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A POSSIBLE MECHANISM OF POLYACETYLENE: MEMBRANE CYTOTOXICITY

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ABSTRACT: *The effects of polyacetylenes on living membranes, rat erythrocyte and murine leukemic L1210 cell as well as artificial lipid bilayer were determined to investigate the cytotoxic mechanism of polyacetylenes against cancer cell lines. As results, panaxydol and panaxynol caused erythrocyte hemolysis dose-dependently while panaxytriol had no lysis. For liposomes composed of phosphatidyl choline (PC) and phosphatidic acid(PA), all three polyacetylenes supressed the osmotic behavior at the same degree. Panaxytriol gave least supression for liposome of PC/PA/Cholestorol. Electron microscopic observations demonstrated that panaxydol caused nonspecific injury to L1210 by affecting cellular membrane, nuclear envelope and mitochondria. It is concluded that cytotoxicities of polyacetylenes may be related to their damage to the membranes and panaxytriol gives least damage by its polarity and a little disturbance for cholesterol of lipid bilayer.*

Keywords: *Polyacetylene, Panax ginseng C.A. Meyer, Erythrocyte, Liposome, L1210 cell.*

INTRODUCTION

Polyacetylenes are distributed in seven families of plant kingdom, namely Araliaceae, Campanulaceae, Compositae, Pittosporaceae, Oleaceae, Santalaceae and Umbelliferae (Hansen and Boll, 1986). Their biological properites make them of interest to plant pathologists and pharmacologists. Since panaxynol was first isolated from ginseng root in 1964 (Takahashi *et al.*, 1964), series of polyacetylenes including C_{17}-or C_{14}-polyacetylene and C_{17}-compounds having chlorine or acetyl group were isolated (Poplawski *et al.*, 1980; Dabrowski *et al.*, 1980; Kitagawa *et al.*, 1983; Shim *et al.*, 1983; Kim *et al.*, 1988a; Ahn *et al.*, 1988). The biological studies has not been activated until pertroleum ether extract of ginseng was reported to show cytotoxicities against some cancer cell lines such as L5178Y, HeLa cell and Sarcoma 180 (Hwang and Cha, 1978) and to inhibit macromolecular synthesis in Sarcoma 180 (Yoon *et al.*, 1980). Recent investigation showed that panaxydol, panaxynol and panaxytriol in-

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hibited synthesis of DNA, RNA and Protein and the rate of inhibition was related to their cytotoxicities in L1210 (Kim *et al.*, 1988b). Besides cytotoxicities against cancer cell lines, antioxidant activities of panaxyne A and C(Lee, 1984), panaxydol, panaxynol and panaxytriol were reported (Kim(Jun) *et al.*, 1988).

Extracts of the leaves of *Schefflera digitata*, which were used by Maoris in New Zealand for treatment of skin disease, were found to contain falcarindiol, polyacetylenic compound (Muir *et al.*, 1982). It was observed that falcarindiol destroyed the plasma membrane of dermatophytes (Muir *et al.*, 1982) and artificial lipid bilayer membrane (Garrod *et al.*, 1979). Its antifungal activity led to the hypothesis that falcarindiol may be similar to the polyene antibiotics in its affinity for sterols (Kinsky *et al.*, 1967). Garrod *et al.* (1979) reported that the destructive ability of falcarindiol is presumably due to its hydrophobic nature and no sterol dependence.

In the present study, the effects of polyacetylenes on living membranes as well as artifical one were determined to investigate the cytotoxic mechanism of polyacetylenes against cancer cell lines. As a model of living membrane, rat erythrocyte and murine leukemic L1210 cell were used. For an artificial lipid bilayer, two kinds of liposomes were prepared from phospholipids with or without cholesterol and used for the study.

MATERIALS AND METHODS

Materials

Eggyolk lecithin was purchased from Sigma Co. and purified by alumina column chromatography. Other chemicals were obtained from the following sources; Tris (hydroxy methtyl) aminomethane from Kanto Chemical Co., Inc., Ethylenediamine tetraacetic acid disodium salt from Nakarai Chemical Co.; Fisher's medium and horse serum from GIBCO laboratories; Cholesterol, phosphatidic acid, penicillin-G and streptomicin disodium salt from Sigma Chemical Co.; Silica gel 60(70-230 mesh, ASTM) and silica gel 60F 254 plate (0.2mm) from Merck Chemical Co.; solvents for HPLC from J.T. Baker Chemical Co. All other reagents used were of guaranteed reagent grade commercially available.

Isolation of polyacetylenes from *Panax ginseng C.A. Meyer*

Polyacetylene compounds, panaxydol, panaxynol and panaxytriol were isolated from petroleum ether extract of ginseng root. Briefly, concentrated extract was repeatedly chromatographed over a silica gel column and active fractions were identified by thin-layer chromatography. Preparative HPLC was used for separation of pure compounds. Detailed methods were described by Kim (1988). Esters of fatty acids were also isolated from ginseng root and used only for erythrocyte study. The structures of polyacetylenes used in this study are shown in Table 1.

Effect of polyacetylenes on erythrocytes

Fresh rat bood was mixed with isotonic saline solution (154 mM NaCl containing 0.5% EDTA) 1:9(v/v) and centrifuged for 5 min on a microcentrifuge. The supernatant was discarded and the pellet resuspended in fresh saline, approximately five times

Table 1. Structures of polyacetylene compounds.

Compound	Structure
Panaxydol	C_7H_{15}-CH-CH-CH_2-$(C\equiv C)_2$-CH-CH=CH_2 (O; OH)
Panaxynol	C_7H_{15}-CH=CH-CH_2-$(C\equiv C)_2$-CH-CH=CH_2 (OH)
Panaxytriol	C_7H_{15}-CH-CH-CH_2-$(C\equiv C)_2$-CH-CH=CH_2 (OH OH; OH)

the pellet volume, and the process repeated (Garrod *et al.*, 1979). Three treatments were carried out in plastic micro-centrifuge tubes set up as follows: (i) 1 m*l* saline, 20 μl ethanol, (ii) 1 m*l* saline, 20 μl ethanol containing polyacetylenes or fatty acid esters and (iii) 1.02 m*l* distilled water. To all three treatments, 25 μl of washed erythrocytes in saline was added and this was considered at time zero. The tubes were all shaken gently and left for 15 min at 22°C prior to centrifugation, to remove intact cells, and spectrophotometric determination of the hemoglobin present in the supernatant fluid was carried out between 500 to 600 nm. % hemolysis was determined by a comparison of the absorbance at 577 nm with that obtained when erythrocytes were completely lysed.

Preparation of liposomes

Liposomes were prepared by the method of Yu and Jo(1984) but made with different ratio of phospholipids. Two kinds of liposomes were prepared from phospholipids with or without cholesterol. Egg phosphatidyl choline(PC), phosphatidic acid(PA) and/or cholesterol(Ch) in chloroform were mixed in the molar ratio of 2:0.3:1 and chloroform was then removed and further dried under vacuum. To this dry film, aqueous solution of 60 mM glucose (2 mM EDTA, 10 mM Tris-HCl buffer, pH 7.4) was added and the lipid was suspended by a vortex mixer. The dispersion equilibrated within an hour. Fresh liposomes were prepared at each experiment. To see the osmotic behavior in the absence of phosphatidic acid, other liposomes made of phosphatidyl choline and of phosphatidyl choline:cholesterol (2:1) were also prepared.

Osmotic behavior of multilamellar liposomes

Stock dispersion(0.05 m*l*) formed in 60 mM glucose solution was diluted with 3 m*l* of glucose solution of various concentrations to give the desired concentration gradients. Cin/Cout, the ratio of glucose concentration of liposome stock dispersion to that of dilution medium, was varied from 0.1 to 2.0. To see the effect of polyacetylenes on liposomes, polyacetylenes (30 μg/m*l*) was added to the glucose solution of various concentration beforehand. After 1 hr incubation, the absorbance at 450 nm was measured using a Beckman DU-6 Spectrophotometer. Temperature was maintained at 20 ± 2°C throughout the experiment.

Cell culture and sample treatment

Murine L1210 leukemic cells were grown and maintained as a suspension culture in Fisher's medium (GIBCO) consisting of 10% horse serum, $NaHCO_3$ (1.125 g/L), penicillin (100,000 unit/L), and streptomycin (100 mg/L) which was adjusted to pH 7.2. The cells were cultured at 37°C in 95% air; 5% CO_2 with a doubling time of 12-14 hr. To determine the effect of polyacetylenes on the cell, 5 ml of cell suspension (6.5×10^5 cell/ml) with treatment of panaxydol (10 μg/ml) was incubated for 10 hr. After incubation, cells were collected by centrifugation.

Electron microscopic examination

Cells were placed in a glutaraldehyde fixative (3% glutaraldehyde in 0.1M phosphate buffer, pH 7.4) overnight at 4°C, rinsed in buffer and postfixed in 1% osmium tetraoxide in the same buffer for 2 hr at 4°C. Following dehydration through a series of graded ethanol and propylene oxide, the tissues were embedded in Epon 812 (Electron Microscopy Science, Fort Washington, PA). Thin section were cut with an Ultramicrotome (Sorvall MT2-B, Ivan Sorvall Inc., Norwalk, CT), stained with uranyl acetate for 30 min and lead citrate for 10-15 min, and examined in an electron microscope (Model H-500, Hitachi, Ltd., Tokyo, Japan).

RESULTS

Effect of polyacetylenes on erythrocytes

Hemolysis of rat erythrocytes by polyacetylenes or esters of fatty acids was determined by a comparisom of the absorbance recorded with that obtained when erythrocytes were completely lysed. Total lysis was accomplished in the distilled water treatment; after centrifugation, the absorbance of this preparation at 577 nm was 0.9. When samples were treated at concentration of 30 μg/ml (Fig. 1), the 2% ethanol control or panaxytriol treatments showed no absorption between 500 to 600 nm since no hemolysis had occurred in these tubes. Panaxydol showed the largest hemolysis and then panaxynol and esters of fatty acids from ginseng roots did. As shown in Fig. 2, panaxydol, panaxynol and esters of fatty acids increased hemolysis dose-dependently. At concentration of 30 μg/ml, panaxydol caused approximately 67% lysis in 15 min at 22°C while panaxynol and esters of fatty acids showed 26% and 8% lysis, respectively.

Effect of polyacetylenes on the osmotic behavior of liposomes

An ideal osmotic behavior of liposomes was described as a linear relationship between the reciprocal 3/2 s power of absorbance at 450 nm and the osmotic gradient across the membrane (Yu and Jo, 1984). The multilamellar liposomes composed of PC with or without cholesterol showed no linear relationship with the osmotic gradient across the membrane (Fig. 3). However, addition of phosphatidic acid(PA) caused to show osmotic behavior of these two liposomes. The osmotic behavior was suppressed by cholesterol. For liposomes composed of PC and PA, all three polyacetylenes sup-

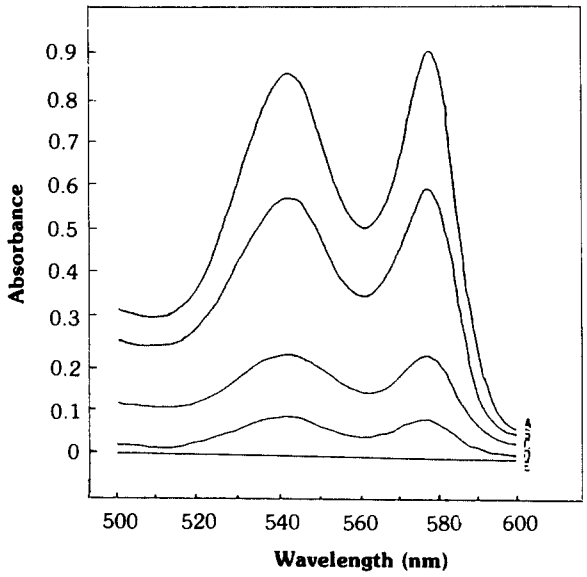

Fig. 1. Absorbance due to hemoglobin in supernatant fluids of: A, erythrocytes plus water; B, erythracytes plus saline containing 2% ethanol and panaxydol; C, erythrocytes with panaxynol in saline and 2% ethanol; D, erythrocytes with fatty ester in saline and 2% ethanol and E, erythrocytes plus saline containing 2% ethanol with or without panaxytriol.

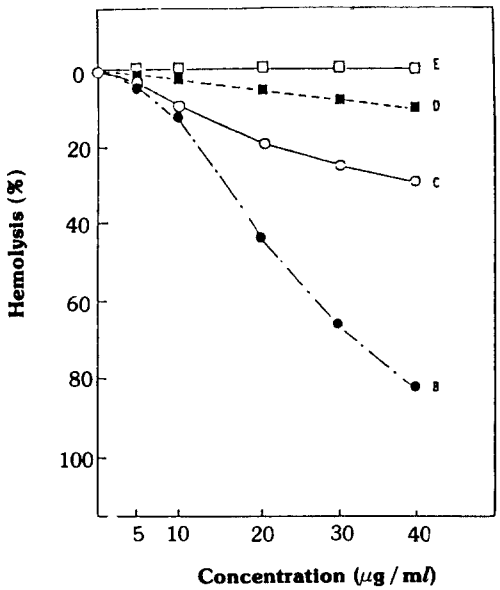

Fig. 2. Effect of various concentrations of polyacetylene compounds and fatty esters on erythrocyte hemolysis. Erythrocytes were incubated with saline containing 2% ethanol and polyacetylenes or fatty esters and % hemolysis was calculated assuming erythrocytes plus water as 100% hemolyzed control. B, erythrocytes with panaxydol; C, erythrocytes with panaxynol; D, erythrocytes with fatty ester and E, erythrocytes with or without panaxytriol.

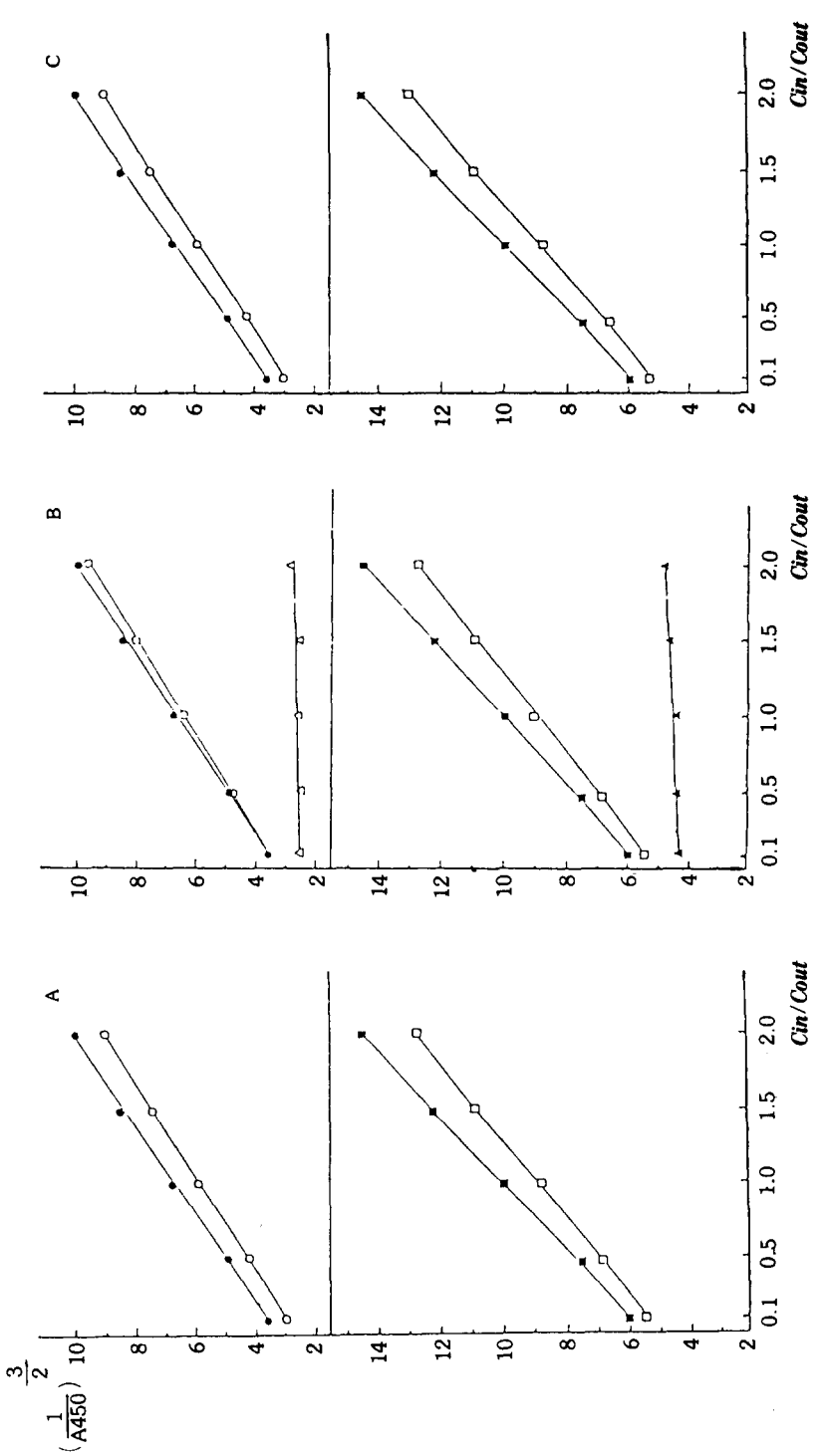

Fig. 3. Effect of polyacetylenes on the osmotic behavior of PC/Ch/PA(●) and PC/PA(■) liposomes, respectively. Blank symbols represent each polyacetylene-treated liposomes. A; panaxydol, B; panaxytriol, C; panaxynol. Neither PC/Ch(△) nor PC(▲) liposomes show osmotic behaviors.

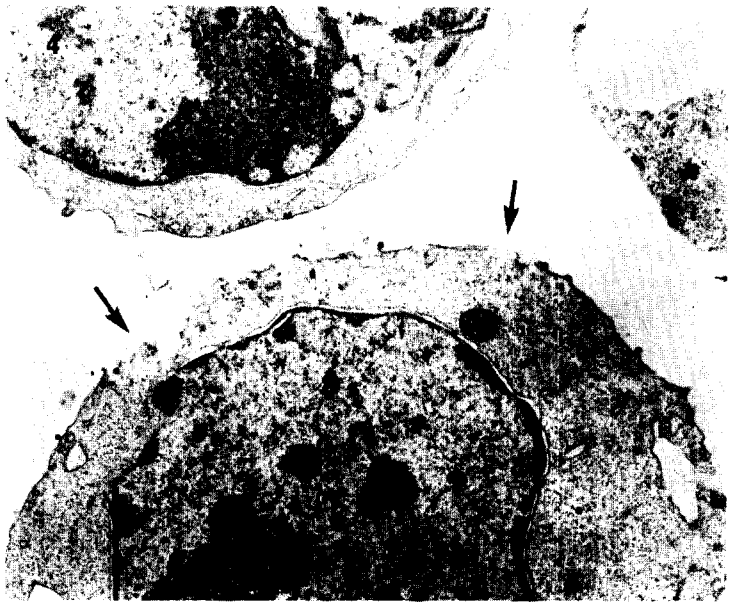

Fig. 4. Electron micrograph of L1210 cell incubated with panaxydol for 10 hr. Note damage of cellular membranes. × 10,000.

pressed the osmotic behavior at the same degree while panaxytriol showed least suppression among polyacetylenes for the osmotic behavior of PC/Ch/PA liposomes.

Effect of panaxydol on the morphology of L1210 cell

Cell membranes appear as three-layered structures: two dark layers sandwiching a lucent middle layer. The two dense lines represent the proteins and the hydrophilic portions of lipid molecules. The inner lucent layer represents the two hydrophobic ends of a phospholipid bilayer facing each other (Hand and Holmstedt, 1981). When panaxydol was incubated with L1210 for 10 hr., cellular membranes were broken partially (Fig. 4. and 5) and mitochondria were swollen and cristae were lost (Fig. 5 and 6). Since cytochromes and other enzymes, involved in cellular respiration in the presence of oxygen, are exclusively located at the surface of cristae (Hand and Holmstedt, 1981; Robbins *et al.*, 1984), diminished cristae may cause functional damage to the cell. In addition, nuclear envelopes which are two layers of trilaminar membranes were hardly seen in some cases of panaxydol-treated cells (Fig. 6) while sizes of nucleus and cells were not changed as compared to untreated control. These findings demonstrated that panaxydol caused nonspecific injury to L1210 cell at the concentration of 10 μg/ml for 10 hr-incubation.

DISCUSSION

Cellular membranes create a semipermeable barrier between the environment and

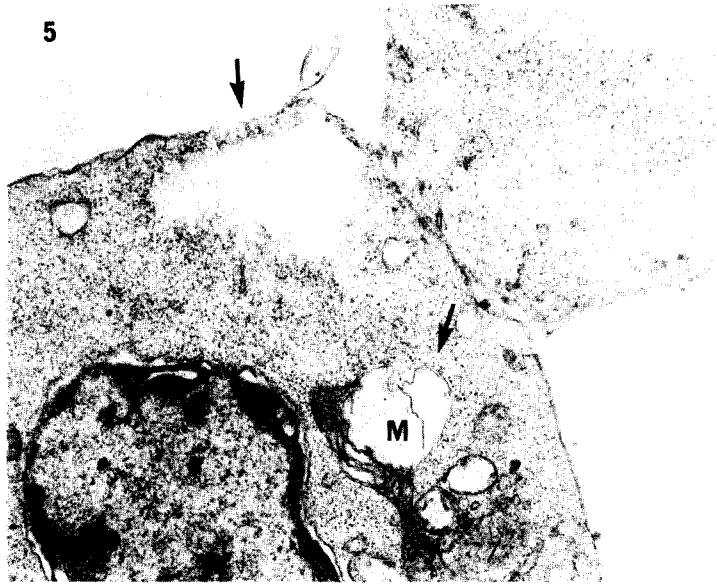

Fig. 5. Mitochondria(M) were swollen and cristae were lost by panaxydol. Cellular membranes were also damaged. × 15,000.

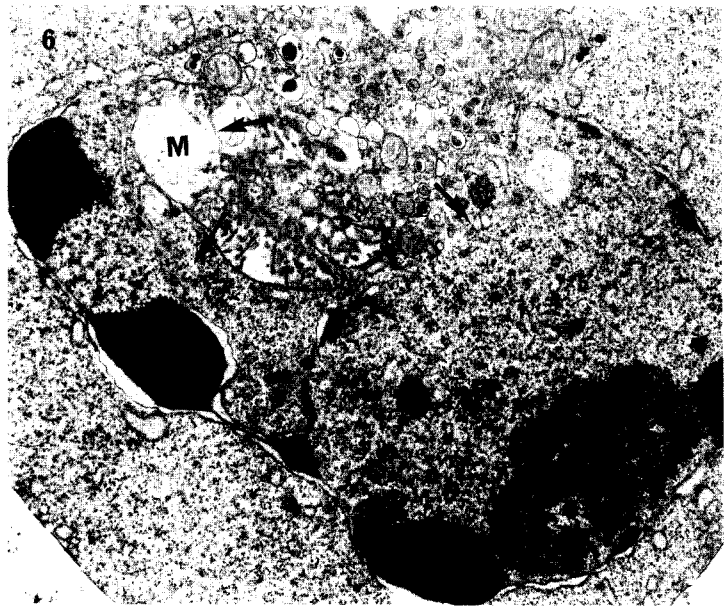

Fig. 6. Nuclear envelope was hardly seen (arrows) and damaged mitochondria were shown. × 15,000.

the cytosol. This allows differential uptake by the cell and diffusion of substances across the membrane. The nature of transmembrane transport of matters depends on the size, chemical composition, and electric charge of the substances (Han and Holmstedt, 1981). From electron microscopic observations, panaxydol led to the membrane damage of cell and nucleus which may be one of causes for cytotoxicity in L1210 cell. Erythrocyte experiment results showed that panaxydol and panaxynol increased hemolysis with concentration while panaxytriol did not cause lysis. Falcarindiol was adsorbed on the hydrophobic groups of the phospholipids in the biological membranes and formed mixed falcarindiol-phospholipid micelles (Garrod *et al.*, 1979). If the destructive ability comes from its hydrophobic nature (Garrod *et al.*, 1979), our results that panaxytriol did not interfere with erythrocyte membrane could be supported by its polarity. Between panaxydol and panaxynol, panaxydol has an epoxide which probably gives more damage to the cell membrane. Free fatty acids as anticancer agents have been shown to contribute to cytotoxic properties of effector cells, such as lymphocytes (Okudaira *et al.*, 1970) and macrophages (Chait *et al.*, 1982) by directly affecting the tumor cell surface (Siegel *et al.*, 1987). In this study, esters of fatty acids isolated from ginseng root caused hemolysis for erythrocyte dose-dependently but gave less damages than panaxydol or panaxynol.

Liposomes are useful model systems for studying many membrane related phenomena, especially properties of the lipid barrier of biological membranes. In this system, the mechanism of action of certain drugs can be studied without interference of other membrane and cell functions (Yu and Jo, 1984). In this study, liposomes were prepared with phosphatidyl choline: cholesterol (2: 1) to make a perfect model of rat erythrocyte and added 10% of phosphatidic acid for the sum of phosphatidyl choline and cholesterol to give a linear relationship between absorbance and the osmotic gradient across the membrane. Even though panaxydol, panaxynol and panaxytriol could not break the artifical membranes, they suppressed the osmotic behavior of PC/PA liposome. For liposome of PC/Ch/PA, panaxytriol had little effect on its osmotic behavior. Therefore, it is concluded that panaxytriol gives least damage to the membranes by its polority and a little disturbance for cholesterol of lipid bilayer and the cytotoxicities of polyacetylenes against L1210 might be related to membrane damage.

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