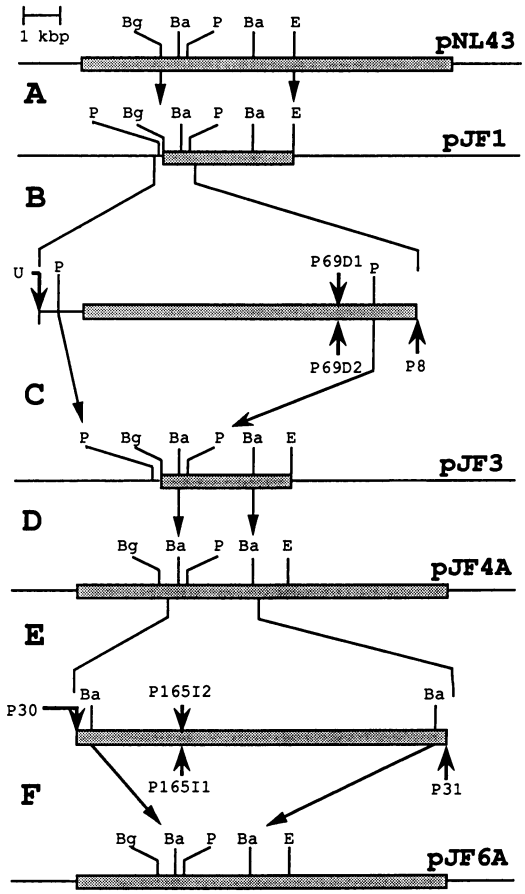

FIG. 1. Construction of molecular clones of HIV-1 carrying the Asp-69 mutations (pJF4A) or both the Asp-69 and Ile-165 mutations (pJF6A). Shaded bars depict HIV sequences. Vector sequences are shown as lines. A 3.6-kbp *Bgl*II-*Eco*RI restriction fragment carrying the pNL4-3 *pol* gene was first subcloned into the *Bam*HI-*Eco*RI-digested vector, pUC118 (step A), to generate pJF1. PCR was then used to perform site-directed mutagenesis by the overlap extension technique (9). The primer pairs P8 and P69D1, and P69D2 and the M13 universal sequencing primer (U) (28) (positions indicated by bold vertical arrows), were used to generate DNA fragments with overlapping termini which contained the Asp-69 mutations. Both fragments were included in a second PCR with the outer primers, P8 and the universal primer, to produce a 924-bp fragment (step B), which was digested with *Pst*I and used to replace the corresponding fragment of pJF1 to create pJF3 (step C). The 1.9-kbp *Bal*I fragment from pJF3 was then exchanged into pNL4-3 to produce pJF4A (step D). pJF4A was then used in the PCR with the primer pairs P165I1 and P30 and P165I2 and P31 (bold vertical arrows) to create fragments with overlapping termini containing the Ile-165 mutation. Both were included in a second PCR along with the outer primers P30 and 31 to generate a fragment of 2,062 bp (step E). This PCR product was then digested with *Bal*I and exchanged into *Bal*I-digested pNL4-3, yielding pJF6A (step F). Restriction sites: Bg, *Bgl*II; Ba, *Bal*I; Bm, *Bam*HI; P, *Pst*I; E, *Eco*RI.

A

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      63
NL4-3 IKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKQKKSVTVLDVGDAYFSVPLDK
PRE                     K                   K
POST        D           K                   K
      123
NL4-3 DFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQCSMTKILEPFRKQNPDIVIYQ
PRE               V                         S
POST              V                         S  I
      183
NL4-3 YMDDLYVGSDLEIGQHRTKIEELRQHLLRWGFTTPDKKHQKEPPFLWMGYELHPDKWTVQ
PRE
POST
      243
NL4-3 PIVLPEKDSWTVNDIQKLVGKLNWASQIYAGIKVRQL
PRE                                 P    K
POST                                P    K
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B

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                2745            2765      3033            3053
PRE-ddC  PR8    --------------------      --------------------
         PR19   --------------------      --------------------
         PR28   --g-----------------      ----------t---------
         PR36   -g------------------      --------------------
         PR37   --------------------      --------------------
         PR40   ----------a---------      --------------------
         NUC    AAAGACAGTACTAAATGGAGA      AGTAGCATGACAAAAATCTTA
         PEP    K  D  S  T  K  W  R       S  S  M  T  K  I  L
                          69                        165

POST-ddC PO5    --------------------      ----------c---------
         PO12   --------------------      --------------------
         PO21   --------------------      --------------------
         PO16   ---------acc---------      ----------c---------
         PO18   --------------------      ----c-----c---------
         PO25   --------------------      --------------------
         PO28   --------------------      --------------------
         PO30   --------------------      ----------c---------
         PO32   --------------------      ----------c---------
         PO41   --------------------      --------------------
         PO47   --g-----------------      --------------------
         NUC    AAAGACAGTGATAAATGGAGA      AGTAGCATGATAAAAATCTTA
         PEP    K  D  S  D  K  W  R       S  S  M  I  K  I  L
                          69                        165
```

FIG. 2. Alignment of reverse transcriptase, and *pol* gene, sequences. (A) Consensus peptide sequence from the amplified *pol* region. Pre- and post-ddC sequences are compared with the corresponding sequence of laboratory strain NL4-3; only differences from NL4-3 are indicated. (B) Nucleotide sequences of pre- and post-ddC clones in the immediate vicinity of residues 69 and 165. Amino acid residues are counted from the NH_2-terminal proline of reverse transcriptase, and nucleotide coordinates refer to the HIV-1 NL4-3 sequence (22). Dashes indicate agreement with the corresponding consensus (at the bottom of each set), and lowercase letters indicate nucleotides not in agreement. Boldface type indicates positions involved in AZT resistance (positions 67 and 70 [16]) and residues preferentially occurring in the post-ddC samples examined (D [Asp]-69 and I [Ile]-165).

Nucleotide sequence accession number. Complete sequence data have been submitted to GenBank under accession numbers M83276 through M83292.

RESULTS

Two peripheral blood mononuclear cell samples were obtained, one prior to ddC therapy (pre-ddC) and one after 80 weeks of therapy (post-ddC). PCR amplification products from both samples were cloned, and 6 and 11 clones from the pre- and post-ddC samples, respectively, were sequenced. The consensus amino acid sequences are shown in Fig. 2A and compared with that of the laboratory HIV strain NL4-3. The post-ddC consensus had two changes relative to the pre-ddC consensus: Thr-69 to Asp and Thr-165 to Ile. The pre- and post-ddC sequences differed from NL4-3 at six positions. Five of the six changes are to amino acids present at these positions in other wild-type isolates (22); the conservative Ile-135-to-Val change has not been reported previously.

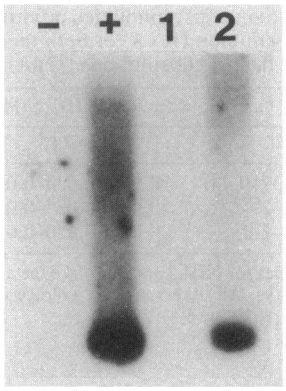

FIG. 3. Hybridization of P69D1 with PCR products from pre- and post-ddC PBM samples. Lanes: −, negative control; +, positive control; 1, DNA amplified from the pre-ddC PBM sample; 2, DNA amplified from the post-ddC PBM sample. Controls are plasmids carrying cloned *pol* gene fragments containing the wild-type sequence (−) or the Asp-69 mutations (+). Plasmids were digested with *Kpn*I and *Pst*I to release the *pol* fragment. All lanes contain approximately the same amount of DNA, except the negative control, which contains about five times as much as the others.

Nucleotide sequences in the regions of reverse transcriptase positions 69 and 165 are shown in Fig. 2B. Note that the Thr-69-to-Asp mutation requires a two-nucleotide change, ACT to GAT.

Colony hybridizations with oligonucleotides corresponding to the Asp-69 (P69D1) and Ile-165 (P165I1) mutations revealed that 18 of 23 clones from the post-ddC sample, but none of 8 clones from the pre-ddC sample, carried the Asp-69 mutations and that 15 of 23 post-ddC clones, but only 1 of 8 pre-ddC clones, carried the Ile-165 mutation (data not shown). Since the Ile-165 mutation occurred in at least one clone from both the pre- and post-ddC amplifications, it is highly unlikely to have been a PCR-induced artifact (10). The Asp-69 mutation was found only in the post-ddC clones. However, the product of a second PCR amplification performed on the post-ddC PBM DNA sample hybridized strongly to P69D1 (Fig. 3), ruling out the possibility that it was a PCR-induced artifact.

One sequenced clone from the pre-ddC sample was found to contain a single-base mutation in the Thr-69 codon (ACT to AAT), causing a change to asparagine (Asn) (Fig. 2B). Colony hybridizations with an oligonucleotide (P69N) specific for this mutation (it did not hybridize to the double mutation or the wild-type sequence) showed that 1 of the 23 post-ddC clones also carried this mutation; sequencing of the clone confirmed this result (data not shown).

To determine whether Asp-69 caused resistance to ddC, we used site-directed mutagenesis to introduce this double mutation into a plasmid (pNL4-3) carrying an infectious molecular clone of strain NL4-3 (1), yielding the mutant plasmid pJF4A (see Materials and Methods). Stocks of mutant and wild-type virus were used to assay susceptibility to ddC by plaque reduction on $T4^+$ HeLa monolayers. As summarized in Table 2, the Asp-69 mutation caused a fivefold decrease in ddC susceptibility.

Another plasmid construct, pJF6A, contained both the Asp-69 mutations and the Ile-165 mutation. The corresponding virus had a 50% inhibitory dose (ID_{50}) similar to that of JF4A (Table 2). The amino acid substitutions carried by

TABLE 2. Antiviral susceptibilities of viruses carrying the Asp-69 substitution (JF4A) or both the Asp-69 and Ile-165 substitutions (JF6A)[a]

Strain	Susceptibility (ID_{50} [μM]) to:		
	ddC	AZT	ddI
NL4-3	0.41 (0.37–0.44)	0.026 (0.024–0.027)	2.3 (2.1–2.4)
JF4A	2.2 (2.0–2.4)	0.028 (0.025–0.030)	2.6 (2.5–2.7)
JF6A	1.6 (1.4–1.8)	0.026 (0.020–0.032)	2.3 (1.7–2.9)

[a] The ddC ID_{50} values for NL4-3 and JF4A are the average of four separate plaque reduction assays. All others are the average of at least two assays. Ranges are shown in parentheses.

JF4A and JF6A had no effect on the susceptibility of the virus to AZT or ddI (Table 2).

DISCUSSION

A causal relationship between the development of resistant virus and clinical deterioration has not yet been demonstrated even for AZT. Nevertheless, we chose to study this patient because his clinical course was consistent with the possibility that mutations had occurred to cause a decrease in susceptibility to ddC. The Thr-69-to-Asp double mutation, found only in the PCR products from the post-ddC peripheral blood mononuclear cell sample, appeared to be a likely candidate. Asp-69 is adjacent to one residue (Lys-70) involved in AZT resistance and close to another (Asp-67) (16). Thus the region of reverse transcriptase from 67 to 70 had already been implicated in nucleoside triphosphate binding. Furthermore, Asp-69 was absent not only from the pre-treatment samples from this patient, and from NL43 (Fig. 2), but also from all of 22 other HIV-1 reverse transcriptase sequences previously reported (7, 16, 22).

The selectivity of the changes involved is remarkable. The Lys-70-to-Arg mutation causes an eightfold decrease in AZT susceptibility without affecting ddC susceptibility (13, 15), whereas we have shown that the Thr-69-to-Asp mutation causes a comparable decrease in ddC susceptibility without affecting AZT susceptibility. The lack of cross-resistance is encouraging with regard to the potential usefulness of combination therapy to delay or prevent outgrowth of resistant virus.

The 5-fold increase in the ID_{50} caused by the Asp-69 substitution is considerably lower than the 100-fold increase found for AZT when viruses containing all four AZT resistance amino acid substitutions were tested (16). The Asp-69 mutation may be a step toward development of higher-level resistance; evidence to this effect exists for the AZT resistance mutations involving amino acid residues 70 and 215 (15, 16). There may also be mutations conferring additional ddC resistance in portions of the gene which were not included in the PCR products amplified from our patient. Unfortunately, virus isolates from the patient studied are not available.

The Thr-69 (ACT)-to-Asn (AAT) mutation is a likely intermediate in the pathway to the Asp-69 (GAT) mutation. Whether it is a compulsory intermediate and whether it may prove to be a marker for propensity of a patient's viral population to develop ddC resistance remain to be seen.

The Ile-165 mutation did not confer additional resistance when tested in combination with Asp-69. We infer that Ile-165 is unrelated to ddC resistance. This is based partly on comparison with AZT resistance mutations, which tend to be additive or synergistic (15). In addition, Ile-165, unlike other resistance mutations thus far characterized (15, 27), occurred in a pretreatment sample. We are currently examining DNA from a second ddC-treated patient. Preliminary results indicate that Asp-69 occurs in post-ddC but not pre-ddC samples and that Ile-165 is absent from both (8). This strengthens the inference that Asp-69 is, and Ile-165 is not, a ddC resistance mutation.

After submission of this paper, St. Clair et al. (27) reported another putative ddC resistance mutation, Leu-74 to Val, which, unlike Asp-69, conferred cross-resistance to ddI. Val-74 also antagonized the effect of AZT resistance mutations. Much work must be done to catalog the spectrum of clinically significant *pol* gene resistance mutations, their interactions, and their effects on response to antiretroviral therapy.

ACKNOWLEDGMENTS

This work was supported by grant AI 25914 under the AIDS Clinical Trial Group Program of the National Institute of Allergy and Infectious Diseases. J.E.F. is an AmFAR/Pediatric AIDS Foundation Scholar. R.M.H. was supported in part by a Rutgers University Fellowship.

CEM.SS and $T4^+$ HeLa cells were obtained through the NIH AIDS Research and Reference Reagent Program; these cell lines were donated by P. L. Nara and R. Axel, respectively. The plasmid pNL4-3 was obtained from A. Rabson with permission from M. Martin. AZT was donated by the Burroughs Wellcome Co., and ddC was donated by Hoffmann-LaRoche Inc. We thank the members of the Robert Wood Johnson AIDS Clinical Trials Unit for their help.

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