Efficient Cytoplasmic Delivery of a Fluorescent Dye by pH-sensitive Immunoliposomes

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ABSTRACT We previously showed that liposomes composed of dioleoylphosphatidylethanolamine and palmitoyl-homocysteine (8:2) are highly fusion competent when exposed to an acidic environment of pH <6.5. (Connor, J., M. B. Yatvin, and L. Huang, 1984, *Proc. Natl. Acad. Sci. USA.* 81:1715–1718). Palmitoyl anti-H2K^k was incorporated into these pH-sensitive liposomes by a modified reserve-phase evaporation method. Mouse L929 cells (k haplotype) treated with immunoliposomes composed of dioleoylphosphatidylethanolamine/palmitoyl-homocysteine (8:2) with an entrapped fluorescent dye, calcein, showed diffused fluorescence throughout the cytoplasm. Measurements by use of a microscope-associated photometer gave an approximate value of 50 μM for the cytoplasmic calcein concentration. This concentration represents an efficient delivery of the aqueous content of the immunoliposome. Cells treated with immunoliposomes composed of dioleoylphosphatidylcholine (pH-insensitive liposomes) showed only punctate fluorescence. The cytoplasmic delivery of calcein by the pH-sensitive immunoliposomes could be inhibited by chloroquine or by incubation at 20°C. These results suggest that the efficient cytoplasmic delivery involves the endocytic pathway, particularly the acidic organelles such as the endosomes and/or lysosomes. One possibility is that the immunoliposomes fuse with the endosome membranes from within the endosomes, thus releasing the contents into the cytoplasm. This nontoxic method should be widely applicable to the intracellular delivery of biomolecules into living cells.

The development of an effective, nontoxic method for the delivery of macromolecules to the cytoplasm of living cells is important for studies of control mechanisms of cellular processes. The versatility of liposomes, in their composition, size, and ability to encapsulate many different macromolecules, makes them attractive carriers for cellular delivery. Work in this lab, involving a model system for studying membrane fusion, led to the devleopment of a liposome composition of dioleoylphosphatidylethanolamine/palmitoyl homocysteine (DOPE/PHC)[1] (8:2) that undergoes fusion at acidic pH's (1). Concurrent with this model study, our laboratory established a reproducible cell targeting system, which involves the incorporation of a fatty acid derivatized monoclonal antibody into liposomes (2). This allows for the specific binding of the immunoliposomes to target cells (3). Upon binding the immunoliposome is endocytosed and eventually delivered to the lysosomes (4). The processing pathway includes an intermediate step at which the immunoliposomes encounter an acidic environment in the endosomes with a pH that varies from 4 to 6 (5).

In the work presented in this paper we have combined the established monoclonal antibody targeting system with the pH sensitive liposomes to generate pH-sensitive immunoliposomes that become fusion competent at acidic pH's. Thus, if the liposomes were endocytosed and proceeded into the endosome vesicles they would encounter an acidic environment and become fusion competent. If a fusion reaction occurred between the liposome and the endosome membranes the contents of the liposome would be released into the cell cytoplasm. The experiments in this paper were designed to test this hypothesis.

MATERIALS AND METHODS

Materials: PHC was synthesized and purified as described (6). DOPE and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Calcein and chloroquine were obtained from Sigma Chemical Co. (St. Louis, MO).

[1] *Abbreviations used in this paper:* DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; PHC, palmitoyl homocysteine.

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Antibody Preparation: Anti-H2K^k antibody from a mouse hybridoma cell line 11-4.1 was purified, labeled with ^{125}I, and derivatized with *N*-hydroxysuccinimide ester of palmitic acid, as described by Huang et al. (2).

Liposome Preparation: Reverse-phase evaporation vesicles were prepared as follows. Solvent-free lipid films containing DOPE/PHC (8:2) or DOPC were suspended in phosphate-buffered saline (PBS) containing 60 mM calcein. A trace amount of hexadecyl [^{3}H]cholestanyl ether was included in the lipid mixture to facilitate the monitoring of the lipid. A critical ratio of 65 μl buffer per 5 μmol lipid must be maintained in order to form a stable emulsion with the organic phase. The lipid suspension was sonicated at room temperature for 10 min with a bath sonicator (Laboratory Supplies Co. Inc., Hicksville, NY) and the pH was adjusted to 7.6. The sonicated liposomes were transfered to a 25-ml round-bottom flask, and 4 ml of a 3:1 (vol/vol) mixture of chloroform/ethyl ether was added. The mixture was briefly sonicated (~30 s) to form a stable emulsion. The emulsion mixture was rotovaped at 30°C with a water aspirator using a Buchi Rotavapor-R (Buchi Laboratoriums Technik AG, Switzerland) until all of the organic solvent was removed. The resulting reverse-phase evaporation vesicles were incubated in a fume hood for 1 h to remove any residual organic solvent. PBS was then added to the lipid suspension to bring the final concentration to 10 mM.

Antibody Incorporation: Immunoliposomes were prepared by a modification of the methods developed by Shen et al. (7). Palmitoyl antibody, in PBS containing 0.15% deoxycholate at an antibody concentration of 1 mg/ml, was mixed with reverse-phase evaporation vesicles at a lipid-to-antibody weight ratio of 10. 5% (vol/vol) of ethyl ether was added to the vortexing antibody–liposome mixture; this final solution was dialyzed against three changes of 4 L PBS to remove ethyl ether, deoxycholate, and untrapped calcein. The resulting immunoliposomes were run on a 5-ml 5–20% linear sucrose gradient spun at 46,000 rpm for 5 h to evaluate the efficiency of incorporation. Sizing of immunoliposomes was done by measurements obtained from electron micrographs. The immunoliposomes were negatively stained with 0.5% aqueous uranyl acetate and viewed in a Hitachi 600 electron microscope (Hitachi Ltd., Tokyo) at 75 kV. Size histograms were produced from micrographs taken from various preparations of liposomes.

Cell Incubations: Mouse L929 cells (k haplotype) and A31 cells (d haplotype) were grown on glass coverslips which had been pretreated with a 1% solution of gelatin. The medium used for the incubation experiments consisted of PBS containing 1 mM Ca^{++}, 1 mM Mg^{++}, and 16 mM D-glucose. Immunoliposomes (DOPE/PHC or DOPC) at a lipid concentration of 50 μg/ml were incubated with both types of cell at 4°C for 1.5 h. The cells were washed three times with medium and incubated in fresh medium for 30 min more at 4°C to reduce the nonspecific binding.

After the second 4°C incubation the cell were again washed three times with buffer and then observed under a Leitz Orthoplan epiluminescence microscope equipped with an Orthomat-W camera. Both phase-contrast and fluorescent pictures were taken; all of the fluorescent pictures were taken with the same exposure time (1.5 min).

In parallel experiments cells that had been treated with liposomes at 4°C as above were then incubated at either 20 or 37°C for 2 h, washed, and then photographed under the fluorescence microscope. In another series of experiments the cells were incubated with 50 μM chloroquine before the 4°C binding step, and after all washing steps the fresh incubation buffer was also supplemented with 50 μM chloroquine. After incubation with immunoliposomes at 4°C these cells were then incubated at 37°C for 2 h in the presence of chloroquine, washed, and then photographed under the fluorescence microscope.

Quantitation of Cytoplasmic Fluorescence Intensity: Calcein in concentrations from 1 μM to 1 mM was entrapped in large (>20 μm) multilamellar liposomes composed of DOPC (8). A calibration curve was established using a Leitz Wetzlar MPV-2 Microscope Photometer, by measuring the fluorescence intensity from an area of 4 μm^2 of the flattened liposomes that contained varying concentrations of calcein. At least two different areas of 25 liposomes were measured for each calcein concentration. The results showed a linear calibration curve over the concentration range of 1 μM to 1 mM (data not shown). The fluorescence intensity from a spot of same surface area in the cytoplasm of the cells incubated at 37°C with pH-sensitive immunoliposomes was measured for two different areas of 15 cells. The cytoplasmic dye concentration was calculated from the calibration curve under the assumption that the thickness of the cytoplasm is about the same as that of the flattened liposomes. In reality the cytoplasm was probably thinner than the liposomes. Therefore, the estimated dye concentration in cytoplasm represents a lower limit of the actual value. Fluorescence intensity determination of untreated cells showed no measurable autofluorescence. The cytoplasmic dye concentrations of the control experiments were not measured, since the calcein fluorescence appeared in granules and was not evenly distributed in the cytoplasm. The fluorescence intensity of the punctate granules did not fall along the established calibration curve.

RESULTS

Immunoliposomes

The average size of the immunoliposomes composed of DOPE/PHC (8:2), as determined by negative stain electron microscopy, was 1,400 ± 400 Å. The results of the sucrose gradient centrifugation showed a co-migration of the liposomes and the monoclonal antibody, which appeared at the top of the gradient. Radioactive marker counting indicated an 80% incorporation of the derivatized antibody into the liposomes.

4°C Binding

Previous work of this laboratory (4) demonstrated that there was specific targeting of immunoliposome with the anti-H2K^k antibody to k-haplotype target cells whereas the d haplotype cells showed no specific binding. Fig. 1 shows the binding of calcein-entrapped DOPE/PHC (8:2) and DOPC immunoliposomes to L929 cells. The immunoliposomes bound specifically to the target cells that show an external ring of fluorescence. Incubation of immunoliposome with A-31 cells displayed no detectable fluorescence of the cells. Cells incubated with liposomes without antibody showed no binding of liposomes to either cell type as determined by no visible fluorescence (photographs not shown). Cells incubated with free calcein (0.3 mM) showed no uptake of dye.

20°C Incubation

Fig. 2, *A* and *B* are photographs of L929 cells treated with immunoliposomes at 4°C, washed, and then incubated at 20°C. Punctate or granular fluorescence appeared inside the cells. Little or no diffused fluorescence in the cytoplasm was observed. It is clear that the liposomes were internalized by the cell, but no cytoplasmic release of dye was observed. The internalized immunoliposomes and their contents remained in the endosome/lysosomes. No significant difference was found between the DOPE/PHC and DOPC immunoliposomes.

37°C Incubation

In these experiments both DOPE/PHC and DOPC immunoliposomes were bound to target cells at 4°C and then incubated at 37°C to allow endocytosis of the bound immunoliposomes. Figure 3*A* shows photographs of cells treated with the pH-sensitive DOPE/PHC liposomes. The diffused fluorescence observed in these cells clearly indicates a cytoplasmic delivery of calcein. Cells also showed a dark nucleus shadow, which indicates that calcein did not penetrate the nuclear membrane. The calcein released into the cytoplasm would still maintain its charged nature and should therefore be unable to permeate the nuclear membrane. The calcein may also associate with cytoplasmic macromolecules, which could block its entrance into the nucleus. Using the microscope photometer, we measured the relative fluorescent emission of the cytoplasmic calcein for a number of target cells. Based on the fluorescence intensity calibration curve the concentration of the calcein in the cellular cytoplasm was 50 ± 20 μM ($n = 30$). The average volume of the cell cytoplasm was ~10^3 μm^3, and that of the immunoliposomes was ~1×10^{-3} μm^3. The calcein concentration in the immunoliposomes was 60 mM, which is about 1,000-fold higher than the concentration found in the cytoplasm. This result indicates that

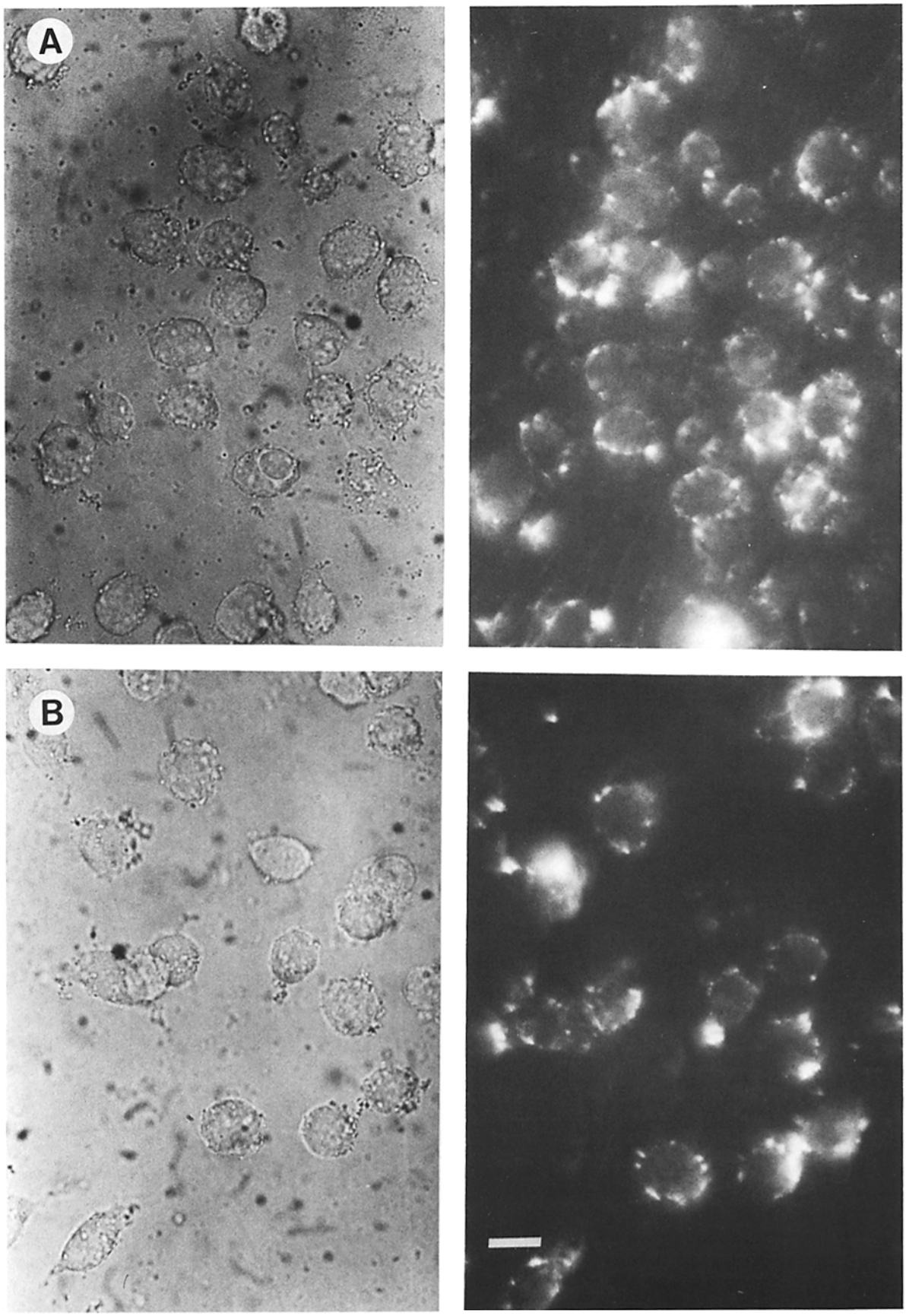

FIGURE 1 L929 cells incubated with calcein entrapped immunoliposomes at 4°C as described in Materials and Methods. *A*, DOPE/PHC (8:2) immunoliposomes; *B*, DOPC immunoliposomes. Bar, 10 μm. × 1,000.

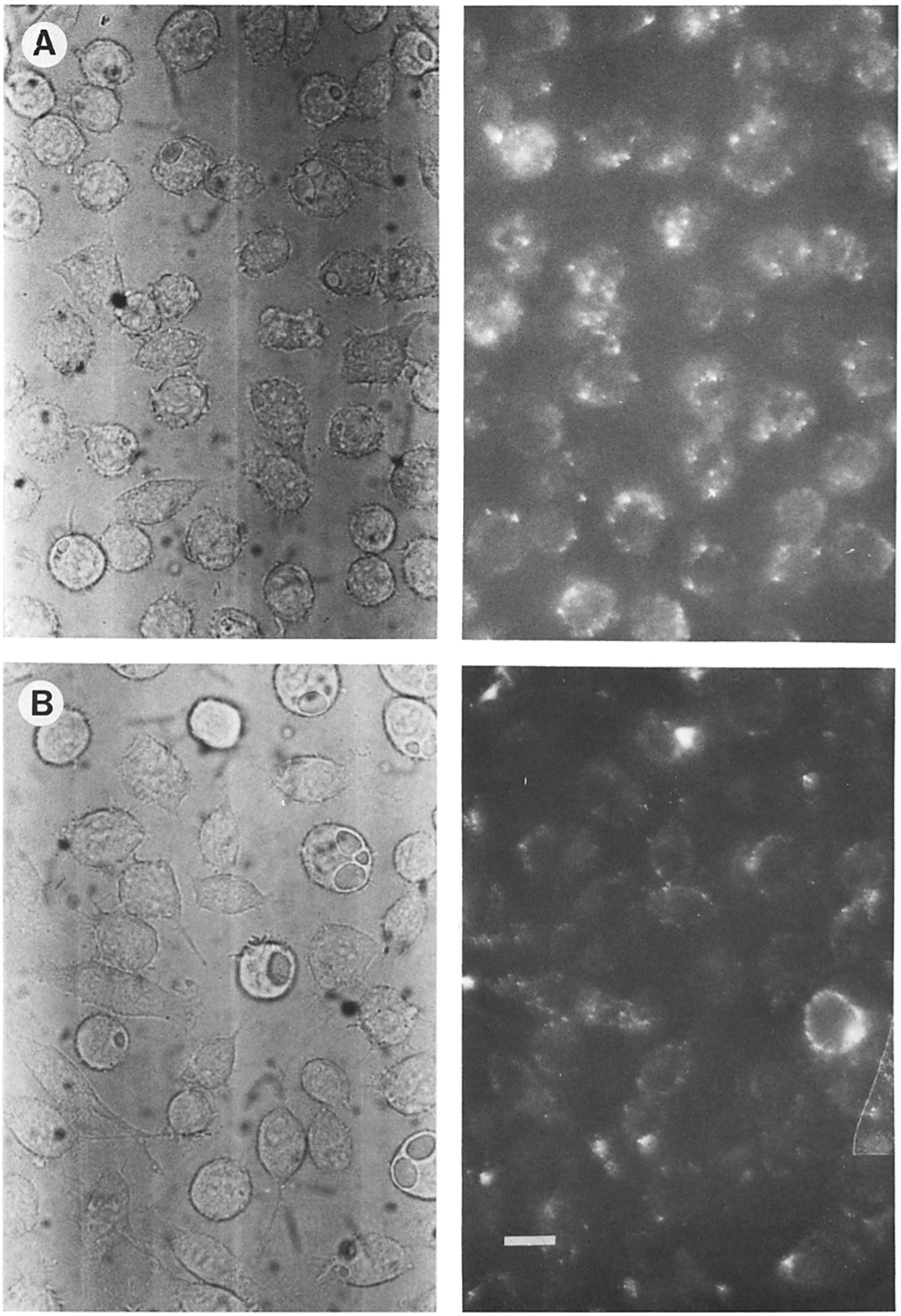

FIGURE 2 L929 cells incubated with immunoliposomes at 4°C for 1.5 h, washed, and then incubated at 20°C for 2.0 h as described in Materials and Methods. *A*, DOPE/PHC (8:2) immunoliposomes; *B*, DOPC immunoliposomes. Bar, 10 μm. × 1,000.

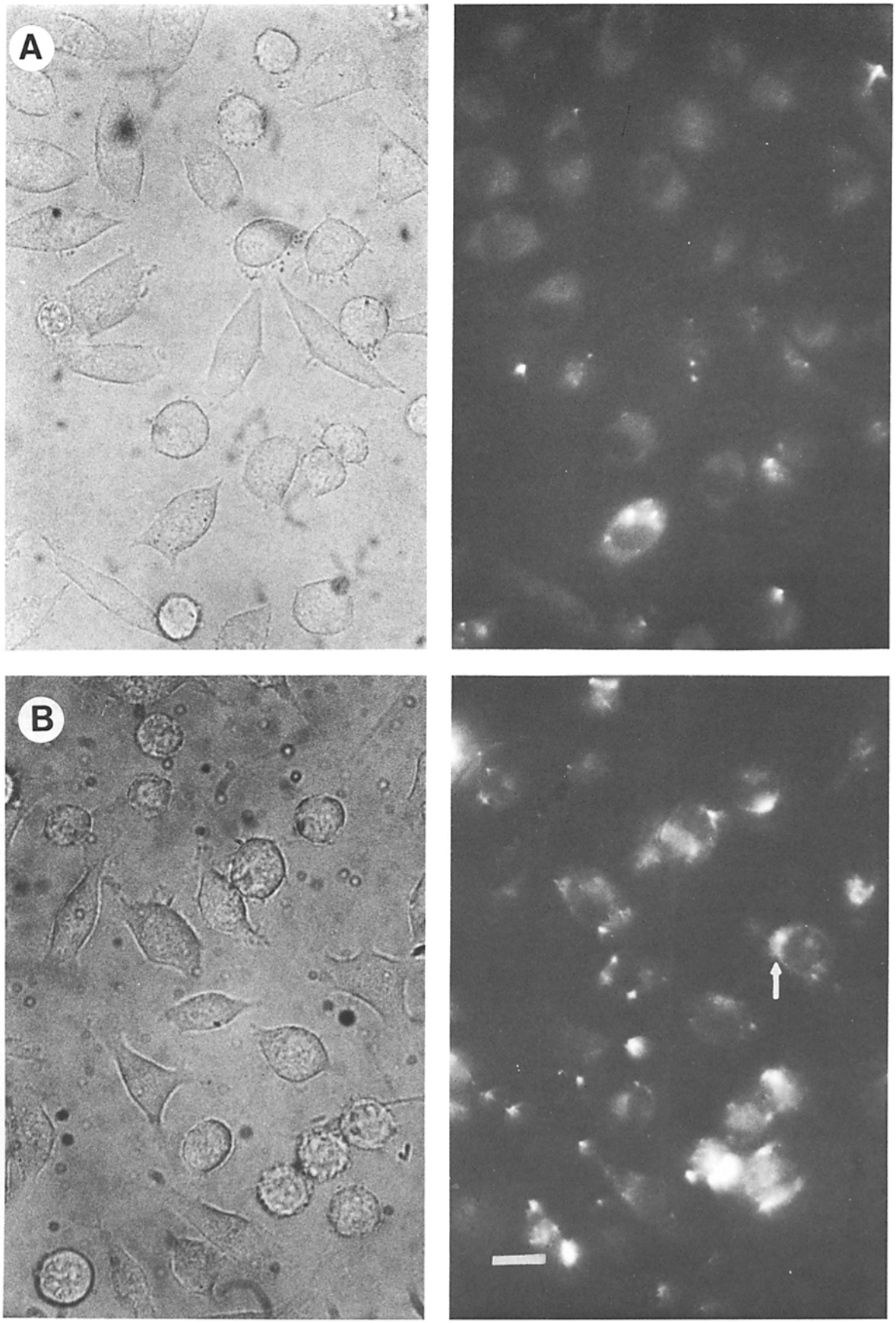

FIGURE 3 L929 cells incubated with immunoliposomes at 4°C for 1.5 h, washed, and then incubated at 37°C for 2.0 h as described in Materials and Methods. *A*, DOPE/PHC (8:2) immunoliposomes; *B*, DOPC immunoliposomes. Evidence of paranuclear fluorescence can be seen in B (↑). Bar, 10 μm. × 1,000.

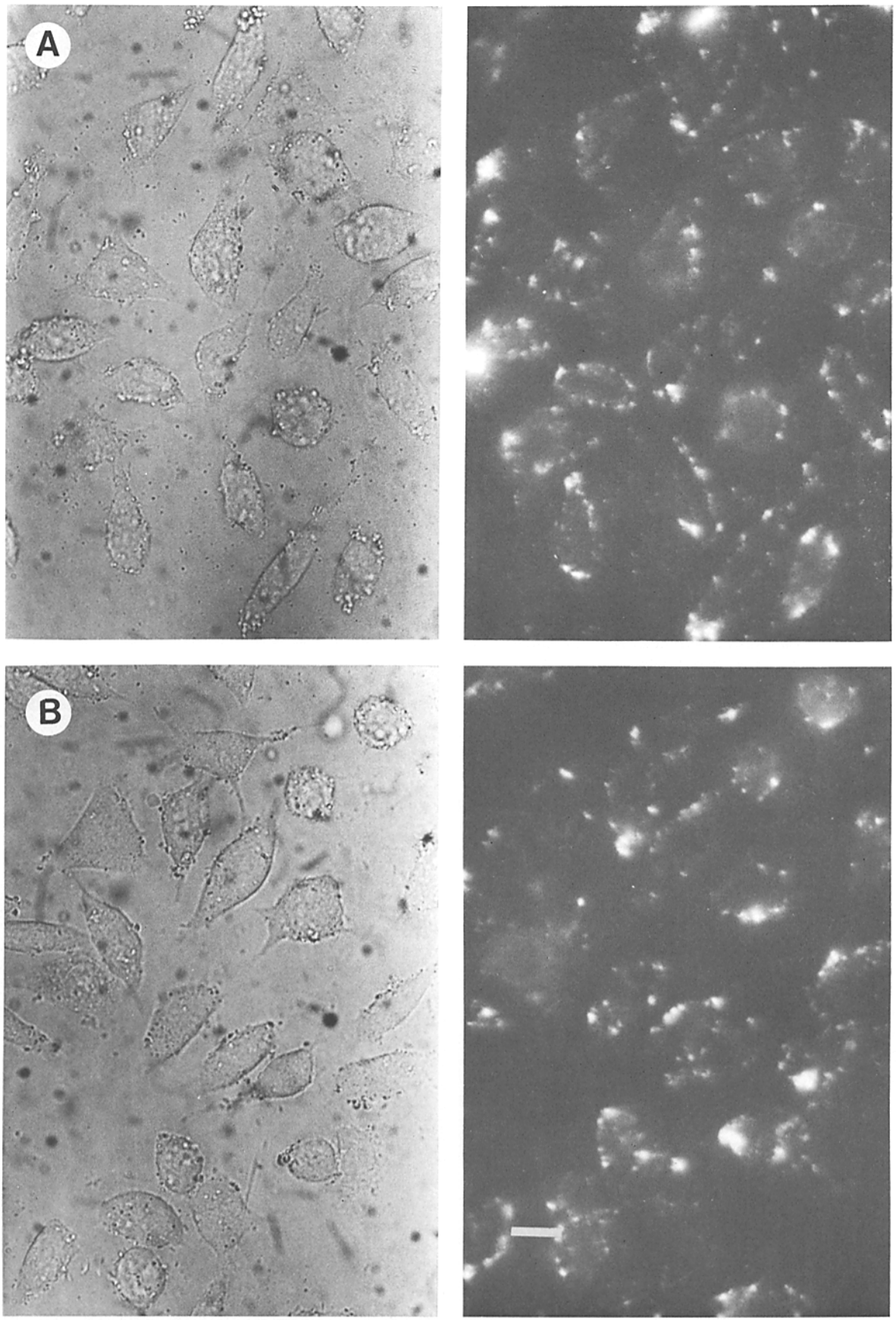

FIGURE 4 Effect of chloroquine on the calcein delivery by immunoliposomes. Immunoliposomes were incubated with L929 cells that had been constantly exposed to 50 μM chloroquine. Immunoliposomes were incubated at 4°C for 1.5 h, washed, and then incubated at 37°C for 2.0 h as described in Materials and Methods. *A*, DOPE/PHC (8:2) immunoliposomes; *B*, DOPC immunoliposomes. Bar, 10 μm. × 1,000.

about one thousand liposomes had released their entrapped calcein into the cytoplasm of a cell.

Cells treated with the pH-insensitive DOPC liposomes did not show any release of dye (Fig. 3*B*). Only punctate fluorescence was observed with these cells, indicating that the calcein was retained in the endosome/lyosome system. Cells treated with DOPC immunoliposomes did display capping of fluorescence, which has been observed in early work involving targeting of these liposomes (3). Paranuclear fluorescence was also seen in some of the cells treated with DOPC immunoliposomes, indicating delivery of the liposome and its contents to the lysosomes had occurred. Cells incubated with free calcein (0.3 mM) at 37°C showed some punctate but no diffused fluorescence, probably as a result of pinocytosis.

Incubation with Chloroquine

Chloroquine is a weak base that is readily taken up by cells, and is partitioned into acidic organelles and raises their pH (9–11). It has been demonstrated that chloroquine does not interfere with the receptor-mediated internalization of ligands (9, 11, 12). Because incubation of target cells with chloroquine blocks the acidification of the endosomes and lysosomes, upon endocytosis the immunoliposomes should encounter a less acidic pH.

Immunoliposomes were bound at 4°C to chloroquine-treated cells, and the incubation temperature was then raised to 37°C to activate endocytosis of the liposomes. Fig. 4, *A* and *B*, shows cells that received such treatment. Similarly to the 20°C incubation, the fluorescence appeared punctate both on the periphery of the plasma membrane and inside the cells, which indicates that there was little or no release of the liposome-entrapped calcein. This is expected if the calcein delivery is dependent upon an acid-induced fusion of the immunoliposomes with the endosome membrane. The loss of cytoplasmic delivery with the neutralization of the endosome clearly supports this hypothesis. The cells treated with DOPC immunoliposomes also displayed internal punctate fluorescence similar to that seen with the 20°C incubation.

DISCUSSION

We previously showed that liposomes composed of DOPE/PHC (8:2) become highly fusion active at acidic pH's (1). The pH for half-maximal fusion lies at ~6.4, whereas almost 100% fusion occurs at pH 4.8 or lower. Although the mechanism of liposome fusion is not known, it probably involves the formation of the hexagonal phase of DOPE when PHC is protonated at the low pH. Since the pH range at which the liposome becomes fusion competent falls in the same range as the endosome and lysosome pH, the liposome may become fusion active if they are delivered to these organelles. We previously showed that palmitoyl antibody can be incorporated into liposome membranes and the immunoliposomes are rapidly endocytosed by the target cells by a process that resembles the receptor-mediated endocytosis (4). If fusion of the liposome with the endosome membrane occurs, one would expect to see the release of the liposome content into the cytoplasm of cells. We have used a fluorescent dye, calcein, for this purpose. It is a water-soluble and self-quenching dye. It does not permeate cell membrane due to the high charge content (5).

Immunoliposomes were bound to target cells at 4°C, washed, and then incubated at 37°C so that normal endocytosis and cellular processing, including endosome acidification, would occur. Cells incubated with liposomes composed of DOPE/PHC, which become fusion competent at acidic pH's, display diffused fluorescence throughout the cytoplasm, indicating a release of the entrapped calcein (Fig. 3*A*). Since the calcein cannot penetrate through the endosome membrane the dye must have been released directly into the cytoplasm probably by a fusion reaction between the liposome and the endosome membranes. The inability of calcein to cross membranes can also be seen by the lack of fluorescence in the nucleus. The pH-insensitive DOPC liposomes showed little or no diffused fluorescence after the 37°C incubation. Some cells showed high degree of paranuclear fluorescence, indicating this type of liposomes are delivered to the lysosomes.

The cytoplasmic delivery of calcein by the pH-sensitive liposomes could be blocked by chloroquine (Fig. 4*A*). Since chloroquine effectively raises the pH of the endosomes/lysosomes (9–11), this result strongly indicates the importance of the acidic environment for calcein release. Incubation at 20°C also prevents the dye release by the pH-sensitive liposomes, but the liposomes appear to be intracellular (Fig. 2*A*). Studies with mutants of Semliki Forest Virus have shown that at 20°C cells actively endocytose but that the fusion of endosomes with lysosomes is blocked (13, 14); concurrent with this blocking effect is a large degree of heterogeneity of the endosome pH (15). The lack of dye release from the pH-sensitive liposomes may indicate that the liposomes are located in those endosomes whose pH's are not sufficiently acidic. Alternatively, the dye release may take place in the lysosomes, which would be blocked if the endosome–lysosome fusion did not occur at 20°C.

It is not clear from the present study if the liposomes actually fused with with endosome or lysosome membranes. We also do not know the fate of the liposomes after the dye release. In any case, the cells appeared to be morphologically normal and maintained normal doubling time after the treatment with the pH-sensitive immunoliposomes. If the liposomes fuse with the endosome or lysosome membrane as we suspect, the situation is very similar to the infection pathway of the enveloped virus such as the Semliki Forest, influenza, and vesicular stomatitis viruses. The viral membrane fuse with the endosome membrane in response to the acidic pH (references 16–20). The difference here is that the driving force for fusion in liposomes is a weakly acidic lipid such as PHC, whereas the driving force for the viruses is the viral glycoproteins.

The efficiency of this pH-sensitive immunoliposome delivery system is apparent from the high number of liposomes ($\sim 10^3$ liposomes/cell) that released their contents to the cytoplasm of the cell. This high level of delivery should prove very useful in future studies. For example, to know that immunoliposomes encapsulated with a monoclonal antibody at 10 mg/ml could deliver to each cell $\sim 10^4$ antibody molecules would obviously be very helpful in studying various cellular functions. There are a great many potential uses for this pH-sensitive immunoliposome system, depending only upon the ability to trap the desired molecule.

In conclusion, we have designed a liposome system that efficiently delivers the contents to the cytoplasm of the cells in a target-specific manner. Since water-soluble molecules can be easily encapsulated in liposomes, this system should be very useful for the cytoplasmic delivery of drugs, enzymes,

antibody, nucleic acids, and other biologically active molecules into the living cells.

An article appeared after this paper was submitted for publication which described cytoplasmic delivery of calcein and fluorescently labeled dextran by pH-sensitive liposomes free of antibody (21).

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REFERENCES

1. Connor, J., M. B. Yatvin, and L. Huang. 1984. pH-sensitive liposomes: acid-induced fusion. *Proc. Natl. Acad. Sci. USA.* 81:1715–1718.
2. Huang, A., Y. S. Tsao, S. J. Kennel, and L. Huang. 1982. Characterization of antibody covalently coupled to liposomes. *Biochim. Biophys. Acta.* 716:140–150.
3. Huang, A., L. Huang, and S. J. Kennel. 1980. Monoclonal antibody covalently coupled with fatty acid. *J. Biol. Chem.* 255:8015–8018.
4. Huang, A., S. J. Kennel, and L. Huang. 1983. Interaction of immunoliposomes with target cells. *J. Biol. Chem.* 258:14034–14040.
5. Straubinger, R. M., K. Hong, D. S. Friend, and D. Papahadjopoulos. 1983. Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles. *Cell.* 32:1069–1079.
6. Yatvin, M. B., W. Kreutz, B. A. Horweitz, and M. Shinitzky. 1980. pH-sensitive liposomes: possible clinical implications. *Science (Wash. DC).* 210:1253–1255.
7. Shen, D. F., A. Huang, and L. Huang. 1982. An improved method for covalent attachment of antibody to liposomes. *Biochim. Biophys. Acta.* 689:31–37.
8. Bangham, A. D., M. M. Standish, and J. C. Watkins. 1965. Diffusion of univalent ions across lamellae of swollen phospholipid. *J. Mol. Biol.* 13:130–136.
9. Galloway, C. J., G. E. Dean, M. Marsh, G. Rudnick, and I. Mellman. 1981. Acidification of macrophage and fibroblast endocytic vesicles in vitro. *Proc. Natl. Acad. Sci. USA.* 80:3334–3338.
10. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327–3331.
11. Helenius, A., M. Marsh, and J. White. 1982. Inhibition of Semliki Forest Virus penetration by lysosomotropic weak bases. *J. Gen. Virol.* 58:47–61.
12. Ho, S. C., and L. Huang. 1983. A novel cytochemical marker for liposome decomposition in lysosomes. *J. Histochem. Cytochem.* 31:404–410.
13. Marsh, M. 1984. The entry of enveloped viruses into cells by endocytosis. *Biochem. J.* 218:1–10.
14. Helenius, A., and M. Marsh. 1982. Endocytosis of enveloped animal viruses. *Ciba Found. Symp.* 92:59–76.
15. Geisow, M. J., and W. H. Evans. 1984. pH in endosomes. *Exp. Cell. Res.* 150:36–46.
16. Eidelman, O., R. Schlegel, T. S. Tralka, and R. J. Blumenthal, 1984. pH-dependent fusion induced by vesicular stromatitis virus glycoprotein reconstructed into phospholipid vesicles. *J. Biol. Chem.* 259:4622–4628.
17. White, J., and A. Helenius. 1980. pH-dependent fusion between the Semliki Forest Virus membrane and liposomes. *Proc. Natl. Acad. Sci. USA.* 77:3273–3277.
18. Maeda, T., K. Kawasaki, and S. I. Ohnishi. 1981. Interaction of influenza virus hemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2. *Proc. Natl. Acad. Sci. USA.* 78:4133–4137.
19. Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing $\alpha 2$ macroglobulin. *Cell.* 28:643–752.
20. March, M., K. Matlin, K. Simons, J. Reggio, J. White, J. Kartenbeck, and A. Helenius. 1982. Are lysosomes a site of enveloped virus penetration? *Cold Spring Harbor Symp. Quant. Biol.* 46:835–843.
21. Straubinger, R. M., N. Duzgunes, D. Papahadjopoulos. 1985. pH-sensitive liposomes mediate cytoplasmic delivery of encapsulated macromolecules. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 179:148–154.