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Liposome Uptake into Human Colon Adenocarcinoma Cells in Monolayer, Spinner, and Trypsinized Cultures[1] (41520)

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Abstract. The purpose of this study was to begin investigating the nature of liposome interactions with colon tumor cells. Thus, experiments were performed to study the uptake and incorporation of multilamellar and of reverse-phase evaporation liposomes of neutral charge into monolayers, suspended spinner cultures, and trypsinized cells of a human colon adenocarcinoma cell line, LS174T. The results showed that the same tumor cells cultured under each condition exhibited a distinct pattern of vesicle uptake as determined at 0, 15, 30, 60, and 120 min. In monolayer cultures of LS174T cells, the uptake of liposomes bearing [^{3}H]actinomycin D in the lipid bilayers was linear throughout the incubation period. In contrast, in trypsinized and spinner suspension cultures, uptake of liposomes was biphasic. There was a proportional uptake of both liposome (labeled with [^{3}H]phosphatidylcholine or [^{14}C]cholesterol) and of actinomycin D (trace labeled with ^{3}H) into the cells under all culture conditions, indicating quantitative delivery of the drug with the intact lipid vesicle. Although the amount of actinomycin D presented to tumor cells by the two liposomes was equivalent, reverse-phase evaporation liposomes were more effective than multilamellar vesicles in inhibiting uridine uptake. In the presence of excess liposomes (10 times the uptake studies), saturation of the tumor cell surface occurred by 120 min. However, the liposomes remained accessible to enzymatic removal for 60 min. Liposome-saturated tumor cells remained refractory to further binding of liposomes for at least 2 hr. The results thus revealed that differences in cell uptake were due to the state of the target cells and not the liposome types, or their differential leakage of labels.

Liposomes are artificial, phospholipid vesicles that have proven to be exceptionally versatile and effective biological carriers of diverse macromolecular substances (1, 6). In both *in vitro* and *in vivo* experimental systems liposomes have been shown to transport or present antigens (7–11), enzymes (12, 13), ions (2, 14), chelating agents (15–17), and anti-cancer drugs (18, 19) to cells with greater efficiency than that achieved with the free substances alone. Results in animal tumor studies and in limited human studies (1, 6, 20, 21) have suggested that liposomes provide a potential means for improving the therapeutic indices of drugs in human neoplasms. However, translation of *in vitro* and *in vivo* experimental studies into clinical applications will not be easy. In addition to the applications noted above, a large volume of literature is extant which focuses on liposome–cell interactions. These reports include determining requirements and/or characteristics for liposome size uniformity (22), for appropriate liposome surface charge and/or chemical composition for specific application (23, 24), for directed homing to target tissues (25), of liposome interactions with humoral elements (26), as well as cellular elements (27) from peripheral blood, and for controlled and effective drug release at target sites (28). It is clear that liposome–cell inter-

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actions are affected by numerous variables. In the present studies, the manner in which the cells were maintained and processed for liposome studies affected the subsequent results. This report describes the uptake of multilamellar (MLV)[3] and reverse-phase evaporation vesicles (REV) by a cultured human colon adenocarcinoma cell line, LS174T. The results revealed a difference in the pattern of liposome uptake into a cultured human colon tumor cell when maintained as a monolayer, spinner-suspended, or trypsinized cell preparation.

Materials and Methods. *Preparation of tumor cells.* An established human colon adenocarcinoma cell line, LS174T, developed in this laboratory and previously described (29, 30), was cultured in Eagle's minimal essential medium with Hanks' salts (MEM) supplemented with 10% fetal bovine serum (MEM 10; Grand Island Biological Co., GIBCO, Grand Island, N.Y.) and with penicillin (100 U/ml) and streptomycin (100 μg/ml). Tumor cell suspensions of LS174T were prepared from stock monolayer cultures by incubation with 0.05% trypsin (GIBCO) in 0.02% ethylenediaminetetraacetic acid (EDTA), pH 7.4, for 15 to 30 min at 37°. Trypsinization was terminated by the addition of 10 ml fresh, warmed (37°) MEM 10. The cells were utilized immediately as "trypsinized" cells and resuspended in MEM 10 for experiments utilizing spinner-cultured cells, or for seeding onto tissue culture plates for monolayer studies. The spinner cells were cultured in 100 ml MEM 10 at a concentration of 2×10^5 cells/ml overnight (to foster regeneration of trypsin-sensitive molecules) and then used in incubation with liposomes.

Liposome preparation. MLV liposomes containing actinomycin D were prepared with the drug in the lipid bilayers of the liposomal membranes. Twenty milligrams of phosphatidylcholine and 10 mg cholesterol were combined, as previously described (31), with 2.5 mg unlabeled Act D (from Cosmegen with mannitol removed; Merck, Sharp and Dohme, West Point, Pa) and a tracer of 15 μCi [^{3}H]Act D (3 Ci/mmole; Schwartz/Mann, Orangeburg, NY) in 10 ml chloroform. Alternatively, trace labels of 10 μCi [^{14}C]cholesterol (58.4 μCi/mmole, Amersham) and/or 20 μCi [^{3}H]phosphatidylcholine (40–60 μCi/mmole, Amersham) were used. This solution was dispensed into a 100-ml round-bottom flask and dried in a rotary evaporator under negative pressure in a 37° water bath, resulting in a thin lipid film. Five milliliters of 8 mM calcium chloride was added to the flask and stirred gently for approximately 15 min in a 37° water bath to produce multilamellar vesicles. This preparation was collected by centrifugation (700*g*) for 5 min at ambient temperature, washed twice (with 5 and 7.5 ml 8 m*M* $CaCl_2$), and resuspended in 5 ml 8 m*M* $CaCl_2$, and stored under nitrogen gas until used. The amount of Act D associated with the vesicles was determined by measuring the incorporated radioactive tracer. Lipid concentrations were estimated by performing phosphorus assays on the liposome preparations (32).

Reverse-phase evaporation liposomes were prepared by first placing 20 mg phosphatidylcholine and 10 mg cholesterol in a 50-ml round-bottom flask and removing the solvent under negative pressure in a rotary evaporator (33). The dried lipids were redissolved in 2.3 ml diethyl ether and then 10 mg unlabeled Act D (from Cosmegan) and a trace of 10 μCi [^{3}H]Act D (3 Ci/m mol; Schwartz Mann) were added in 0.8 ml aqueous volume. Alternatively, trace labels of [^{14}C]cholesterol or [^{3}H]phosphatidylcholine were used. This mixture was sonicated for 4 min at a setting of 6.5 (35 W) in a bath-type sonicator (Heat Systems, Inc.) at 4°. The mixture was then returned to the rotary evaporator and the organic phase was removed under negative pressure at 23°. The REVs were collected and washed.

Liposome uptake into monolayer cultures. For monolayer cultures, 10^4 LS174T cells in 0.2 ml MEM 10 were seeded into each well of a sterile flat-bottomed 96-well Microtest II culture plate (Falcon Plastics, Oxnard,

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[3] Abbreviations used: The term "liposomes" was recommended by the New York Academy of Science's Conference (1) to represent all classes of artificial, lipid vesicles. The original liposomes described by Bangham *et al.* (2) were multilamellar vesicles (MLV). REV, reverse-phase evaporation vesicle; Act D, actinomycin D; FBS, fetal bovine serum; MEM 10, minimal essential medium with Hanks' salts and 10% FBS; PBS, phosphate-buffered saline, pH 7.4.

Calif.) and grown at 37° in 5% CO_2 and humidified air for 48 hr to form an adherent monolayer of approximately 3×10^4 cells. Stock Act D liposome preparations (6 mg lipid/ml) were washed once in 10 ml MEM and diluted to the desired final concentration in MEM. Aliquots of 0.1 ml drug-free neutral liposomes, [^{3}H]Act D liposomes (or trace-labeled lipids), or equivalent amounts of free drug were each added to six replicate wells containing twice-rinsed (with MEM) LS174T monolayer cells or cell-free control wells and incubated without agitation at 37° for 0, 15, 30, 60, and 120 min. At the end of each incubation period, the media containing free liposomes or excess drug were aspirated, the wells rinsed, and the monolayer cells trypsinized for 15 min at 37° (0.2 ml of 0.05% trypsin in 0.02% EDTA). The dispersed cells were collected onto glass fiber filters by vacuum filtration with the multiple automated sample harvester (MASH II; Microbiological Associates, Bethesda, Md.) and processed for liquid scintillation counting. Cell counts and viabilities (greater than 90% viable) were determined from an adjacent row (pool of six wells) of cells. In experiments evaluating the effect of Act D on [^{3}H]uridine incorporation monolayer cells were treated with various amounts of Act D-bearing MLV or REV, rinsed three times after the incubation periods, refed with 0.2 ml MEM 10 per well, and exposed to 1 μCi [^{3}H]uridine (Research Products International (RPI), Elk Grove, Ill.) in 20 μl MEM for 24 hr. The cells were then trypsinized and harvested.

Liposome uptake into trypsinized cells. The stock of MLV [^{3}H]Act D liposome preparation or free drug was diluted in MEM to twice the desired concentration and admixed in MEM with an equal volume 10^6 freshly trypsin-suspended LS174T cells/ml. One-tenth-milliliter aliquots of this mixture (providing 50×10^3 cells) were added to each well of a culture plate, and incubated without agitation for varying periods in 5% CO_2 at 37°. During the 2-hr study, the LS174T cells remain rounded and do not spread. The liposome–cell mixture settled in the wells and thus provided close apposition of cells with liposomes. Rocking of the plates under these conditions did not enhance uptake rates. In experiments that measure the effect of Act D liposomes on [^{3}H]uridine incorporation, cells and liposome dilutions were admixed for incubation in 50-ml plastic centrifuge tubes. At the end of each reaction period, aliquots of the liposome–cell mixture equivalent to the amount in 15 wells were removed, layered onto 10% sucrose in normal saline, and centrifuged at 800*g* for 20 min to separate the cells from free liposomes, which remain at the top of the sucrose–saline interface. The cell pellets were washed once in MEM and diluted in 3 ml MEM 10; 0.2-ml aliquots were then dispersed into each of 12 wells of a Microtest plate. The cells in each well were exposed to 1 μCi [^{3}H]uridine for 24 hr and harvested.

Liposome uptake into suspended cell cultures. In experiments using trypsin-suspended, spinner-maintained tumor cells, MLV or REV liposomes were added to the cells and incubated in stationary culture. The cells were harvested without further trypsinization.

In order to assess the kinetics of binding and uptake of liposomes to the LS174T cells, one-tenth ml 0.1% trypsin[4] in 0.04% EDTA (this is 2× the normal concentration) was added in some experiments to each well 15 min prior to the end of the incubation to treat the 0.1-ml volume of suspended liposome–cell mixtures. Six wells were pooled for cell counts and viability assessment by trypan blue dye exclusion (viabilities ranged from 80 to greater than 90%). No attempt was made to remove excess liposomes and both the trypsinized and the untreated cells samples were vacuum harvested as described for the monolayers.

Liposome saturation and desaturation of LS174T cells. Two sets of liposomes were prepared. One set contained [^{14}C]cholesterol liposomes (15 μCi in 30 μl [^{14}C]cholesterol was added to a preparation of liposomes prepared with 15 μg cholesterol); the other set of liposomes was unlabeled.

[4] Gibco Trypsin "1:250," Stock No. 70725, was used because it contains a mixture of bovine pancreatic enzymes. In addition to its proteolytic activities (trypsin, chymotrypsin), this product contains mucolytic enzymes (elastase, 3), RNase (4), esterases (5), amidases (5), as well as enzymes catalyzing hydrolysis of fatty acids (5).

Excess liposomes (480 μg lipid; i.e., 10-fold more than in kinetic studies) in 100 μl were coincubated in round-bottom Linbro plates with 10^5 trypsinized LS174T cells. The plates were incubated for 2, 4, 6, and 8 hr, and for 18, 20, and 22 hr at 37°. At the end of each time period one plate was taken out, vacuum harvested, and counted for ^{14}C activity. The low plating efficiency of the LS174T cells (<50% in 24 hr) was an advantage in these studies. The cells did not adhere tightly to the plates and were readily removed in the vacuum rinsing system of the harvester. To trace the resaturation kinetics unlabeled liposomes were initially comixed (480 μg lipid/ 100 μl MEM 10/well) with 10^5 LS174T cells (100 μl MEM 10/well) and incubated at 37°. At the end of each incubation period, 100 μl ^{14}C-liposomes containing 480 μg lipid was added to the already plated LS174T cells and unlabeled liposomes. After a further incubation of 1 hr the cells were harvested and counted. In the latter experiment, the "cold," unlabeled liposomes present in the preparation competed with the labeled liposomes for the cells. In another experiment, the excess cold liposomes were removed by centrifuging the liposome–cell mixture through 10% sucrose. In the control tubes 4×10^6 LS174T cells in 1 ml were incubated with ^{14}C-liposomes containing 19 mg lipid. Five sets of tubes were set up for 30-min, 2-hr, 4-hr, 6-hr, and 8-hr incubations. At the end of each period the contents of the tube were poured over 5 ml 10% sucrose and spun at 800*g* for 20 min. The supernate was discarded and the pellet resuspended in 1.0 ml MEM 10. The cell suspension was further resuspended in 10 ml scintillation fluor, incubated for 24 hr, and counted in the Beta counter.

As a control for liposome alteration of metabolite uptake, LS174T (10^5 cells/100 μl) cells in monolayer, spinner- and trypsin-suspended cultures (quadruplicates) were treated for various time intervals with drug-free, neutral liposomes (100 μl) corresponding to the range of liposomal lipids used in the REV studies (10, 100, and 400 μg phospholipids); the cultures were incubated at 37° for 2 hr, rinsed twice with MEM, and replenished with 0.2 ml MEM 10. The cells were exposed to either 1 μCi [^{3}H]thymidine

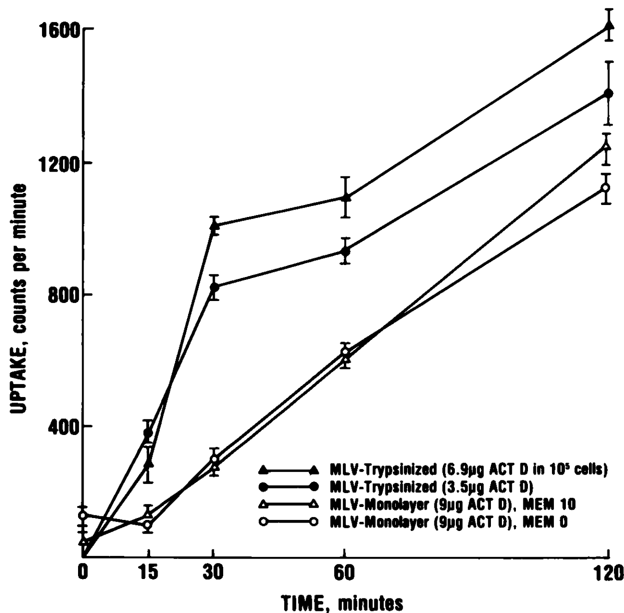

FIG. 1. Colon tumor cell uptake of liposomes containing [^{3}H]actinomycin D. Each point represents the mean of six replicates. Each bar denotes the mean ± SD. Two concentrations of Act D were tested on the trypsinized cells. The amounts of liposomes (45–50 μg lipid) were equivalent in each group. The presence of fetal bovine serum (MEM 10) did not affect the results.

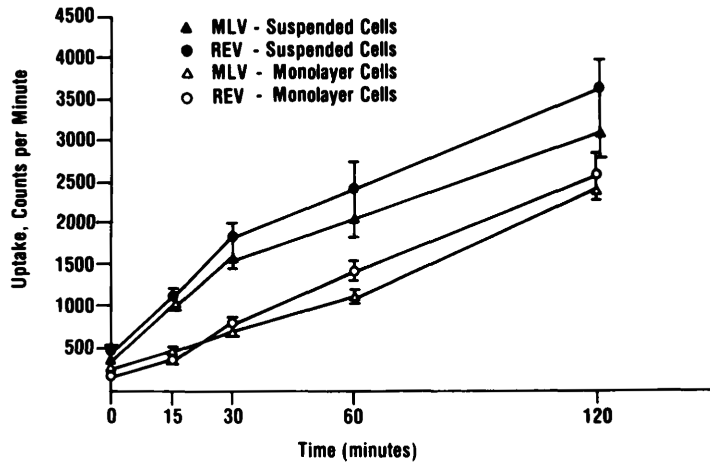

FIG. 2. Colon tumor cell uptake of liposomes (40 μg lipid/10^5 cells) trace-labeled with [^{14}C]cholesterol. Each point represents the mean of six replicates (±SD). The pattern of uptake into spinner-suspended or monolayer cells is identical for either liposome preparation.

(2 μCi/mmole), 1 μCi [^{3}H]uridine (0.5–2 μCi/mmole), or 0.25 μCi [^{3}H]leucine (0.5–2 μCi/mmole) for 24 hr, and harvested. The effect on metabolite uptake was computed as a ratio (index) of experimental cpm/control cpm.

All significant determinations were computed by Student's t test, where significance was demonstrated when $P < 0.05$.

Results. There is a clear difference in liposome uptake between adherent monolayer cells and both suspended trypsinized (Fig. 1) and spinner-cultured (Figs. 2 and 3) cells. "Uptake" (or binding) is meant to denote the

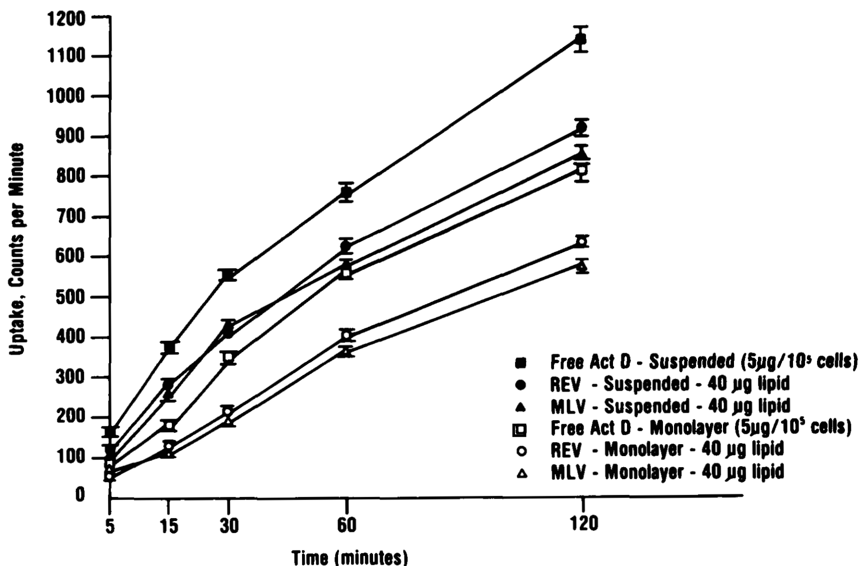

FIG. 3. Colon tumor cell uptake of liposomes (40 μg lipid) bearing 5 μg actinomycin D trace-labeled with [^{3}H]Act D. Each point represents the mean of six replicates (±SD). The pattern of uptake into spinner-suspended or monolayer cells is identical for either liposome preparation. Free Act D (5 μg) alone was readily taken up by the tumor cells.

removal from the media of liposomes which thereafter are associated with the cell fraction. The two uptake patterns were independent of the type of liposome used (REV or MLV), but dependent on the culture conditions. The same patterns were maintained at different Act D doses. In Fig. 1, the initial rate of liposome uptake (as reflected by uptake of [^{3}H]Act D) into trypsinized cells was 800–1000 cpm in 30 min; the subsequent uptake rate onto the trypsinized cells was about 250 cpm in 30 min; comparable to the 300 cpm in 30 min for monolayer cells. The presence of serum did not alter the uptake patterns. Suspended (spinner-cultured) cells behaved similarly to trypsinized cells (Figs. 2 and 3), both exhibiting accelerated initial uptake rates of double-labeled liposomes followed by a reduced rate parallel with the monolayer rates. In Fig. 2, [^{14}C]cholesterol was monitored to evaluate the liposome uptakes. As exhibited in Fig. 1, the uptake patterns of suspended cells were similar to trypsinized cells and different from the monolayer cells. Using the identical amounts of Act D (5 μg) and of liposomes (40 μg lipid) for 10^5 cells, the observation that MLV and REV liposomes behave identically in the uptake studies is also illustrated in Fig. 3; whether tested on monolayer or suspended cells, the uptakes are not significantly different. Note that the free Act D uptake patterns reflect that of suspended cells. The extent of uptake of free Act D was greater than by liposome delivery. This is not surprising since Act D is amphipathic and readily lipid soluble. Further, it might be expected that, given the lipid solubility of the drug and the confinement of Act D when in liposomes, free drug would have greater access to the tumor cells than the liposome-borne drug. However, as seen below, REV liposomes were equally effective as free drug in inhibiting uridine uptake.

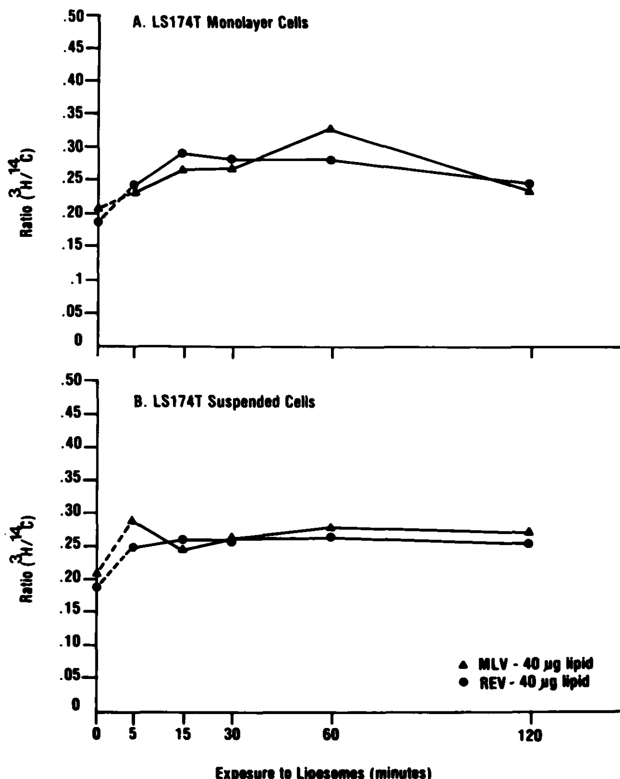

FIG. 4. Ratio of [^{3}H]Actinomycin D: [^{14}C]cholesterol in LS174T cells treated with double-labeled liposomes. Each point represents the ratio computed from results in Figs. 2 and 3.

The integrity (and a measure of Act D leakage) of the Act D-containing liposomes in these studies is demonstrated in Fig. 4 in which double-labeled ([^{3}H]Act D and [^{14}C]cholesterol) liposomes were used to determine the ratio of ^{3}H/^{14}C taken up into LS174T cells. Throughout the 120-min period, the ratios did not change significantly. This approach to evaluating the differential loss of Act D is more relevant than to measure leakage of [^{3}H]Act from liposomes in culture media alone. In an earlier report, we noted a negligible (less than 2%) leakage of [^{3}H]Act D from MLV liposomes after 2 hr in media (31). The same study was not performed on REVs. If any changes can be noted, there appears some loss of cholesterol between 0 time and 5–15 min, reflected by a slight increase in the ^{3}H/^{14}C ratio. Although the same amount of [^{3}H]Act D in REV and MLV liposomes was taken identically into monolayer or suspended cells (Fig. 3), Act D's inhibitory effect on uridine uptake with the two liposomes differed (Fig. 5).

The results suggest a greater effectiveness of REVs over MLVs to deliver their contents to cells as previously reported by Szoka and Papahadjopoulos (33). In contrast to the binding studies in which binding to suspended versus monolayer cells differed regardless of liposome type, the inhibition results reflected differences due to liposome type, and not target type. Since the amount of Act D leakage (as indicated above) from REV and MLV liposomes was not found to differ, the observed results appear related to the efficiency of REVs in releasing their contents to the cytoplasm of both cell prepara-

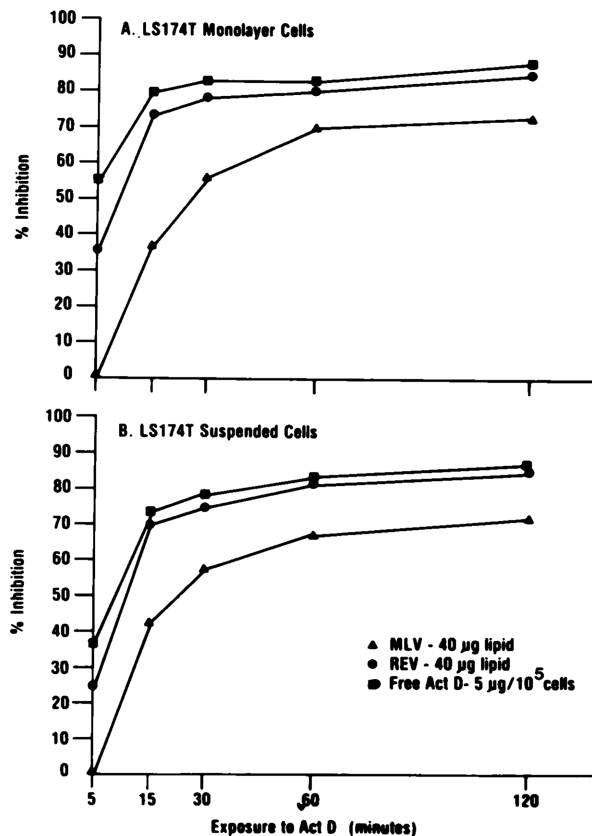

FIG. 5. Inhibition of [^{3}H]uridine uptake in Act D–liposome (5 µg Act D)-treated LS174T cells. Each point represents the mean of six replicates. Significant differences in inhibition by MLV and REV liposomes exist at each time point for both targets.

tions and thus to the nucleus. Utilizing the actual counts per minute (of [^{3}H]uridine), the percentage inhibition of uridine uptake was compared at each time point for both liposomes on each cell type. On either cell type, the percentage inhibition produced by REVs was greater than by MLVs ($P < 0.001$ at 5 and 15 min; $P < 0.05$ at 30, 60, and 120 min). This interpretation must be tempered, however, by the problem of comparing 50% inhibitions with two different maxima, i.e., REV-treated cells were inhibited 85–90%, while treated cells were inhibited only 65–70%.

As indicated in Fig. 5, maximum inhibition of uridine uptake was complete by 60 min. However, uptake of liposomes continued beyond 120 min (Figs. 1–3). Therefore, the kinetics of liposome binding (uptake) and incorporation were studied and are presented in Fig. 6. Because of technical problems of sampling in this assay only monolayer targets could be studied. These results revealed that liposomes were accessible to trypsin activity for up to 60 min.[4] For example, whether REV or MLV liposomes were used, over 70% of the double-labeled liposomes were readily stripped off the tumor cells at 15 min. With MLVs, 50% of the liposomes were inaccessible to trypsin by 30 min. The 50% point for REVs occurred later, at about 60 min. No significant loss of liposomes was detected at 120 min, indicating the completion of incorporation into the cells. "Incorporation" is meant to denote that liposomes are no longer accessible to removal by trypsin. Whether measured with [^{3}H]phosphatidylcholine or [^{14}C]cholesterol in the liposomes, the relative amounts of binding and incorporation were parallel. The results confirmed the integrity and the total transfers of the liposomes in these studies.

The latter results revealed a two-step process of liposome–cell interaction which include binding to the cell followed by incorporation and raised the question as to the saturability of the LS174T colon tumor cells. Hence excess (480 μg) ^{14}C-labeled REV liposomes were cocultured for 0.5 to 22 hr with trypsinized cells. The pattern of liposome uptake and saturation is presented in Fig. 7, as open circles. The data also indicate that, once saturated with cold MLV after a 2-hr incubation, in order for additional liposomes to be taken up by these cells, a 4-hr latent period is necessary for "new" space

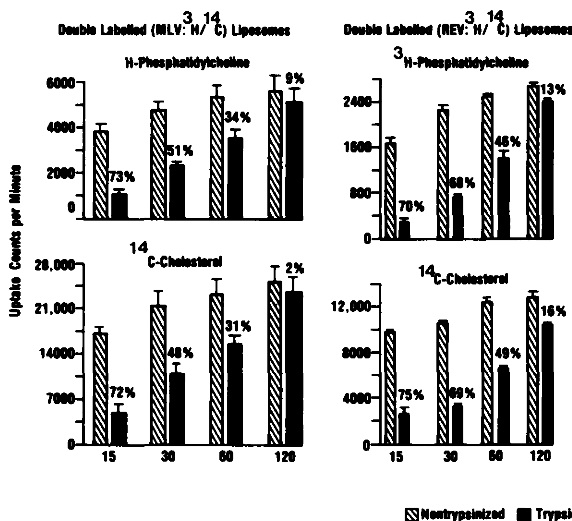

FIG. 6. Kinetics of binding and uptake of double-labeled (^{3}H/^{14}C) liposomes into spinner-cultured LS174T cells. Each determination represents the mean of six replicates. The bars denote ±SD. The figures above the solid "trypsinized" bars represent the amount of percentage decrease in counts after trypsinization. Except for the 31 and 34% decreases with MLVs at 60 min ($P < 0.005$), all other decreases from 15 to 60 min are significant at $P < 0.001$. The decreases at 120 min are not significant.

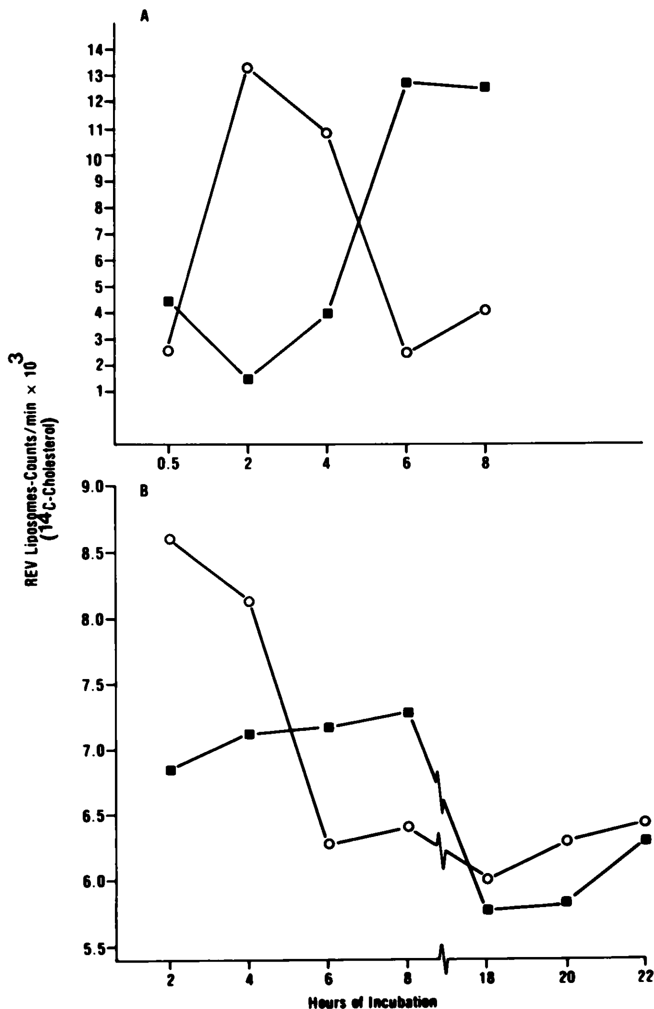

FIG. 7. Desaturation and resaturation of liposome-treated LS174T cells. Each point represents the mean of three replicates. Panel A: The open circles denote saturation and desaturation kinetics of the tumor cells in the presence of ^{14}C-labeled REV liposomes. The closed squares denote the reciprocal experiment in which "cold," unlabeled liposomes are first added to saturate the cells for 2 hr, washed, and then ^{14}C-labeled liposomes are added to determine the kinetics of "hot" replacement of "cold" liposomes. Panel B: The desaturation kinetics represented by open circles is extended to 22 hr. Replacement of cold liposomes by hot (closed squares) is only 50% complete when excess cold liposomes are not removed.

to appear for subsequent uptake of "hot" liposomes (Fig. 7A, black squares). Although the uptake and the decay exhibit similar kinetic patterns, the requirement for the waiting period argues against a *displacement* of surface (nonincorporated) bound liposomes, but for a *replacement* of "processed and released" liposomes. Maximum amounts of liposomes are again seen after another 2 hr, i.e., 6 hr after initial saturation. If the excess cold liposomes were not washed out, a saturation pattern seen in Fig. 7B is developed, where only one-half of the total counts are retrieved (when compared between the 4- and 8-hr points), resulting from 1:1 replacement of cold liposomes by the hot. This result supports the idea that liposome–cell interactions are passive events with no positive

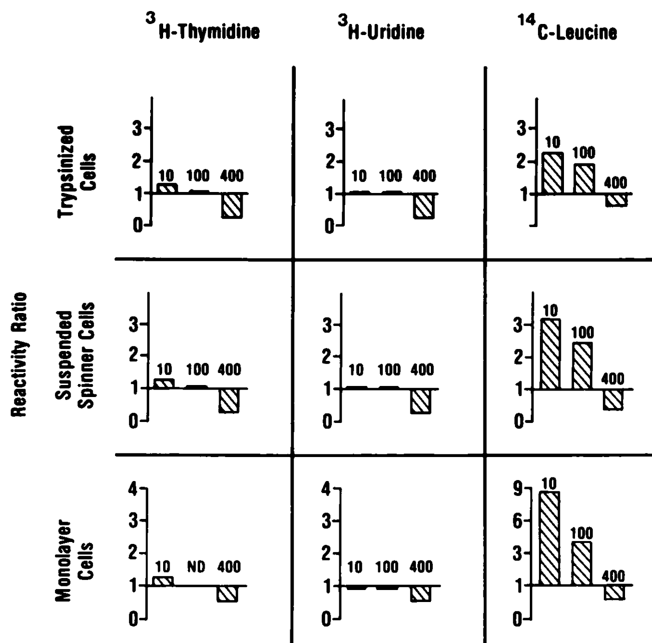

FIG. 8. Effect of empty liposomes on LS174T cells. Tumor cells are treated for 2 hr with 10, 100, or 400 μg REV liposomes. The ratios on the ordinate represent the proportion of radioactive counts contained in tumor cells after overnight incubation with each label. Thus a ratio of 1 indicates identical counts in both liposome-treated or untreated cell groups. Each determination represents a mean of four replicates.

cooperativity developed by the binding of the first liposomes.

Although excess lipids did not appear to affect liposome binding, excess lipids (400 μg) were inhibitory to the cellular uptake of thymidine, uridine, and leucine into all three cell cultures (Fig. 8). At doses of 10 and 100 μg lipid, leucine uptake was significantly enhanced. However, the lower doses did not significantly alter uptake of thymidine or uridine.

Discussion. In this report, evidence was presented demonstrating the pattern of liposome uptake into nonadherent colon tumor cells differed from that into the same cells grown in a monolayer. This was evidenced by the uptake of radioactive lipid labels and/or of Act D onto the tumor cells and confirmed by the inhibition of [^{3}H]uridine incorporation into the cells. The nonadherent cells, whether trypinsized or spinner-cultured, behaved identically in these studies, suggesting that free-floating cells possessed similar inherent differences in liposome interactions from monolayer cells. Thus trypsinization of target cells did not affect the uptake pattern of MLV or REV liposomes into the suspended cells. This lack of trypsin sensitivity to liposome binding was also demonstrated by Blumenthal and colleagues (27) with L-α-dioleoyl phosphatidylcholine liposomes and human peripheral blood lymphocytes. Both trypsinized and nontrypsinized lymphocytes bound liposomes equally. In contrast, other investigators have reported that trypsin pretreatment altered liposome binding (34), or could release liposomes already bound to the cells (35). Based on our studies, the above results are not contradictory. We found that liposome binding (adsorption) onto the tumor cells was reversible with trypsin within the first 60 min of coincubation, suggesting that early adsorption events occurred on membrane surface structures sensitive to trypsin.[4] Presumably only during this time period could displacement experiments be performed, i.e., when liposome binding is reversible. Thereafter, bind-

ing is irreversible. The inability in our studies to "displace" liposomes after 2 hr of saturation is thus consistent with this interpretation. Uptake of liposomes into membranes may be independent of specific surface determinants and due solely to lipid–lipid associations (27).

We can estimate the number of liposomes that can be accommodated on a LS174T tumor cell. Based on a mean diameter of 30 μm for a LS174T cell (29) and a 2-μm maximum diameter (mean diameter, 0.5 μm) for each liposome (17), at least 900 liposomes could be arranged as a monolayer on the surface of each tumor cell. Accordingly, in one set of experiments, out of 8576 cpm added to the reaction mixture, an average of 1423 cpm were bound at saturation or 16.6%. This is equivalent to 6.2 μg lipid bound by 50 $\times 10^3$ tumor cells, or 12.4×10^{-2} ng lipid per cell. The observation that liposome type (MLV or REV) did not alter uptake patterns is consistent with the report by Poste and Papahadjopoulos (24) in which they stated that liposome chemical composition rather than size was important in determining pathway of uptake into cells. The mechanism of uptake was not studied here, but these same authors (24) reported that for fluid vesicles (such as those used in our study), the mode of uptake was by vesicle fusion to the cell membrane. This conclusion was also expressed by Van Renswoude *et al.* (36).

Our results revealed that the differences in uptake were due to the state of the target cells and not the liposome types, or their differential leakage of labels. The difference in liposome uptake by monolayer versus suspended cells may relate to their geometry—a three-dimensional (suspended) cell versus a more flattened (monolayer) cell. In the studies of Papahadjopoulos and his colleagues (37), the uptake of negatively charged liposomes into murine L929 monolayer cells did not peak until 6 hr, whereas the same liposomes incubated with suspended human erythrocyte ghosts plateaued in 1½ hr. Additionally, it is known that cells in stationary culture, such as confluent monolayers, take up liposomes at a much slower rate than actively growing young or sparse cultures (35, 38). Pagano *et al.* (38) reported a 10-fold increase in uptake of neutral egg yolk phosphatidylcholine vesicles into sparse monolayers of V79 hamster cells than into V79 cells reaching confluency. A related phenomenon was observed by Janson *et al.* (35) in which the uptake of positively charged small unilamellar liposomes was 2-fold greater in young mouse L1210 leukemic cultures than in those from a stationary-phase culture. These observations are consistent with the greater surface area exposed on dividing than on quiesent cells, e.g., dividing cells round up and can be readily detached from their substrates to reveal more liposome binding and uptake sites. In contrast quiescent substrate-bound monolayer cells, such as the colon tumor cells, present only one cellular face. However, they exhibit many surface protrubances such as blebs, microvilli, and filipodia (39). These structures provide liposome binding sites, but because of the instability of adequate surface contact may hamper or delay uptake into the cell proper. One might speculate that trypsin sensitivity is exhibited when liposome binding and thus exposure is on such cellular embellishments. It appears that liposome uptake may be dependent on cell culture age, cell density, and cell geometry. In other words, enhanced uptake may be found in young, sparse, and suspended cultured cells, as compared to old, dense, and adherent cell counterparts. Since trypsinization of cells can elicit the initiation of a cycle of cell division, trypsin-dispersed adherent cells may temporarily resemble young, actively growing cultures.

The relatively proportional delivery of liposome–actinomycin D to the tumor cells (see Fig. 4) reflects the stability of the lipophilic Act D bound in this system. In contrast, when the molecule of interest is transported in the aqueous phase of liposomes there is great variation in the ratio of lipid:aqueous marker transferred to the cells; as evidenced by others (36, 38, 40), there is no proportional uptake.

Our study also indicated that the tumor cell uptake of liposomes can affect the uptake of precursors for protein, RNA, and DNA synthesis, both enhancing (leucine) or inhibiting (thymidine, uridine, leucine) the uptake. Ozato *et al.* (41) did not find any alteration of [^{3}H]thymidine uptake at 30 min in murine lymphocytes upon cell interaction

with a series of liposomes including neutral ones of the same type used in our studies. Our results for thymidine are consistent with their report. It is important to note, however, that we found uniform thymidine, uridine, and leucine suppression at high lipid doses (400 μg) but only stimulation of leucine uptake at lower doses (10 and 100 μg). Furthermore, this stimulation by "empty" liposomes exhibited in our study was apparent at 120 min and not at 30 minutes. In unpublished data we showed that following a 2-hr treatment with neutral MLV liposomes, the electrophoretic mobility of LS174T colon tumor cells was increased by 17.5%. These changes may underlie the altered phenotype expressions reported in several studies (42–44). Utilizing the same 1:1 molar ratio of phosphatidylcholine and cholesterol as in the experiments herein, Inbar and Shinitsky (42) reported, for example, that the tumor cell surface (and apparent function) can be altered to appear "normal" by uptake of liposome-borne cholesterol. Alderson and Green (43) incubated bovine lymph node lymphocytes with liposomes to increase cholesterol levels which resulted in a reduced Con A mitogenic response, but not the ability of the cells to bind Con A. Ostro *et al.* (44) demonstrated altered membrane fluidity (by antibody capping) in lymphocytes treated with liposomes. Thus some of the precursor uptake changes found in our results may reflect early biomolecular synthetic shifts accompanying liposome–cell interactions leading to the above-described manifestations.

In summary, the results described in this study reveal the interaction of uncharged liposomes with cultured human colon adenocarcinoma cells and indicate that (a) liposomes are readily taken up into LS174T colon tumor cells; (b) adherent and suspended target cells differ in their kinetics of liposome uptake; (c) the pattern of liposome uptake occurs with a rapid binding step followed by uptake into the cell; (d) the tumor cell surface can be readily saturated with lipid; and (e) liposomal lipid alone can alter the uptake of metabolic precursors.

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