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SELECTIVE CYTOTOXICITY OF TUMOR CELLS INDUCED BY LIPOSOMES CONTAINING PLANT PHOSPHATIDYLINOSITOL

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Liposomes containing highly purified phosphatidylinositol (PI) from plant origin selectively killed tumor cells from 8 out of 9 cultured cell lines, but did not kill 4 types of normal cells. Other phospholipids, including PI or phosphatidylserine from animal origin, synthetic phosphatidic acid, phosphatidylglycerol, or phosphatidylcholine, were not cytotoxic. Cholesterol enrichment of cells, shown by other investigators to inhibit tumor development, was slightly cytotoxic in this system, but the toxic effect of cholesterol was minor compared to the massive cytotoxicity induced by plant PI.

It is well-known that the metabolism of phosphatidylinositol (PI) is associated with certain important cellular functions. Increased intracellular turnover of PI ("PI effect") characteristically occurs in a variety of cells when the cells are stimulated or activated, or when ligand-receptor interactions occur on the cell surface (1-5). The "PI effect" has evoked various theories regarding the intracellular role of PI (1-5). One of the characteristic features of PI in animals (but not in plants) is that the fatty acids consist predominately of arachidonic acid and stearic acid, and PI serves as a reservoir of arachidonate for prostaglandin synthesis (6-8). Phosphatidylinositol turnover and arachidonate utilization may be related. For example, lymphocyte mitogenesis has been reported to be associated with increased PI turnover (9), and breakdown (10), and arachidonic acid release (11,12).

In the course of experiments on the effects of extracellular phospholipids on cells, we have discovered that liposomes comprised of highly purified PI which

Abbreviations: PI, phosphatidyinositol; DPPG, dipalmitoyl phosphatidylglycerol; DPPC, dipalmitoyl phosphatidylcholine; DPPA, dipalmitoyl phosphatidic acid; PS, phosphatidylserine.

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lacks arachidonic acid (plant PI) have a striking cytotoxic effect on malignant transformed cells, but not on normal cells. The cytotoxic effect is so strong that it causes actual disintegration of the transformed cells. In this report we describe the selective cytotoxic effect of plant PI against tumor cells. We also show that tumor cytotoxicity does not occur with liposomes containing animal PI or any of several other acidic phospholipids.

MATERIALS AND METHODS

Cell Cultures. The ten tumor cells that were used in this study included: HL-60 (promyelocytic leukemia cells), Raji (lymphoblastoid-like cells from Burkitt lymphoma), Elaine (an established lymphoid cell line from an infectious mononucleosis patient), WI-38/SV-40 (transformed embryonic lung cells), HT-144 (melanoma cells), N4TG1 and SK-N-MC (neuroblastoma cell lines), U-87-MG (glioblastoma), Ehrlich-lettre (ascites carcinoma), and HT-29 (adenocarcinoma). The four normal cell lines were: WI-38 (embryonic lung cells), Detroit 551 (fibroblasts), HCMC (human colonic mucosal cells), and L cells (fibroblasts). All cell lines were of human origin except for the L cells and Ehrlich-lettre ascites cells (both lines from mouse). All cell lines were obtained from the American Type Culture Collection (Bethesda, MD), except: N4TG1, which was was a gift of Dr. Peter Chiang at WRAIR; L-Cells, purchased from Flow Laboratory; and WI-38, which was obtained from either American Type Culture Collection or M. A. Bioproducts Inc. Each cell line was cultured according to the specifications provided by the source, except that kanamycin was used instead of penicillin/streptomycin in the maintainence of cultures of all cell lines. However, penicillin/streptomycin (100 u/100 µg/ml) was used in the culture medium in the assay of cytotoxicity described below. Growth medium, serum and supplements were obtained from Gibco. Tumor cells were kept in culture for no more than 4-6 weeks, after which new cultures were established from frozen stock. Normal cells were started from frozen stock for each experiment in order to keep the number of passages low.

Assays for cytotoxicity. Four assays for cytotoxicity were used: thymidine uptake, microscopic quantitation, protein analysis (Lowry), and leucine incorporation. All of the assays were mutually consistent and resulted in identical conclusions. Because of this, quantitative results from only two of the assays (microscopy and thymidine uptake) are presented.

Thymidine uptake was assayed as follows. Day 1: Cells in their usual growth medium were plated in 96-well plates (100 µl/well) so that they would reach ca. 60% confluency 18 hrs. later. Suspension cutures were adjusted to ca. 60% of the cell density attained at the end of the log phase of growth. Day 2: Liposomes (10 µl/well) were added to give the concentrations indicated. Samples were done in triplicate or quadruplicate. Day 4: The samples were pulsed for three hours with ^{3}H-methyl-thymidine (1 µCi/10 µl/well) (ICN), 9 Ci/mmole, and then harvested by by using a Mash Cell Harvester (M. A. Bioproducts Inc.). Attached cells were scraped free using the probes of the cell harvester. Microscopic examination confirmed release of the cells. Filters were transferred to 5 ml scintillation vials, 0.3 ml of NCS Tissue Solubilizer (Amersham) was added, and the vials incubated overnight at 25°C. Five ml of spectrafluor (Amersham) was added, and the samples were counted in a Mark III scintillation counter (Searle).

In the microscopic quantitation assay, cells were plated as described above, except that 24-well plates were used. Volumes were increased proportionately, and the cells were treated with liposomes, as described. Using a hemocytometer, viable cells were counted in the presence of trypan blue in order to see intact cells in the presence of massive amounts of cell debris.

Liposomes. Multilamellar vesicles were prepared, as described in detail elsewhere (14), by vortexing dried lipids with a swelling solution consisting of Eagle's minimum essential medium in Earle's balanced salt solution. The proced-

ure was carried out under aseptic conditions. Penicillin/streptomycin was not included since it caused clumping of concentrated liposome suspensions. The liposomes were comprised of phospholipid/cholesterol in a 2/1 molar ratio, except where indicated, and the concentration of the phospholipid was 7.04 mM. The phospholipids were obtained from the following sources: DPPC, DPPG and plant PI (soybean): Sigma; plant PI (pea): Applied Sciences; PS and animal PI (both from bovine brain): Calbiochem-Behring; and DPPA: Supelco. Cholesterol was purchased from Calbiochem-Behring.

Repurification and characterization of Plant PI. Although the plant PI was chromatographically pure when received, to minimize the possibility that our results might have been caused by a contaminant, plant PI (soybean) was carefully repurified by preparative thin layer chromatography using silica gel 60 precoated plates as described by Wassef et al. (15). PI was detected by spraying a portion of the plate with molybdenium blue reagent. Hydrolytic conversion of the PI fatty acids to methyl esters followed by gas chromatographic analysis revealed the following fatty acid compositions: palmitic, 37%; stearic, 6.7%; oleic, 4.3%; linoleic, 47.5%; linolenic, 4.2%. There was no detectable arachidonic acid. Repurification of PI did not result in any change of tumor cell cytotoxic activity.

RESULTS

Cytotoxicity induced by plant PI. All of the cultured cells were treated with liposomes having various lipid compositions. After treatment with liposomes containing plant PI/cholesterol (2/1) it was observed by all four criteria for cytotoxicity that tumor cells, but not normal cells, became irreversibly and lethally damaged. Eventually the tumor cells disintegrated, producing fragments of cell debris. Microscopic examination of plant PI-treated tumor cells (Fig. 1a, 1b, 1c, right frames) illustrated the degree of damage imparted to three of the tumor cell lines, and these were compared to the untreated cells (Fig. 1a, 1b, 1c, left frames). Of ten tumor lines tested, only one, HT-29, was not susceptible to the cytotoxic effect of plant PI. In contrast, among normal cell lines incubated with equivalent concentrations of liposomes containing plant PI, none were killed. As an example of normal cells, mouse L cells are shown in the presence (Fig. 1d, right) and absence (Fig. 1d, left) of the same liposomes.

As shown by quantitative microscopic analysis (Fig. 2a), ca. 50% of the tumor cells were killed by incubation with liposomes containing plant PI at a PI concentration of 120 μM, and 90% were killed by 160 μM PI. In order to evaluate the effects of plant PI on normal and tumor cells in a more convenient quantitative manner, a rapid and reliable assay was developed to assess cytotoxicity by utilizing ^{3}H-methyl-thymidine uptake. Suppression of uptake of ^{3}H-methyl-thymidine was observed in cultures of Raji and WI-38/SV-40 transformed cells after treatment with plant PI (Fig. 2b). In contrast, two normal cell lines, L cells and

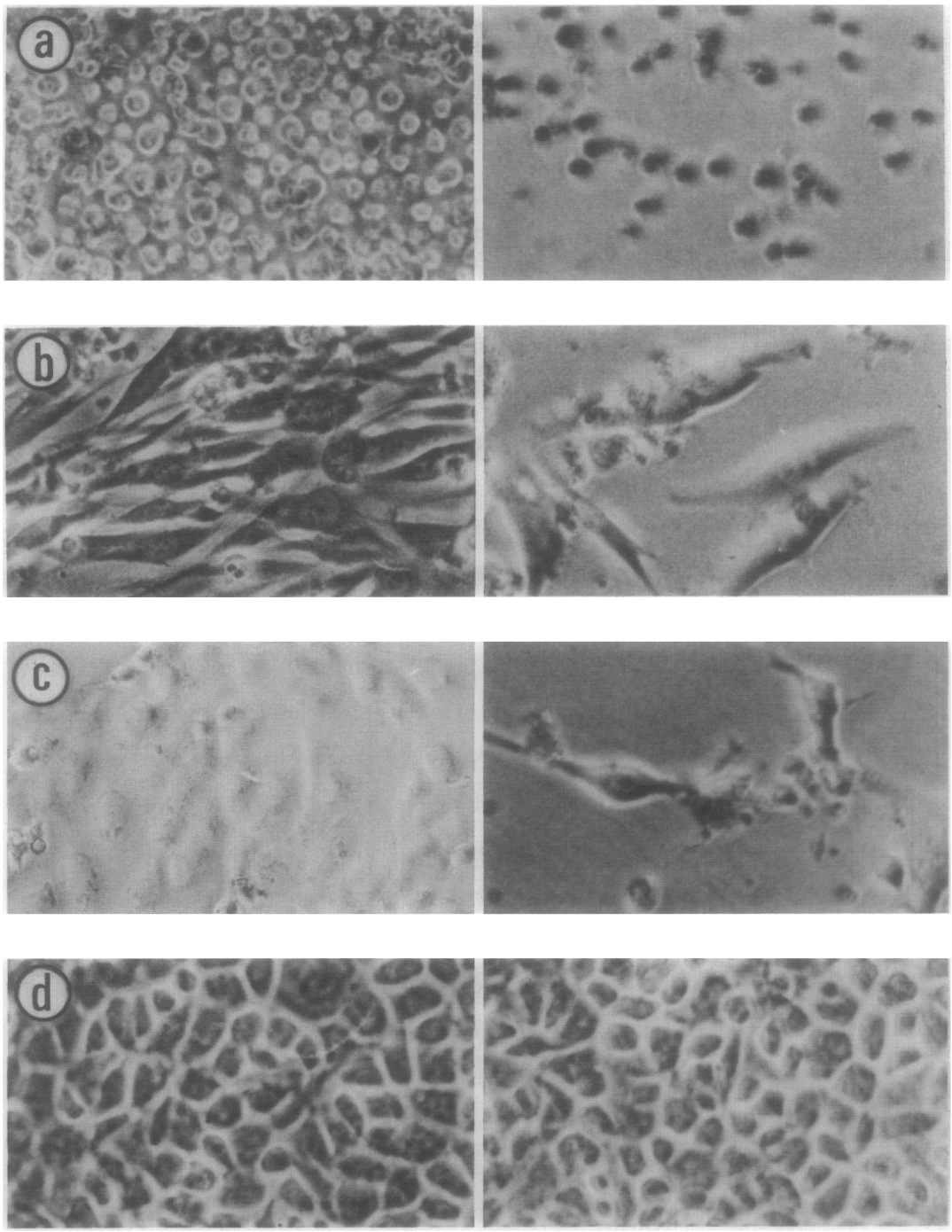

Fig. 1. Photomicrographs of the effect of plant PI on normal and tumor cells. Cells were plated (day 1) so that they would be 60% confluent at the time of liposome (PI/cholesterol, 2/1) addition on day 2. The frames on the left illustrate cells not treated with liposomes, and the frames on the right show the same cells after treatment for 2 days (day 4) with liposomes containing plant PI. The cell lines shown are Raji lymphoblastoid cells (a), HT-144 melanoma cells (b), WI-38/SV-40 transformed lung fibroblasts (c) and mouse L cells (d). Original magnification, 58 x.

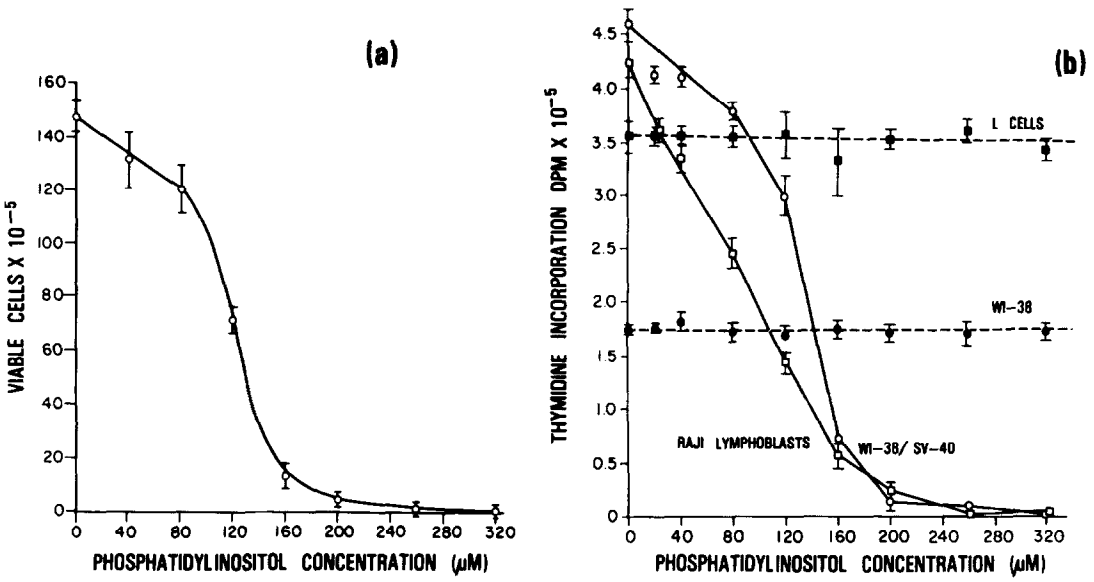

Fig. 2. Cytotoxic effects of plant PI. (a) Microscopic quantitation. Raji lymphoblastoid cells were plated in 24-well cluster sterile plates (0.72 x 10^6 cells/well). Eighteen hours later, liposomes containing plant PI/cholesterol in a 2/1 molar ratio were added at the PI concentrations indicated. After three days, surviving cells were counted, using a hemocytometer, in the presence of trypan blue. (b) Thymidine incorporation. Tumor (———) or normal cells (-----) were plated (96-well cluster sterile plates) so that ca. 60% confluency would be achieved 18 hours later. Liposomes containing plant PI/cholesterol (2/1) were then added. Two days after liposome addition, the cells were pulsed for 3 hours with ^{3}H-methyl thymidine, as described in "Methods". All of the results are reported as DPM x 10^{-5}, except for the WI-38 normal cells where the results are DPM x 10^{-4}. All of the experiments in this study were done in triplicate, and the geometric mean and standard error of the mean are shown. The data are representative of results obtained on at least three separate days.

WI-38 cells, were not affected (Fig. 2b). Identical cytotoxic effects of PI were also found by examining uptake of ^{14}C-leucine, and by measuring total cellular protein (data not shown). The relative sensitivities of various tumor cell lines to killing by plant PI differed only by 3 to 4-fold. In order to quantify the relative sensitivities, the median lethal dose of plant PI in tissue culture was determined. For each tumor cell line, the median lethal PI dose was as follows: N4TG1 (70 μM), Raji (120 μM), Ehrlich-lettre (125 μM), WI-38/SV-40 (135 μM), SK-N-MC (180 μM), HT-144 (205 μM), U-87-MG (240 μM), and HL-60 (265 μM). One other cell line, Elaine, was also sensitive to plant PI, but the range of plant PI concentrations was not examined sufficiently to assign an exact median lethal dose.

Effects of cholesterol. Because of previous reports that suggested that alteration of cell cholesterol by liposomes laden with cholesterol might have cyto-

toxic effects on tumor cells (16-18), we examined the effect of cholesterol in our system. As shown in Fig. 3, we confirmed that increased liposomal cholesterol did increase the cytotoxicity of plant PI in Raji lymphoblastoid cells. There was also a slight cytotoxic effect of cholesterol on L cells (an example of normal cells). This latter cytotoxic effect was observed under conditions where liposomes would be expected either to extract cholesterol from cell membranes (PI/ cholesterol, 2/0), or to increase the cholesterol content of the cell membranes (PI/cholesterol, 2/4). However, when compared with the tumoricidal effect of liposomes containing plant PI, the cholesterol-induced cytotoxicity on tumor or normal cells was minor.

Effect of phospholipid composition. Among the phospholipids tested, a tumoricidal effect was observed only with plant PI. As shown in Fig. 4, other acidic or neutral phospholipids, including animal PI, animal PS, DPPA, DPPG or DPPC, had no effect on any of the normal or tumor cell lines, and several phospholipids even had a slight stimulatory effect.

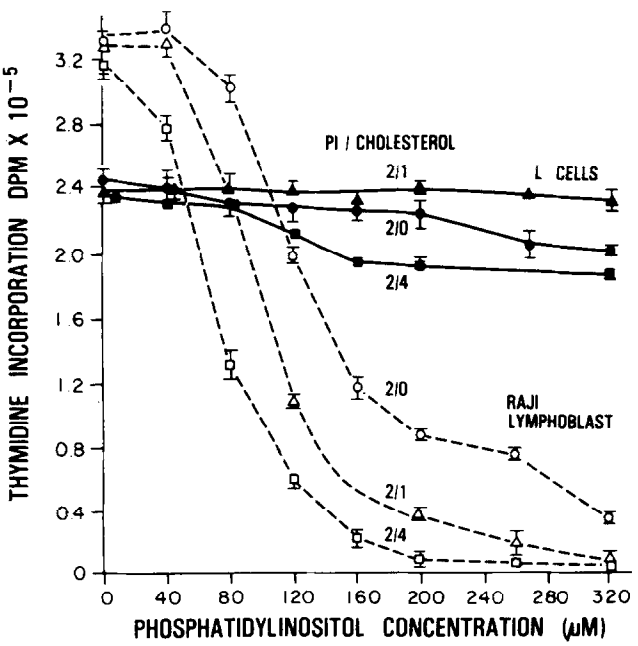

Fig. 3. Influence of cholesterol. Raji lymphoblastoid cells (----) and normal L cells (——) were plated, the liposomes added, and the cells harvested exactly as described in the legend for Fig. 2b. The molar ratios of plant PI/cholesterol are indicated on the figure.

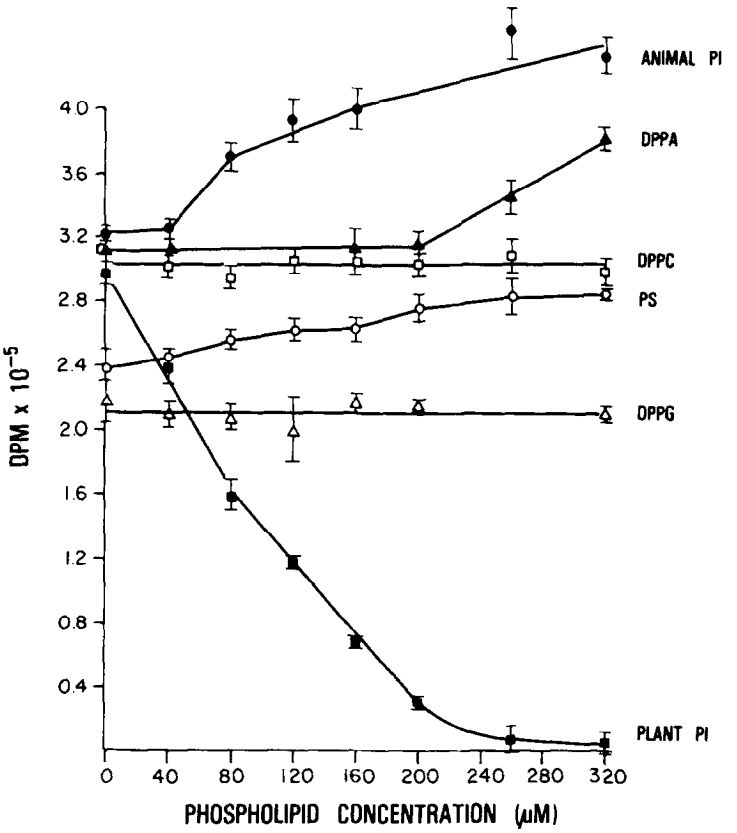

Fig. 4. Comparative effects of different phospholipids. Raji lymphoblastoid cells were plated (0.9 x 10^5 cells/well) in 96-well cluster sterile plates, and 18 hours later the liposomes containing phospholipid/cholesterol (2/1) were added. The liposomal phospholipids are indicated on the figure. The cells were harvested as described in the legend for Fig. 2b.

DISCUSSION

Our results show that liposomes containing PI from plant origin can have potent cytotoxic effects on certain tumor cells, but not on normal cells. Although the mechanism of this phenomenon has not yet been completely elucidated, several clues are available. First, the liposomes obviously did not have nonspecific disruptive effects on all cell membranes, since all four normal cell lines, and one of the ten tumor cell lines, were unaffected. The ability to be killed by plant PI apparently was a characteristic property of the sensitive tumor cells. Second, the cytotoxic effects were not due to alteration of cell surface properties caused by a differential ability of liposomes containing plant PI to bind to cells. By examining the amount of liposomal radioactivity that became associated with the cells, we found that approximately the same amount of each of the liposome types,

regardless of the tumoricidal activity, was taken up by each of the tumor and normal cell types. Cellular uptake of liposomes was determined after inclusion of ^{14}C-cholesteryl oleate as a trace constituent of the liposomes. Third, the effects were not secondary events induced by cholesterol transfer. Cholesterol-loading of cells by liposomes laden with cholesterol has been reported to inhibit the growth of tumor cells (16-18). In this study we did find (Fig. 3) that increased liposomal cholesterol enhanced the cytotoxic effect of plant PI, but the cytotoxicity was still strongly observed even when the liposomes lacked cholesterol. Fourth, liposomes containing a wide variety of phospholipids other than plant PI (Fig. 4) were not cytotoxic to either tumor or normal cells. These results make it unlikely that the cytotoxicity of plant PI was caused by alteration of the fluidity of the plasma membrane by transfer of plant PI from the liposomes. There was no correlation between the potential fluidity differences of the various phospholipids used and the ability to induce cytotoxicity.

Increased turnover of intracellular PI has been associated with a number of cellular functions, including the malignant state (1-5,19-21), but the exact purpose of this increased turnover of PI has not been completely clarified. PI turnover has been proposed as a mechanism that promotes intracellular calcium movement (1-5), and has been linked to cyclic nucleotides (1-5,22), and to functions of protein kinases (23-27). Clearly, each of the above proposed functions of intracellular PI is sufficiently important that alteration of any one of them might have adverse effects on cell viability, and such alterations could be responsible for the cytotoxic effects on tumor cells that we observed.

In the present study, one of the most prominent observations was the absence of suppression by animal PI. An important difference between plant and animal PI is that animal PI contains a high concentration of arachidonic acid. It is possible that the fatty acid composition of plant PI (see Materials and Methods), and particularly the absence of arachidonic acid, may have played a role in tumor cytotoxicity induced by plant PI.

Regardless of the mechanism, the present observations strongly suggest that extracellular presentation of highly purified plant PI can have a killing effect

on many types of tumor cells in tissue culture, and the system may have potential usefulness for elucidating certain novel biochemical aspects of tumor cells. In addition, if extracellular PI is able to participate in the events normally associated with intracellular PI turnover, this may have implications with respect to the intracellular "PI effect" (1-5). Obviously, the discovery of cytotoxic effects of plant PI on tumor cells also raises the potential that this phospholipid might have therapeutic usefulness. Resolution of this latter question must await in vivo studies.

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