Cellular Retention of Liposome-Delivered Anionic Compounds Modulated by a Probenecid-Sensitive Anion Transporter

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Purpose. Drug carriers such as liposomes may enhance the intracellular delivery of therapeutic agents for infectious or neoplastic diseases. However, the mechanisms affecting cellular retention of liposome contents are understood poorly. We tested the hypothesis that retention of anionic compounds may be modulated by a nonspecific probenecid-sensitive anion transport mechanism, and that liposome composition may determine the impact of such transporters on drug retention by cells.

Methods. The fluorescent anionic dye hydroxy-pyrene-[1,3,6]-trisulfonate (HPTS) was transferred to the cytoplasm of cultured CV-1 or J774 cells by direct needle-microinjection or by ATP-induced permeabilization of the plasma membrane, respectively, to investigate whether the cells have anion transport mechanisms capable of extruding HPTS from the cytoplasm. Cellular retention of dye was monitored in the presence and absence of the anion transport inhibitors probenecid or sulfinpyrazone. Liposomes containing HPTS were co-labeled with tetramethylrhodamine-labeled phosphatidylethanolamine (Rho-PE) as a marker of liposome membrane fate, and uptake was investigated using J774 cells.

Results. Needle-injected HPTS underwent both sequestration in early endocytic vesicles and rapid extrusion into the extracellular medium. Probenecid or sulfinpyrazone reduced the extrusion of HPTS. Thus HPTS is a substrate for a probenecid-sensitive anion transporter in J774 and CV1 cells. After delivery via fluid liposomes composed of phosphatidylglycerol:phosphatidylcholine:cholesterol (3:7:5 mole ratio) and co-labeled with Rho-PE, cell-associated HPTS declined more rapidly than did Rho-PE. Exposure of cells to 5 mM probenecid doubled the quantity of HPTS retained by cells, without changing the retention of the Rho-PE membrane marker. In contrast, the effect of probenecid was negligible when gel-phase liposomes of distearoylphosphatidylglycerol:cholesterol (10:5 mole ratio) were used.

Conclusions. Probenecid-sensitive nonspecific anion transporters can mediate the extrusion of model anions delivered *via* liposomes. However, liposome composition modulates the amount of material subject to extrusion from cells, possibly by altering the endocytic compartment in which liposomes release their contents.

KEY WORDS: intracellular drug delivery; drug carriers; liposomes; probenecid-sensitive anion transporter.

INTRODUCTION

Encapsulation of therapeutic agents in liposomes (phospholipid vesicles) can be used to enhance both the selectivity and magnitude of delivery to target cells. In many cancer- and infectious disease applications, maximization of effect requires maximization of drug exposure to the intracellular site of action. However, after the initial uptake of liposomes by cells, components from both the liposome membrane and aqueous compartment undergo efflux from cells. Mechanistic studies of liposome-cell interaction have shown that liposomes can bind to the cell surface (1,2), undergo endocytosis in clathrin-coated pits, and are delivered to progressively-acidifying endosomal/lysosomal compartments of cells (2–5). The mechanisms by which cellular loss of liposomes might occur include: (i) shedding of intact liposomes from the cell-surface, (ii) leakage of aqueous contents from surface-bound liposomes, (iii) regurgitation of intact liposomes or their aqueous contents after endocytosis by cells, during the process of endocytic vesicle recycling, and (iv) efflux of liposome-delivered compounds by carrier-mediated-mechanisms. The leakage or shedding of surface-bound liposomes appears to be well-established, but little is known of other possible efflux mechanisms for liposome contents.

A wide range of anionic compounds have been encapsulated in liposomes, not only for therapeutic applications, but also as probes of liposome-cell interaction and intracellular fate. Mechanisms controlling their intracellular disposition are understood poorly. Macrophages and other cells possess nonspecific anion transporters that redistribute fluorescent dyes or antibiotics delivered into the cytoplasm by scrape-loading or ATP-mediated permeabilization (6–9). Anions are either extruded from cells by carriers in the plasma membrane, or concentrated in early endocytic vesicles *via* an energy-dependent process involving carriers in the endocytic vesicle membrane (6). Using fractionated subcellular organelles, it was observed that the anionic fluorescent dye Lucifer Yellow was taken up by endosomes preferentially over lysosomes (7), suggesting that anion transporters may be most active in plasma membrane and endosomes, but least active in lysosomes. Anion transport was sensitive to inhibition by probenecid (6), a compound commonly used at moderate doses in humans to modulate renal secretion and reabsorption of organic anions.

Here we have investigated the intracellular processing of liposomes, and tested two hypotheses: first, that anion transporters can modulate the cellular retention of liposome-delivered compounds, and second, that liposome composition can determine the extent to which anion transporters redistribute liposome-delivered compounds. The membrane-impermeant anionic dye hydroxy-pyrene-[1,3,6]-trisulfonate (HPTS) has been used previously to report on the acidification of liposome-containing vacuoles during endocytosis (1,4,5). The fluorescence emission of HPTS is either unchanged or increases slightly as a function of pH when excited in the range of 360–415 nm, and is quenched at acid pH when excited at 450 nm (1,4,5,10,11). In the time frame of experiments reported here, HPTS fluorescent properties are not altered by metabolism or degradation (4). HPTS was encapsulated here as a marker of the liposome aqueous space, and the fluorescent phospholipid analog tetramethylrhodamine phosphatidylethanolamine (Rho-PE) was included as a marker for the liposome membrane. J774, a macrophage-like murine tumor cell line, was used as

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a target cell, owing to our interest in developing liposomal formulations of antibiotics for therapy of intracellular infections (12–14).

EXPERIMENTAL PROCEDURES

Materials

Hydroxy-pyrene-[1,3,6]-trisulfonate (HPTS), rhodamine-labeled dextran (Rho-Dex, 40 kDa) and tetramethylrhodamine phosphatidylethanolamine (Rho-PE) were from Molecular Probes (Eugene, OR). Phosphatidylcholine (PC), phosphatidylglycerol (PG), cholesterol (Chol), and distearoylphosphatidylglycerol (DSPG) were from Avanti Polar Lipids (Alabaster, AL). RPMI-1640 and sera were from Fisher Scientific (Pittsburgh, PA). Non-Enzymatic Cell Dissociation Solution® and other reagents were from Sigma (St. Louis, MO).

Preparation of Liposomes

Multilamellar liposomes containing HPTS and Rho-PE were prepared as follows. Lipid mixtures (PG:PC:Chol:Rho-PE, 3:7:5:0.03 or DSPG:Chol:Rho-PE, 10:5:0.03) were dissolved in chloroform and the solvent was removed using a rotary evaporator. The dried lipid film was hydrated with 0.5 mL of an isotonic solution containing 5 mM HPTS in NTE buffer (consisting of 145 mM NaCl, 10 mM TES (N-Tris[hydroxymethyl]-2-aminoethane-sulfonic acid), and 0.1 mM EDTA (ethylenediamine tetraacetate). After vigorous vortex mixing, the liposomes were extruded three times through 0.2 μm polycarbonate membrane filters (15). All processing steps were performed at 56°C for lipid mixtures containing DSPG, and at 37°C for other compositions. Unencapsulated HPTS was removed from liposomes by gel filtration on Sephadex G-75 equilibrated with NTE buffer.

Needle Microinjection of HPTS

CV-1 cells (16) were microinjected with HPTS as detailed previously (17,18). Briefly, cells were grown on round glass coverslips in Dulbecco's Modified Eagle Medium (DMEM) containing 5% newborn bovine serum. For injection, coverslips were mounted in a custom temperature-controlled holder, and placed on an inverted microscope. Cells were incubated during injection with serum-containing DMEM, in the presence or absence of 5 mM probenecid. All operations with cells were performed at 37°C. A glass needle was drawn to a tip of approximately 0.2 μm using a microforge (Kopf, Inc., Tajunga, CA), back-filled with 5 mM HPTS in a buffer of 50 mM HEPES (N(2)hydroxyethyl-piperazine-N'-[2 ethanesulfonic acid]) and 80 mM KCl (pH 7.3), and mounted on a micromanipulator (Narashige, Inc., Japan). In some cases, 1 mg/mL rhodamine-labeled dextran (40 kDa) was also included as a water-soluble, membrane-impermeant macromolecular marker of the cell interior. Several femtoliters of dye were injected into the cytoplasm of selected cells. The time of injection was recorded by videotaping, and the X/Y coordinates of injected cells were recorded so that specific cells could be observed at specific time intervals. After injection, cells were imaged by fluorescence epi-illumination, using a Dage SIT (silicon-intensified target) camera (San Diego, CA) interfaced to a Perceptics image processor (Knoxville, TN). Automatic gain, voltage, and gamma correction functions of the camera were disabled, and were set manually to allow quantitation of intracellular fluorescence from the video images (12). Images were also recorded in S-VHS format on video tape for later digitization. In order to observe recovery from probenecid, the probenecid-containing medium was removed at predetermined times, cells were washed twice with PBS, and medium without drug was added.

Measurement of Cell-Associated Fluorescence

J774, a murine macrophage-like cell line, was obtained from the American Type Culture Collection (Gaithersburg, MD), and was cultured in complete medium (RPMI-1640 supplemented with 5% fetal calf serum and 5% newborn calf serum). Cells were detached from culture flasks using Non-Enzymatic Cell Dissociation Solution® (Sigma), resuspended in complete medium, and plated at 1–5 $\times$ 10^5 cells per well in a 24-well plate. After overnight incubation to allow cells to adhere, cells were incubated for 1 hr at 37°C with fluorescent liposomes. The incubation was terminated by washing cells three times with culture medium and twice with PBS. Cells were then incubated in complete medium in the presence or absence of 5 mM probenecid or 1 mM sulfinpyrazone. After various intervals, cells were washed three times with culture medium and lysed with 1 mL of a solution containing 0.05% Trypsin, 0.53 mM EDTA, and 0.1% Triton X-100 in PBS. The lysates were diluted with 1.5 mL of the same solution, and fluorescence was measured using an SLM 9000C spectrofluorometer (Urbana, IL). HPTS was measured using an excitation wavelength of 460 nm and an emission wavelength of 510 nm. Rhodamine was measured using an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Dye concentrations were determined by comparison to known standards.

RESULTS

Efflux of HPTS by a Probenecid-Sensitive Anion Transporter

The anionic dye HPTS was microinjected directly into the cytoplasm of CV-1 monkey kidney cells, in order to determine whether it is a substrate for nonspecific anion transporters that may modulate the intracellular retention and disposition of some anionic compounds (6–8). Rhodamine-labeled dextran was combined with HPTS to provide a high molecular-weight (40 kDa), water-soluble, membrane-impermeant marker with which to identify injected cells. Needle microinjection was used as an alternative to the gross disruption of plasma membrane integrity induced by scrape-loading or transient permeabilization (6,9), and to reduce the probability that dye would be pinocytosed into vesicles rather than loaded directly into the cytoplasm. CV-1 cells were used because they are large, well-spread endothelioid cells that are easily microinjected (18).

Immediately after initiating injection, both HPTS (Figure 1) and Rho-Dex (not shown) diffused rapidly throughout the cell. Within 20 sec., HPTS could be observed in ruffles at the most distant extremes of the cell (Figure 1D). Within 90 sec., punctate accumulations of HPTS were observed (Figure 1E). Based on previous studies (6), these accretions likely correspond to early endosomes into which the dye was extruded by active anion transporters. Although HPTS diffused rapidly throughout

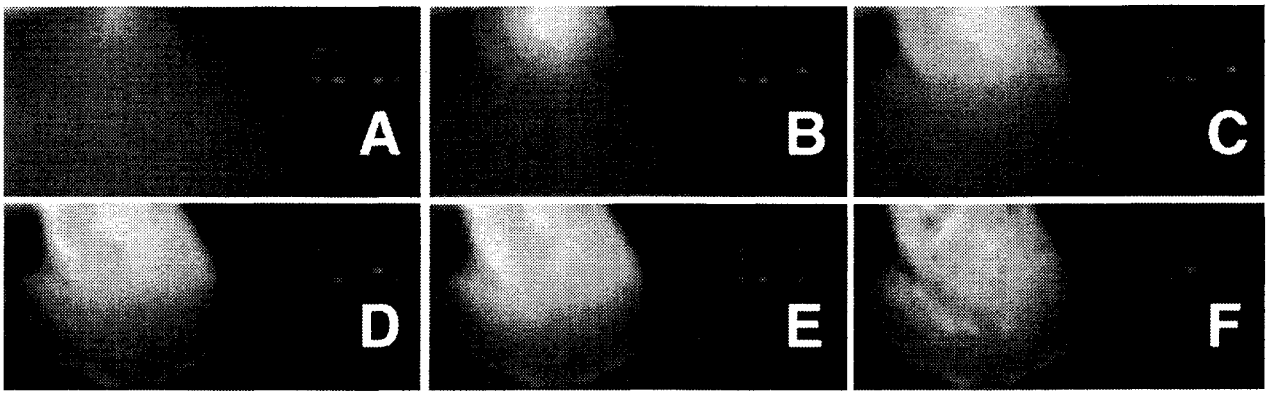

Fig. 1. Intracellular redistribution of the anionic fluorophore HPTS following needle microinjection. The epithelioid cell line CV-1 was grown on glass coverslips, and the coverslips were mounted on an inverted fluorescence microscope in a special holder that allowed immersion of cells in tissue culture medium. A glass micropipette was filled with 5 mM HPTS and 1 mg/mL Rho-D (40 kDa), mounted on a micromanipulator, and the dyes were injected into the cytoplasm under fluorescence illumination at 400 nm. Panels (A–E): intracellular distribution of HPTS 4, 15, 18, 24, and 32 sec. after initiation of injection, respectively; (F) HPTS approx. 1.5 min. after injection. The site of injection is at the top of each panel, and is marked by the intense localized fluorescence in panel B.

the cell, entry of the 40 kDa Rho-Dex probe into the nucleus was retarded (cf. Figure 2A) because of its high molecular weight.

Over a period of 30–60 min, the intensity of intracellular HPTS decreased markedly (Figure 2B), and numerous punctate accretions of fluorescence were observed. Image acquisition at 400 nm, a wavelength at which HPTS fluorescence is largely insensitive to pH (4,10,11), showed that the decrease in HPTS intensity was the result of reduced dye concentration, rather than pH-dependent quenching. Image acquisition at 450 nm, a wavelength at which HPTS fluorescence is decreased at acid pH, showed that the fluorescent punctates were either neutral or slightly acidic in pH when observed immediately after injection (cf. Figure 1F), but were clearly acidic at later times (data not shown).

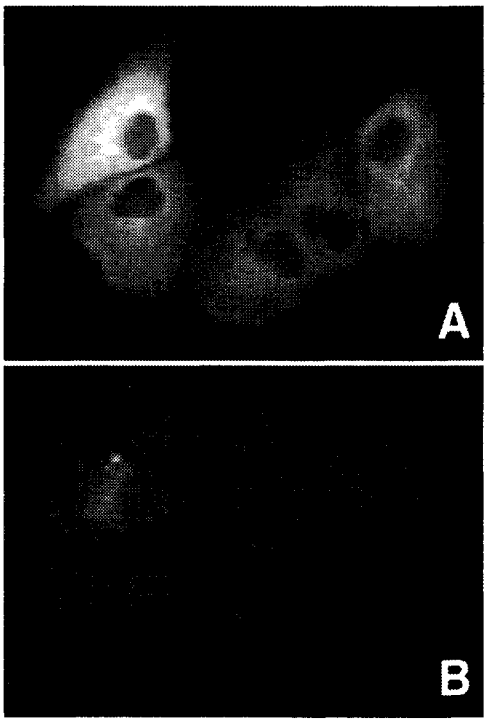

Fig. 2. Cellular retention of HPTS following needle microinjection. CV-1 cells on glass coverslips were microinjected with a solution of 5 mM HPTS and 1 mg/mL Rho-D. Cells were observed for intracellular distribution of (A) Rho-D and (B) HPTS 45 min after injection. HPTS images were acquired at 400 nm. Rho-Dex was retarded in entry into the nucleus because of its higher molecular mass.

In the presence of 5 mM probenecid, the intracellular HPTS fluorescence remained diffusely distributed and intense (Figure 3B) for the duration of probenecid exposure (≤60 min). When probenecid was removed subsequently and replaced with drug-free culture medium, cell-associated HPTS fluorescence declined rapidly (Figure 4B). Within 30–45 min of probenecid removal, the cytoplasmic HPTS concentration fell below the limit of detection in many injected cells (Figure 4B), which could be discriminated from uninjected cells by the presence of the co-injected Rho-Dex marker (Figure 4A).

Although J774 cells were difficult to microinject, it was possible to investigate whether HPTS is a substrate for an anion transporter in those cells also. HPTS was introduced into the cytoplasm by reversible, ATP-mediated permeabilization (Figure 5B) (6,19). Within 20 min of loading, the cytoplasmic HPTS concentration had declined, and the sequestration of HPTS in large intracellular vacuoles was observed (Figure 5C). Within 40–55 min (Figure 5D), the cytoplasmic concentration of HPTS had fallen to background levels, and the remaining cell-associated HPTS appeared to be sequestered within small vacuoles. In the presence of probenecid, cells retained high concentrations of HPTS (not shown; similar to Figure 3B). Thus J774 cells extrude HPTS from the cytoplasm *via* the probenecid-sensitive anion transporter that has been implicated in the export of other anionic fluorescent dyes (6,19) and antibiotics (8).

Cellular Retention of PG:PC:Chol Liposome Contents

To investigate whether organic anion transporters modulate the cellular retention of compounds delivered *via* liposomes, J774 cells were incubated with PG:PC:Chol liposomes containing HPTS. Probenecid (5 mM) or sulfinpyrazone (1 mM) was added to cells at the end of a 1 h incubation with liposomes, to ensure that the presence of the drug did not alter the initial

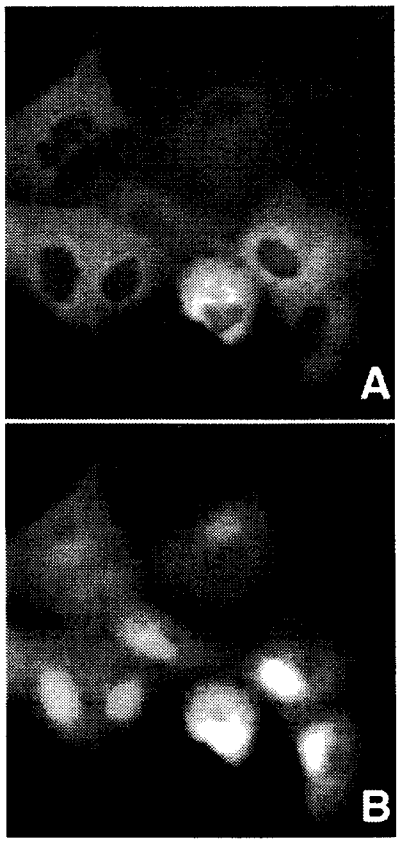

Fig. 3. Effect of probenecid on cytoplasmic retention of HPTS. CV-1 cells were microinjected with 5 mM HPTS and 1 mg/mL Rho-D in the presence of 5 mM probenecid. Cells were observed for intracellular distribution of (A) Rho-D and (B) HPTS approximately 50 min after injection. HPTS images were acquired at 400 nm.

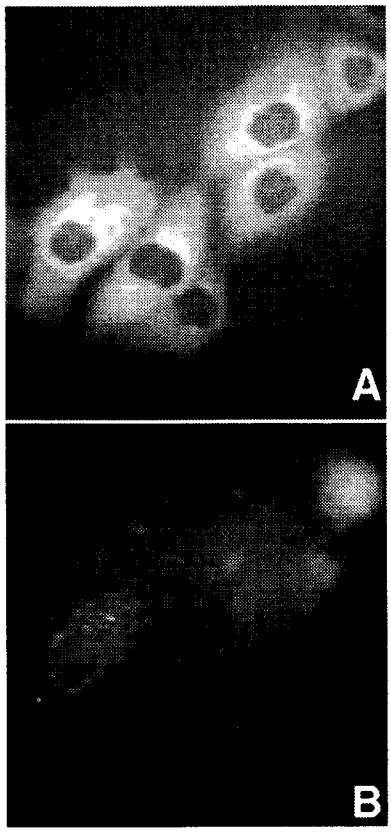

Fig. 4. Cytoplasmic retention of HPTS following removal of probenecid. CV-1 cells were microinjected with 5 mM HPTS and 1 mg/mL Rho-D in the presence of 5 mM probenecid. After 1 h, cells were washed free of probenecid and incubated in growth medium. Approximately 30 min after removal of probenecid, cells were observed for intracellular distribution of (A) Rho-D and (B) HPTS. HPTS images were acquired at 400 nm.

liposome-cell interaction or intracellular disposition. Tetramethylrhodamine-labeled phosphatidylethanolamine (Rho-PE) was included in liposomes as a marker for the fate of the liposome membrane.

In the absence of probenecid, the concentration of cell-associated HPTS decreased progressively after the removal of unbound liposomes (Figure 6A). After 3 h, approximately 58% of the original cell-associated HPTS remained with cells, and after 6 h, 35% of the HPTS was retained (Figure 6A).

In the presence of 5 mM probenecid, the retention of liposomal aqueous contents was greater; 3 h after incubation with liposomes, 65% of HPTS remained with J774 cells, and approximately 58% was retained at 6 h (Figure 6A). Sulfinpyrazone exerted a similar effect (data not shown).

The presence or absence of probenecid or sulfinpyrazone had no discernible effect on cellular retention of the liposomal membrane, based on the behavior of the fluorescent phospholipid Rho-PE (Figure 6B). Three hours after the removal of unbound liposomes, approximately 66% of the initial Rho-PE remained cell-associated, and approximately 45% was cell-associated after 6 h of incubation.

Cellular retention of the liposome aqueous and lipid markers was compared by calculating the HPTS/Rho-PE ratio. In the first hour after removal of unbound liposomes, the HPTS/Rho-PE ratio decreased (Figure 7A), indicating that liposome contents (HPTS) were lost from cells at a greater rate than was the liposomal membrane marker, Rho-PE. Over the succeeding 5 h, the HPTS/Rho-PE ratio remained relatively constant, indicating that the cellular content of both HPTS (Figure 6A) and Rho-PE (Figure 6B) decreased in parallel.

In the presence of probenecid, the initial rate of decline in the HPTS/Rho-PE ratio was reduced (Figure 7A), indicating enhanced retention of the aqueous marker. At 3 h, the HPTS/Rho-PE ratio was nearly equal to the ratio prior to exposure to cells, suggesting that liposomal contents underwent efflux from cells at the same rate as liposomal membrane. After 6 h, the HPTS/Rho-PE ratio exceeded the ratio in the original liposomes, indicating that at later times, a relatively greater fraction of HPTS was retained by cells, compared to the Rho-PE membrane marker.

Cellular Retention of DSPG:Chol Liposome Contents

Liposome composition can modulate both the stability of liposomes and their intracellular fate; PG:PC:Chol liposomes have been used in many delivery applications, but they are somewhat leaky during interaction with cells. The substitution of high phase transition (T_m) phospholipids results in a greater retention of aqueous fluorophores and a greater activity of

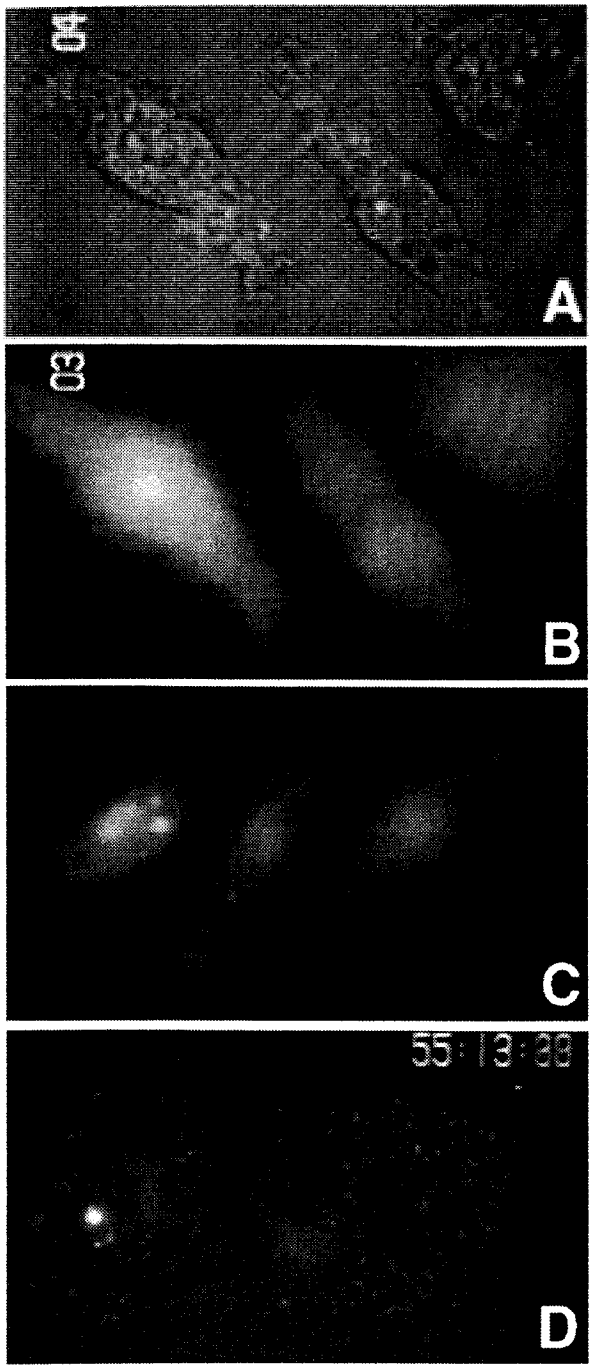

Fig. 5. ATP-induced loading of HPTS in J774 cells. J774 cells were loaded with HPTS (10 μM) by permeabilizing for 5 min with 5 mM ATP^{4-}, and re-sealed at "Time 0" by adding 5 mM $MgSO_4$ from a concentrated stock. (A) Phase contrast image of J774 cells within 3 min of re-sealing; (B) fluorescence image of the same field as in Panel A), acquired at approx. 4 min; (C) fluorescence image at 22 min; (D) fluorescence image 53 min after re-sealing.

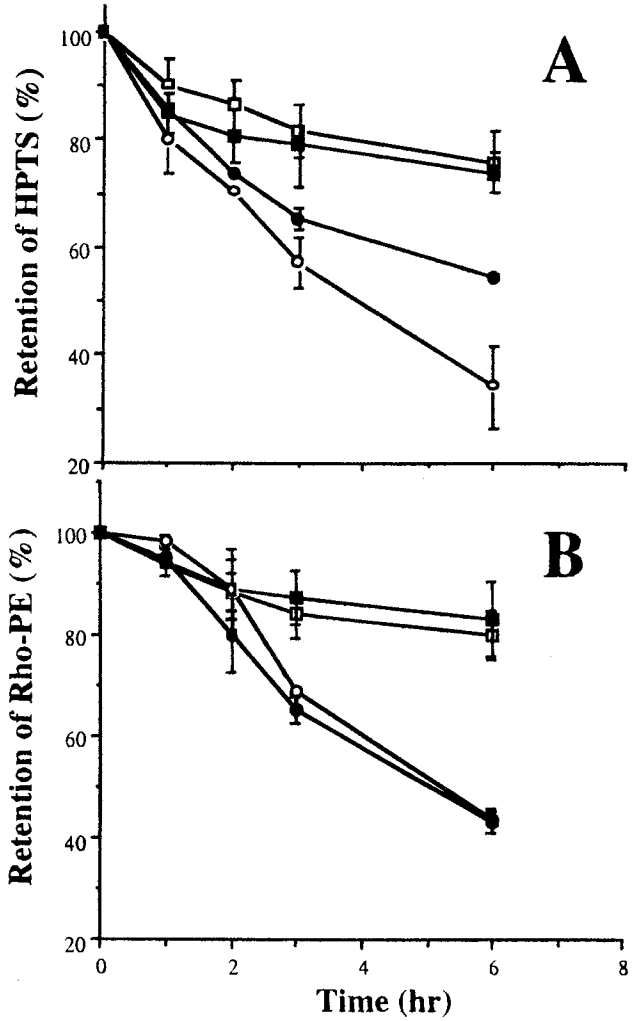

Fig. 6. Effect of probenecid on the retention of HPTS delivered *via* liposomes. J774 cells were incubated with liposomes containing HPTS (5 mM) in the internal aqueous space and Rho-PE in the liposome membrane at 3 mole %. After 1 h, cells were washed to remove unbound liposomes, and re-fed with culture medium in the absence (open symbols) or presence (filled symbols) of 5 mM of probenecid. At the times indicated, cells were washed, lysed with detergent, and cell association of the fluorophores was measured. Cellular content of (A) HPTS and (B) Rho-PE. Circles: PG:PC:Chol (3:7:5) liposomes; Squares: DSPG:Chol (2:1) liposomes. Vertical bars indicate the standard deviation for each point.

capsulated drugs (1,20,21). In addition, the association of osome contents with J774 cells increases with increasing gative charge content of liposomes, as does the activity of capsulated drugs (1,13,21). Liposomes of DSPG:Chol (10:5 l:mol) were prepared in order to investigate the role of the probenecid-sensitive anion pump in the cellular retention of contents delivered by more stable liposomes.

The total cellular uptake of HPTS by J774 cells was greater when dye was encapsulated in DSPG:Chol liposomes (data not shown), and the rate at which J774 cells released HPTS was lower (Figure 6A). Three hours after terminating the interaction of liposomes with cells, approximately 80% of the initially-associated HPTS remained with cells, and at 6 h, more than 75% of the initial HPTS remained.

Probenecid (5 mM) had little effect on the cellular retention of HPTS delivered by DSPG:Chol liposomes (Figure 6A). Probenecid also had little effect on the cellular retention of the Rho-PE liposome membrane marker (Figure 6B).

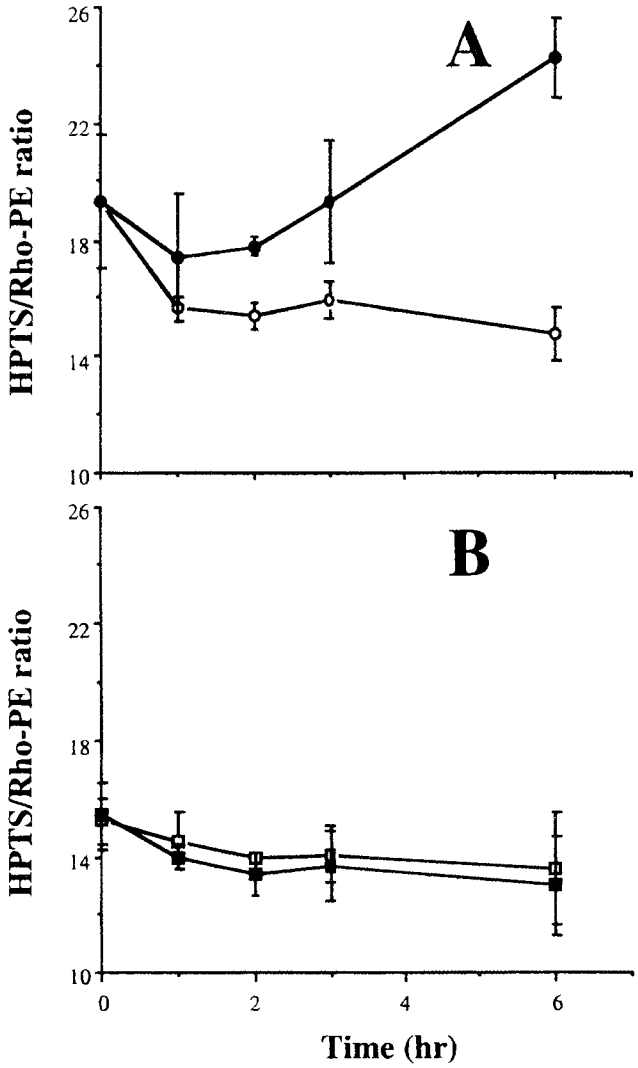

Fig. 7. Effect of probenecid on the HPTS/Rho-PE ratio. J774 cells were incubated with liposomes containing both HPTS and Rho-PE, and composed of (A) PG:PC:Chol or (B) DSPG:Chol. After 1 h, cells were washed and re-fed with culture medium in the absence (open symbols) or presence (filled symbols) of 5 mM of probenecid. At the times indicated, cells were washed, lysed with detergent, and cell-associated fluorophores were quantified. Vertical bars indicate the standard deviation for each point.

When the cellular retention of the aqueous and lipid markers was compared by calculating the HPTS/Rho-PE ratio for DSPG:Chol liposomes, it appeared that the rate of loss of HPTS was only slightly greater than that of Rho-PE (Figure 7B). At 6 h after incubation with cells, the HPTS/Rho-PE ratio was approximately equal to the initial ratio for intact liposomes. The HPTS/Rho-PE ratio also showed that the continuous presence of probenecid did not enhance significantly the retention of HPTS relative to that of lipid label when DSPG:Chol liposomes were used as the carrier.

DISCUSSION

The possible mechanisms for cellular loss of liposomes and their contents includes: (i) release of intact, surface-bound liposomes prior to internalization, (ii) leakage of contents from surface-bound liposomes, (iii) regurgitation of liposomes or their contents during the process of endocytic vesicle recycling, and (iv) selective efflux of specific components *via* cellular transport mechanisms. Of these mechanisms, cell-mediated leakage has received the greatest attention. The observations presented here suggest an additional, significant role for intrinsic cellular transport mechanisms in the selective release of liposome-delivered compounds.

We found that HPTS, an anionic fluorophore used previously to report the intracellular pH encountered by liposomes during endocytosis (1,4,5), is a substrate for a probenecid-inhibitable anion transporter that functions in both J774 macrophages and CV-1 endothelioid cells. After delivery to J774 cells *via* PG:PC:Chol liposomes, HPTS was extruded rapidly from cells, and a significant fraction of the cell-associated dye was lost to the external medium within 6 h. Cellular retention of the liposome membrane marker, Rho-PE, was greater than the retention of HPTS. Such an observation would also be consistent with a mechanism of HPTS leakage from liposomes bound to the cell surface. However, the anion transport inhibitor probenecid reduced HPTS efflux to a rate matching that at which the Rho-PE liposome membrane label was released from cells. Probenecid would not be expected to have impact on simple cell-mediated liposome leakage.

The simplest explanation for the continued, coordinated release of HPTS and Rho-PE from cells in the presence of probenecid would be mechanisms by which the constituents of a single liposome could be released from cells in a quantal fashion. Mechanisms consistent with this pattern of release would include (i) or (iii) above: loss of intact liposomes from the cell surface or regurgitation of endocytosed liposomes. Previous work exploiting the pH-reporting properties of HPTS has shown that liposomes are internalized rapidly by J774 cells (2,5). The continued release of HPTS and Rho-PE in the presence of probenecid, occurring at times after internalization of liposomes would be virtually complete (2,5), suggests that endocytic regurgitation may occur also. This hypothesis requires further investigation.

The HPTS/Rho-PE ratio at 6 h (Figure 7) showed that at later times, the retention of liposome aqueous contents can exceed the retention of liposome membrane markers. Preliminary data (not shown) suggests that over longer times, Rho-PE may be metabolized to a more water-soluble form that can be released from cells.

The vectorial transport of anionic fluorophores and antibiotics has been observed to proceed from the cytoplasm to the extracellular medium, or from the cytoplasm to the lumen of nascent, non-acidic endosomes (6–9). However, liposome-delivered dye is localized within endosomes; to explain probenecid-sensitive efflux of liposome contents from the cell, one logical hypothesis is that anion transport is not inherently vectorial, but rather responds to the electrochemical driving force and the sequestration of co-transported or anti-ported ions such as H^+ or OH^- (6). By this reasoning, the transporter may be able to exchange liposome-delivered dye across the endosome membrane into the cytoplasm, from which compartment the plasma membrane transporters would extrude dye to the cell exterior.

The therapeutic consequences of the observed anion transport activity may be complex. Liposomes are avidly taken up

by macrophages *in vivo*, and have been investigated widely for the delivery of antibiotics to macrophages infected with intracellular pathogenic microorganisms (13,22–24). For anionic antibiotics having low membrane permeability, such as fluoroquinolones and β-lactams, delivery in liposomes has been reported to enhance antimicrobial activity against intracellular bacterial pathogens such as *Salmonella* (25,26) and *Mycobacteria* (13,24). The action of an anion transporter to extrude antibiotics from endocytic vesicles to the cytoplasm might reduce activity against intracellular pathogens by reducing the concentration of drug within intracellular vesicles. Alternatively, anion transport might enhance antibiotic delivery by subsequently redistributing antibiotics from the cytoplasm into non-lysosomal, bacterium-containing vesicles to which direct, liposome-mediated antibiotic delivery has not occurred.

Overall, the cellular transport mechanisms that modulate retention of liposome-delivered compounds are poorly-characterized. The studies presented here suggest that a nearly ubiquitous nonspecific anion transporter can redistribute anions delivered *via* liposomes into the endocytic apparatus of target cells. The impact of these cellular processes on therapeutics is unknown. However, specific changes in liposome formulation can modulate the extent to which anionic compounds undergo efflux *via* anion transport mechanisms.

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