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Interactions of the HIV-1 reverse transcriptase 'AZT-resistant' mutant with substrates and AZT-TP

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To investigate the biochemical basis of the HIV-1 resistance to AZT we obtained the RT mutant containing four amino acid substitutions by an oligonucleotide-directed mutagenesis technique. Enzymatic properties of the wild type and mutant RTs were compared. 'AZT-resistant' mutations in RT were shown to be associated with the reduced capability of AZT-TP to block the DNA- but not RNA-directed DNA synthesis.

HIV-1; Reverse transcriptase; 'AZT-resistant' mutation

1. INTRODUCTION

AZT is the only drug routinely available for AIDS and advanced AIDS-related complex (ARC) therapy [1,2]. The 5′-triphosphate of AZT presumably inhibits HIV replication through the termination of nascent DNA chains synthesized by HIV reverse transcriptase [3,4]. However, HIV isolates from patients who were treated with AZT for more than 6 months show reduced sensitivity to this drug [5]. Sequence analysis of such HIV isolates demonstrated the presence of several common mutations in the region coding for RT, which confer them a resistance to AZT. Amino-acid substitutions D67 → N, K70 → R, T215 → F(Y) and (in most cases) K219 → Q were found in RT from the AZT-resistant HIV isolates [6]. Kinetic analysis and primer extension experiments did not reveal significant distinctions between the w.t. and mutant RTs [6]. In this paper we demonstrate that 'AZT-resistant' mutations in RT are associated with the reduced capability of AZT-TP to block the DNA-directed DNA synthesis.

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Abbreviations. HIV-1, human immunodeficiency virus-1; RT, reverse transcriptase; CM, 'complete' mutant, containing four amino acid substitutions; AZT, AZT-MP and AZT-TP, 3′-azido-2′,3′-dideoxythymidine and its 5′-mono- and triphosphate; RNAtp, RNA template-primer; DNAtp-1, DNA template-primer 1, DNAtp-2, DNA template-primer 2; w.t., wild type.

2. MATERIALS AND METHODS

HIV-1 RT was purified from *E coli* BL21 (DE3) cells [7] harboring the expression plasmid pBRP-5. The latter was obtained by placing the HIV-1 RT gene under the control of T7 RNA polymerase promoter in the pET-3a plasmid. 'AZT-resistant' 'complete' mutant (D67 → N, K70 → R, T215 → F, K219 → Q) was generated by an oligonucleotide-directed mutagenesis technique according to Kunkel et al [8]. The mutations were verified by dideoxy-sequencing [9]. (The detailed description of pBRT-5 construction and mutagenesis will be published elsewhere.) AZT-TP was the gift of Dr. M.K. Kukhanova (Institute of Molecular Biology, Moscow). Oligonucleotides were synthesized by the phosphotriester method. Poly(rA), oligo(dT)$_{18}$ and dNTPs were purchased from Boehringer Mannheim. ^{32}P-labeled dNTPs were obtained from the Physico-Energetical Institute (Obninsk, Russia).

Heteropolymer RNA and DNA template-primers were obtained as follows. The 120 bp *Sal*GI–*Sph*I fragment of pBR322 was cloned into pTZ-18/19 vectors to give the pPV-18 and pPV-19 plasmids, respectively. Single-stranded DNA was isolated from pPV-18-harboring *E. coli* cells according to [10] and annealed with a 20 bases-long synthetic deoxyribooligonucleotide primer 5′-GCTCTCCCTTATGCGACTCC-3′ to give a DNAtp-1. To obtain RNAtp, RNA synthesized by a run-off transcription [11] of *Sal*GI-digested pPV-19 plasmid with T7 RNA polymerase was annealed with the same primer. To get DNAtp-2, the reverse sequence primer was annealed to the same single stranded DNA

RT was assayed under standard conditions (37°C, 20 min) [12] in 20 μl samples containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 5% glycerol, 0.01% Nonidet NP40, 50–100 ng of enzyme, a template–primer complex at an appropriate concentration, and dTTP (in the case of poly(rA) · oligo(dT)), or a mixture of four dNTPs (in the case of heteropolymer template). Kinetic parameters were calculated using ENZFITTER program (Elsevier Biosoft).

5′-^{32}P-end labeled primer was used in electrophoretic experiments. Standard reactions (see above) were stopped by adding a formamide dye [9], boiled for 5 min and loaded onto 12% polyacrylamide–7 M urea gels. The fidelity of DNA synthesis was tested in the same reaction mixtures in the absence of one of the four dNTPs [13]. The processivity of the DNA synthesis was examined using the salmon sperm DNA as a trap according to [14].

Table I

K_m (μM) and V_{max} (μmol/min · mg of protein) values for dTTP of the w.t. and CM reverse transcriptases

RT	Template–primer					
	Poly(rA) dT_{18}		RNAtp		DNAtp-1	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
w.t.	3.1 ± 0.6	12.0 ± 3.1	0.4 ± 0.1	1.7 ± 0.3	1.0 ± 0.2	3.4 ± 0 6
CM	2.9 ± 0.5	1.8 ± 0.5	0 2 ± 0.05	0.7 ± 0.2	0.7 ± 0.1	2.1 ± 0.5

The saturating concentrations of templates and 30 μM concentrations of dNTPs other than dTTP were used.

3. RESULTS AND DISCUSSION

Though the association between the distinct mutations in the RT gene and HIV-1 resistance to AZT was clearly demonstrated [6], the true biochemical basis of this phenomenon remains still obscure. K_m^{dTTP} and $K_i^{AZT\text{-}TP}$ values for the w.t. RT and 'complete' mutant which was isolated from AZT resistant virus were shown to be the same when poly(rA) · oligo(dT) was used as a template [6]. To further investigate its properties, we obtained CM by an oligonucleotide-directed mutagenesis technique and carried out experiments with ribo- and deoxyribonucleotide heteropolymer templates-primers (RNAtp and DNAtp-1). These templates had the same 100 base-long nucleotide sequences immediately following the 3′-end of the primer. The first nucleotide to be incorporated into the growing DNA chain was dTMP. Table I gives the kinetic parameters for the w.t.RT and CM RT for dTTP. As seen from Table I, K_m values differ only slightly, whereas V_{max} for CM is lowered by a factor of 1.5–10. The K_m^{dTTP} value on poly(rA)·oligo(dT) is close to that reported previously [6,15,16].

The K_m values of the enzymes for several templates are listed in Table II. A clear-cut distinction between DNA and RNA templates is found: in the case of CM the values for RNA templates are significantly reduced whereas for DNAtp-1 they are close. The data presented in Tables I and II suggest that mutations affect somehow the template binding whereas the dNTP binding remains virtually unchanged. These results are in accordance with recent X-ray data [17] pointing to the fact that the mutations are located in the template binding region of RT.

Table III shows the results on inhibition of the w.t. and mutant RTs by AZT-TP. Inhibition of the w.t. RT was shown to be competitive versus dTTP [15,16]. It can be seen from Table III that the K_i values depend on the template used, and therefore the inhibition cannot be defined as a 'pure competitive'. For RNAtp, the K_i value of the mutant is increased by a factor of about ten pointing to the weakening of the AZT-TP affinity to the enzyme–template complex.

Gel electrophoresis experiments were performed for further characterization of CM properties. Figs. 1 and 2 illustrate the results for RNAtp and DNAtp-1, respectively. One can see that the mutant can elongate the primer on both RNA and DNA templates with an efficiency approximating that of the w.t. enzyme (Fig. 1C, lanes 9, 10; Fig. 2C, lanes 1 and 2). The tests for processivity of DNA synthesis also showed no pronounced distinctions between the w.t. RT and CM (data not shown).

The primer extension experiments in the presence of AZT-TP revealed the marked distinction between the w.t. and mutant enzymes. AZT-TP used as the only substrate (i.e. in the absence of other dNTPs) was adequately incorporated into DNA chains in the RNA-directed synthesis catalyzed by both the w.t. and mutant RTs (Fig. 1A). In contrast to this, CM was unable to incorporate AZT-MP in the range of concentrations from 0.005 to 50 μM when DNAtp-1 was used as a template–primer (Fig. 2A). A similar pattern was ob-

Table II

K_m (nM) values for templates of the w.t. and CM reverse transcriptases

RT	Template–primer		
	Poly(rA) dT_{18}	RNAtp	DNAtp-1
w.t.	146.0 ± 7.0	1,450.0 ± 200	3.6 ± 0.8
CM	54.0 ± 15.0	190.0 ± 30	2.4 ± 0.5

The saturating concentrations of dNTPs (30 μM) were used.

Table III

Inhibition of the w.t. and CM reverse transcriptases by AZT-TP

RT	Template–primer		
	Poly(rA) dT_{18}	RNAtp	DNAtp-1
w.t.	0.030 ± 0.002	0 120 ± 0.030	0.110 ± 0 020
CM	0.090 ± 0.003	1.560 ± 0.650	0.420 ± 0.010

K_i (μM) were calculated according to competitive mode of inhibition versus dTTP The saturating concentrations of substrates were used.

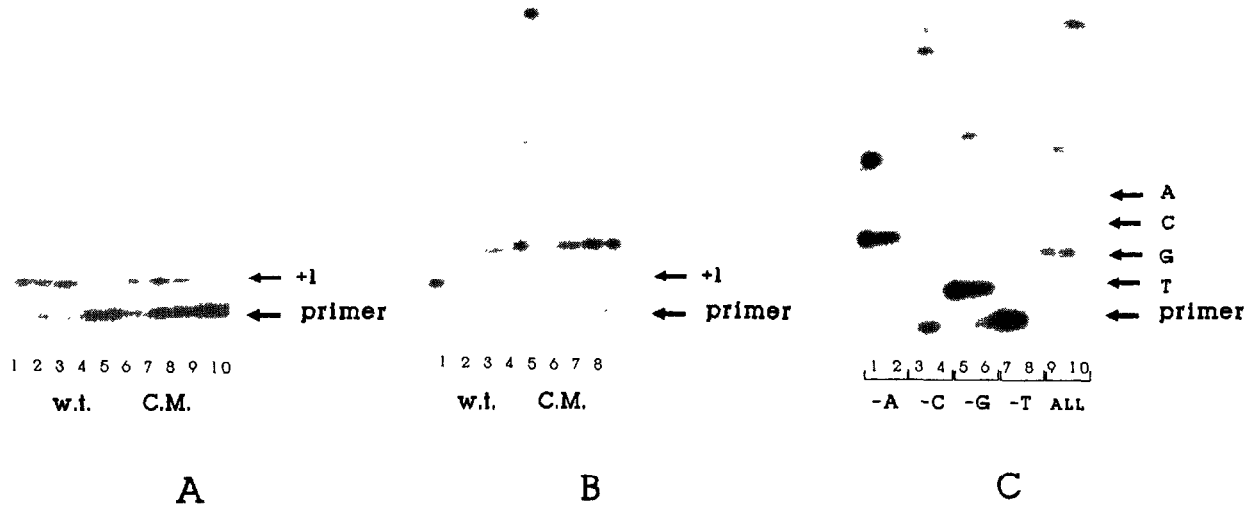

Fig. 1. RNA-directed DNA synthesis by w.t. and CM RTs. ^{32}P-labeled RNAtp (see section 2) was used as a template–primer. (A) Primer extension by the w.t. (lanes 1–5) and CM (lanes 6–10) RTs in the presence of AZT-TP as the only substrate. AZT-TP concentrations (μM): 0.005 (lanes 5, 10); 0.05 (lanes 4, 9); 0.5 (lanes 3, 8); 5.0 (lanes 2, 7); 50.0 (lanes 1, 6). (B) Primer extension by the w.t. (lanes 1–4) and CM (lanes 5–8) RTs in the presence of AZT-TP and 25 μM each of dNTPs. AZT-TP concentrations (μM): 0.05 (lanes 4, 8); 0.5 (lanes 3, 7); 5.0 (lanes 2, 6); 50.0 (lanes 1, 5). (C) The fidelity of the DNA synthesis by the w.t. (odd lanes) and CM (even lanes) RTs. All samples contained 25 μM of each dNTP except for the indicated. The positions of primer and first four dNMPs to be incorporated are shown at the right margin.

served in the presence of dNTPs with dTTP omitted (data not shown). No detectable termination by AZT-TP was observed in the DNA- but not RNA-directed synthesis when all four dNTPs were present (Figs. 1B and 2B).

DNAtp-2 was used as an alternative DNA template–

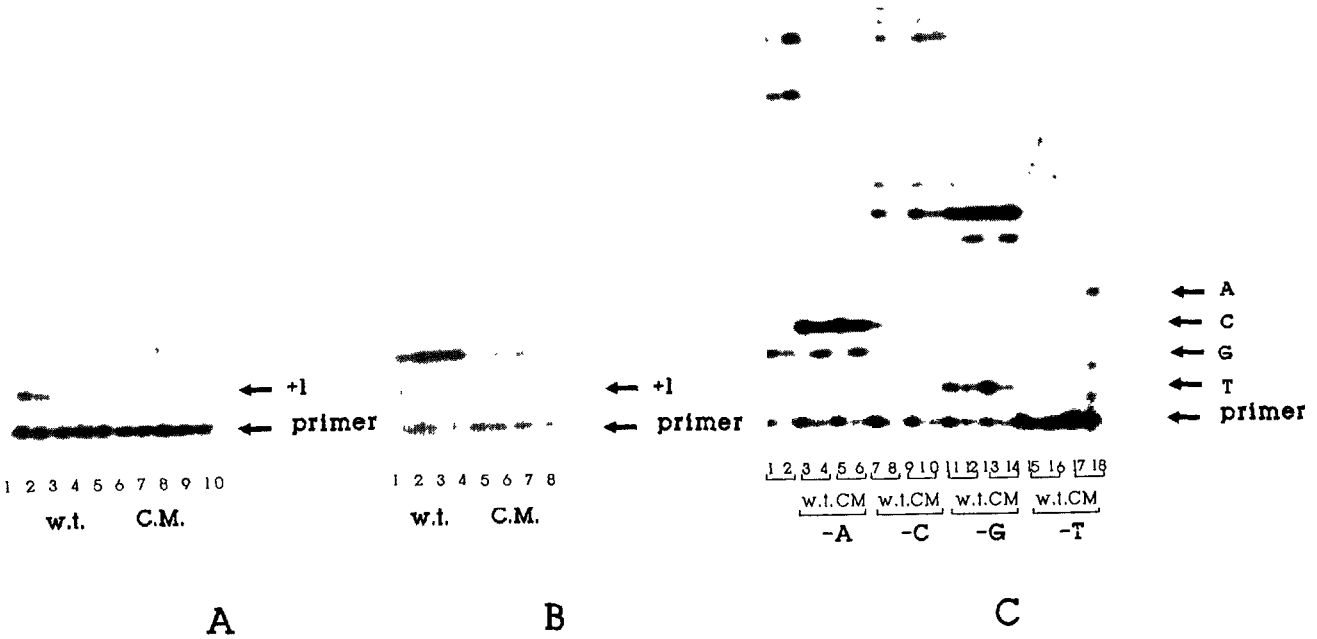

Fig. 2. DNA-directed DNA synthesis by w.t. and CM RTs. ^{32}P-labeled DNAtp-1 (see section 2) was used as a template–primer. (A) Primer extension by the w.t. (lanes 1–5) and CM (lanes 6–10) RTs in the presence of AZT-TP as the only substrate. AZT-TP concentrations (μM): 0.005 (lanes 5, 10); 0.05 (lanes 4, 9); 0.5 (lanes 3, 8); 5.0 (lanes 2, 7); 50.0 (lanes 1, 6). (B) Primer extension by the w.t. (lanes 1–4) and CM (lanes 5–8) RTs in the presence of AZT-TP and 25 μM each of dNTPs. AZT-TP concentrations (μM): 0.05 (lanes 4, 8); 0.5 (lanes 3, 7); 5.0 (lanes 2, 6); 50.0 (lanes 1, 5). (C) The fidelity of the DNA synthesis. The lanes 1 (w.t.) and 2 (CM) are control of synthesis in the presence of all four dNTPs. From the third lane the samples contained either 12.5 μM (odd lanes) or 50 μM (even lanes) of each dNTP, except for the indicated.

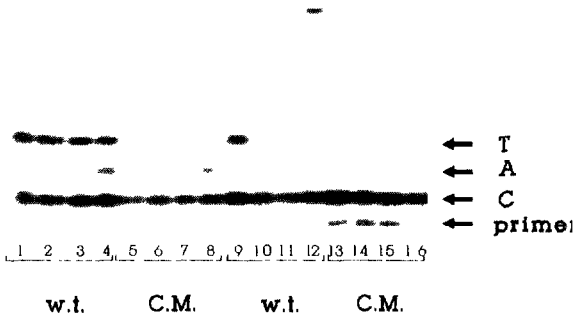

Fig. 3. DNA-directed DNA synthesis by w.t. and CM RTs. ^{32}P-labeled DNAtp-2 (see section 2) was used as a template–primer. Primer extension by w.t. (lanes 1–4) and CM (lanes 5–8) RTs in the presence of AZT-TP and 25 μM each of dNTPs, when dTTP omitting, and in the presence of all dNTPs (lanes 9–12 = w.t., lanes 13–16 = CM). AZT-TP concentrations (μM): 0.05 (lanes 4, 8, 12, 16), 0.5 (lanes 3, 7, 11, 15); 5.0 (lanes 2, 6, 10, 14); 50.0 (lanes 1, 5, 9, 13). The strong stop on dCMP at position +1 was observed for both the w.t. and CM.

primer. In this case dTMP was the third nucleotide to be incorporated into the growing DNA chain (Fig. 3). When dTTP was omitted from the reaction mixture, only traces of AZT-MP incorporation by CM were detected. In the presence of all four dNTPs no detectable incorporation of azidonucleotide was demonstrated over the wide range of AZT-TP concentrations.

It is noteworthy that the reduced capability of CM to use AZT-TP as a substrate is not connected with variations in fidelity of DNA synthesis. As seen from the Figs. 1C and 2C, no visible distinctions between the w.t. RT and CM in the DNA synthesis are detected when one of the four dNTPs is omitted. It is precisely the interactions of the enzyme with AZT-TP which are affected by the mutations. An indirect evidence in favour of this hypothesis is the sensitivity of AZT-resistant HIV-1 isolates to an alternative 'terminator-like' drug, dideoxycytidine [18].

The data obtained show that the mutations result in moderate decreases in the V_{max} values with both RNA and DNA templates. Distinct changes of the template affinity were detected for heteropolymer RNA only. The AZT-TP affinity of CM was decreased, once again the effect being more pronounced when using the heteropolymer RNA template. In this regard the latter is more suitable than a commonly used poly(rA) · oligo(dT).

It is evident that the mutations affect the template binding only (as X-ray data also suggest), whereas dNTP's interactions remain practically unchanged. The mutations result in an increase in RT affinity to RNA template, whereas the DNA template affinity remains the same. Taking into account a large difference in the K_m values for RNA and DNA templates of the same nucleotide sequences, one can suggest that some structural distinctions between the w.t. RT complexes with RNA and DNA exist. It seems that the mutations contribute to these distinctions so that AZT-TP utilization by CM in RNA- and DNA-directed synthesis has considerable differences. In the latter case, the binding of AZT-TP in the dNTP site of the enzyme results in the formation of unproductive ES complex (this effect is more pronounced when dTMP is the first nucleotide to be incorporated) and reveals itself in the inhibitory effect.

Relying on the data obtained some considerations can be applied to the original rise of the HIV-1 resistance to AZT. According to current concept, both RNA- and DNA-directed DNA synthesis in HIV-1 replication is catalyzed by RT [19]. These stages are variously affected by mutations. A more than ten-fold reduction in the AZT-TP affinity of the mutant enzyme results in a drop in the chain termination during the course of the RNA-directed synthesis. In the second stage of replication (in the presence of all four dNTPs) no effective termination happens at all and therefore the production of complete DNA copies of the viral RNA, even if at a lower rate, is a distinct possibility.

The data obtained suggest an association between RT mutations and changes in its enzymatic properties. However, it is not inconceivable that additional mechanisms are also involved in the total AZT therapeutic effect.

REFERENCES

[1] Yarchoan, R., Klecker, R.W., Weinhold, K.J., Markham, P.D., Lyerly, H.K., Durack, D.T., Gelmann, E., Nusinoff-Lehrmann, S., Blum, R.M., Barry, D.W., Shearer, G.M., Fischl, M.A., Mitsua, H., Gallo, R.C., Collins, J.M., Bolognesi, D.P., Myers, C.E. and Broder, S. (1986) Lancet 1, 575–580.
[2] Mitsua, H., Yarchoan, R. and Broder, S. (1990) Science 249, 1533–1544.
[3] St. Clair, M.H., Richards, C.A., Spector, T., Weinhold, K.J., Miller, W.H., Langlois, A.J. and Furman, P.A. (1987) Antimicrob. Agents Chemother. 31, 1972–1977.
[4] Matthes, E., Lehman, Ch., Scholz, D., von Janta-Lipinski, M., Gaertner, K., Rosental, H.A. and Langen, P. (1987) Biochem. Biophys. Res. Commun. 148, 7885–7890
[5] Larder, B.A., Darby, G. and Richman, D.D. (1989) Science 243, 1731–1736.
[6] Larder, B.A. and Kemp, S.D. (1989) Science 243, 1155–1158.
[7] Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Methods Enzymol. 185, 60–89.
[8] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 367–382.
[9] Saenger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[10] Vieira, J. and Messing, J. (1987) Methods Enzymol. 153, 3–11.
[11] Sampson, J.R. and Uhlenbeck, O. (1988) Proc. Natl. Acad. Sci. USA 85, 1033.
[12] Rechinsky, V.O., Barbashov, S.F., Degtyarev, I.L., Vorobyev, S.M., Lyakhov, D.L., Mishin, A.A., Kostyuk, D.A., Starov, A.I., Matsevich, A.R. and Kochetkov, S.N. (1991) Mol. Biol. (Moscow) 25, 1248–1257.
[13] Preston, B.D., Poiesz, B.J. and Loeb, L.A. (1988) Science 242, 1168–1171.

[14] Dudding, L.R., Nkabinde, N.C. and Mizrahi, V. (1991) Biochemistry 30, 10498–10506.
[15] Kedar, P.S., Abbots, J., Kovacs, T., Lesiak, K., Torrence, P. and Wilson, S.H. (1990) Biochemistry 29, 3603–3611.
[16] Parker, W.B., White, E.L., Shaddix, S.C., Ross, L.J., Buckheit, R.W., Germany, J.M., Secrist III, J.A., Vince, R. and Shannon, W.M. (1991) J. Biol. Chem 266, 1754–1763.
[17] Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A and Steitz, T.A. (1992) Science 256, 1783–1790.
[18] St Clair, M.H., Martin, M.H., Tudor-Williams, G., Bach, M.C., Vavro, C.L., King, D.M., Kellam, P., Kemp, S.D. and Larder, B.A. (1991) Science 253, 1557–1559.
[19] Peliska, J.A. and Benkovic, S.J. (1992) Current Biol. 2, 521–523.