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Immunoliposomes with different acid sensitivities as probes for the cellular endocytic pathway

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By combining dioleoylphosphatidylethanolamine (DOPE) with oleic acid (OA), palmitoylhomocysteine (PHC) or dipalmitoylsuccinylglycerol (DPSG) we have prepared pH-sensitive liposomes with different acid sensitivities. DOPE/OA liposomes are the most acid sensitive, while DOPE/DPSG liposomes are the least acid sensitive. Incubation of DOPE/OA liposomes with mouse L929 cells reduces the pH-sensitivity of these liposomes by altering the lipid composition. Using diphtheria toxin fragment A as a marker for cytoplasmic delivery, we find that the delivery kinetics of pH-sensitive immunoliposomes closely correlates with the modified acid sensitivities of the liposomes. Immunoliposomes encounter pH 6–6.2 with a $t_{1/2}$ of 5–15 min after internalization. By contrast, acidification of the endosomes to pH 5.0 takes longer ($t_{1/2} \approx 25$ min). We also used a whole cell null point technique (Yamishiro and Maxfield (1987) J. Cell Biol. 105, 2713–2721) to directly determine the average pH encountered by the endocytosed immunoliposomes. We find that acidification determined by the null point method proceeds less rapidly than that estimated from DTA delivery data. This is likely due to the fact that the measured DTA delivery is done by those liposomes which first arrive at the endosomes with sufficient acidity. Our data suggests that DOPE/PHC immunoliposomes deliver at the early endosome while DOPE/DPSG immunoliposomes deliver at the late endosomes. The DOPE/OA immunoliposomes, with the altered composition and acid sensitivity, deliver with a kinetics intermediate between the other two immunoliposomes. Thus, pH-sensitive liposomes represent useful probes for studying the kinetics of endosome acidification.

Introduction

Endocytosis is a major route for cellular uptake of extracellular materials. Many types of molecules including low density lipoprotein [1], transferrin [2], growth factors [3] and viruses are taken up by endocytic pathway. Receptors and their ligands first cluster in clathrin coated pits [4]. These pits then pinch off from the plasma membrane to form coated vesicles which, after loss of their clathrin coat, fuse with the early endosomes. From the early endosomes some receptors such as transferrin recycle back to the cell surface [4], while others are transported to later elements in the pathway including the late endosome and the lysosome [4]. Recently, it has been demonstrated that early and late endosomes are biochemically distinct organelles which are kinetically related yet exhibit quite different luminal acidities [5]. Internalized ligands reach the early endosome with a $t_{1/2}$ of 2–5 min and are acidified to approximately pH 6–6.5 [5]. Accumulation of ligands in the late endosomes proceeds asynchronously with a $t_{1/2}$ of 10–25 min and acidification to pH 5.0–5.5 [5]. The lysosome is reached with a $t_{1/2}$ of approx. 35 min exposing the ligand to pH 4–5 [5]. It should be noted that the precise acidification kinetics is dependent on the method of detection and, perhaps, also on the type of cell used. Other investigators have reported similar but not identical acidification kinetics [6–9].

The primary route of liposome uptake by cultured cells is the endocytic pathway [10,11]. Liposomes and immunoliposomes (liposomes bearing covalently attached antibody) are taken up through endocytosis and

Abbreviations: OA, oleic acid; PS, phosphatidylserine; PC, phosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPSG, dipalmitoylsuccinylglycerol; PHC, palmitoylhomocysteine; NBD-PE, *N*-(nitrobenz-2-oxa-1,3-diazol-4-yl)-PE; N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-PE; FPE, *N*-fluoresceinthiocarbamoyldipalmitoyl-PE; DTA, diphtheria toxin A fragment; PBS, phosphate-buffered saline; CE, hexadecyl cholestanyl ether.

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encounter the progressively more acidic pH values of the endocytic organelles. Once the lysosome is reached, the liposome, (or immunoliposome) along with its contents, is degraded [11]. Recently, we and others have developed liposomes which can exploit the acidification properties of the endocytic organelles and release their contents into the cytoplasm following liposome-endosome fusion [12–14]. These liposomes are stable at neutral pH and become fusion active and leaky at weakly acidic pH values [15]. Such pH-sensitive liposomes [14] and immunoliposomes have been shown to mediate the cytoplasmic delivery of a variety of encapsulated solutes including fluorescent dyes [12], cytotoxic drugs [13], plasmid DNA [16,17] and diphtheria toxin A fragment (DTA) [18]. The process of delivery by pH-sensitive immunoliposomes involves antibody-mediated binding of the immunoliposomes to antigen expressing cells, uptake of the immunoliposomes into the endocytic pathway and acid induced fusion of the liposomes with the cellular endosomes.

Recently, we have characterized the physical behavior of three types of pH-sensitive liposomes. The liposomes share a common design in which dioleoylphosphatidylethanolamine (DOPE) is combined with a protonatable amphipathic lipid. DOPE alone will not form bilayers under physiological conditions [19], however, stable bilayers form when DOPE is combined with deprotonated acidic lipids [15]. We have found that different DOPE/amphiphile combinations yield liposomes with different stabilities and different acid sensitivities. As described above, the endocytosis of pH-sensitive liposomes has been demonstrated and the endocytic pathway has been shown to be composed of organelles with different luminal acidities which are encountered sequentially. The goal of the studies presented here was to determine if the physical properties of various pH-sensitive liposomes are reflected in different delivery kinetics following endocytosis. Our data suggests that pH-sensitive liposomes may provide nontoxic carriers of defined composition and behavior which may be useful probes for studying the endocytic pathway.

Experimental procedures

Materials

DOPE, dioleoylphosphatidylcholine (DOPC), bovine brain phosphatidylserine (PS), *N*-(nitrobenz-2-oxa-1,3-diazol-4-yl)-PE (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-PE (N-Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). OA was purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]OA was purchased from New England Nuclear. *N*-Fluoresceinthiocarbamoyldipalmitoyl-PE (FPE) was purchased from Molecular Probes (Eugene, OR). PHC was synthesized as described [20]. [^{3}H]PHC was prepared by using [^{3}H]*N*-hydroxysuccinimide ester of palmitic acid as a starting material [21]. DPSG was synthesized as described [34]. [^{14}C]DPSG was synthesized using [^{14}C]-succinic anhydride as a starting material. Diphtheria toxin was obtained from Connaught Laboratories (Ontario, Canada). DTA was purified as described [18]. Calcein was obtained from Sigma Chemical Co. (St. Louis, MO).

Antibody preparation

Anti-H2K^{k} antibody from mouse hybridoma cell line 11-4.1 was purified, labeled with ^{125}I and derivatized with the *N*-hydroxysuccinimide ester of palmitic acid, as described by Huang et al. [21].

Liposome and immunoliposome preparation

Liposomes or immunoliposomes were prepared by a reverse-phase evaporation method with modifications [12,22]. Briefly, solvent free lipid films at a DOPE/amphiphile ratio of 4:1 (mol/mol) were suspended in phosphate-buffered saline (PBS) (pH 8.0). For calcein-containing liposomes the lipid was suspended in PBS (pH 8.0) containing 50 mM calcein. A trace amount of hexadecyl [^{3}H]cholestanyl ether ([^{3}H]CE) was included in the lipid mixture to facilitate evaluation of the lipid. The lipid suspension was sonicated for 10 min in a bath type sonicator (Laboratory Supplies Co. Inc. Hicksville, NY). The pH was found to decrease upon sonication and was readjusted to pH = 8.0 by addition of 0.1 M NaOH. The liposomes were then sonicated for an additional 10 min. If DTA was to be entrapped DTA (50 μg per 10 μmol lipid) was added. The sonicated liposomes were then transferred to a pear-shaped flask and diethyl ether added to a 3:1 (v/v) ratio to the aqueous phase. The mixture was sonicated 20–30 s to form a stable emulsion. The emulsion was then rotary evaporated at 30°C under vacuum (water aspiration) using a Buchi Rotvaper-R (Buchi Laboratoriums Technik AG, Switzerland) until a liquified gel was formed. The resulting liposomes were diluted with PBS to a final lipid concentration of 5 mM. Immunoliposomes were prepared by adding palmitoyl antibody, in PBS (pH 8.0) containing 0.15% deoxycholate, to the liquified gel. The lipid to antibody ratio was 10:1 (w/w). Diethyl ether at 2% (v/v) was added to facilitate antibody incorporation [22] and this final mixture was vortexed and dialyzed for 25 h against 12 l PBS (pH 8.0). The liposomes and immunoliposomes were then extruded five times through 0.1 μm nuclepore filters.

Unencapsulated calcein or DTA were separated from liposomes and immunoliposomes by passage over Bio-Gel A0.5M column equilibrated with PBS (pH 7.5).

Lipid mixing assay

Liposomes prepared to contain 1 mol% N-NBD-PE and 0.5 mol% N-Rh-PE ('labeled vesicles') were in-

cubated with a 3-fold excess of unlabeled vesicles. Acid-induced lipid mixing was assayed as described by Connor et al. [15].

Acid-induced leakage

Liposomes containing calcein were diluted to a final lipid concentration of 50 μM in PBS (pH 7.5) and assayed for acid-induced calcein release as described [15].

Immunoliposome delivery kinetics

L929 cells were plated into 96-well plates at 10^5 cells/ml and allowed to attach overnight. The cells were then washed with ice-cold McCoy's medium containing 10% calf serum and incubated at 4°C for 30 min. The cells were then gently washed three times with ice-cold serum-free McCoy's medium. DTA-containing immunoliposomes in serum-free McCoy's were added to the cells and incubated for 35 min at 4°C on a shaker plate. The final DTA concentration was $5 \cdot 10^{-3}$ mg/ml at a final lipid concentration of 45–50 μg/ml. This concentration of liposomal DTA was chosen, because previous experiments showed that the cellular protein synthesis was inhibited maximally (70–80%) at this concentration. After 35 min at 4°C, the cells were gently washed five times with ice-cold serum-free McCoy's medium. To allow endocytosis to begin, the cold serum-free medium was replaced with prewarmed (37°C) McCoy's medium containing 10% calf serum. At various times (0–60 min) after warmup to 37°C, McCoy's medium containing calf serum and 10 mM NH_4Cl was added to the cells to inhibit endosome acidification. At 17 h after warmup, [^{3}H]leucine (New England Nuclear) in McCoy's medium containing 10% calf serum and 10 mM NH_4Cl, was added to the cells (1 μCi/well) and incubated a further 6 h. The cells were then harvested onto glass fiber filters using an automated cell harvester (Cambrige PHD, Cambridge Technologies) and processed by scintillation counting. Immunoliposome-treated cells were compared to controls (untreated) at each time point (0% maximal toxicity). Maximal toxicity (100%) refers to the toxicity obtained in experiments where no NH_4Cl was used.

Lipid exchange

Liposomes were prepared as described above to contain [^{3}H]CE as a marker for total lipid. For DOPE/OA and DOPE/DPSG the appropriate ^{14}C-labeled the amphiphiles were also included in the liposome membranes. For DOPE/PHC, two separate liposome preparations had to be prepared: one containing [^{3}H]CE (total lipid marker) and one containing [^{3}H]PHC.

Labeled liposomes (45 μg/ml total lipid) were added to L929 cells which had been grown in 6-well plates (10^5 cells/ml) and incubated with agitation at 37°C for 30 min. The cell medium was then harvested and the cells were dissolved in 0.2% Triton X-100. Total lipid recovery and recovery of OA, DPSG or PHC were then evaluated by scintillation counting.

Cell-induced leakage

Calcein-containing liposomes of the indicated compositions (45 μg/ml total lipid) were incubated with L929 cells for 30 min at 37°C. The cell medium was harvested and the liposomes evaluated for calcein self-quenching using the following equation:

$$\% \text{ quenching} = (1 - F_0/F_T) \cdot 100$$

where F_0 and F_T are the measured fluorescence intensities of liposomes before and after addition of deoxycholate to 0.2% (v/v).

'Null point' acidification assay

The pH of immunoliposome containing compartments was measured using a null point method [23]. Immunoliposomes composed of DOPC and PS (4:1, mol/mol) were made to contain 2 mol% FPE by the reverse-phase-evaporation method described above. L929 cells were grown on glass cover slips (11 × 30 mm) and washed with Mes-buffered saline (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM glucose and 20 mM Mes (pH 7.4)). FPE containing immunoliposomes (50 μg/ml total lipid) was added to the cells and incubated at 37°C. At various times of incubation the cells were washed five times with ice cold Mes-buffered saline (pH 7.4) and placed on ice. The cover slips were then placed in a teflon cover slip adapter which allows fluorescence measurements to be made on cells attached to the cover slip. This adapter holds the cover slip at a 60° angle with respect to the fluorescence cuvette face. The cover slip was placed in 2.5 ml of Mes-buffered saline (pH 7.4) and initial fluorescence measurement made at 490 nm excitation and 516 nm emission. After the initial fluorescence measurements were made, the media was replaced with Mes-buffered saline, preadjusted to pH 6.8, 6.5, or 6.0. Fluorescence intensity measurements were made after this change in extracellular pH (F_{ex}). A mixture of ammonium acetate and methylamine (70 mM each final concentration) was added to equilibrate the intracellular pH with that of the external medium [23]. Fluorescence intensity measurements were made 2 min after addition of ammonium acetate/methylamine (F_{in}). All fluorescence measurements were made at 25°C. The percentage change in fluorescence intensity was calculated as described [23] as:

$$\Delta I = \frac{F_{in} - F_{ex}}{F_{ex}} \cdot 100\%$$

and plotted vs. extracellular pH.

Results

Acid sensitivity of the liposomes

Fig. 1 shows a comparison of the acid sensitivity of pH-sensitive liposomes of three different compositions. We have used the lipid mixing assay described by Connor and Huang [15] to assay for acid-induced destabilization of pH-sensitive liposomes. The destabilization of pH-sensitive liposomes involves fusion and collapse into nonbilayer structures such as hexagonal phase [19]. As shown in Fig. 1, DOPE/oleic acid (DOPE/OA) liposomes exhibit 50% maximal lipid mixing (pH_{50}) at approximately pH 6.9. DOPE/palmitoylhomocysteine (DOPE/PHC) liposomes exhibit a pH_{50} at approximately pH 6.25, while liposomes composed of DOPE/dipalmitoylsuccinylglycerol (DOPE/DPSG) exhibit a pH_{50} of approximately pH 5.0 (Fig. 1). Thus, the three liposome compositions gave well-defined but different acid sensitivity. Since all the pH-sensitive liposome compositions contain the same amount of DOPE, the different acid sensitivities reflect structural differences in the amphiphilic lipid combined with DOPE. In particular, the intrinsic pK_a values of the acidic lipids are probably different.

Delivery kinetics of the liposomes

Based on the results of our acid-induced destabilization studies and what is known about the kinetics of endocytosis, one would predict DOPE/OA liposomes should deliver earlier in the endocytic pathway than DOPE/PHC liposomes which should in turn, deliver earlier than liposomes composed of DOPE/DPSG. In order to test this hypothesis, we prepared immunoliposomes composed of DOPE/OA, DOPE/PHC and DOPE/DPSG. All preparations contained DTA which we have previously shown to be effectively delivered by pH-sensitive liposomes in a process involving immunoliposome endocytosis and immunoliposome-endosome

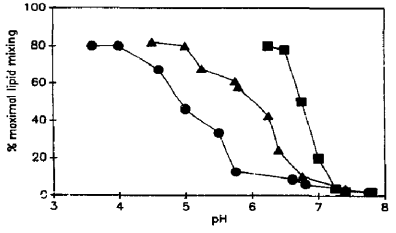

Fig. 1. Acid-induced destabilization of liposomes. Lipid mixing at different pH values was assayed as described in Methods for: DOPE/OA (4:1, mol/mol) liposomes (■), DOPE/PHC (4:1, mol/mol) liposomes (▲) and DOPE:DPSG (4:1, mol/mol) liposomes (●).

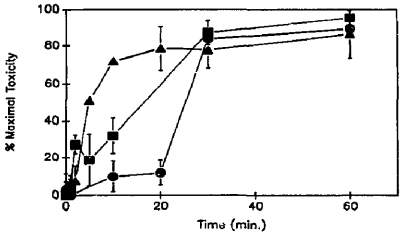

Fig. 2. Kinetics of DTA delivery by pH-sensitive immunoliposomes. % maximal toxicity was assayed as described in Methods and plotted vs. time of incubation at 37°C prior to NH_4Cl addition. ▲, DOPE/PHC immunoliposomes; ■, DOPE/OA immunoliposomes, and ●, DOPE/DSPG immunoliposomes.

fusion [18]. In order to evaluate the kinetics of delivery we first incubated cells at 4°C to inhibit endocytosis. The different immunoliposome preparations were then added to the cells and allowed to bind at 4°C. We have previously shown that immunoliposomes are bound but not endocytosed at 4°C [7,8]. The cells were then washed to remove unbound immunoliposomes and warmed to 37°C to allow endocytosis and endosome/lysosome acidification to proceed. Acidification was stopped after various times at 37°C by addition of NH_4Cl which we have previously shown to be effective in blocking immunoliposome-endosome fusion [18]. Using this assay we can determine the $t_{1/2}$ (time required for 50% maximum toxicity) of delivery by the different immunoliposome compositions.

In support of the hypothesis described above, delivery of DTA by DOPE/DPSG immunoliposomes could be completely blocked by NH_4Cl at time points up to 20 min after warmup to 37°C (Fig. 2). At time points longer than 20 min, DTA delivery was not blocked. This indicates that delivery via DOPE/DPSG immunoliposomes occurs in an acidified endocytic organelle which is reached with a $t_{1/2}$ of approx. 25 min. DOPE/PHC immunoliposome delivery kinetics also supported our hypothesis. As seen in Fig. 2, DOPE/PHC liposomes were not blockable by NH_4Cl at time points greater than 2 min. The $t_{1/2}$ for delivery by DOPE/PHC immunoliposomes was approximately 5 min (Fig. 2). The delivery kinetics of DOPE/OA immunoliposomes deviated significantly from our hypothesis. DOPE/OA immunoliposomes exhibit a $t_{1/2}$ for delivery of approximately 15 min (Fig. 2) which is intermediate between DOPE/PHC and DOPE/DPSG. According to the pH_{50} of the DOPE/OA liposome, one would expect that the $t_{1/2}$ for delivery would be less than 5 min.

Cell-induced alterations of immunoliposomes

In order to determine why DOPE/OA immunoliposome delivery kinetics deviated from our original hypothesis, we investigated the possibility that L929 cells were altering the composition and thus the behavior of the immunoliposomes. Earlier work in our laboratory has shown that incubation in serum-free McCoy's medium does not alter the composition or stability DOPE/OA immunoliposomes (data not shown). However, it is known that long-chain fatty acids, such as OA, can desorb very readily from liposomes membranes and transfer rapidly between lipid membranes [24]. Liposome-cell transfer of OA has also been demonstrated [25]. To investigate whether OA may be undergoing immunoliposome-to-cell transfer, we examined the transfer of [^{14}C]OA from liposomes in the presence of L929 cells. In order to reduce the contribution of endocytosis to [^{14}C]OA (and total lipid) uptake, we used liposomes rather than immunoliposomes since the former are not readily endocytosed [11]. As shown in Fig. 3, approx. 99% of total lipid was recoverable after 30 min incubation with L929 cells. By contrast, only 71% of total OA was recovered from the cell media. While only 1% of total lipid became cell associated, 29% of total OA was associated with the cells (Fig. 3). PHC transfers less readily from liposomes to cells (Fig. 3). After 30 min incubation with cells, 96% of total PHC could be recovered in the cell media with approx. 4% being cell associated (Fig. 3). Of the three types of liposomes, those prepared with DPSG exhibited the least liposome-to-cell transfer of amphiphilic lipid. After 30 min at 37°C in the presence of cells, 99% of total DPSG was recoverable from the cell medium (Fig. 3). Slightly less than 1% of total DPSG became cell-associated under these conditions (Fig. 3).

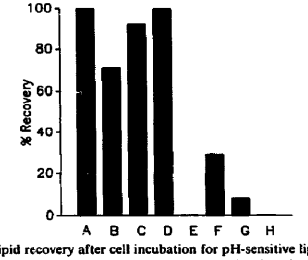

Fig. 3. Lipid recovery after cell incubation for pH-sensitive liposomes. Liposomes of the indicated compositions were incubated with L929 cells for 30 min at 37° C. Then lipid recovery in cell medium (A–D) and cell associated lipid (E–H) was assayed using radioactive lipids as described in Methods. A, E: total lipid recovery; B, F: OA recovery; C, G: PHC recovery; D, H: DPSG recovery.

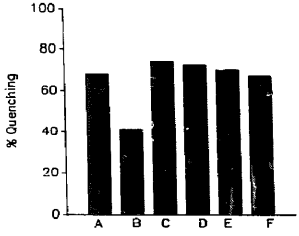

Fig. 4. Cell induced leakage from pH-sensitive liposomes. pH-sensitive liposomes containing calcein were incubated with L929 cells and calcein release assayed as described in Methods. DOPE/OA liposomes in (A) absence and (B) presence of L929 cells, DOPE/DPSG liposomes in (C) absence or (D) presence of L929 cells and DOPE/PHC liposomes in (E) absence or (F) presence of L929 cells.

We also investigated the possibility that L929 cells may destabilize pH-sensitive liposomes. For these studies we encapsulated calcein, a self-quenching fluorescent dye, in liposomes of various compositions. We then assayed for calcein release from liposomes incubated at 37°C in the absence or presence of L929 cells. When calcein is encapsulated in liposomes, its fluorescence is high quenched due to collisional energy transfer. However, when the calcein leaks out of the liposomes, fluorescence increases and quenching decreases. When DOPE/OA liposomes containing calcein were incubated at 37°C in the absence of L929 cells, the calcein was 70% quenched (Fig. 4). Incubation of DOPE/OA liposomes with L929 cells for 30 min at 37°C reduced the calcein quenching to 41% (Fig. 4). Even after 5 min incubation with cells, DOPE/OA liposomes exhibited 45% quenching (data not shown). This indicates that cell-induced leakage from DOPE/OA liposomes occurs rather rapidly. DOPE/PHC liposomes exhibited less leakage after cell incubation. For this composition, 75% quenching of calcein was obtained when liposomes were incubated in the absence of cells (Fig. 4). Incubation of DOPE/PHC liposomes with L929 cells reduced the calcein quenching to 67% (Fig. 4) indicating that some leakage had taken place. DOPE/PHC liposomes are clearly less leaky than their DOPE/OA counterparts upon cell incubation. DOPE/DPSG liposomes were then least leaky composition we tested. After incubation at 37°C in the absence of cells, calcein in DOPE/DPSG liposomes was 74% quenched (Fig. 4). After 30 min at 37°C in the presence of cells, the fluorescence quenching was only reduced to 72.3% (Fig. 4).

To further investigate the liposome–cell interaction we examined the acid sensitivity of pH-sensitive liposomes which had been incubated with L929 cells.

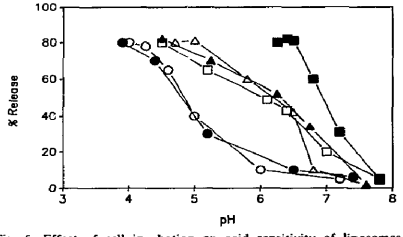

Fig. 5. Effect of cell incubation on acid sensitivity of liposomes. Liposomes which had been incubated 30 min at 37° C in the absence (closed symbols) or presence (open symbols) of L929 cells were harvested and assayed for acid-induced leakage as described in Methods. ■, □: DOPE/OA liposomes; ▲, △: DOPE/PHC liposomes; and ●, ○: DOPE/DPSG liposomes.

Calcein-containing pH-sensitive liposomes composed of either DOPE/OA, DOPE/PHC or DOPE/DPSG were incubated 30 min in the presence or absence of cells. The liposomes were recovered and assayed for acid-induced leakage of calcein. Free calcein was not separated from the liposomes and did not affect our pH-sensitivity measurements. As shown in Fig. 5, the acid sensitivity of DOPE/DPSG and DOPE/PHC liposomes was unaffected by cell incubation. DOPE/OA liposomes which had not been incubated with cells exhibited a pH_{50} for release of approx. pH 6.9 (Fig. 5). By contrast DOPE/OA liposomes incubated in the presence of cells had a pH_{50} for release of approx. pH 6.0 (Fig. 5). This effect may be due to transfer of lipid from the cell to the liposome [26] in addition to the liposome-to-cell transfer of OA.

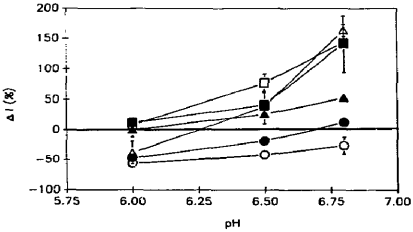

Fig. 6. Time course of L929 cell endosome acidification measured by null point method. L929 cells were incubated at 37° C in the presence of DOPC/PS/FPE liposomes for: 5 min (○), 10 min (●), 15 min (△), 20 min (▲), 30 min (■), or 60 min (□). ΔI is calculated as described in Materials and Methods. Each point represents the mean ± S.D. for three experiments.

TABLE I

Acidification of endocytic compartments determined by null point method or DTA delivery in L929 cells

Incubation time (min)	pH (null point) [a]	pH (DTA delivery)
0	7.4 [b]	7.4 [b]
5	> 6.8	6.2 [c]
10	6.7 ± 0.1	–
15	6.2 ± 0.1	6.0 [c]
20	6.0 ± 0.1	–
25	–	5.0 [c]
30	< 6.0	–
60	< 6.0	–

[a] Calculated from Fig. 6 using the pH at which $\Delta I = 0$ [31].
[b] Extracellular pH.
[c] Calculated from the pH_{50} (Fig. 1 and Fig. 5) and $t_{1/2}$ for DTA delivery (Fig. 2).

Acidification kinetics by null point method

The whole cell null point method for pH measurement was originally developed by Yamishiro and Maxfield [23] as a way to accurately measure the pH of intracellular compartments after short periods of endocytosis. The method relies on the pH-dependent fluorescence of fluorescein which increases with increasing pH. pH determination is based on whether the endosome pH is above or below the extracellular 'test' pH [23]. Because the method depends exclusively on the change in fluorescein fluorescence after addition of ammonium acetate and methylamine, this technique compensates for cell surface (non-internalized) fluorescence. The time course for acidification of immunoliposome-containing L929 cell endosomes is shown in Fig. 6. The change in fluorescein fluorescence is plotted vs. the extracellular pH. From this graph, the average pH encountered by the endocytosed immunoliposome can be calculated as the pH where $\Delta I = 0$ (Table I). According to our delivery data (Fig. 2 and Table I), immunoliposomes reach approximately pH 6.2 with a $t_{1/2}$ of 5 min. By contrast, pH measurement by the null point method shows that at 5 minutes the endosomal pH is still > 6.8 (Fig. 6; Table I). Using the null point method, we find that acidification to pH 6.2. requires 15 min (Fig. 6, Table I). DTA delivery kinetics data suggests that immunoliposomes reach pH ≈ 6.0 with a $t_{1/2}$ of 15 min. Null point pH measurements show that 20 min are required to reach pH 6.0 (Fig. 1). In all cases, the acidification kinetics are more rapid when estimated from DTA delivery data than when measured by the whole cell null point method.

Discussion

Previously we have shown that pH-sensitive liposomes are effective delivery vehicles for introducing

molecules of interest into cultured cells [12,13,16–18]. These immunoliposomes have been shown to deliver their contents via acid-induced fusion with the endosome membrane [16–18]. In the present study we have prepared three types of pH-sensitive immunoliposomes with differing acid sensitivities and examined their interactions with cultured cells. We find that the immunoliposome compositions which fuse at less acidic pH values release at early time points after endocytosis, while liposomes which fuse under more acidic conditions deliver at later time points. We suggest that the intracellular sites of delivery may differ for the different pH-sensitive immunoliposomes.

In the present study, we used DTA as a marker for cytoplasmic delivery. DTA is a potent protein synthesis inhibitor, however in its free form DTA cannot cross cell membranes and is non-toxic [18]. We have previously shown that pH-sensitive immunoliposomes can effectively deliver DTA via a liposome-endosome fusion event following the endocytosis of the immunoliposome [18]. As shown above, DOPE/PHC liposomes have a pH_{50} for fusion of pH 6.25 (Fig. 1). DOPE/PHC immunoliposomes deliver DTA with a $t_{1/2}$ of 5 min (Fig. 2). By comparison, DOPE/DPSG liposomes exhibit a pH_{50} for fusion of pH 5.3 (Fig. 1). Immunoliposomes of this composition require approx. 25 min to deliver DTA sufficient to produce 50% maximal toxicity (Fig. 2).

DOPE/OA liposomes exhibited a pH_{50} of pH 6.9 in the absence of cells (Fig. 1) but in the presence of cells the pH_{50} was altered to pH 6.0 (Fig. 5). The alteration in pH_{50} is due to an alteration in the lipid composition of the immunoliposome (Fig. 3). Rapid loss of liposomal OA occurs due to fatty acid transfer from liposome-to-cell. Partial leakage of liposome contents also occurs (Fig. 4), probably due to loss of stabilizing fatty acid. The alteration of pH_{50} to lower pH values may be due to cell-to-liposome lipid transfer of either cholesterol or phospholipid, or both [26]. In support of this conclusion, addition of cholesterol or phosphatidylcholine reduces the acid sensitivity of pH-sensitive liposomes [15,27]. The delivery kinetics of DOPE/OA immunoliposomes reflects the altered pH_{50} value. As shown in Fig. 5, the pH_{50} value of DOPE/OA liposomes after cell incubation is intermediate between the pH_{50} values for DOPE/DPSG (pH 5.3) and DOPE/PHC (pH_{50} 6.25). The delivery kinetics for DOPE/OA immunoliposomes is also intermediate between DOPE/PHC (5 min and DOPE/DPSG ($\approx$ 25 min) immunoliposomes.

The data presented here suggest the following working model. Immunoliposomes of all three compositions first bind to the cell surface. At the cell surface, exchange of OA (liposome-to-cell) and reciprocal exchange of cell lipid (cell-to-liposome) takes place, altering the composition of DOPE/OA immunoliposomes. DOPE/OA immunoliposomes also become leaky at the cell surface and may release at least part of their contents extracellularly. DOPE/PHC and DOPE/DPSG immunoliposomes undergo less alteration of their compositions and do not leak significantly. Upon endocytosis, the immunoliposomes are taken into the cells and within 5 min encounter a compartment with a luminal pH of approx. 6.2. At this compartment, DOPE/PHC immunoliposomes fuse and release DTA into the cytoplasm. After approximately 15 min of internalization, the immunoliposomes encounter a pH of approx. 6.0, allowing DOPE/OA immunoliposomes to deliver DTA. As endocytosis proceeds the pH encountered by the immunoliposomes progressively decreases. At approx. 25 min after internalization, the immunoliposomes encounter a pH of approx. 5.0. This pH induces DOPE/DPSG immunoliposome fusion and delivery of DTA to the cell cytoplasm.

Our data suggest that DOPE/PHC immunoliposomes deliver at the early endosome. By contrast, our data with DOPE/DPSG immunoliposomes is consistent with delivery at the late endosome. DTA delivery by DOPE/OA immunoliposomes is complicated by the cell-induced change in immunoliposome composition and physical behavior. However, the $t_{1/2}$ of delivery by DOPE/OA immunoliposomes is intermediate between the $t_{1/2}$ values obtained for DOPE/DPSG and DOPE/PHC immunoliposomes. This may reflect delivery at a compartment of intermediate acidity between the early and late endosomes and may support a 'maturation model' of endocytosis [28]. However, this interpretation must be made with caution since there may be heterogenous populations of DOPE/OA immunoliposomes which have different acid sensitivities. In this scenario, the cell-induced alteration of DOPE/OA immunoliposomes leads to the production of some 'high pH_{50}' and some 'low pH_{50}' population of DOPE/OA immunoliposomes. The high pH_{50} population could deliver at the early endosome while the low pH_{50} population could deliver at the late endosome. Neither our pH_{50} measurements (Figs. 1 and 5) nor our delivery work (Fig. 2) can rule out this possibility since both sets of data reflect averages over the total liposome population. Another possibility is that the $t_{1/2}$ of delivery by DOPE/OA immunoliposomes reflects the maximum time spent in the early endosome (time required to reach pH = 6.0) or the minimum time required to reach the late endosome. We are currently investigating these possibilities.

Our results also shed light on the endosome acidification kinetics of L929 cells. To our knowledge, this has not been previously studied. Using Semliki Forest Virus (SFV) and SFV mutants with altered fusion pH thresholds, Kielian et al. [8,9] probed the endosome acidification kinetics of BHK-21 cells. In their study, wild type (wt) SFV (fusion threshold of pH 6.2) and fus

1 mutant SFV (fusion threshold of pH 5.3) were used to monitor endosome acidification. They found that the wt threshold pH was reached with a $t_{1/2}$ of 15 min, while reaching the fus 1 threshold pH required longer times ($t_{1/2} \approx 45$ min) after endocytosis. In our system, the pH_{50} of DOPE/PHC is very close to that of wt SFV while the pH_{50} of DOPE/DPSG is similar to that of fus 1 (Fig. 1 and Kielian et al. [8,9]). We find that L929 cell endosomes reach pH 6.2 with a $t_{1/2}$ of 5 min, pH 6.0 with a $t_{1/2}$ of 15 min and reach pH 5.0 with a $t_{1/2}$ of 25 min. Therefore, it appears that endosome acidification in L929 cells proceeds more rapidly than in BHK-21 cells [8,9]. This interpretation must be made with caution. Since we do not know the exact amount of DTA delivered to the cells, the cytotoxicity we observe reflects fusion by only a small population of liposomes which have rapidly arrived at a compartment with sufficient acidity. This leads to an underestimate of the average time required for endosome acidification in L929 cells (Table I).

This conclusion is supported by our data obtained using the null point method for determining endocytic pH (Fig. 6; Table I). In all cases, the acidification estimated from DTA delivery occurs more rapidly than the acidification kinetics determined using the null point method. The null point method measures the average pH encountered by the entire internalized immunoliposome population [23]. By contrast, DTA delivery may only allow observation of the first immunoliposomes to reach a certain pH value or endocytic compartment. Despite the discrepancies, both sets of data show that immunoliposomes encounter progressively more acidic pH values after internalization. Comparison of the data also suggests that uptake and delivery by pH-sensitive immunoliposomes may be markedly asynchronous.

Many toxins and viruses require endocytosis and acidification in order to penetrate into the cell cytoplasm [8,9,29]. By taking advantage of this fact, a number of mutant cell lines with various defects in endocytosis have been isolated [30,31]. In addition, drugs, such as chloroquine, have been used to select for endosome acidification mutants [32]. Certain ligands, such as transferrin or LDL may also be useful for isolating mutants of the early [5] or late [4] endosomes, respectively. However, the use of either transferrin or LDL to select for mutants would require that cytotoxic drugs or macromolecules be coupled to the ligand. This may modify their routing and/or uptake.

The liposomes described here may also be useful for isolating mutant cells with altered endosome functions. For example, DOPE/PHC immunoliposomes deliver DTA early in the endocytic pathway and therefore kill cells with normal early endosome acidification. This suggests the possibility that this liposome composition may be useful for selecting for early endosome mutants. In like manner, DOPE/DPSG immunoliposomes may be useful for selecting mutants of late endosome acidification. Studies using such mutants would allow further elucidation of the normal roles of early and late endosomes. pH-sensitive immunoliposomes have several advantages over other methods of mutant selection. In particular, liposomes of well-defined composition and physical behavior can be prepared and large amounts of biologically active solutes can be encapsulated in a non-toxic carrier. Immunoliposome uptake is also highly specific and the delivery process very efficient [12]. We except that these pH-sensitive liposomes will be quite useful tools for studying endocytosis and are currently investigating these possibilities.

Specifically, these immunoliposomes may be ideal tools to help elucidate the role of endocytosis in antigen processing and presentation. Recently it has been suggested that antigenic peptides may bind to the class II major histocompatability antigen at an endosomal site before being presented on the cell surface [33]. The actual endosome population involved is not known. One possible strategy to elucidate the site of class II antigen binding is to use pH-sensitive immunoliposomes of defined pH_{50} to deliver antigenic peptide. Delivery of peptide by DOPE/PHC immunoliposomes (early delivery) and DOPE/DPSG immunoliposomes (late delivery) may be one way to determine the endosomal compartments involved in antigen presentation.

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