The First Specific Antibody against Cytotoxic Polyacetylenic Alcohol, Panaxynol

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Antitumor polyacetylenic alcohol, panaxynol, was isolated and purified from a powder of the root of *Panax ginseng* C. A. MEYER. Panaxynol inhibited the growth of various kinds of cultured tumor cell lines in a dose-dependent manner. In this paper we demonstrated the first specific antibody production against panaxynol. Anti-panaxynol antibody was elicited in rabbits by immunization with panaxynol hemisuccinate–bovine serum albumin conjugate (panaxynol hemisuccinate–BSA conjugate). An enzyme immunoassay (EIA) for the determination of panaxynol was established using a double-antibody technique. The EIA was highly specific against panaxynol although the antibody showed a minimal cross-reactivity with other types of polyacetylenic alcohol, *i.e.* panaxydol (12.0%) and panaxytriol (0.77%). Panaxynol at a concentration as low as 6.4 ng/ml can be detected. Using this assay we reconfirmed the rapid consumption of panaxynol by target tumor cells in an *in vitro*-culture system. The anti-panaxynol antibody may be a valuable tool for studies of the biological properties of polyacetylenic compounds.

Keywords polyacetylenic alcohol; panaxynol; enzyme immunoassay; *Panax ginseng*

Introduction

It is well known that some plants contain several types of polyacetylenic compounds.[1)] However, there have been few reports on biological properties of polyacetylenic compounds. *Panax ginseng* C. A. MEYER contains a moderate amount of polyacetylenic alcohol.[2)] For thousands of years the roots of *Panax ginseng* have been used as an analeptic, stomachic and erythropoietic agent in Asian countries, and is now used in Japan as a commercial medical drug.

Recently, several investigators demonstrated that polyacetylenic compounds isolated from the root of *Panax ginseng* C. A. MEYER suppressed *in vitro*-tumor cell growth.[3)] We also reported that three polyacetylenic alcohols, *i.e.* panaxynol, panaxydol and panaxytriol, isolated from *Panax ginseng*, inhibited the *in vitro*-growth of cultured tumor cells.[4)] Panaxytriol suppressed the growth of B16 melanoma transplanted into mice.[5)] But, antitumor mechanisms are still unknown. These findings show a significant and urgent necessity for studies on biological properties of polyacetylenic compounds.

We tried to produce a specific antibody against panaxynol in order to specify the biological properties and pharmacokinetics of the polyacetylenic alcohol.

Materials and Methods

Preparation of Polyacetylenic Alcohol Polyacetylenic alcohol, panaxynol, panaxydol and panaxytriol were isolated and purified from the powder of heat-treated roots of *Panax ginseng* C. A. MEYER, which is used in Japan as a commercial medical drug by the name of Korean Red Ginseng Powder (Nikkan Korai Ninjin Co., Ltd., Kobe, Japan).[4)] Figure 1 shows the chemical structure of panaxynol.

Reagents *cis*-Dehydromatricaria ester was isolated as previously reported.[1d)] β-D-Galactosidase (β-Gal; EC 3.2.1.23) from *Escherichia coli* was obtained from Boehringer Mannheim (Mannheim, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Dojin Chemical Co. (Kumamoto, Japan).

Equipment The IR spectrum was recorded with a Hitachi 270-30 spectrometer (Tokyo, Japan). Proton nuclear magnetic resonance (^{1}H-NMR) spectrum was taken with a JEOL JNM-FX-100 spectrometer (Tokyo, Japan) at 100 MHz using tetramethylsilane as an internal standard. Enzymic activity was measured with a Shimadzu RF-540 spectrofluorophotometer (Kyoto, Japan).

Preparation of the Immunogen for Panaxynol A solution of panaxynol (100 mg, 0.41 mmol) and succinic anhydride (410 mg, 4.1 mmol) in pyridine (2 ml) was stirred overnight at room temperature. After the addition of water, the resulting mixture was extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated down. The residue was chromatographed on a column of Silica gel 60 (2 × 18 cm) with a mixed solvent of chloroform and ethyl acetate (1 : 1, v/v). In this solvent system, the unreacted panaxynol was first eluted from the column, followed by panaxynol hemisuccinate. A total of 77 mg (64.8%) of panaxynol hemisuccinate as a yellowish oil was obtained. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 2256 (C≡C), 1716—1746 (COOH). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, s, 17-CH_3), 1.27 (12H, s, –$(CH_2)_6$–), 2.69 (4H, s, –$COCH_2CH_2CO$–), 3.00, 3.06 (2H, d, J = 5.9 Hz, 8-H).

1-Ethyl-3,3-dimethylaminopropyl-carbodiimide hydrochloride (EDPC) (11 mg, 58 μmol) and *N*-hydroxysuccinimide (6.7 mg, 58 μmol) were added to a solution of panaxynol hemisuccinate (10 mg, 29 μmol) in 95% dioxane (2 ml), and the resulting solution was allowed to stand at room temperature for 2 h. The reaction mixture was extracted with ethyl acetate, washed with water, dried over anhydrous Na_2SO_4, and evaporated down to give succinimidyl panaxynol hemisuccinate (11.4 mg) as a yellowish oil. The resulting succinimidyl panaxynol hemisuccinate was used without further purification for preparing the conjugates with bovine serum albumin (BSA) and β-Gal, respectively, as the panaxynol immunogen and the tracer in the enzyme immunoassay (EIA).

A solution of BSA (10 mg, 0.15 μmol) in 50 mM phosphate buffer (pH 7.3, 2 ml) was mixed with a solution of succinimidyl panaxynol hemisuccinate (6.6 mg, 15 μmol) in *N*,*N*-dimethylformamide (DMF, 2 ml), and incubated overnight at 4 °C. The reaction mixture was dialyzed successively for 96 h against 50, 25, 15 and 10% DMF–H_2O and H_2O. The purified conjugate was lyophilized and used as an immunogen for the EIA. Using the trinitrobenzene sulfonic acid method for the determination of the primary amine,[6)] the conjugate was estimated to contain about 29.5 molecules of panaxynol per BSA molecule.

The scheme of preparation of the immunogen for panaxynol was shown in Fig. 2.

Antibody Production in Rabbits One ml of a saline suspension of 1 mg of panaxynol hemisuccinate–BSA was emulsified with an equal volume of complete Freund's adjuvant. The white female rabbits were each given multiple s.c. injections over sites along both sides of their backs. Booster

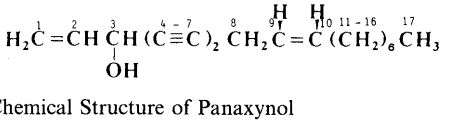

Fig. 1. Chemical Structure of Panaxynol

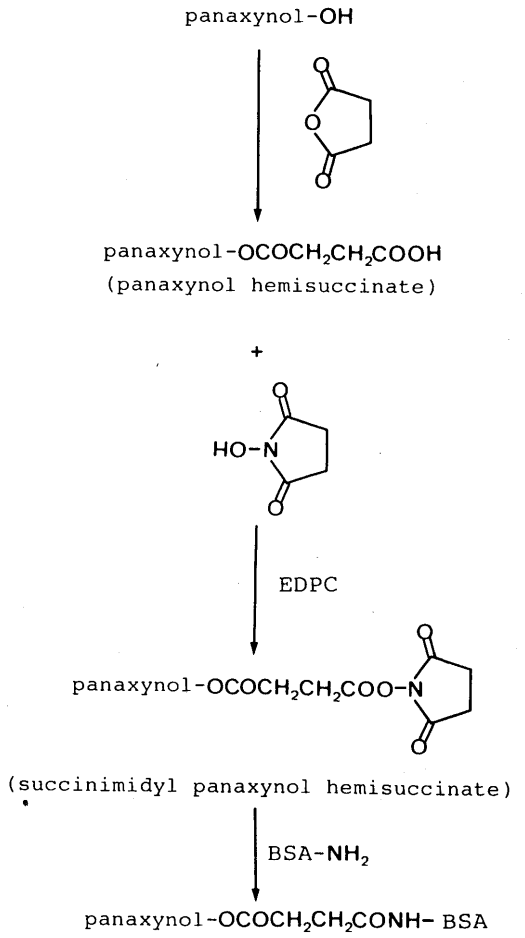

Fig. 2. Scheme for Synthesizing Immunogen for Panaxynol

injections were then given three times at biweekly intervals, using one-half the amount of the dose of the first immunization. The rabbits were bled from an ear vein 2 weeks after each injection. Sera were separated by centrifugation, heated at 55 °C for 30 min, and stored at −20 °C.

Preparation of Panaxynol–β-Gal Conjugate Panaxynol was labeled by binding to β-Gal, essentially by the same principle used for preparation of the panaxynol immunogen. In brief, 50 μl DMF solution containing succinimidyl panaxynol hemisuccinate (approximately 6.2 μg, 14.0 nmol) was mixed with β-Gal (78 μg, 0.14 nmol) in 200 μl 50 mM phosphate buffer (pH 7.3), and incubated overnight at 4 °C. The mixture was chromatographed on a column of Sepharose 6B (2 × 35 cm) using 20 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA, and 0.1% NaN_3 (buffer A) to remove any small molecular compounds remaining. Three-ml fractions were collected, and fractions 16 to 18, representatively the main peak of the pure enzyme activity, were chosen as a label in the EIA.

Measurement of β-Gal Activity Fifty μl of diluted enzyme solution were incubated with 150 μl of 0.1 mM 7-β-D-galactopyranosyloxy-4-methylcoumarin in buffer A at 30 °C for 1 h. The reaction was stopped by adding 2.5 ml of 0.2 M glycine–NaOH buffer (pH 10.3), and the 7-hydroxy-4-methylcoumarin liberated was read spectrofluorometrically at wavelengths of 365 nm for excitation and 448 nm for emission. The amount of the conjugate was expressed in units of β-Gal activity, defining 1 unit of the enzyme activity as the amount that hydrolyzes 1 μmol of the substrate per min.[7]

EIA Method The double-antibody technique[8] was used for the separation of antibody-bound and free antigen. Experimental conditions for EIA were extensively examined and an effective assay system was established as follows. An antiserum, panaxynol–β-Gal and unlabeled panaxynol were diluted in buffer B (0.06 M sodium phosphate, pH 7.4, containing 0.01 M EDTA, 0.1% BSA, 0.1% NaN_3). Fifty μl of panaxynol–β-Gal conjugate (314 μU), 50 μl of panaxynol or sample, as appropriate, and 50 μl of a 1:3000 solution of the antiserum were mixed, and the mixture was incubated at 25 °C for 14 h. Then 50 μl of a 10% solution of goat anti-rabbit IgG and 50 μl of a 1% solution of normal rabbit serum were added. After further incubation for 3 h, the immune precipitate was washed twice by the addition of 1.0 ml of buffer A and centrifuged at 2500 rpm for 15 min in a refrigerated centrifuge. The supernatant was decanted, and the enzyme activity in the immune precipitate was measured.

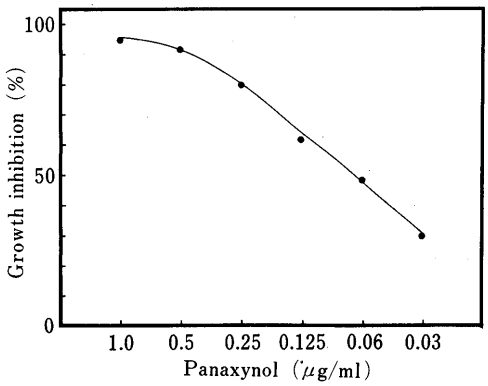

Fig. 3. Effect of Panaxynol on MK-1 Cell Growth *in Vitro*

Fifty microliters of cell suspension (5×10^4 cells/ml) and 50 μl of panaxynol containing medium were plated in flat-bottomed microtiter wells and incubated for 48 h in a 5% CO_2 incubator at 37 °C. % growth inhibition

$$= \left(1 - \frac{\text{tumor cells in RPMI 1640 with panaxynol } (OD_{590})}{\text{tumor cells in RPMI 1640 without panaxynol } (OD_{590})}\right) \times 100$$

Results are the mean of four parallel wells.

Tumor Cell Line Nude mouse-transplantable human gastric adenocarcinoma cells (MK-1 cells) was maintained in RPMI 1640 containing 10% fetal calf serum (FCS).

Cell Growth Cellular growth was determined using the previously described MTT-microculture tetrazolium assay.[9] Briefly, 50 μl of tumor cells (5×10^4 cells/ml) and 50 μl of panaxynol solution were plated in flat-bottomed microtiter wells and incubated for 48 h at 37 °C in a humidified atomosphere of 5% CO_2 in air. After the culture, 10 μl of MTT (5 mg/ml) were added to the microculture wells. After 2-h incubation at 37 °C, 100 μl were removed from each well, to which 100 μl of 100% dimethyl sulfoxide (DMSO) were added to solubilize the MTT-formazan product. Absorbance at 590 nm was measured with a Dynatech model MR 600 microplate reader. Percent cell growth inhibition = (1-tumor cells in RPMI 1640 with panaxynol (OD_{590})/tumor cells in RPMI 1640 without panaxynol $(OD_{590})) \times 100$.

Results

Effect of Panaxynol on *in Vitro*-Tumor Cell Growth We examined the effect of panaxynol on the growth of MK-1 human gastric carcinoma cells. Panaxynol inhibited the growth of MK-1 cells in a dose-dependent fashion as shown in Fig. 3. ED_{50} which indicates the concentration of panaxynol required to obtain 50% growth inhibition of MK-1 cells was 0.07 μg/ml.

Antibody Response Antibodies to panaxynol were produced in each of the two rabbits immunized with the panaxynol-hemisuccinate–BSA conjugate. Subsequent booster injections increased the antiserum titers considerably. The titers in the serum samples were measured in terms of enzyme activity of panaxynol–β-Gal bound to solutions of antiserum obtained by the EIA. The antibody titer increased gradually with booster injections and reached a maximum 8 weeks after the first immunization. Judging from the binding value, a 3000 times diluted solution of the highest titered serum was chosen for EIA.

EIA for Panaxynol A dose–response standard curve obtained in Buffer B is shown in Fig. 4. The limits of drug detection by the EIA were between 1.28 ng and 20 μg/ml of panaxynol. For practical purposes, the working range was arbitrarily set between 6.4 ng and 4 μg/ml based on the

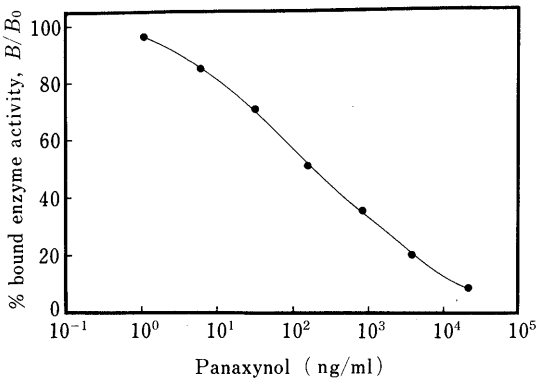

Fig. 4. Standard Curve for Panaxynol with Anti-panaxynol Antiserum

The curve shows the amount (percentage) of bound enzyme activity for various doses of panaxynol (B) as a ratio of that bound using panaxynol–β-Gal alone (B_0).

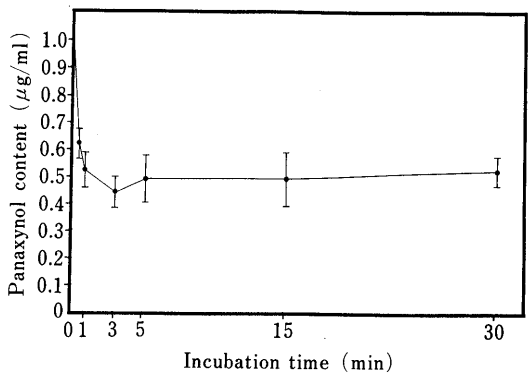

Fig. 5. Absorption of Panaxynol by MK-1 Cells

MK-1 cells (5×10^5 cells/ml) were incubated with panaxynol at a concentration of 1 μg/ml at 37 °C. Cell-free supernatants were determined by this EIA. Results are the mean ± S.D. of three experiments.

TABLE I. Precision of EIA for Panaxynol

	Added (ng/ml)	Estimated (ng/ml)	C.V. (%)
Intraassay	6.4	6.5 ± 1.0	15.4
	32.0	32.6 ± 3.0	9.2
	160.0	157.4 ± 14.0	8.9
	800.0	810.0 ± 74.8	9.2
	4000.0	3940.0 ± 606.6	15.4
Interassay	6.4	6.8 ± 0.9	13.2
	32.0	32.5 ± 3.0	9.2
	160.0	153.3 ± 16.3	10.6
	800.0	821.4 ± 72.1	8.8
	4000.0	4008.0 ± 577.3	14.4

Values represent the mean ± S.D. of a total of 5 experiments.

TABLE II. Specificity of Anti-panaxynol Serum

Compound structure and name		% cross-reaction (50%)
$CH_2=CHCH(C\equiv C)_2CH_2CH=CH(CH_2)_6CH_3$ (OH)	Panaxynol	100
$CH_2=CHCH(C\equiv C)_2CH_2CH=CH(CH_2)_6CH_3$ ($OCO(CH_2)_2COOH$)	Panaxynol hemisuccinate	100
$CH_2=CHCH(C\equiv C)_2CH_2CH$–$CH(CH_2)_6CH_3$ (OH, O)	Panaxydol	12.0
$CH_2=CHCH(C\equiv C)_2CH_2CH$–$CH(CH_2)_6CH_3$ (OH, OH OH)	Panaxytriol	0.77
$CH_3(CH_2)_8CH_2(C\equiv C)_2CH_2(CH_2)_7COOH$	10,12-Tricosadiynoic acid	1.28
$CH_3(C\equiv C)_3CH=CHCOOCH_3$	*cis*-Dehydromatricaria ester	<0.15
$CH_2=CH(CH_2)_6CH_3$	1-Nonene	<0.08
$CH_2=CHCH_2$ (OH)	Allyl alcohol	<0.08
$CH_3CH_2CHC\equiv CH$ (OH)	1-Pentyn-3-ol	<0.08
$HC\equiv CCH_2(CH_2)_8CH_3$	1-Dodecyne	<0.15

precision data for the EIA (Table I). The coefficients of variation for intra- and interassays between panaxynol concentrations of 6.4 ng to 4 μg at five different levels each were 8.9 to 15.4% and 8.8 to 14.4%, respectively.

Specificity of the Antibody The specificity of anti-panaxynol serum was determined by measuring the displacement of bound panaxynol–β-Gal by panaxydol, panaxytriol and compounds of a similar structure. The anti-panaxynol antibody showed 12.0% cross-reaction with panaxydol, 1.28% with 10,12-tricosadiynoic acid and 0.77% with panaxytriol, in terms of the amount of each compound required for 50% inhibition of binding. No detectable cross-reaction, however, was found in *cis*-dehydromatricaria ester, 1-nonene, allyl alcohol, 1-pentyn-3-ol and 1-dodecyne.

Consumption of Panaxynol by MK-1 Cells Our previous studies suggest that a similar polyacetylenic alcohol, panaxytriol, may be rapidly absorbed by target tumor cells in an *in vitro*-culture system.[5b] To confirm the phenomenon, the following experiment was performed. Cell-free supernatants were collected at varying intervals from the mixed culture of panaxynol and MK-1 cells. A remaining panaxynol content in the supernatants were measured with EIA. One μg of panaxynol were added to 1 ml of MK-1 cells suspension (5×10^5 cells/ml), and suspension was incubated at 37°C. Panaxynol content in the culture supernatant decreased very rapidly to 0.61, 0.53 and 0.53 μg/ml at 0.5, 1 and 30 min, respectively (Fig. 5).

Discussion

In order to make it possible to study the biological properties and pharmacokinetics of the polyacetylenic compounds, a specific and sensitive EIA for a polyacetylenic alcohol, panaxynol, was developed. To our knowledge, there is no report demonstrating specific antibody production against polyacetylenic compounds. It is one probable reason that polyacetylenic compounds have no suitable reactive structure for making immunogen such as the compound–BSA conjugate. Panaxynol has one hydroxyl group at the C-3 position as shown in Fig. 1. The hydroxyl group is readily available for introducing a reactive group into the molecule. Therefore, we chose to introduce a carboxylic group at the site. Panaxynol immunogen and panaxynol–β-Gal conjugate (as a tracer) were prepared using the hydroxysuccinimide ester method[10] as shown in Fig. 2. It is of interest that the panaxynol–BSA conjugate retains a significant cytotoxic activity against tumor cells (unpublished data).

An optimal assay procedure was established for the panaxynol EIA that proved to be so sensitive that panaxynol concentration of >6.4 ng/ml could be measured re-

producibly (Fig. 4, Table I). This sensitivity appears to be about 20 times more sensitive than the previous gas chromatography.[11] And many samples can be treated at one stage in EIA. Development of the panaxynol EIA made it easier to study the pharmacokinetics of panaxynol as shown in Fig. 5.

The antibody specificity was directed mainly toward panaxynol, although there is a slight cross-reactivity with a panaxynol analogues, panaxydol (12.0%). And, only negligible cross-reactivity with another panaxynol analogue, panaxytriol (0.77%) was found (Table II). As shown in Table II, these compounds are structurally different only in C-9,10 positions. Namely, the respective C-9,10 positions of panaxynol, panaxydol and panaxytriol are double bond, epoxy and glycol type. In addition, no significant cross-reactivity (1.28%) with 10,12-tricosadiynoic acid, in which the diacetylene position is similar to that of panaxynol, was found. It might therefore be said that the antibody-recognition site is the double bond moiety and is at the diacetylene moiety of the panaxynol molecule.

Polyacetylenic compounds such as panaxynol, panaxydol and panaxytriol have been expected as a new type of antitumor substance. *Panax ginseng* C. A. MEYER, which has been widely used in Asian countries as a commercial medical drug, contains a moderate amount of these polyacetylenic compounds. However, there are few reports concerning the chemical, pharmacological and biological studies of the polyacetylenic compounds. This panaxynol EIA may provide many opportunities for the study of polyacetylenic compounds.

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