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Research Article

Modulation of Human Ovarian Tumor Cell Sensitivity to *N*-(Phosphonacetyl)-L-Aspartate (PALA) by Liposome Drug Carriers

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Carrier-based formulations of cytotoxic agents may be highly efficacious for intracavitary therapy of malignancies which reside in or metastasize to the peritoneal cavity. *N*-(Phosphonacetyl)-L-aspartic acid (PALA) is a transition-state inhibitor of aspartate transcarbamylase which has shown enhanced activity against several cell lines upon encapsulation in liposomes. We have examined the growth inhibitory effects of PALA-containing liposome formulations against four human ovarian cancer cell lines (Ovcar-3, Hey-1b, A90, and A121a) that have significantly different growth characteristics. With the optimal liposome formulation defined in the present studies, the potency of encapsulated PALA was 22- to 570-fold greater than that of free PALA, depending on the cell line. Control liposomes containing buffer, rather than PALA, did not inhibit cell growth. Fluorescence studies of liposome–cell interaction suggest that high liposome negative surface charge density and high phase transition temperature increase both cellular association and retention of liposome contents. Briefer exposure of tumor cells to treatment accentuates the advantage of liposome formulations; on Hey-1b cells, the cytostatic effect of 1-hr exposure to PALA-liposomes is 900-fold greater than is the equivalent exposure to free PALA. The considerable increase in *in vitro* potency of PALA–liposome formulations, coupled with potential pharmacokinetic advantages *in vivo* (i.e., intraperitoneal retention of liposome-associated drug versus rapid clearance of free PALA), suggests the possibility of enhanced antitumor activity of liposome-encapsulated PALA for both single-agent and combination chemotherapy.

KEY WORDS: *N*-phosphonacetyl-L-aspartate (PALA); liposome; endocytosis; human ovarian carcinoma; aspartate transcarbamylase.

INTRODUCTION

Direct intracavitary administration of chemotherapeutic agents has been proposed as a means to exploit pharmacokinetics for improved therapy of cancers that arise in or metastasize to the peritoneal cavity (1,2). Although drugs with the appropriate pharmacology and pharmacokinetic profiles have been identified, clinical success of intracavitary therapy has been limited (reviewed in Ref. 3). Rapid absorption of drugs from the peritoneal cavity into the systemic circulation is a major hindrance to establishing high local concentrations of drug at the site of disease. Owing to the greatly prolonged residence time of particles in the peritoneal cavity, compared to low molecular mass compounds, as well as the obstruction of lymphatic drainage that occurs in ovarian cancer (4,5), colloidal or particulate drug carriers may enhance the approach of intracavitary therapy (6–8).

Liposomes (phospholipid vesicles) are spontaneously forming microparticulate carriers that are in clinical trial or under study as drug carriers for treatment of several neoplastic and infectious diseases (reviewed in Ref. 9). The widening variety of liposome-encapsulated drugs entering clinical trial reflects an emerging understanding of the safety, utility, and the methodology to produce this experimental drug carrier system. However, development of clinically useful formulations that take advantage of certain unique features of the carrier has been hampered by the fact that few clinically used agents have properties that complement the mechanisms by which liposomes mediate intracellular delivery.

PALA[3] [*N*-(phosphonacetyl)-L-aspartate], a transition-state inhibitor of aspartate transcarbamylase (ATCase), may

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[3] *Abbreviations used:* ATCase, aspartate transcarbamylase; Chol, cholesterol; DPPG, dipalmitoylphosphatidylglycerol; DSPC, distearoylphosphatidylcholine; DSPG, distearoylphosphatidylglycerol; PALA, *N*-(phosphonacetyl)-L-aspartic acid; PBS, Dulbecco's phosphate-buffered saline; PBS-CM, PBS supplemented with 5 m*M* Ca^{2+} and 5 m*M* Mg^{2+}; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; HPTS, 8-hydroxy-1,3,6-pyrene-trisulfonate; IC_{50}, drug concentration giving 50% inhibition of cell growth.

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be the most nearly-ideal liposome-dependent agent yet described (10,29). Free PALA has been studied extensively in human clinical trials (11) but has shown somewhat disappointing activity as a single agent. This limited success of free PALA might be related to the proposed mechanism for its cellular uptake: free PALA enters tumor cells by fluid-phase endocytosis and transfers into the cytoplasm after acidification of endocytic vesicles (12). Although fluid-phase endocytosis is very limited in its efficiency, such a mechanism for drug delivery complements the mechanism by which liposomes interact with cells. Liposomes are endocytosed and deliver high drug concentrations into the endocytic apparatus of cells. Endocytic vesicles are a progressively acidifying compartment, from which weakly acidic compounds can escape to the cytoplasm (13,14). Experimentally, PALA has shown some of the properties of a lysosomotropic drug; these may explain the significant increase observed in the cytotoxicity of liposome-encapsulated PALA (10).

Our previous work has shown that encapsulation of prototype liposome-dependent drugs gave only small improvements in cytotoxicity to human ovarian tumor cells *in vitro* (15). In order to test the hypothesis that liposome-encapsulated PALA may be superior for therapy of intraperitoneal malignancies, we have developed, analyzed, and optimized PALA-liposome formulations in an *in vitro* system. The model system we have chosen employs human ovarian cancer cell lines established from patients (a) prior to treatment (A121a) (16), (b) clinically resistant to a single chemotherapeutic agent (A90 and Hey-1b) (16,17), or (c) clinically resistant to a combination chemotherapy regimen (Ovcar-3) (18). These human ovarian cancer cell lines have several major advantages over murine or nontumor cell lines. First, they originated from the surface germinal epithelium of the ovary, which is the usual progenitor of epithelial tumors of the ovary. Second, implantation of these cell lines in athymic nude mice produces tumors which retain morphologic, karyotypic, and drug sensitivity characteristics of the original cells from which they were derived (19). Thus the *in vitro* studies presented here represent a requisite first step in the development of formulations for testing against *in vivo* models of human ovarian cancer.

MATERIALS AND METHODS

Reagents. *N*-(Phosphonacetyl)-L-aspartic acid was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol was purchased from Sigma (St. Louis, MO) and was recrystallized three times from methanol. Phospholipids and cholesterol were stored in chloroform under argon at −70°C. HPTS (8-hydroxy-1,3,6-pyrene-trisulfonate) was purchased from Molecular Probes (Junction City, OR). Growth media, 0.5% trypsin solution, 10,000 units/ml penicillin and streptomycin, and fetal bovine serum were purchased from GIBCO (Grand Island, NY). Sephadex G75 was obtained from Sigma.

Cell Culture. A90 and A121a, human epithelioid ovarian tumor cell lines of ascites tumor origin, were obtained from the Roswell Park Cancer Institute (Buffalo, NY), originally from the laboratory of Dr. K. Crickard (Roswell Park Cancer Institute/Buffalo General Hospital). The Hey-1b human ovarian tumor cell line was obtained from Dr. A. Marks (University of Toronto, Canada). Ovcar-3 was obtained from Dr. T. Hamilton (National Cancer Institute, Bethesda, MD). Colon-26, a murine tumor cell line, was obtained from Dr. E. Mayhew (Roswell Park Cancer Institute/SUNY–Buffalo). These cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin. The murine macrophage-like cell line J774 was obtained from the American Type Culture Collection/NIH repository. CV-1, an established line of African green monkey kidney cell was obtained originally from the laboratory of Dr. P. Berg (Stanford University, Palo Alto, CA). The J774 and CV-1 cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin.

Liposome Preparation. Liposomes were prepared by the reverse-phase evaporation procedure (REV) of Szoka and Papahadjopoulos (20). Modifications of the procedure for small-scale preparation are detailed in Ref. 21. Typically, 30 μmol of phospholipid, 1.5 ml of aqueous solute, and 2 ml diethyl or diisopropyl ether were used for a single preparation. PALA was captured at 45 m*M*. HPTS, a membrane-impermeant, aqueous fluorescent marker (14,22,23), was captured at 35 m*M*. Liposomes were extruded through polycarbonate filters of 0.2-μm pore diameter (24) to produce a more homogeneous size distribution and were separated from unencapsulated material by gel chromatography on Sephadex G-75 (Pharmacia). Phospholipid concentration was determined by phosphorous assay (25) and encapsulation of PALA was determined by phosphorous assay after extraction from liposomes (26).

Growth Inhibition Experiment. Cells were plated at a density of 2×10^4 cells/ml in 24-well plates (Costar) and allowed to attach overnight. Triplicate wells were exposed to various treatments, and cells were enumerated 72 hr later. "Wash experiments" (cf. Fig. 4) were performed in a manner similar to growth inhibition experiments except that cells were washed free of drug after defined periods of exposure (1–72 hr) and were returned to culture in complete medium for the remainder of the growth period.

Cell-Associated Fluorescence. Cells (1×10^6) were plated in 60-mm tissue culture dishes in complete growth medium. After 24 hr, HPTS-containing liposomes were added at a ratio of 50 nmol of phospholipid per 1×10^6 cells/ml, either in complete growth medium or after washing with Dulbecco's phosphate-buffered saline (PBS). At defined time points, cells were washed free of unbound liposomes and assayed for HPTS fluorescence. For experiments of a design similar to wash experiments, cells were washed free of HPTS-containing liposomes and incubated in complete growth medium for additional time before assay for total cell-associated fluorescence.

Fluorometric Analysis. HPTS-containing cell samples in PBS were solubilized with 0.1% Triton X-100 and the pH was adjusted to >10 for maximum fluorescence yield. Fluorescence was monitored with an Aminco fluorometer (excitation, 450 nm/emission, 510 nm), and HPTS concentration was determined by comparison to a standard curve.

RESULTS

Growth Inhibition Potency of Free PALA on Human Ovarian Tumor Cell Lines

Four human ovarian tumor cell lines were assayed for their sensitivity to free PALA. The potency of PALA is known to vary with cell growth rate and ATCase content (27). With a continuous 72-hr exposure to free drug, the concentration required for 50% inhibition of cell growth (IC_{50}) is similar for Hey-1b and A121a human ovarian cancer cells (Table I). In contrast, the tumor cell lines A90 and Ovcar-3 are 8- to 10-fold less sensitive to PALA and are the least sensitive of all cell lines tested. Thus human ovarian tumor lines display a substantial range of sensitivity to the free drug.

Additional cell lines were tested to allow comparison of the present work with previous studies (10) or to anticipate future experiments on the antitumor efficacy of PALA liposomes. Colon-26, a murine colon tumor cell line, is twofold more sensitive to free PALA than Hey-1b, the most sensitive ovarian cancer cell line. Of the cell lines derived from normal tissue, CV-1 (African green monkey kidney) is relatively resistant to free PALA, with an IC_{50} about twofold lower than the most PALA-resistant ovarian tumor lines. In contrast, J774, a murine macrophage-like cell line, is the most sensitive of all cell lines tested, 10-fold more sensitive than the most sensitive tumor cell line (Colon-26). Thus, the panel of cells selected for the present studies varies more than 200-fold in *in vitro* sensitivity to free PALA. The variation among the human ovarian tumor lines is smaller, but nonetheless substantial, with 10-fold differences between the most- and the least-susceptible cell lines.

Effects of PALA Encapsulated in Liposomes

Previous work developing liposomes for delivery to the human ovarian tumor cell line Ovcar-3 achieved a 2.5- to 5-fold increase in the potency of encapsulated methotrexate-γ-aspartate (MTX-γ-Asp) or 5-fluoroorotate (FO), respectively (15), compared to free drug. In the present work, we find that phosphatidylglycerol:phosphatidylcholine:cholesterol (PG:PC:Chol, 2:8:5 mole ratio) liposomes mediate a similar increase in the potency of PALA on the Ovcar-3 cell line (Fig. 1), approximately 3.5-fold. Other human ovarian tumor lines varied considerably in their sensitivity to PALA in PG:PC:Chol (2:8:5) liposomes (Fig. 1). Hey-1b showed the smallest liposome-enhanced potency of PALA (2-fold), while A121a showed the greatest enhancement of potency by liposomes (28-fold), compared to free drug. A121a also was the cell line most sensitive to PALA encapsulated in PG:PC:Chol liposomes. Encapsulation increased PALA potency 7-fold on A90, one of the cell lines most resistant to free PALA.

Table I. Growth-Inhibitory Activity of Phosphonacetyl-L-Aspartate *in Vitro* on Normal and Tumor Cell Lines

Cell line	IC_{50} (μM)[a]	SD (μM)[b]
Ovcar-3	50.9	8.80
A90	40.0	8.16
A121a	5.75	3.30
Hey-1b	4.50	1.87
Colon-26	2.10	0.27
CV-1	28.0	9.70
J-774	0.24	0.06

[a] Concentration of drug resulting in 50% inhibition of cell growth; cells were exposed to drug continuously over the period of assay.
[b] SD: standard deviation.

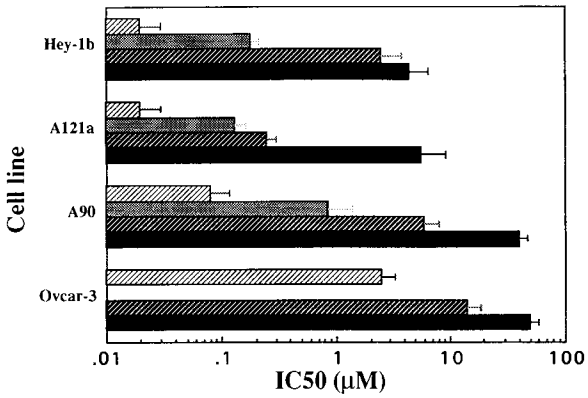

Fig. 1. Sensitivity of human ovarian tumor lines to free and encapsulated PALA. Cells were propagated and treated with free or encapsulated PALA as described under Materials and Methods. The IC_{50} value for each concentration–effect curve was calculated graphically. All experimental points were determined in triplicate, and experiments were repeated at least five times. Filled bars, free PALA; dark hatched bar, PALA in PG:PC:Chol (2:8:5) liposomes; stippled bar, PALA in PG:Chol (2:1) liposomes; light hatched bar, PALA in DSPG:Chol (2:1) liposomes.

Effects of Liposome Surface Charge and Lipid Acyl Chain Composition on Cytostatic Potency of PALA-Liposomes

Previous work has demonstrated that highly negatively charged liposomes are efficient in delivering drugs or macromolecules into cells (10,28–30). Therefore, we tested the effect of high negative-charge phospholipid content on the cellular delivery of PALA to tumor cell lines. Depending on the cell line, increasing the PG content of cholesterol-containing liposomes from 20 to 100% of the phospholipid content (i.e., PG:Chol, 2:1) results in a 2- to 14-fold further increase in the potency of encapsulated PALA, compared to liposomes of PG:PC:Chol (2:8:5) (Fig. 1). The greatest increase in PALA potency (14-fold), obtained by increasing liposome negative charge, was observed in Hey-1b, the cell line showing the lowest sensitivity to PALA in PG:PC:Chol liposomes.

Phospholipid acyl chain composition has a major impact on liposome stability (31). Previously it was found that liposomes in which dipalmitoylphosphatidylglycerol (DPPG) is substituted for phosphatidylserine (PS) are more stable in serum-containing media (15,30). Here, substitution of distearoylphosphatidylglycerol (DSPG) for PG in cholesterol-rich liposomes resulted in a considerable (7- to 13-fold) further increase in the potency of encapsulated PALA (Fig. 1). In the case of Hey-1b and A121a cells, there was a 7.5- to 9-fold increase in the potency of PALA encapsulated in DSPG:Chol liposomes, compared to PG:Chol liposomes. The total gain in potency compared to free PALA was 225- to 280-fold for the two human ovarian tumor lines most sensi-

tive to PALA (Hey-1b and A121a, respectively). For the two lines most resistant to PALA, the total gain in PALA potency was more variable; A90 showed more than a 500-fold increase, while Ovcar-3 showed a 20-fold increase in potency compared to free drug.

A systematic study was undertaken to investigate the relationship between the liposome content of negatively charged phospholipids and the potency of PALA encapsulated in high-phase transition ("gel-phase") liposomes. Previous studies using fluorescence assays have shown that cell lines vary with respect to the extent and rate of endocytosis for fluid liposomes of different fractional negative charge (32).

Liposomes were prepared of distearoyl phospholipids and cholesterol at a molar ratio of 2:1. Liposome phospholipid composition ranged from 100% DSPG to 100% distearoylphosphatidylcholine (DSPC), with several intermediate DSPG:DSPC ratios. Figure 2 shows the dependence of PALA cytostatic potency on liposome negative charge. For all cell lines, PALA potency increases with increasing negative charge; for most, >50 mol% DSPG is required for the most significant gains in potency. The epithelioid line CV-1 is the exception, with ≥25 mol% DSPG resulting in the maximal increase in potency, about 150-fold. This result correlates with previous studies that show maximal endocytosis by CV-1 of fluid liposomes having ≥10 mol% negative charge (32).

For the tumor lines Hey-1b, A90, A121a, and Colon-26, PALA in liposomes having ≤50 mol% negative charge is equipotent to or less potent than free PALA. Highly stable liposomes having too little charge for efficient interaction with those cells may sequester drug in the extracellular medium and, thus, render it less active than free PALA. Colon-26 and Hey-1b show the odd behavior that liposomes having 10 mol% negative charge are less potent than liposomes with 0 mol% charge (i.e., DSPC:Chol, 2:1); the latter liposomes should interact with cells even less efficiently than liposomes of 10% charge. We surmise that liposomes having a small fraction of negative charge are comparatively less leaky to PALA, which exists as a multivalent anion at neutral pH; 100% DSPC liposomes may leak a higher fraction of their contents, and leaked drug would be taken up as free PALA.

The macrophage-like line J774 is the most remarkable in terms of the dependence of PALA potency on liposome negative charge; PALA liposomes of ≤50 mol% charge are less potent than free PALA, and 100% DSPG:Chol (2:1) are more than 1200-fold more potent than free drug. In the range of 50 to 75 mol% negative charge, PALA potency changes approximately 3 orders of magnitude. Previous studies on the endocytic rate for fluid liposomes did not include data for 75% charge but did show the general trend that J774 cells require high negative charge for efficient internalization of liposomes (32).

Growth Inhibitory Properties of Buffer-Containing Liposomes

Reformulation of PALA in DSPG:Chol (2:1) liposomes resulted in large increases in drug cytostatic effect. Experiments were undertaken to determine the inherent cytostatic effect of the liposome carrier and whether the carrier could

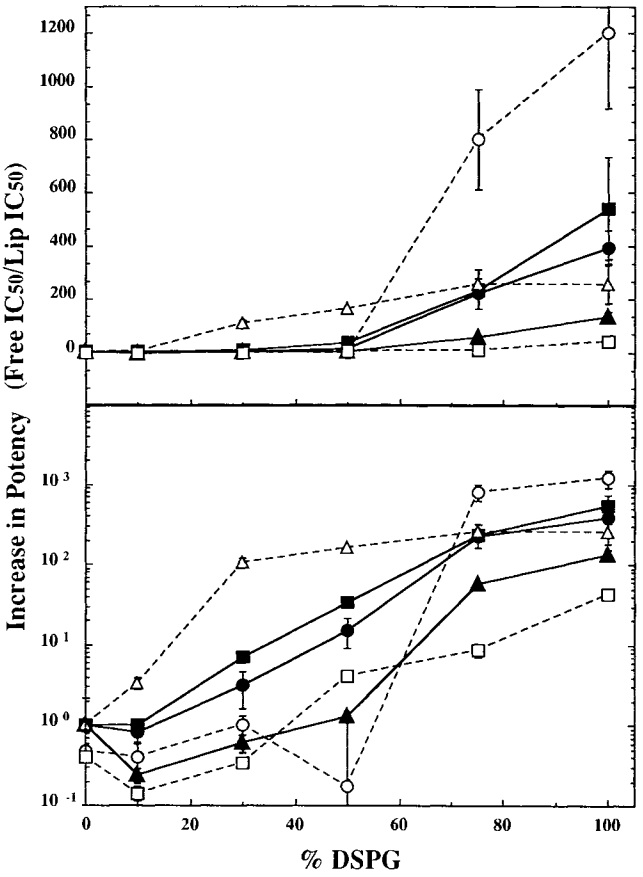

Fig. 2. Effect of negative surface charge density on cytostatic potency of PALA-containing liposomes. Ovarian and nonovarian cell lines were plated for growth inhibition experiments as described. Triplicate wells were exposed to various concentrations of free PALA or encapsulated PALA, and cells were enumerated after 72 hr. The IC_{50} value for each concentration–effect curve was calculated graphically. All experimental points were determined in triplicate, and experiments were repeated at least three times. Potency was calculated as the ratio of the IC_{50} for free PALA relative to PALA-containing liposomes. For most points the standard deviation is smaller than the symbols used in the figure. Solid lines, human ovarian tumor cell lines; dashed lines, nonhuman/nontumor cell lines. Filled squares, A90 cell line; filled circles, A121a cells; filled triangles, Hey-1b cells; open squares, Colon-26 cells; open triangles, CV-1 cells; open circles, J774 cells.

increase the effect of free PALA. The concentration of liposomal phospholipid to which each cell line was exposed at the PALA-liposome IC_{50} was calculated for each cell line. J774, the line most sensitive to PALA in DSPG:Chol liposomes, was exposed to 0.8 n*M* phospholipid at the PALA-liposome IC_{50}; most cell lines were exposed to 80–200 n*M* phospholipid at the PALA-liposome IC_{50}. Hey-1b cells (160 n*M* phospholipid at the IC_{50}) were chosen as representative for two types of experiment: first, cells were exposed to high concentrations of buffer-loaded liposomes in order to determine the IC_{50} of liposomes alone; second, buffer-loaded liposomes were mixed with free PALA and the IC_{50} was determined. The results showed no effect of DSPG:Chol liposomes on Hey-1b growth at any concentration ≤250 μ*M* phospholipid, the highest concentration tested (data not shown). Second, 10 μ*M* liposomal phospholipid had no

effect on the sensitivity of Hey-1b to free PALA (data not shown).

Association of Liposome Aqueous Contents with Human Ovarian Tumor Cell Lines

The relative efficiency of PALA encapsulation was similar for the liposome formulations used in the present studies, so that the large differences in potency must reflect other differences among formulations, such as the extent of liposome–cell binding, contents leakage, or the efficiency of intracellular PALA delivery. Therefore, we examined the cellular uptake and retention of liposome contents as a function of liposome composition. Liposomes of the same composition as those used in Fig. 1, but containing 8-hydroxy-1,3,6-pyrene-trisulfonate (HPTS), a polar aqueous marker for liposome contents (14,22,23), were incubated for 30 min with cells, washed, and cultured in liposome-free medium for defined intervals. Figure 3 shows that both initial association and subsequent retention of liposome contents vary with ovarian tumor cell line, incubation time, and liposome formulation.

Hey-1b (Fig. 3A) and A121a (Fig. 3C) cells showed the highest initial uptake of PG:PC:Chol (2:8:5) liposomes; A90 cells accumulated about 50% less, relative to the other cell lines (Fig. 3B). When HPTS-liposome uptake after 30-min incubation is compared with IC_{50} values for PALA-containing liposomes of the same composition (Fig. 1), no correlation is apparent; Hey-1b and A121a cell lines show similar HPTS accumulation and similar sensitivity to free PALA (Fig. 1), but the potency of PALA in PG:PC:Chol liposomes differs nearly 10-fold for the two cell lines.

Over the initial 4 hr following removal of free liposomes, liposome contents were lost from all three cell lines. Hey-1b (Fig. 3A) showed the most drastic loss of HPTS (fourfold), while decreases were twofold or less for the other cell lines (Figs. 3B and C). When the rank order retention of HPTS at 4 hr is compared with IC_{50} values for PALA encapsulated in equivalent liposomes (PG:PC:Chol, 2:8:5; cf. Fig. 1), a correlation exists among the three cell lines; Hey-1b and A90 retained similar amounts of liposome contents and were the least sensitive to PALA in PG:PC:Chol liposomes, while A121a retained the most HPTS and was the most sensitive to PALA in PG:PC:Chol liposomes.

Increasing the mole fraction of PG in PC:Chol liposomes from 20 to 100% increased both the initial amount of cell-associated HPTS, as well as the amount retained by all cell lines 4 hr after removal of unbound liposomes (Fig. 3). Similarly, the IC_{50} of PALA in PG:Chol (2:1) liposomes decreased. Hey-1b again showed the most drastic loss of contents (fourfold), while decreases were two- to threefold for the other cell lines. Rank-order cellular retention of HPTS 4 hr after removal of free liposomes correlates with rank-order IC_{50} of PALA (Fig. 1) but does not correlate with the initial (30-min) cellular accumulation of liposomes (Fig. 3).

Substitution of DSPG for PG resulted in still higher initial uptake of HPTS liposomes for all three ovarian tumor lines (Fig. 3). Compared to the uptake of PG:PC:Chol (2:8:5) liposomes, association of DSPG:Chol (2:1) liposomes was nearly 20-fold higher in all cases. Furthermore, the fraction of liposome contents lost from the cells was less than for either of the "fluid" liposome compositions; Hey-1b and A121a cells lost only 30% of the initially associated HPTS over 4 hr, and cell-associated HPTS decreased by 50% for the A90 cell line. For the time points ≥4 hr after removal of unbound liposomes, rank-order retention of HPTS correlates with rank-order IC_{50} of PALA in DSPG:Chol liposomes (Fig. 1).

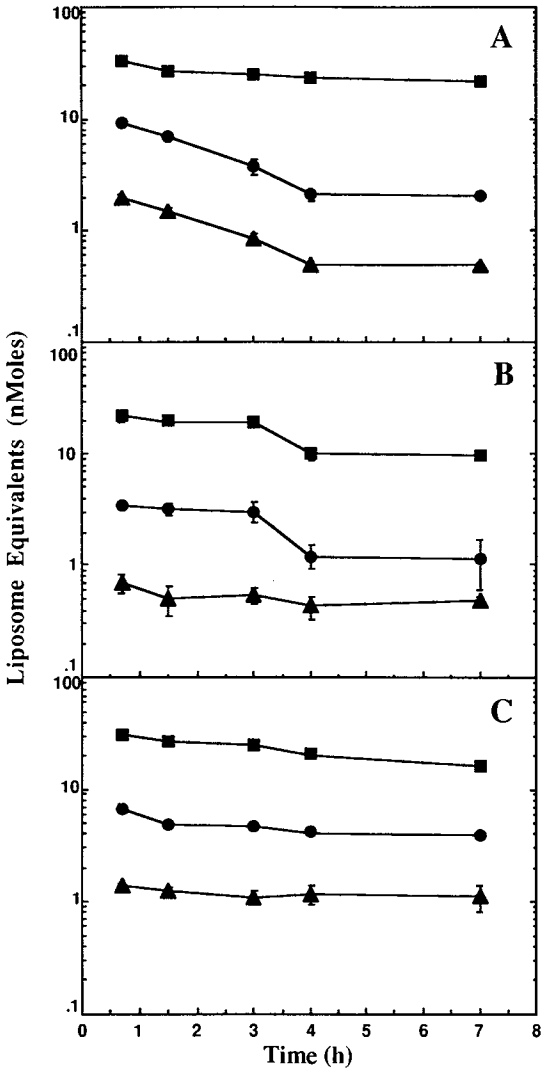

Fig. 3. Uptake and retention of liposome contents by human ovarian tumor cells. One million cells were plated in 60-mm tissue culture dishes in complete growth medium. After 24 hr, cells were washed and incubated for 30 min at 37°C in PBS-CM containing 50 nmol/mL of HPTS-containing liposomes. After 30 min, cells were washed free of unbound liposomes and incubated further in complete growth medium. At the time indicated, triplicate cell monolayers were washed and assayed for total associated fluorescence. Vertical bars indicate the standard deviation for each triplicate determination. The results are expressed as the quantity of phospholipid (liposomes) that would contain the amount of cell-associated HPTS detected, in order to normalize for the variation in the initial HPTS:phospholipid ratio of different formulations. Cells were counted at each point, and the results are normalized to nmol/10^6 cells. The results are not adjusted to compensate for differences in cell-mediated liposome leakage. Triangles, PG:PC:Chol (2:8:5) liposomes; circles, PG:Chol (2:1) liposomes; squares, DSPG:Chol (2:1) liposomes. (A) Hey-1b cells; (B) A90 cells; (C) A121a cells.

Effects of Duration of Treatment on the Potency of Free and Encapsulated PALA

Continuous exposure of tumor cells to the therapeutic agent may be one important respect in which these *in vitro* experiments do not simulate conditions likely to exist in intracavitary therapy. Clearance of solutes and particles from the peritoneum reduces the exposure time of tumor cells to free drug or liposomes, even though clearance is impeded in the case of ascites tumors (4,5). Previous studies have shown that reducing exposure time of cells to free drug or liposomes enhances the relative difference in their respective growth-inhibitory effects (15,33). Presumably, rapid binding of liposomes to cells would supply a surface-bound reservoir of drug to be internalized with time.

To investigate the effect of shortened treatment periods on PALA growth-inhibitory effects, Hey-1b cells were exposed for defined intervals to free or encapsulated PALA, washed, and permitted to grow for a total of 72 hr. Figure 4A shows that free PALA has an IC_{50} greater than 78 μM after a 1-hr exposure. With a 3-hr exposure, free drug has an IC_{50} of 30.7 μM. Over an exposure period of 72 hr, the IC_{50} for free PALA decreases nearly 20-fold. In striking contrast, PALA encapsulated in DSPG:Chol liposomes has an IC_{50} of 0.09 μM after 1 hr of exposure, nearly 900-fold lower than that of free drug after the equivalent exposure. That value is nearly 50-fold lower than the optimal IC_{50} achieved with exposure to free drug for 72 hr (Fig. 4A). With increasing periods of exposure, the potency of DSPG:Chol liposomes increases; over 72 hr, the IC_{50} decreases by approximately 450-fold, relative to free PALA. Thus, the greatest differential toxicity between free drug and drug encapsulated in liposomes is observed with shorter exposure times (1–6 hr) (Fig. 4B). Furthermore, DSPG liposomes establish a major proportion of their maximal growth-inhibitory effect with relatively brief periods of exposure to tumor cells. Such a result may predict a considerable widening of therapeutic index in the treatment of intraperitoneal tumors with PALA-containing DSPG liposomes.

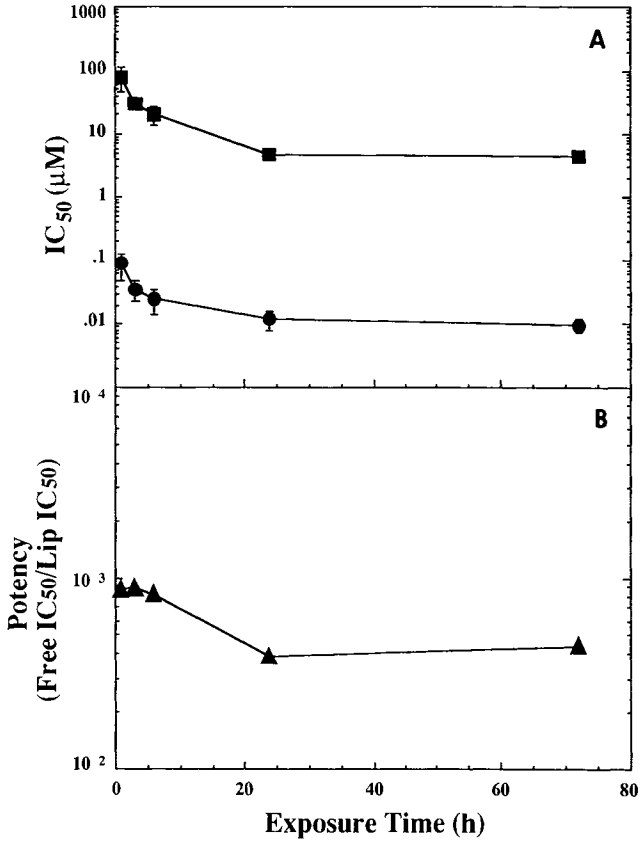

Fig. 4. Time-dependent establishment of the cytostatic effect of free and encapsulated PALA. Hey-1b cells were prepared as under Materials and Methods and were incubated in various concentrations of free PALA or PALA encapsulated in DSPG:Chol (2:1) liposomes. At the time indicated, cells were washed free of drug or liposomes and incubated in growth medium for a total of 72 hr. Cells were enumerated, and the IC_{50} was determined graphically. IC_{50} determinations were performed in triplicate and repeated at least three times. For most points the standard deviation is smaller than the symbols used in the figure. Squares, free PALA; circles, PALA encapsulated in DSPG:Chol (2:1) liposomes; triangles, potency was calculated as the ratio of the IC_{50} for free PALA relative to PALA-containing liposomes.

DISCUSSION

The potential for improving therapy of intraperitoneal cancers by direct intracavitary treatment is supported by both theoretical analysis and empirical data (1–3). Several criteria have been advanced to guide the selection of chemotherapeutic agents that may be appropriate for intraperitoneal administration (reviewed in Ref. 3); among the most important are (i) retarded clearance from the peritoneal cavity, (ii) low peripheral toxicity once the agent has entered the circulation, either by rapid metabolism or by elimination, and (iii) antitumor activity when the agent encounters the tumor cells by direct contact, since systemic delivery may be reduced.

Particulate drug carriers may enhance the efficacy of intracavitary therapy by several mechanisms, and PALA-containing liposomes represent a major advancement in the development of appropriate drug-liposome formulations for such an application. First, particles such as liposomes are retarded in their efflux from the peritoneal cavity (6–8,34). Second, PALA is cleared rapidly from the systemic circulation by renal filtration (35); this should reduce toxicity to critical normal tissues if liposomes leak their contents. Third, the liposome formulations developed here are greatly improved in their ability to interact with tumor cells and deliver PALA to its intracellular site of action.

Just as intracavitary therapy requires the selection of agents with specific characteristics, selection of the appropriate agents for formulation in liposomes likewise is guided by principles that reflect our improved understanding of the mechanism of liposome-cell interaction. Practical examples of "liposome-dependent" drugs (29) have been limited by the fact that few clinically used agents have properties that complement the mechanism by which liposomes deliver their contents to cells. Methotrexate-γ-aspartate, 5-fluoroorotate, and PALA are examples of liposome-dependent agents. However, only PALA has had extensive human clinical investigation (11). Thus, there may be a strong rationale for the reformulation of PALA in liposomes for chemotherapy of human disease, particularly for intracavitary administration.

In the present work, we have evaluated several liposome compositions and physical parameters for their ability to enhance PALA potency against Hey-1b, A121a, A90, and Ovcar-3 human ovarian carcinoma cells. We have found that the enhanced growth-inhibitory activity of encapsulated PALA varies greatly with liposome composition. The maximum growth-inhibitory activity of PALA was observed with DSPG:Chol liposomes. Because of important pharmacokinetic effects *in vivo*, such as the prolonged retention of liposome particles and the rapid clearance of free drug, it is likely that these substantial increases in potency may translate into further increases in therapeutic index when tested for antitumor activity.

In our previous work (15), the maximal liposome-mediated enhancement of methotrexate-γ-aspartate was approximately three- to fivefold on the cell line Ovcar-3. In the present work, similar results were obtained when PALA was substituted for methotrexate-γ-aspartate, suggesting that the efficiency of drug delivery, rather than the liposome-dependent agent per se, was the major limiting parameter. The greatest enhancement of drug potency observed previously was achieved by coupling a tumor-targeting antibody, OC-125, to the liposome surface (15). The potency of methotrexate-γ-aspartate in OC-125-targeted liposomes was eightfold greater than that of free drug. In the present work, encapsulation of PALA in gel-state liposomes of high negative charge resulted in a 20-fold enhancement in potency in Ovcar-3. Thus refinement of the liposome formulation has produced a simple, potent delivery system that may surpass the activity of antibody-directed liposomes under certain conditions.

The present work extends our previous work (15) by expanding the number of human ovarian tumor cell lines in which we evaluated liposome–cell interaction and drug delivery. With the optimal liposome formulation, the smallest enhancement in PALA potency was 22-fold on Ovcar-3. All other human ovarian tumor cell lines were more avid in their uptake of liposomes, and the potency of encapsulated PALA was as much as 570-fold greater than that of free drug. Thus the Ovcar-3 tumor line used previously represents the most liposome-resistant human ovarian tumor identified to date, and encapsulation of PALA in DSPG:Chol liposomes represents a significant advancement in overcoming that resistance to drug delivery.

Our results comparing potency of PALA-containing liposomes and cell association of fluorescent liposomes suggest that both initial association of liposomes with cells and subsequent retention of contents are major parameters determining potency. Changes in liposome formulation enhance both the association of liposomes with tumor cells and the cellular retention of liposome-delivered material. The maximal rate at which cells lose liposome contents occurs within the first four hours after liposome–cell interaction. We observed that 4–7 hr after removal of unbound liposomes, the rate of loss of cell-associated liposome contents reaches a minimum. The formulation-dependent differences in cell-retained fluorescence ≥4 hr after initial liposome–cell interaction correlate approximately with the differences in potency of PALA encapsulated in similar formulations. Hey-1b, A121a, and A90 show 12- to 25-fold higher retention of liposomal contents when the formulation is changed from PG:PC:Chol to DSPG:Chol, and this appears to translate into 200- to 250-fold (for Hey and A121a) and 500-fold (for A90) increases in PALA potency when the liposome composition is changed from PG:PC:Chol to DSPG:Chol.

Experiments to examine the relationship between the fractional liposome negative charge and the potency of encapsulated PALA reveal striking differences among different cell types. CV-1 cells required ≤25% negative charge for maximal PALA potency, while J774 cells required ≥75% negative charge. These results agree well with drug delivery experiments (10) and with previous experiments (32) that compared the role of negative charge in determining the extent of endocytosis for these cell lines, albeit using fluid, rather than gel-phase liposomes. This correlation lends further support to the hypothesis that enhancement of endocytosis is a major mechanism by which reformulation of PALA in liposomes of high negative charge enhances potency.

Although the optimal liposome formulations used in this study generally mediated large increases in PALA potency (compared to free drug), the largest differential between free and liposome-encapsulated drug was observed with shorter exposure times. DSPG:Chol liposomes establish a greater fraction of their maximal cytostatic effect more quickly than does free drug. For Hey-1b cells, DSPG:Chol liposomes are 900-fold more potent than free drug after just 1 hr of exposure. Over an additional 71 hr of exposure, the potency of liposomes increases just 9-fold, while that of free drug increases 20-fold. We believe that binding and endocytosis of liposomes to these ovarian tumor cells are rapid and that the difference in potency between liposomes and free drug arises from the fact cells may accumulate cytoplasmic PALA more quickly after liposomes have established a high concentration of drug within the cellular endocytic apparatus. Additionally, surface-bound liposomes may represent a reservoir of encapsulated drug that cells internalize with time.

As a result of both reduced particle clearance from the peritoneum, as well as the binding of liposomes to tumor cells, we expect that pharmacokinetic experiments with PALA-containing liposomes *in vivo* will show sustained, tumor-associated drug levels and reduced peripheral toxicity, compared to rapid clearance of free drug from the peritoneum and higher toxic side effects. The simple formulations used in the present studies are similar to formulations already under investigation in human clinical trials, suggesting that promising therapeutic results in model systems could be translated expeditiously to experimental therapy of human ovarian cancer.

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