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〈*Original Article*〉

RNA-dependent DNA Polymerase (RT) Activity of Bacterial DNA polymerases

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ABSTRACT

RNA-dependent DNA polymerase (RT) is an enzyme that synthesizes DNA from an RNA template in retroviruses. The Klenow fragment (KF), part of *E. coli* DNA polymerase I (Pol I), is similar to RT molecule and exhibits RT activity. The HIV-1 RT inhibitor, AZT, has been reported to be bactericidal against some species of bacteria. This finding indicates that AZT targets bacterial polymerase which possesses RT activity. In this study, we analyzed the RT activities of *E. coli*, *Shigella sonnei* I, *Vibrio cholerae* non-O1, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Except in *Staphylococcus aureus*, AZT-TP inhibited bacterial RT activities. Expect in *P. aeruginosa*, these results corresponded to the effect of AZT against these bacterial species. We further determined the similarity of bacterial DNA polymerases by examination using inhibitory antibodies obtained from a rabbit immunized with *E. coli* KF (anti-KF). Anti-KF inhibited the RT activities of *E. coli* and *S. sonnei* I. Our findings indicate that the bacterial DNA polymerases which possess RT activities may be targets of antibiotic therapy.

Introduction

Reverse transcriptase (RT), an RNA-dependent DNA polymerase, is an enzyme that synthesizes DNA from an RNA template in retroviruses (BALTIMORE et al., 1970; GALLO et al., 1970). In bacteria, RT encoded in the retron has been reported as a mobile element for producing multicopy single-stranded DNA of the RNA-DNA complex of unknown functions (TEMIN., 1989). The activity of RNA-dependent DNA polymerization is also found in other DNA polymerases. One of these enzymes is *Escherichia coli* DNA polymerase I (Pol I), which is the first DNA-dependent DNA polymerase to be purified; Pol I has been extensively studied (EGAR et al., 1991; RICCHTTI et al., 1993). The biological significance of the RT activity of Pol I as well as that of retron RT has not yet been clarified. The Klenow fragment (KF) is part of *E. coli* Pol I, a monomer of 68 kDa, and exhibits DNA-dependent DNA polymerase (DDDP) and RT activities (LOEB et al., 1973; NAKANO et al., 1994; RICCHTTI et al., 1993). Recent studies of DNA polymerases, particularly KF and human immunodeficiency virus (HIV) RT, have led to the proposal that nucleic acid polymerases show fundamental similarities in structure and catalytic mechanism

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(JOYCE et al,. 1995; STEITZ et al., 1993). Those findings explain why an inhibitor of HIV-1 RT possesses an antibacterial effect in *E. coli* (EGAR et al., 1991).

HIV-RT inhibitors are divided into two groups (KOHLSTAEDT., 1992); nucleotide analogs and non-nucleotide analogs. Nucleotide analogs, such as dideoxythymidine (AZT), dideoxycytidine (ddC) and dideoxyinosine (ddI), have been designed and applied to the clinical treatment of HIV infections (MITSUYA et al., 1990). Among nucleotide analogs, AZT is a potent bactericidal agent against many members of the family *Enterobacteriaceae*, including strains of *E. coli*, *Salmonella typhimurium*, *Klebshiella pneumoniae*, *Shigella flexneri*, and *Enterobacter aerogenes* (ELWELL et al., 1987). Elwell et al. (1987) reported that AZT is a substrate of *E. coli* deoxythymidine (dThd) kinase, and that AZT triphosphate (AZT-TP), an active form of AZT (HUANG et al., 1995), functions in chain termination in the DDDP activity of KF. Their findings indicate that AZT targets *E. coli* Pol I, which is structurally similar to the RT molecule, and suggest that AZT-sensitive bacterial DNA polymerases possess RT activity. Since it is not clear whether DNA polymerases of other bacteria possess RT activity, we measure the RT activity of DNA polymerases in five bacterial species, and examine the response to AZT and the antigenic similarity of these enzymes.

Materials and methods

Bacteria and preparation of crude bacterial homogenate

The bacteria listed in Table 1 were cultured overnight at 37 ℃ in heart infusion broth. Bacterial colonies were suspended in 0.15 M phosphate-buffered saline (pH 7.2) with McFarland #2, and the suspension was vortexed with 3-mm-diameter glass beads for 10 min to prepare a homogenous suspension. The suspension was centrifuged at 1,360 x g for 15 min, and the pellet was resuspended in a lysis buffer containing 0.5% Triton X-100 in 50 mM Tris-HCl (pH 7.8). To lyse bacterial cells, the suspension was sonicated in ice-cold water bath for 15 min using an ultrasonic washer (W-208, Masuda, Tokyo, JAPAN). The bacterial solution was then centrifuged at 1,360 x g for 15 min. The supernatant was used in the measurement of polymerase activity. In some experiments, bacterial homogenates were prepared with BugBuster reagent (Merck Tokyo, Tokyo, Japan). Briefly, bacterial cells were harvested from a liquid culture and then centrifuged at 10,000 x g for 10 min. For small-scale extraction (1.5 ml or less), centrifugation was performed using a 1.5-ml tube at 14,000–16,000 x g for 15 min. The cell pellet was resuspended in BugBuster reagent at a concentration of 1 gram per 5 ml reagent. The supernatant was used in the measurement as above.

Polymerase assay

Polymerase activity was assayed using a non-radioisotopic assay kit (NAKANO et al., 1994). The samples were mixed with 2X lysis buffer containing 0.5% Triton X-100 in 50 mM Tris-HCl buffer (pH 7.8). Recombinant KF (rKF) was purchased from Takara Co. (Shiga, Japan) and recombinant HIV-1 RT (rHIV-1 RT) from Seikagaku Co. (Tokyo, Japan). These polymerases were used as positive controls for DNA polymerases. The non-RI assay kit for RT and DDDP consisted of 1) aminated microtiter plates (Sumitomo BakeLite Inc. Tokyo, Japan) containing fixed poly(rA)-oligo(dT) for RT or poly(dA)-oligo(dT) for DDDP, 2) a 2X polymerase chain reaction mixture consisting of 6 μM biotin-11-dUTP, 235 μM dTTP, 18 mM $MgCl_2$, 0.9 mM dithiothreitol (DTT), 2.16 mM glutathione (reduced), 0.36% Triton X-100, 3.6% ethyleneglycol, 252 mM KCl and 0.02% NaN3 in 180 mM HEPES buffer (pH 7.8), 3) 83 ng/ml alkaline phosphatase (ALP)-labeled streptavidin (SA) in 50 mM Tris-HCl buffer (pH 7.5), 4) ALP substrate solution containing 1 mg/ml para-nitrophenylphosphate and 1 mM $MgCl_2$ in 0.5 M diethanolamine-HCl buffer (pH 9.5), 5) 5 M NaCl RT stopping solution, 6) 1 N NaOH as ALP stopping solution, and 7) washing buffer containing 0.02% Tween 20, 0.1 M NaCl, and 0.05 M $MgCl_2$ in 0.05 M Tris-HCl buffer (pH 7.5). A 50-ml portion of each sample was mixed with the same volume of the 2X polymerase reaction mixture in a well with immobilized template primers and incubated at 37 ℃ for 1 hr. Polymerase reaction was terminated by adding 10 μl of RT stopping solution, and placing the mixture at room temperature for 5 min. The well was washed with the washing buffer. SA-ALP solution (100 μl) was added followed by incubation at 37 ℃ for 60 min. The well was washed again with the washing buffer. After washing, 150 μl of ALP substrate solution was added and the microplate was incubated at 37 ℃

for 30 min prior to the addition of 50 μl of ALP stopping solution. Optical density (O.D) was measured using a microplate reader (NJ 2001, Nippon InterMed Inc. Tokyo, Japan) at a wavelength of 405 nm.

All the experiments were performed in triplicate, and the mean O.D was determined. The buffers were measured in triplicate, and the mean ± 2SD was determined as the cutoff value.

Polyclonal antibody against Klenow fragment (anti- KF)

A bulk of purified recombinant *E. coli* DNA polymerase I (Klenow fragment) was purchased from Takara Co. (Shiga, Japan). A 10-week-old male rabbit was immunized by the subcutaneous injection of 0.20 mg of Klenow fragment on the 0th, 14th and 38th day. The production of an antibody against the polymerase was confirmed by Western blot analysis. In brief, 10 μl of polymerase solution was electrophoresed in a 10% NuPAGE Bis-Tris gel (Invitrogen, Tokyo, Japan) plate and blotted onto a PVDF membrane (Millipore, MA 01821, USA). The membrane was reacted with rabbit immune serum and HRP-labeled goat anti-rabbit IgG (Cappel, Ohio, USA). We used this rabbit immune serum as anti-KF.

Results and discussion

Measurement of DNA polymerase activity of rKF and E. coli *lysate*

To determine whether our assay is suitable for the detection of DNA polymerase activity, the activities of rKF and *E. coli* lysates, and rHIV-1 RT were measured (**Fig. 1**). A positive signal for DDDP activity was detected in the poly(dA)-immobilized wells with rKF and *E. coli* lysates, but not in the wells with rHIV-RT. In the poly(rA) immobilized wells, a positive signal for RT activity was detected for all the samples. Although a previous report (RICCHTTI et al., 1993) indicated that KF expresses a higher DNA-polymerase activity with a poly(dA) template than with a poly(rA) template, and that the polymerase activities for RT and DDDP are not comparable to each other in this non-RI RT assay. The probable reason for this is that the amount of immobilized poly(dA) template is not same as the amount of poly(rA) template. The DDDP and RT activities of rKF increased with incubation time (**Fig. 1-a**). Both activities of *E. coli* lysate also increased in a time-dependent manner at least in the initial 45 min of incubation (**Fig. 1-b**). rHIV-1 RT showed an increase in RT activity, but not in DDDP activity (**Fig. 1-c**), as Masquelier et al. (1991) reported.

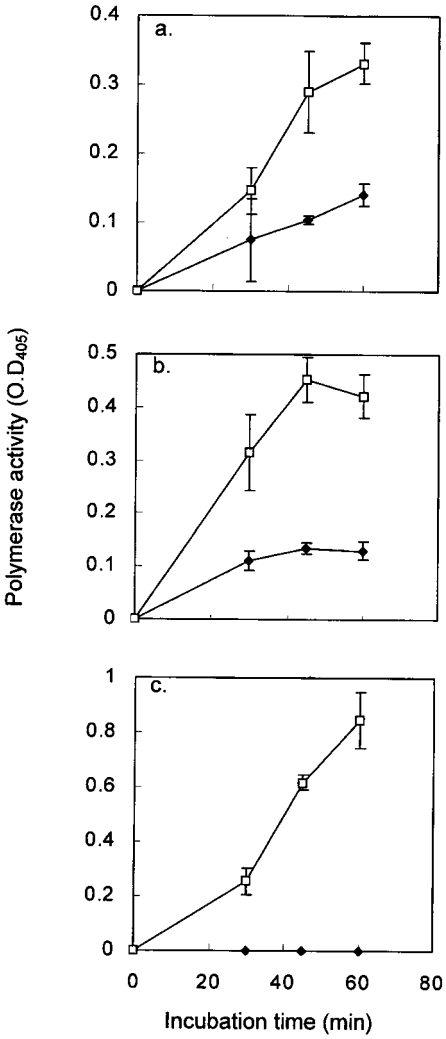

Fig. 1: Time course of polymerase activities. DNA polymerase activities of rKF, *E. coli* lysate and rHIV-1 RT were measured upon extended incubation. RT (□) and DDDP (◆) activities were evaluated on each photograph. (a) Both DDDP and RT activities of rKF increased with incubation time. (b) DDDP and RT activities of E. coli lysate increased in a time-dependent manner within 45 minutes. (c) rHIV-1 RT revealed an increase in RT activity only.

To determine whether the assay is quantitative, DNA polymerase activity was determined at different concentrations (**Fig. 2**). The polymerization reactions of rKF (**Fig. 2-a**), *E. coli* lysate (**Fig. 2-b**), and rHIV-1 RT (**Fig. 2-c**) were enhanced in a concentration-dependent manner. The R^2 values

for the dependence were between 0.965 and 0.996. Because both RT and DDDP activities were quantitatively measured in purified DNA polymerases and *E. coli* lysate, we used the non-RI RT assay in subsequent experiments.

To determine whether a signal for DNA polymerase can be inhibited by an RT inhibitor, the rKF and *E. coli* lysates were analyzed in the presence of AZT-TP (Sigma), the active form of AZT (**Fig. 3**). The percentage inhibitions of the

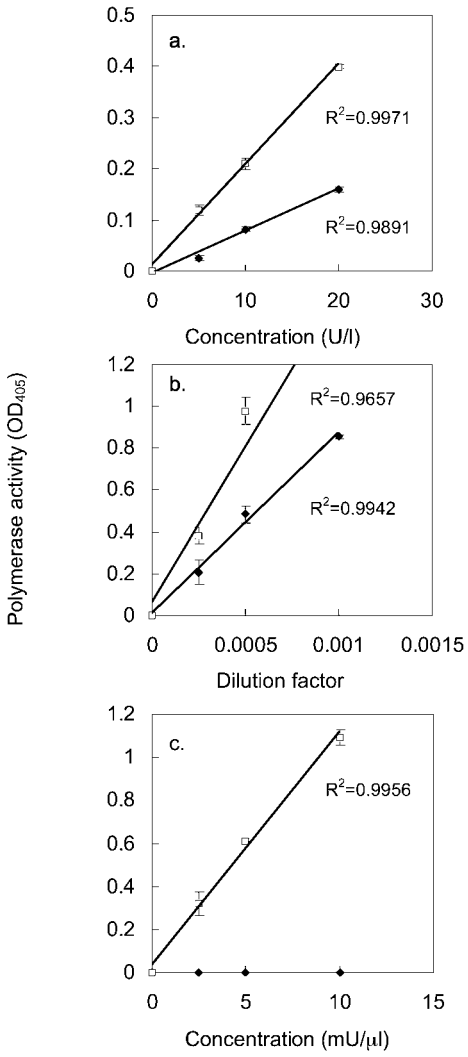

Fig. 2: Concentration dependence of polymerase activities of rKF and *E. coli* lysate and rHIV-1 RT.
To evaluate whether the assay is quantitative, polymerases at different concentrations were examined in terms of their activities. RT (□) and DDDP (◆) activities were evaluated on each photograph. The polymerization reactions of rKF (a), *E. coli* lysate (b) and rHIV-1 RT (c) were enhanced in a concentration-dependent manner. The R^2 values for the dependence were between 0.965 and 0.996.

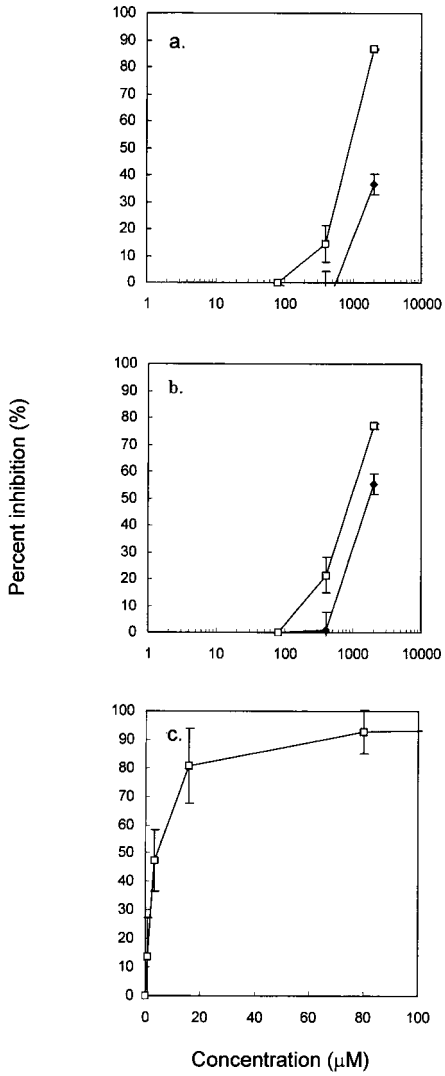

Fig. 3: Inhibition of DDDP and RT activities in the presence of AZT-TP.
RT (□) and DDDP (◆) activities of rKF (a), *E. coli* lysate (b) and HIV-1 RT (c) in the presence of AZT-TP were evaluated in vitro. (a) AZT-TP higher than 400 mM showed the concentration-dependent inhibition of both DDDP and RT activities of rKF. (b) AZT-TP higher than 400 mM showed the concentration-dependent inhibition of both DDDP and RT activities of E. coli lysate. (c) AZT-TP lower than 100 mM revealed the concentration-dependent inhibition of rHIV-RT activity.

signals for both DDDP and RT activities of rKF increased at concentrations higher than 400 mM (Fig. 3-a). When the DNA-polymerizing activity of *E. coli* lysate was measured in the presence of AZT-TP at different concentrations, the percentage inhibitions of the signals for both DDDP and RT activities increased in a concentration-dependent manner (Fig. 3-b). AZT-TP increased the percentage inhibition of the signal for rHIV-1 RT activity in a concentration-dependent manner (Fig. 3-c), as previously reported (MASQUELIER et al., 1991). Since rHIV-1 RT did not catalyze the DDDP reaction, its effect on DDDP activity could not be evaluated. These findings indicate that the assay system can detect DNA polymerase activity inhibited by an RT inhibitor.

Polymerase activities of other bacterial species

Elwell et al. (1987) reported that an RT inhibitor blocks the growth of *E. coli* and other species of bacteria. Their findings indicate that other bacteria may possess a DNA polymerase similar to *E. coli* Pol I, that is, a polymerase that exhibits both RT and DDDP activities. To determine RT activity in bacteria, we analyzed the lysates of five bacterial species for RT and DDDP activities (Table 1). We determined the cutoff values to be 0.130 and 0.147 for RT and DDDP activities, respectively. Both DDDP and RT activities were detected in the extracts of all the five bacterial species examined.

RT activities may be induced by the retrotranscribing enzymes of retrons. Retroelements in bacteria were discovered in studies of a novel satellite DNA, called the multicopy single-stranded DNA (msDNA), in *Myxococcus xanthus*, a gram-negative soil bacterium (DHUNDALE et al., 1987) Further studies of the biosynthesis of msDNA led to the proposal that msDNA is synthesized by RT (DHUNDALE et al., 1985). The entire genetic unit responsible for msDNA synthesis is the retron, which is assumed to be the most primitive retroelement. RT activity in the gram-negative rods examined might be expressed by retron RT. We preliminarily compared RT activity between retron-positive and -negative *V. cholerae* O139 strains, and found that

Table 1. Polymerase activities of various bacterial species

Species	Strain/origin	Polymerase activity (OD_{405}) DDDP			RT		
Escherichia coli	JC2	0.434	±	0.016	0.927	±	0.044
Shigella sonnei I	AQ2-7060	0.413	±	0.028	0.924	±	0.036
Vibrio cholerae non-O1	AQ1397	0.556	±	0.029	0.802	±	0.015
Pseudomonas aeruginosa	ATCC27853	0.510	±	0.106	0.740	±	0.037
Staphylococcus aureus	FDA 209P	0.392	±	0.010	0.302	±	0.007

Cutoff value: 0.130 for RT, 0.147 for DDDP
AQ strains were provided by Osaka Quarantine Station, Japan.

Table 2. Effect of AZT-TP on lysates of several bacterial species

Species	Strain/origin	% inhibition DDDP			RT		
Escherichia coli	JC2	33.6	±	1.5	42.5	±	3.4
Shigella sonnei I	AQ2-7060	15.3	±	4.6	25.7	±	3.4
Vibrio cholerae non-O1	AQ1397	0.0			39.8	±	2.4
Pseudomonas aeruginosa	ATCC27853	0.0			25.7	±	7.4
Staphylococcus aureus	FDA 209P	0.0			0.0		
rKlenow fragment		35.2.	±	4.3	45.0	±	6.2
rHIV-RT		N.T			*91.3	±	5.4

Polymerase activities without (-) and with (+) 1000 mM AZT-TP (*200 mM AZT-TP) were evaluated and listed as % inhibition.
N.T: not tested.

extracts from both strains exhibit RT activity (data not shown). These findings indicate that RT activity in bacterial extracts is not always derived from retron RT.

To confirm whether the polymerase activities of the five bacterial species are inhibited by AZT-TP, the DDDP and RT activities of these species were measured in the presence of 1,000 mM AZT-TP, and the percentage of inhibition was calculated (Table 2). For *E. coli* and *S. sonnei* I, both the DDDP and RT activities were inhibited by AZT-TP. For *V. cholerae* non-O1 and *P. aeruginosa*, RT activity was inhibited, but not DDDP activity. For *S. aureus*, neither DDDP nor RT activities were inhibited. Expect in *P. aeruginosa*, these findings support the notion that *Enterobacteriaceae* bacteria are sensitive to AZT but not *P. aeruginosa*, as Elwell (1987) et al. reported. In *P. aeruginosa*, its growth was not inhibited by AZT, however, AZT-TP inhibited RT activity in its lysate. This discrepancy may be explained by an efflux pump of the bacterium.

Lysate of several bacterial species against anti- KF

We further compared bacterial DNA polymerases by examination using inhibitory antibodies obtained from a rabbit immunized with *E. coli* KF (paper submitted elsewhere). The antibody efficiently inhibited the DDDP and RT activities of KF and *E. coli* lysates, but not the HIV-1 RT activities (Table 3). For *S. sonnei* I, both RT and DDDP activity was inhibited at the same level as that for *E. coli*. This result supported the idea that *Shigella spp.* are known to belong to the *E. coli* genomospecies (BRENNER et al., 1972) For *V. cholerae* non-O1, *P. aeruginosa* and *S. aureus*, neither DDDP nor RT activities were inhibited significantly. For *E. coli*, *S. sonnei* I, and *S. aureus*, this result corresponded to the case of the AZT-TP treated strains. Thus, the anti-KF may have species specificity.

Table 3. Lysates of bacterial species against anti-KF

Species	Strain/origin	% inhibition					
		DDDP			RT		
Escherichia coli	JC2	82.0	±	2.3	88.8	±	5.3
Shigella sonnei I	AQ2-7060	71.4	±	2.8	79.7	±	3.2
Vibrio cholerae non-O1	AQ1397	45.1	±	2.6	16.8	±	7.5
Pseudomonas aeruginosa	ATCC27853	26.3	±	3.3	0.0		
Staphylococcus aureus	FDA 209P	0.0			3.6	±	1.6
rKlenow fragment		82.0	±	5.7	88.6	±	4.5
rHIV-RT		N.T			6.9	±	8.4

Polymerase activities without and with anti-KF were evaluated and listed as % inhibition.

In this study, we confirmed the previously reported findings that human pathogenic bacteria including *E. coli* are sensitive to AZT, and that the RT activity of KF is inhibited by AZT-TP in comparison with native nucleic acid polymerase in the *E. coli* lysate. Furthermore, we discovered that many human pathogenic bacteria have enzymes that exhibit RT activities, and that the RT activities of some of them are inhibited by RT inhibitors. These results lead to the new concept of antibiotic functions. Since quinolone inhibits DNA polymerization by targeting DNA gyrase (MIZUUCHI et al., 1978), the concept of using an RT inhibitor as an antibiotic is new. Since the AZT-TP concentration required for inducing the desired effect, RT inhibitors are not suitable for use as therapeutic agents for bacterial infection. However, further investigation might lead to the development of ideal RT inhibitors for antibacterial agents. We will also search for an effective drug on the basis of previous HIV-1 studies, as AZT was initially developed as an antineoplastic drug (MITSUYA et al., 1985) and successfully applied to the treatment of AIDS. To date, many RT inhibitors have been developed but many of them have been rejected for use in the treatment of HIV-1 infection. Such rejected analogs can be re-examined for their efficacy against bacteria. When a specific inhibitor is discovered, it can be used to clarify the function of bacterial and retron RTs, even if it is not suitable for antibiotic therapy.

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