A Highly Sensitive Enzyme-Linked Immunosorbent Assay (ELISA) for Antitumor Polyacetylenic Alcohol, Panaxytriol

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A new type of antitumor polyacetylenic alcohol, panaxytriol, was isolated from the roots of *Panax ginseng* C. A. MEYER. A highly sensitive enzyme-linked immunosorbent assay (ELISA) for the determination of panaxytriol was developed, which is capable of measuring as low as 25.6 pg/ml. Anti-panaxytriol antibody was obtained by immunizing rabbits with panaxytriol conjugated with bovine serum albumin using the *N*-succinimidyl ester method. An enzyme marker was similarly prepared by coupling panaxytriol with horseradish peroxidase. The specificity of this ELISA seems to be primarily toward both the glycol moiety and the diacetylene moiety of the panaxytriol, showing a slight cross-reaction with the other panaxytriol analogues which are structurally different only in C-9,10 positions, but no cross-reaction with the 1,2-decanediol or 3-nonyl-1-ol. The values for panaxytriol concentration detected by this assay were comparable with those detected by the gas chromatography method. The ELISA was about 5000 times more sensitive in detecting panaxytriol. Using this assay, panaxytriol levels were easily determined in the blood of rats. The ELISA may be a valuable tool for studies of the biological and pharmacological properties of the polyacetylenic alcohol, panaxytriol.

Keywords panaxytriol; enzyme-linked immunosorbent assay; polyacetylenic alcohol; antitumor substance

For thousands of years the roots of *Panax ginseng* C. A. MEYER have been used as an analeptic, stomachic and erythropoietic agent in Asian countries, and are now used in Japan as a commercial medical drug. Many reports have demonstrated that *Panax ginseng* C. A. MEYER contains several types of polyacetylenic alcohols,¹⁾ and that these alcohols suppressed the *in vitro* growth of cultured tumor cells.²⁾ Panaxytriol also suppressed the growth of B16 melanoma transplanted into mice³⁾ and showed a stimulative effect on the antitumor activity of mitomycin C in cultured tumor cells.⁴⁾ Although panaxytriol has been viewed as a new type of antitumor substance, there are no reports concerning the pharmacokinetics of polyacetylenic alcohols. We succeeded in producing the first specific antibodies against the polyacetylenic alcohols panaxynol⁵⁾ and panaxytriol. This paper describes the methodology for the antibody production, the labeling of panaxytriol with horseradish peroxidase (HRP) to act as a tracer, the characterization of antibody specificity, and the measurement of panaxytriol by enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Reagents Panaxytriol, panaxynol and panaxydol were isolated and purified from a powder of heat-treated roots of *Panax ginseng* C. A. MEYER, red ginseng, as reported previously.^{2a,b)} Red ginseng powder was provided by Nikkan Korai Ninnjin Co., Ltd. (Kobe, Japan). Panaxydol chlorohydrine and 1,16-heptadecadiene-4,6-diyne-3,9,10-triol were obtained from Tsumura Co., Ltd. (Tokyo, Japan). Panaxytriol acetone was synthesized by the method of Kitagawa *et al.*⁶⁾ HRP (for enzyme immunoassay) and 3,3',5,5'-tetramethylbenzidine (TMB) were

obtained from Boehringer Mannheim (Mannheim, Germany).

Tumor Cell Line Nude mouse-transplantable human gastric adenocarcinoma cells (MK-1 cells) were maintained in RPMI 1640 containing 10% fetal calf serum (FCS). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

Clonogenic Tumor Cell Survival The *in vitro*-cytotoxicity of panaxytriol was determined using MK-1 cells as target cells. The growing cells were trypsinized and collected. Panaxytriol was added to MK-1 cell suspensions. The suspensions were plated (100 cells/well, 34.6 × 17 mm) and incubated at 37 °C for 8–10 d, at which time surviving colonies were stained with methylene blue and counted. Survival ratio of MK-1 cells = (No. of colonies in drug-containing medium/No. of colonies in drug-free medium) × 100.

Preparation of the Immunogen for Panaxytriol Panaxytriol was conjugated to BSA, essentially by the same principle as used for the previous preparation of panaxynol immunogen.⁵⁾ A solution of panaxytriol (264 mg, 0.95 mmol) and succinic anhydride (95 mg, 0.95 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 Na₂SO₄, and reduced by evaporation. The residue was chromatographed on a column of Silica gel 60 (2 × 20 cm) with a mixed solvent of ethyl acetate and *n*-hexane (2:1, v/v). In this solvent system, the unreacted panaxytriol was first eluted from the column, followed by panaxytriol hemisuccinate. A total of 55 mg (15.3%) of panaxytriol hemisuccinate as a yellowish oil was obtained.

1-Ethyl-3,3-dimethylaminopropyl-carbodiimide hydrochloride (EDPC) (10 mg, 52 μmol) and *N*-hydroxysuccin-

imide (6 mg, 52 μmol) were added to a solution of panaxytriol hemisuccinate (approximately 10 mg, 26 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 panaxytriol hemisuccinate as a yellowish oil. The resulting succinimidyl panaxytriol hemisuccinate was used without further purification to prepare the conjugates with bovine serum albumin (BSA) and HRP, respectively, as the panaxytriol immunogen and the tracer in ELISA.

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 panaxytriol hemisuccinate (approximately 7.5 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 . The purified conjugate was lyophilized and used as an immunogen for ELISA. Using the trinitrobenzene sulfonic acid method to determine the primary amine,⁷ the conjugate was estimated to contain about 37.5 molecules of panaxytriol per BSA molecule.

Preparation of Anti-panaxytriol Antibody One ml of a saline suspension of 1 mg of panaxytriol-BSA conjugate was emulsified with an equal volume of complete Freund's adjuvant. White female rabbits were each given multiple s.c. injections over sites along both sides of their backs. Booster 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 10 weeks after immunization began. Fractions of IgG were extracted from the sera with 50% saturated ammonium sulfate and chromatographed on a column of DEAE-Sephacel (2.1 $\times$ 23 cm) using 17.5 mM phosphate buffer (pH 6.8) as an eluent.⁸ The fractions passed through the column were lyophilized and used as anti-panaxytriol IgG for ELISA.

Preparation of the Panaxytriol-HRP Conjugate Panaxytriol was labeled by binding to HRP, essentially by the same principle as used for preparation of the panaxytriol immunogen. In brief, 250 μl DMF solution containing succinimidyl panaxytriol hemisuccinate (approximately 2 mg, 4 μmol) was mixed with HRP (0.5 mg, 12.5 nmol) in 500 μl of 50 mM phosphate buffer (pH 7.3), and incubated overnight at 4 °C. The mixture was chromatographed on a column of Sephadex G-100 (2 $\times$ 40 cm) using 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1% BSA (buffer A) to remove any small molecular compounds remaining. Four-ml fractions were collected, and fractions 10 to 13, representing the main peak of pure enzyme activity, were chosen as a label in the ELISA.

ELISA for Determination of Panaxytriol ELISA is based on the principle of competition between enzyme-labeled and unlabeled panaxytriol for an immobilized antibody, followed by measurement of the marker enzyme activity of the immunocomplex bound to the solid phase. Briefly, the wells in microtiter plates (Nunc F Immuno-plates I; Nunc, Roskilde, Denmark) were coated by loading 150 μl anti-panaxytriol IgG (4 $\mu\text{g}/\text{ml}$) in 10 mM Tris-HCl buffer (pH 8.5) containing 10 mM NaCl and

10 mM NaN_3 and allowing this to stand overnight at 4 °C. After the plates had been washed twice with 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.05% (v/v) Tween 20 (PBS-T), they were incubated with 200 μl of 2% BSA-PBS for 20 min at 37 °C to prevent nonspecific adsorption. The anti-panaxytriol IgG-coated wells were then filled with 50 μl of either panaxytriol, drug-treated samples, or PBS as a control, followed immediately by 50 μl of the pooled panaxytriol-HRP conjugate that had been diluted 1:2000 in buffer A. The wells were then incubated overnight at 4 °C and once again washed briskly with PBS-T.

The amount of enzyme conjugate bound to each well was then measured by the addition of 150 μl 0.42 mM TMB in 0.05 M acetate-citric acid buffer (pH 5.5) containing 3% dimethyl sulfoxide, 0.01% hydrogen peroxide, followed by incubation of the wells at 37 °C for a suitable period. Enzymatic activity was stopped by the addition of 50 μl 2.0 M H_2SO_4 to each well, and the resulting color intensity was measured spectrophotometrically at 450 nm using an ELISA analyzer (Molecular Devices; California, U.S.A.).

Pharmacokinetic Evaluation Three male Wistar rats weighing 300–400 g were given 1 mg/kg panaxytriol by i.v. bolus injection into the cervical vein. Blood was obtained from the same cervical vein before administration of the drug and at intervals thereafter, and the serum was stored at –20 °C until assayed for panaxytriol concentration. The serum was diluted with buffer A to obtain panaxytriol concentration appropriate for a measurement by ELISA. Serum half-lives were calculated using a non-linear least-squares regression program, MULTI.⁹

RESULTS

Effect of Panaxytriol on *in Vitro*-Tumor Cell Growth

We examined the effect of panaxytriol on the colony growth of MK-1 cells by clonogenic assay, and found that it inhibited growth of these cells in a dose-dependent fashion as shown in Fig. 1. The concentration of panaxytriol required to obtain 50% colony growth inhibition of MK-1 cells was 8.5 ng/ml.

ELISA for Panaxytriol The optimal quantities and optimal incubation time for each reaction were established.

Fig. 1. Effect of Panaxytriol on the Colony Growth of MK-1 Cells

The *in vitro*-cytotoxicity was determined by clonogenic assay as described in Materials and Methods. Results are the mean $\pm$ S.D. of three parallel wells.

A standard dose-response curve obtained using the buffer system is shown in Fig. 2. The limits of drug detection by ELISA were between 25.6 pg and 400 ng/ml of panaxytriol. For practical purposes, the working range was arbitrarily set between 128 pg and 80 ng/ml based on the precision data for the ELISA (unpublished data) which reveal this developed ELISA to be a reproducible technique. The coefficients of variation for intra- and interassays between panaxytriol concentrations of 128 pg to 80 ng/ml at five different levels each were 4.1 to 14.1% and 3.9 to 9.2%, respectively.

Standard curves were also constructed with various volumes of normal pooled drug-free serum added (0.05 to 50 μ l). Standard curves using more than 2.5 μ l of serum differed in shape from that in the buffer system. The limits of drug detection by the ELISA were between 128 pg and 400

Fig. 2. Standard Curve for Panaxytriol with Anti-panaxytriol Antibody

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

ng/ml of panaxytriol (data not shown). However, less than 2.5 μ l of serum produced no observable effect, and thus the buffer system standard curve was used for panaxytriol quantification of the sample at considerably high dilutions.

Specificity of the Antibody The antibody specificity was determined by measuring the displacement of bound panaxytriol-HRP by other similar compounds. Values of the cross-reactivity were defined as the ratio of each compound to panaxytriol in the concentrations required for 50% inhibition of panaxytriol-HRP binding to the antibody. The anti-panaxytriol antibody showed 100% cross-reaction with 1,16-heptadecadiene-4,6-diyne-3,9,10-triol, 24.1% with panaxydol chlorohydrine, 2.7% with panaxydol, 0.12% with panaxytriol acetone and 0.04% with panaxynol. No detectable cross-reaction, however, was found in 1,2-decanediol or 3-nonyl-1-ol (Table I).

Comparison of ELISA and GC The ELISA method was compared with the previous gas chromatograph (GC) method¹⁰⁾ by using specific quantities of panaxytriol in human serum. The GC technique analyzed 10 samples of various concentrations of panaxytriol ranging from 0.625 to 40 μ g/ml, and showed a linear relationship between the peak height of the panaxytriol chromatogram and the injected panaxytriol dose. ELISA determination was done using these panaxytriol samples, properly diluted to the drug-concentration range detectable by ELISA. Figure 3 shows that there was good correlation between the values determined by the two methods, and the plot was linear as predicted by the equation $Y=0.82X+0.77$, where Y is the concentration value determined by GC analysis and X is that determined by ELISA; the correlation coefficient was 0.996 ($n=10$). Since sensitivity of the ELISA is 25.6 pg/ml as described above, the ELISA is about 5000 times more sensitive than GC.

Measurement of Panaxytriol in Rat Serum by ELISA

TABLE I. Specificity of Anti-panaxytriol Antibody

Compound structure and name	% cross-reaction (50%)
$\begin{array}{ccccccc} 1 & 2 & 3 & 4-7 & 8 & 9 & 10 & 11-16 & 17 \\ \text{CH}_2 & = & \text{CHCH} & (\text{C}\equiv\text{C})_2 & \text{CH}_2 & \text{CH} & - & \text{CH} & (\text{CH}_2)_6 & \text{CH}_3 \\ & & & & & & & & & \\ & & \text{OH} & & & \text{OH} & \text{OH} & & & \end{array}$	Panaxytriol
$\begin{array}{ccccccc} \text{CH}_2 & = & \text{CHCH} & (\text{C}\equiv\text{C})_2 & \text{CH}_2 & \text{CH} & - & \text{CH} & (\text{CH}_2)_5 & \text{CH} = \text{CH}_2 \\ & & & & & & & & & \\ & & \text{OH} & & & \text{OH} & \text{OH} & & & \end{array}$	1,16-Heptadecadiene-4,6-diyne-3,9,10-triol
$\begin{array}{ccccccc} \text{CH}_2 & = & \text{CHCH} & (\text{C}\equiv\text{C})_2 & \text{CH}_2 & \text{CH} & - & \text{CH} & (\text{CH}_2)_6 & \text{CH}_3 \\ & & & & & & & & & \\ & & \text{OH} & & & \text{OH} & \text{Cl} & & & \end{array}$	Panaxydol chlorohydrine
$\begin{array}{ccccccc} \text{CH}_2 & = & \text{CHCH} & (\text{C}\equiv\text{C})_2 & \text{CH}_2 & \text{CH} & - & \text{CH} & (\text{CH}_2)_6 & \text{CH}_3 \\ & & & & & & & & & \\ & & \text{OH} & & & \text{OH} & \text{O} & & & \end{array}$	Panaxydol
$\begin{array}{ccccccc} \text{CH}_2 & = & \text{CHCH} & (\text{C}\equiv\text{C})_2 & \text{CH}_2 & \text{CH} & - & \text{CH} & (\text{CH}_2)_6 & \text{CH}_3 \\ & & & & & & & & & \\ & & \text{OH} & & & \text{O} & \text{O} & & & \\ & & & & & & & & & \\ & & & & & \text{C} & & & & \\ & & & & & & & & & \\ & & & & & \text{CH}_3 & \text{CH}_3 & & & \end{array}$	Panaxytriol acetone
$\begin{array}{ccccccc} \text{CH}_2 & = & \text{CHCH} & (\text{C}\equiv\text{C})_2 & \text{CH}_2 & \text{CH} & = & \text{CH} & (\text{CH}_2)_6 & \text{CH}_3 \\ & & & & & & & & & \\ & & \text{OH} & & & & & & & \end{array}$	Panaxynol
$\begin{array}{ccccccc} \text{CH}_2 & \text{CH} & (\text{CH}_2)_7 & \text{CH}_3 \\ & & & \\ \text{OH} & \text{OH} & & \end{array}$	1,2-Decanediol
$\begin{array}{ccccccc} \text{CH}_3 & (\text{CH}_2)_4 & \text{C}\equiv\text{C} & \text{CH}_2 & \text{CH}_2 \\ & & & & \\ & & \text{OH} & & \end{array}$	3-Nonyl-1-ol

Fig. 3. Correlation of Panaxytriol Quantification by the Present ELISA and GC Methods

Fig. 4. Serum Panaxytriol Levels in Rats after a Single i.v. Administration of Panaxytriol

Three rats, each weighing about 300–400 g, were given panaxytriol at a concentration of 1 mg/kg. At the intervals, shown blood was collected and serum panaxytriol was measured by ELISA. Results are the mean $\pm$ S.D. of the three rats.

Panaxytriol was administered by rapid i.v. injection into the cervical vein of three rats at a dose of 1 mg/kg. Blood was obtained from the same cervical vein before administration of the drug and at 2, 5, 10, 15, 30, 45, 60, 90, 120 and 180 min after treatment. The serum was then diluted with buffer A to obtain a final panaxytriol concentration in the sample, such that the diluted serum would contain between 128 pg and 80 ng/ml of panaxytriol. The disappearance of panaxytriol immunoreactivity from the serum is indicated in Fig. 4. For 3 h after drug administration, the decline of panaxytriol in serum was well described by a biphasic pattern of pharmacokinetics, with an average serum α -half-life of 5 min and a serum β -half-life of 51.1 min calculated for the three rats, showing that the serum drug level initially declined very rapidly but gradually slowed after administration.

DISCUSSION

We first developed an ELISA for determination of the

antitumor polyacetylenic alcohol, panaxytriol. *Panax ginseng* C. A. MEYER contained several types of polyacetylenic alcohols such as panaxytriol, panaxynol and panaxydol¹⁾ which are in broad use in Asian countries as a commercial medical drug. Several investigators recently demonstrated that polyacetylenic alcohols inhibited both *in vitro* tumor cell growth²⁾ and the growth of B16 melanoma transplanted into mice.³⁾ Polyacetylenic alcohols possess the unusual property of being soluble in both water and organic solvents, and their site of action may be a surface membrane. In fact, panaxytriol induces a decreased membrane fluidity in target tumor cells.⁴⁾ These results indicate that polyacetylenic alcohol may be a new type of antitumor substance. We earlier reported a method using GC for determination of panaxytriol.¹⁰⁾ Although that technique is highly specific, its sensitivity is not adequate (>125 ng/ml) for pharmacokinetic study in animals and human. There are thus no reports demonstrating the serum levels of panaxytriol in animals to whom *Panax ginseng* was administered orally. Since we previously succeeded in producing specific antibodies against the polyacetylenic alcohol, panaxynol, panaxytriol immunogen and panaxytriol–HRP conjugate (as a tracer) were prepared by essentially the same procedure as the immunogen for panaxynol.⁵⁾ Using both anti-panaxytriol IgG and panaxytriol–HRP conjugate, an ELISA was developed capable of measuring as low as 25.6 pg/ml panaxytriol in buffer solution (Fig. 2). Its sensitivity appears to be about 5000 times greater than that of the GC method¹⁰⁾; although sensitivity is 25.6 pg/ml in the buffer system, it is 128 pg/ml in an experimental animal system. When panaxytriol (1 mg/kg) was administered orally to rats it was rapidly absorbed, reaching a mean peak concentration in serum of about 50 ng/ml at 25 min after dosing (data not shown). It is of interest that panaxytriol is clearly absorbed from the gastrointestinal tract. This ELISA may be sensitive enough to quantify panaxytriol for pharmacokinetic study in experimental animals.

The specificity of this ELISA is shown in Table I and Fig. 3. The cross reactivities of anti-panaxytriol antibody with panaxytriol analogues were examined. The antibody showed high affinity for 1,16-heptadecadiene-4,6-diyne-3,9,10-triol which is structurally different only in the C-16, 17 positions. But the antibody showed very limited reactivity with the other panaxytriol analogues which are different only in the C-9,10 positions. No reactivity was found between the antibody and 1,2-decanediol or 3-nonyn-1-ol. These results indicate that the antibody-recognition sites are both the glycol moiety and the diacetylene moiety of the panaxytriol molecule. Since the antibody recognizes very unique structures of panaxytriol, this ELISA seems to be highly specific. It is well recognized that the GC method is highly specific, and this ELISA method was compared with the GC technique.¹⁰⁾ There was good correlation; the correlation coefficient was 0.996 between the two (Fig. 3). This finding also supports the high specificity of ELISA.

The ELISA presented here is sensitive, specific, reproducible, and adaptable enough for analyses of a large number of samples. The assay should be a valuable tool

in studies of the biological and pharmacological properties of panaxytriol.

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