Differential Inhibitory Effects of Various Herb Extracts on the Activities of Reverse Transcriptase and Various Deoxyribonucleic Acid (DNA) Polymerases

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Forty preparations of the extracts from 28 kinds of Asian herbs were tested for ability to inhibit the activities of murine retroviral reverse transcriptase and human deoxyribonucleic acid (DNA) polymerases. Among the 40 extracts, 35 inhibited reverse transcriptase activity and 29 inhibited DNA polymerase α activity. The inhibitory potencies of these extracts were expressed as the 50% inhibition concentrations (IC_{50}), at which the enzyme activities were inhibited by 50%. Very strong inhibitions were observed with the extracts from *Millettia pachycarpa* (Leguminosae) and *Mallotus apelta* (Euphorbiaceae) as shown by their low IC_{50} values for reverse transcriptase (0.4—0.5 μg/ml) and DNA polymerase α (0.9—1.4 μg/ml). Enzyme kinetic analysis revealed that the mode of inhibition of reverse transcriptase by these two extracts was competitive with respect to the template·primer [poly(rA)·oligo(dT)] and noncompetitive with respect to deoxythymidine triphosphate (dTTP) substrate. Besides reverse transcriptase and DNA polymerase α, DNA polymerase I and ribonucleic acid (RNA) polymerase from *E. coli* were inhibited by these two extracts. These results indicate that the herb extracts contain as yet unidentified substance(s) which inhibit the activities of reverse transcriptase and cellular DNA polymerases.

Keywords herb extract; reverse transcriptase; DNA polymerase; enzyme inhibition; *Millettia pachycarpa*; *Mallotus apelta*

Reverse transcriptase has been regarded as one of the appropriate targets for antiretroviral agents since ribonucleic acid (RNA)-dependent deoxyribonucleic acid (DNA) synthesis (provirus synthesis) is prerequisite for the infection of host cells by retroviruses. Many antiretroviral compounds have, therefore, been designed for the inhibition of reverse transcriptase activity. Unfortunately, however, most of the antiretroviral compounds so far proven to be effective also inhibit the cellular DNA polymerase(s). This fact seems to explain at least a part of the serious side effects observed among acquired immunodeficiency syndrome (AIDS) patients receiving these drugs. Suramin, for example, inhibits reverse transcriptases from various sources,[1] and suppresses infection by and proliferation of human immunodeficiency virus *in vitro*.[2] This compound, however, is cytotoxic to cultured lymphoblastic cells,[3] and often causes side effects such as renal insufficiency, *etc.* when administered to AIDS patients. Suramin has also been shown to inhibit various kinds of DNA polymerases purified from human cells and *E. coli*,[4] in agreement with its cytotoxic effect on cultured cells.[3] Such inhibitory effects on cellular DNA polymerases were also observed with 21-tungsto-9-antimoniate (HPA23),[5] Evans Blue and aurintricarboxylic acid.[6] High selectivity for the inhibition of reverse tanscriptase is, therefore, a necessary condition for efficient antiretroviral drugs.

There are some natural compounds which have been reported to be inhibitory to reverse transcriptase and various DNA polymerase activities. For example, some tannins inhibit reverse transcriptase from avian myeloblastosis virus,[7] and gossypol, which has been isolated from cotton seed, inhibits DNA polymerase from HeLa cells.[8] In order to find more selective compounds for the inhibition of human retrovirus replication, but with lower toxicity to the host cells, we have been looking for inhibitory substances from natural sources including insects and plants.

In this report, we present the results of screening tests of 40 preparations of herb extracts for the ability to inhibit reverse transcriptase preferentially over DNA polymerase α, which plays the most important role in eukaryotic cellular DNA replication. Serveral kinds of extracts have been shown to have differential inhibitory effects on the activities of reverse transcriptase and other DNA polymerases including α-polymerase.

Results

Screening Test of Various Herb Extracts for the Inhibition of Reverse Transcriptase and DNA Polymerase α The first screening test was carried out at extract concentrations between 100 and 500 μg/ml. Nineteen out of the 40 extracts were found to strongly inhibit reverse transcriptase activity and 18 inhibited DNA polymerase α activity almost completely (more than 95% inhibition was achieved at a concentration of 100 μg/ml). The second screening test was performed at concentrations between 10 and 50 μg/ml with the extracts which showed strong inhibitions in the first screening test. Thirteen extracts inhibited reverse transcriptase activity and 5 inhibited DNA polymerase α activity by more than 90% at a concentration of 10 μg/ml. The third test was performed in the concentration range of 1 to 5 μg/ml. Seven extracts inhibited reverse transcriptase activity by more than 80% at a concentration of 5 μg/ml. As to DNA polymerase α, 2 extracts inhibited the enzyme activity to a similar extent. The 50% inhibition doses (IC_{50}) were determined from the dose–response curves and are summarized in Table I.

Kinetic Analysis of the Inhibition of Reverse Transcriptase by the Extracts from *Millettia pachycarpa* and *Mallotus apelta* Among the extracts examined, those from *Millettia pachycarpa* and *Mallotus apelta* exhibited especially strong inhibitory effects on reverse transcriptase as shown by their low IC_{50} values (0.4—0.5 μg/ml). Therefore, the mode of inhibition of reverse transcriptase by these extracts was analyzed by changing the concentrations of either the template·primer or deoxythymidine triphosphate (dTTP) substrate. The results with the extract of *Millettia pa-*

TABLE I. List of the Original Plants and the Inhibitory Effects of their Extracts on Reverse Transcriptase and DNA Polymerase α

Name of the original plant	Family name	IC_{50} (μg/ml) RT	Pol α
Polyporus umbellatus	Polyporaceae	160	140
Selaginella doederleinii	Selaginellaceae	10	9.0
Ethanol extract[a]		320	—
Nephrolepis cordifolia	Davalliaceae	3.5	34
Ethanol extract		6.0	15
Euryale ferox	Nympheaceae	3.2	5.0
Paeonia lactiflora	Paeoniaceae	2.0	10
Ethanol extract		1.3	8.0
Epimedium macranthum	Berberidaceae	8.0	20
Ethanol extract		80	500
Stellaria dichotoma	Caryophyllaceae	220	—
Pseudostellaria rhaphanorrhiza	Caryophyllaceae	—	—
Polygonum multiflorum	Polygonaceae	2.5	16
Astragalus mongholicus	Leguminosae	500	—
Dolichos lablab	Leguminosae	—	—
Millettia pachycarpa	Leguminosae	0.4	0.9
Ethanol extract		1.6	7.5
Ligusrum lucidum	Oleaceae	50	60
Cistanche salsa	Orobanchaceae	80	150
Ethanol extract		80	—
Cornus officinalis	Cornaceae	27	12
Kalopanax pictus	Araliaceae	3.6	15
Acanthopanax trifoliatus	Araliaceae	1.8	3.7
Ethanol extract		18	—
Ethanol extract of *Ficus carica*	Moraceae	140	300
Morus alba	Moraceae	80	80
Breynia fruticosa	Euphorbiaceae	2.0	5.0
Mallotus apelta	Euphorbiaceae	0.5	1.4
Ardisia crenata	Myrsinaceae	13	50
Ethanol extract		80	75
Cynomorium coccineum	Cynomoriaceae	1.0	8.0
Ethanol extract		2.7	14
Anemarrhena asphodelcides	Liliaceae	50	25
Ethanol extract		340	80
Polygonatum sibiricum	Liliaceae	100	—
Polygonatum officinale	Liliaceae	—	—
Ethanol extract		—	—
Dendrobium denneanum	Orchidaceae	180	280
Ethanol extract		60	60
Coix lachryma-jobi	Gramineae	—	—

a) Ethanol extracts were compared to the corresponding water extracts. —: no or slight inhibition.

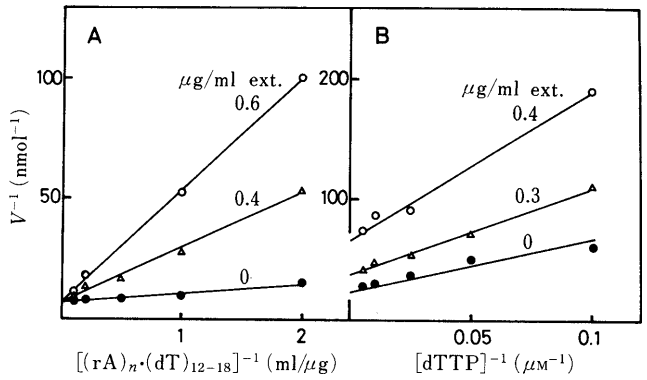

Fig. 1. Analysis of Inhibition of Reverse Transcriptase by the Extract from *Millettia pachycarpa*

Reactions were carried out for 30 min under the conditions described in Experimental, except that various concentrations of poly(rA)·oligo(dT) (A) or [^{3}H]dTTP (B) were used in the presence of various concentrations of the extract as indicated in the figure. The specific radioactivity of [^{3}H]dTTP was 400 cpm/pmol. The figure shows double-reciprocal plots.

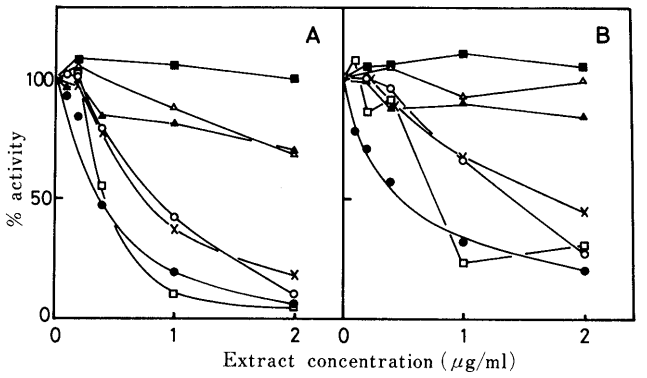

Fig. 2. Effects of the Extracts from *Millettia pachycarpa* and *Mallotus apelta* on the Activities of Various DNA and RNA Polymerases

Reactions were carried out for 30 min under the conditions described in Experimental in the presence of various concentrations of the extracts from *Millettia pachycarpa* (A) and *Mallotus apelta* (B) as indicated in the figure. The enzymes tested and the symbols used are as follows: reverse transcriptase (●), DNA polymerase α (○), DNA polymerase β (▲), DNA polymerase γ (△), TdT (■), DNA polymerase I (□) and *E. coli* RNA polymerase (×). The specific radioactivities of [^{3}H]dTTP and [^{3}H]dGTP were 400 cpm/pmol except in the case of DNA polymerase γ (6000 cpm/pmol), and that of [^{3}H]GTP was 40 cpm/pmol. The 100% values (pmol) were 5.5 (●), 56.3 (○), 15.8 (▲), 1.6 (△), 230.5 (■) 64.0 (□), and 20.5 (×).

chycarpa are shown in Fig. 1A and B. The double-reciprocal plots revealed that the mode of inhibition by the extract was competitive with respect to poly(rA)·oligo(dT) but non-competitive with respect to dTTP. Similar results were obtained with *Mallotus apelta* (data not shown).

Inhibition of Various DNA and RNA Polymerases by the Extracts from *Millettia pachycarpa* and *Mallotus apelta*

The inhibitory effects of these extracts on the activities of various kinds of DNA and RNA polymerases were investigated to examine the enzyme specificity of the inhibition. As shown in Fig. 2A and B, both extracts exhibited similar inhibition profiles (dose–response curves). In addition to murine reverse transcriptase and human KB III DNA polymerase α, the extracts were found to inhibit the activities of DNA polymerase I and RNA polymerase of *E. coli*. However, KB III DNA polymerase β and γ and calf thymus terminal deoxynucleotidyltransferase (TdT) were resistant to inhibition by these extracts. Thus, among the DNA polymerases tested, reverse transcriptase was the most sensitive to inhibition by both extracts.

Discussion

As shown in Table I, many kinds of herb extracts have been proven to be inhibitory to retroviral reverse transcriptase and human DNA polymerase α. Among 28 kinds of herbs tested, 12 herbs were extracted independently with water and ethanol to compare the efficiency of these extraction solvents. Eight out of 12 water extracts showed stronger inhibitory effects on the activity of reverse transcriptase than did the corresponding ethanol extracts. Stronger inhibitions by water extracts than those by ethanol extracts were also observed with DNA polymerase α.

Many extracts have been shown to be inhibitory to both reverse transcriptase and DNA polymerase α, though there are differences in the degree of inhibition. On the whole, however, these extracts are more inhibitory to reverse transcriptase than to cellular DNA polymerases, including α-polymerase, suggesting the feasibility of selective inhibition of reverse transcriptase *in vivo* if appropriate extract concentrations are chosen.

The results presented in this paper indicate that in-

TABLE II. Assay Conditions for Various DNA and RNA Polymerases[a]

DNA and RNA polymerase	Template·primer	Concentration[b] (μg/ml)	Buffer[c]	pH	[^{3}H]dNTP and concentration (μM)	dNTPs and concentration (μM)	Divalent cation and concentration (mM)	Monovalent cation and concentration (mM)
α	Activated DNA	80	Tris	7.5	dTTP, 10	Other 3, 10 each	Mg^{2+}, 4	—
β	$(rA)_n \cdot (dT)_{12-18}$	10 (1:2)[d]	Tris	8.5	dTTP, 10		Mn^{2+}, 0.2	K^+, 100
γ	$(rA)_n \cdot (dT)_{12-18}$	10 (10:1)	Tris	7.5	dTTP, 1		Mn^{2+}, 0.1	K^+, 70
TdT	$(dA)_{12-18}$	6	KPi[e]	6.5	dGTP, 10		Mn^{2+}, 5	K^+, 50
Reverse transcriptase	$(rA)_n \cdot (dT)_{12-18}$	10 (1:1)	Tris	8	dTTP, 10		Mn^{2+}, 0.2	K^+, 50
Polymerase I	Activated DNA	2	Tris	7.5	dTTP, 10	Other 3, 10 each	Mn^{2+}, 0.2	K^+, 100
RNA polymerase	$(dC)_n$	40	Tris	8	GTP, 1000		Mg^{2+}, 8	—

a) All reaction mixture contained 5 mM dithiothreitol and 15% (v/v) glycerol. *b*) With respect to the template when synthetic homopolymer was used. *c*) All buffer concentrations were 50 mM. *d*) Numbers in parentheses are base ratios of the template to the primer. *e*) KPi, potassium phosphate.

hibitors of DNA polymerases are widely distributed among various plants. Tannin is one candidate: it is known to denature proteins. It has also been reported that some ellagitannins inhibit reverse transcriptase of avian myeloblastosis virus *in vitro*.[7] However, other unknown substances in the herb extracts may inhibit the activities of reverse transcriptase and/or DNA polymerase α. Our finding that the extracts from *Millettia pachycarpa* and *Mallotus apelta* showed very strong inhibitory effects on both reverse transcriptase and DNA polymerases indicate the possible existence of some novel inhibitors in these herbs. Further studies on separation and identification of the inhibitory substance(s) are in progress in our laboratories with the aim of finding new antiretroviral and/or anticancer compound(s).

Experimental

Chemicals The sources of chemicals used in this work were as follows: [^{3}H]dNTP's from the Radiochemical Centre, Amersham, England; unlabeled nucleotides, poly(rA), poly(dC), oligo(dT) and oligo(dA) from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; activated calf thymus DNA from Worthington Biochem. Corp., Freehold, N. J., U.S.A.; and diethylaminoethyl (DEAE) cellulose paper discs (DE81, i.d. 23 mm) from Whatman Ltd., Springfield Mill, Maidstone, Kent, England.

Preparation of Herb Extracts The herbs used in the present study were collected in Japanese and other Asian markets. These herbs were divided into several classes according to the plant taxonomy and their scientific and family names are listed in Table I. Air-dried herbs were cut into small pieces and washed with cold distilled water. About 50 g of each material was put into 500 ml of water, and boiled for 1 h, then the water fraction was recovered by filtration. The residue was put into another 500 ml of water, and processed again for extraction in the same manner as above. These filtrates were combined and concentrated at below 45 °C by using a rotary evaporator, followed by lyophilization. Ethanol extracts were prepared as follows: about 50 g of air-dried herb powder was put into 300 ml of 95% ethanol, and boiled for 2 h under reflux, then the ethanol fraction was recovered by filtration; the residue was treated twice with 200 ml each of 95% ethanol in the same fashion as above; the filtrates were combined and concentrated *in vacuo*.

Preparation of DNA Polymerases DNA polymerases α, β and γ were purified from cultured human KB III cells as previously described for DNA polymerases α,[9] β[10] and γ[11] with some modifications. Rauscher leukemia virus (RLV) was obtained from the culture medium of an established virus-producing cell line, R-17,[12] and reverse transcriptase was purified according to the method described earlier.[13] Terminal deoxynucleotidyltransferase (TdT) was purified from calf thymus as described previously.[14] Highly purified preparations of *E. coli* DNA polymerase I and RNA polymerase were purchased from P-L Biochemicals, Milwaukee, Wis., U.S.A.

Assay for DNA Polymerase Activities DNA polymerase α and DNA polymerase I were assayed with activated calf thymus DNA as the template·primer. Reverse transcriptase and DNA polymerases β and γ were assayed with poly(rA)·oligo(dT) as the template·primer. TdT was assayed with oligo(dA) as the primer and RNA polymerase was assayed with poly(dC) as the template. Details of the assay conditions, which were optimized with respect to each enzyme species, were described previously[15] and are summarized in Table II. All incubations (50 μl) were carried out at 37 °C for 30 min, and the reaction was stopped by adding 20 μl of 0.2 M ethylenediaminetetraacetic acid (EDTA) and immersing the mixture in ice. Then, 50 μl of the mixture was transferred to a DE81 filter paper disc and processed for radioactivity counting as previously described.[16]

Acknowledgement We thank Mrs. S. Shinmura for typing the manuscript.

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