Screening of Inhibitor of Eukaryotic DNA Polymerases Produced by Microorganisms

YOSHIYUKI MIZUSHINA, HISAAKI YAGI[a], NOBUKAZU TANAKA, TAKAYOSHI KUROSAWA, HIROKAZU SETO, KEIKO KATSUMI, MEGUMI ONOUE, HANA ISHIDA, ASAYO ISEKI, TAKAYUKI NARA, KENGO MOROHASHI, TADASHI HORIE, YOUICHI ONOMURA, MITSUHIRO NARUSAWA, NORIKAZU AOYAGI, KIYOSHI TAKAMI, MASAKAZU YAMAOKA[a], YASUNORI INOUE, AKIO MATSUKAGE[b], SHONEN YOSHIDA[c] and KENGO SAKAGUCHI*

Department of Applied Biological Science, Faculty of Science and Technology, Science University of Tokyo, Noda-shi, Chiba-ken 278, Japan
[a] Laboratory of Microbial Resource, National Institute of Bioscience and Human-Technology, Tsukuba-shi, Ibaraki-ken 305, Japan
[b] Laboratory of Cell Biology, Aichi Cancer Center Research Institute, Nagoya-shi, Aichi-ken 464, Japan
[c] Laboratory of Cancer Cell Biology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya-shi, Aichi-ken 466, Japan

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DNA polymerases of eukaryotes have been divided into five general classes (α, β, γ, δ and ε), by analogy with mammalian polymerases[1]. If inhibitors of each polymerases were available, the *in vivo* roles of those enzymes could be elucidated more precisely. We have, therefore, established an assay method to detect DNA polymerase inhibitors. This method consists of two screenings. The first screen is an *in vitro* test using enzyme-based screening, using immnoaffinity-purified DNA polymerase α and recombinant rat DNA polymease β, purified as described in earlier papers[2,3]. Secondly screening is *in vivo* test using mammalian cultured cell, *Drosophila melanogaster* and higher plant *Arabidopsis*.

Colonies of actinomycetes and fungi and mycelia of basidiomycetes from field soil were collected, and subjected to the screening. As shown in Table 1, mycelia from actinomycetes (about 1,500 strains), fungi (200 strains) and basidiomycetes (200 strains) were homogenized in a Waring blender, and acetone-soluble compounds were extracted. The extracts were routinely tested in the *in vitro* DNA polymerase assay system as described below.

Neutralized crude samples were dissolved in methanol and sonicated for 30 seconds. Four μl of sonicated sample was then mixed with 16 μl of each DNA polymerase (0.05 units), and held at 0°C for 10 minutes. The inhibitor-enzyme mixture (8 μl) was then added to 16 μl of pol. β standard reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM $MgCl_2$, 10 μg/ml poly(dA), 5 μg/ml $(dT)_{12\sim18}$, 10 μM [^{3}H]-dTTP (100 cpm/pmol), 15% (v/v) glycerol and 150 mM KCl. After incubation at 37°C for 60 minutes, the radioactive DNA product was collected on a DEAE-cellulose paper (DE81) disc as described by LINDELL *et al.* (1970)[4].

In the first screening, 6 actinomycetes, 1 fungus and 12 mushroom strains were found to produce inhibitors, and of these, the product of a species of the *Polyporaceae* family appeared to be most promising. The active principle of the *Polyporaceae* strain was purified by *n*-hexane extraction and alumina column chromatography followed by partitioning in chloroform-water (pH 2). Unexpectedly, the active principle was identified by ^{1}H, ^{13}C and ^{1}H-^{13}C COSY NMR and FAB-MS as linoleic acid, an essential fatty acid in higher organisms.

To establish the secondly screening system, the effects of linoleic acid (commercially-purchased 18:2 Δ9~12 *cis*) on the *in vivo* growth of cultured mammalian cells, adults and progeny of *Drosophila melanogaster* and an *Arabidopsis* plant were investigated. For mammalian cell lines, Chinese hamster Don D-6 and mouse lymphoma L5178Y were used. The methods for cell culture are described in earlier reports[5~7]. Linoleic acid was added directly to the culture media, and its influence on cell growth was observed daily microscopically. *Drosophila melanogaster* lines tested were Oregon R and M361. Various concentrations of linoleic acid were suspended in 3% aqueous sucrose and given in their food for a week. The influence on death rate and abnormality

Table 1. Screening strains for producing DNA polymerase inhibitors.

Organisms	Total strains	Inhibitor-producing strains (%)		
		Inhibitory effect		
		++	+	±
Actinomycetes	1,511	6 (0.40)	10 (0.66)	23 (1.52)
Fungi	200	1 (0.50)	3 (1.50)	7 (3.50)
Basidiomycetes	200	12 (6.0)	18 (9.0)	33 (16.5)

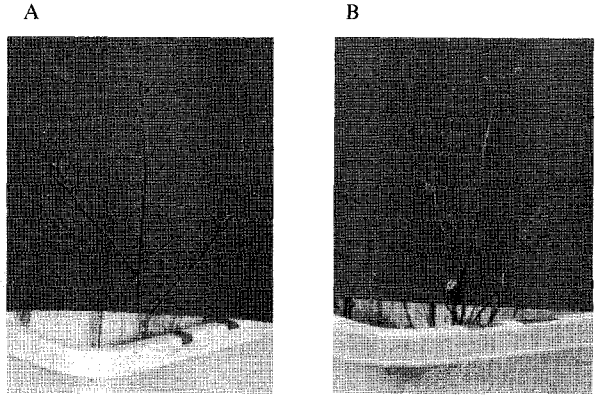
Table 2. Effect of linoleic acid on the activities of various DNA polymerases (0.05 units each).

Enzyme	Concentration (μg/ml)	Inhibitory activity (%)
Mammalian pol. α	25.0	95.3
Mammalian pol. β	25.0	94.8
Higher plant pol. I (α like)	25.0	38.2
Higher plant pol. II (β like)	25.0	44.1
E. coli pol. I (Klenow fragment)	25.0	0.2

DNA polymerases; calf thymus DNA polymerase α, rat recombinant DNA polymerase β, cauliflower DNA polymerase I, cauliflower DNA polymerase II and *Escherichia coli* DNA polymerase I (Klenow fragment).

Fig. 1. Effect of linoleic acid on the growth of *Arabidopsis*.

A: addition of 50% acetone only, B: addition of 20 mg/ml linoleic acid in 50% acetone.



of their progeny were then examined. The wild-type *Arabidopsis* ecotype used was *Landsberg erecta* (*Ler*), and were grown on soil as described by MAYER *et al.*[8] in green house under the long day light condition. On the 18th day after germination, 10 μl of a solution of linoleic acid in 50% acetone at various concentrations were placed on the terminal bud. Since normal blossoming occurs on day 26, the effect of inhibitor on flowering were observed.

As shown in Table 2, linoleic acid showed inhibitory activity against mammalian pol. α (52% decrease in Vmax at 5 μg/ml) and pol. β (250% increase of *Km* at 10 μg/ml). However, it showed no inhibitory activity against *Escherichia coli* pol. I (Klenow fragment), nor pol. I (α-like) and pol. II (β-like) of cauliflower.

The cultured mammalian cell lines (Chinese hamster Don D-6 and mouse L5178Y) were also unaffected by the *in vitro* inhibitor, except at extremely high inhibitor concentration (20 mg/ml). Linoleic acid did not show any effects on the death rate of either adult *Drosophila* flies or their progenies. This result is not unexpected because linoleic acid is basically a diet rich in fat. Since esterified linoleic acid does not produce inhibitory effect *in vitro*, the fatty acid might be rapidly esterified and hydrolyzed as soon as the cells incorporate it.

On the other hand, linoleic acid interfered with seed formation of the *Arabidopsis* plant (Fig. 1), although it is universally present in higher plant seeds. The linoleic acid concentration used was also 20 mg/ml, but only 10 μl of the solution was applied to a shoot of a young *Arabidopsis* at 18 days after germination. One day later, the added linoleic acid on the shoot was washed out. Small portions of the added linoleic acid could, therefore, be incorporated into the tissue, suggesting that the incorporated amount may be closer to the *in vitro* effective dose. The effects were concentrated in the formation or development of the seeds. The seed formation in the silique that were initially differentiated was most strongly affected by linoleic acid, with the effects gradually diminishing in later stages (data not shown). The linoleic acid appeared to have no effect on the development and growth of other tissues, although it was added some time prior to the flowering stages.

Thus, a screening system for DNA polymerase inhibitor was established in combination of *in vitro*, cell-culture and *in vivo* tests. Though the first example of the active principle was common fatty acid, some of novel microbial products are expected to be isolated and evaluated by the screening system.

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