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pH-sensitive immunoliposomes mediate target-cell-specific delivery and controlled expression of a foreign gene in mouse

(gene therapy/nude mouse/liposome composition/phosphatidylethanolamine)

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ABSTRACT **A plasmid containing the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene under the control of a mammalian cAMP-regulated promoter was entrapped in H-2K^k antibody-coated liposomes composed of dioleoyl phosphatidylethanolamine, cholesterol, and oleic acid (pH-sensitive immunoliposomes). The entrapped or free DNA was injected intraperitoneally into immunodeficient (nude) BALB/c mice bearing ascites tumor generated by H-2K^k-positive RDM-4 lymphoma cells. About 20% of the injected immunoliposomes were taken up by the target RDM-4 cells. Uptake was much less when liposomes without antibody were used. The presence of the targeting antibody on liposomes also significantly decreased the nonspecific uptake of liposomes by the spleen. Significant CAT enzyme activity was detected in RDM-4 cells from mice treated with DNA entrapped in the pH-sensitive immunoliposomes. Furthermore, CAT expression in RDM-4 cells was under the control of cAMP, as only the cells from mice injected with 8-bromo-cAMP and 3-isobutyl-1-methylxanthine showed CAT activity. CAT activity in liver and spleen was much lower (by factors of 12 and 5, respectively) than in the RDM-4 cells, and the activities in these reticuloendothelial organs were not regulated by cAMP. CAT activity in RDM-4 cells from mice injected with DNA entrapped in pH-insensitive immunoliposomes (containing phosphatidylcholine in place of phosphatidylethanolamine) was approximately one-fourth that in RDM-4 cells from mice injected with pH-sensitive immunoliposomes, indicating the superior delivery efficiency of the pH-sensitive liposomes. These results are discussed in terms of the DNA-carrier potential of immunoliposomes in therapy of cancer and genetic diseases.**

Correction of genetic disorders by gene therapy is one of the developing areas in medicine (1). The exogenous normal gene that is introduced may replace or coexist with the defective gene and produce normal gene product. Successful therapy of a genetic disorder requires knowledge of the structure, function, and regulation of the gene to be introduced into the deficient cell, as well as an efficient and specific means of delivering the gene to the target cell. Despite its great potential in medicine and biotechnology, gene therapy has not yet been used widely, mainly due to the poor efficiency of DNA delivery. Current methods of delivery of new genetic information into cells *in vitro* (for review, see ref. 1) include cell fusion, chromosome-mediated insertion, microcell-mediated gene transfer, liposome DNA carriers, spheroplast fusion, DNA-mediated gene transfer, microinjection, infection with recombinant RNA viruses, and infection with recombinant DNA viruses. However, most of these techniques are not applicable for use in animals or humans because of low efficiency, instability of introduced genes, introduction of extraneous or undesirable genetic information, and lack of target specificity.

To improve the efficiency of delivery of biologically functional molecules, pH-sensitive liposomes have been developed in several laboratories including ours (2, 3). These liposomes release their contents into the cytoplasm of target cells after they fuse with the endosomal membrane (4). In previous studies, we used a water-soluble fluorescent dye, calcein, as a convenient marker for observing cytoplasmic delivery (5) and used the herpes simplex virus thymidine kinase gene as a selectable marker for gene transfer (6). The results indicated that the liposome contents were released into the cytoplasm and that the transferred DNA can be expressed with high efficiency in target cells.

To increase specific binding of liposomes to target cells, acylated monoclonal antibodies were incorporated into the lipid bilayer of the liposome (7). The present study is designed to test the DNA-delivery potential of the pH-sensitive immunoliposomes in an animal model. We used RDM-4 lymphoma cells (provided by M. F. Mescher, Division of Membrane Biology, Medical Biology Institute, La Jolla, CA) as the target in this model system. These cells, which express the mouse major histocompatibility antigen H2-K^k, were grown as ascites tumor in the immunodeficient nude mouse of the BALB/c background (which expresses no H2-K^k antigen). The *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene was used as a convenient marker for observing gene transfer. This gene was placed under the control of a promoter that contained the cAMP regulatory sequence to test whether the expression of the foreign gene in the target cells could be regulated by an external signal such as cAMP.

MATERIALS AND METHODS

Materials. Dioleoyl phosphatidylethanolamine ([Ole$_2$]PtdEtn) and dioleoyl phosphatidylcholine ([Ole$_2$]PtdCho) were purchased from Avanti Polar Lipids. Oleic acid, cholesterol, acetyl coenzyme A, *n*-octyl glucoside, 8-bromo-cAMP (8-Br-cAMP), and 3-isobutyl-1-methylxanthine (iBuMeXan) were obtained from Sigma. SM-2 beads were purchased from Bio-Rad. [*dichloroacetyl*-1,2-^{14}C]Chloramphenicol was purchased from New England Nuclear. Anti-H2-K^k antibody (mouse IgG2a) was isolated from ascites fluid generated by hybridoma 11-4.1 (7) and was purified by protein A-Sepharose affinity chromatography (7). It was radioiodinated with ^{125}I and acylated with *N*-hydroxysuccinimide ester of pal-

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Abbreviations: CAT, chloramphenicol acetyltransferase; [^{3}H]CE, hexadecyl [^{3}H]cholestanyl ether; [Ole$_2$]PtdEtn, dioleoyl phosphatidylethanolamine; [Ole$_2$]PtdCho, dioleoyl phosphatidylcholine; iBuMeXan, 3-isobutyl-1-methylxanthine.

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mitic acid as described (7). BALB/c nude mice were purchased from Life Sciences (St. Petersburg, FL).

Plasmid. Plasmid pBBO.6-CAT (4.6 kilobase pairs) was a gift of W. D. Wicks (Department of Biochemistry, University of Tennessee, Knoxville). It was constructed from the plasmid pAZ1009 (8) by replacing the promoter region with a 621-base-pair upstream sequence of the rat phospho*enol*pyruvate carboxykinase gene, which contains a cAMP regulatory sequence (9). Details of the plasmid construction will be published elsewhere. Plasmid DNA was prepared and purified by standard techniques (10).

Liposome Preparation. Methods used for the preparation of large unilamellar immunoliposomes were based on the procedure of Philippot *et al.* (11), with modifications. Lipid films of various compositions (total lipid 10 μmol) were formed under a nitrogen stream and suspended in 10 mM Hepes/1 mM EGTA/150 mM NaCl, pH 8. Hexadecyl [^{3}H]cholestanyl ether ([^{3}H]CE) was included in the lipid mixture to monitor the lipid (12). The lipid suspension was sonicated with a bath sonicator (Laboratory Supplies, Hicksville, NY) and the pH was adjusted to 8. Palmitoylated anti-H2-K^k (1/25th of total lipid by weight), octyl glucoside (100 μmol), and DNA (150 μg) were added, and the mixture (final volume, 0.34 ml) was dialyzed at 4°C against 100 ml of 10 mM Tris·HCl/1 mM EDTA/150 mM NaCl (pH 8) and 1 g of washed SM-2 beads (13) overnight without stirring the dialysis buffer. Stirring was started the next morning. After 24 hr in dialysis, the buffer and beads were replaced with fresh ones and the dialysis was continued for an additional 24 hr. The liposomes were extruded through a polycarbonate filter of 0.2-μm pore diameter (Nuclepore) to obtain liposomes of uniform size distribution. Subsequently, the liposomes were separated from free DNA by gel filtration using a column of autoclaved Sepharose CL-2B (Pharmacia).

Inoculation of Mice. RDM-4 lymphoma cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.01% sodium pyruvate. RDM-4 cells (10^7) in 0.5 ml of phosphate-buffered isotonic saline (pH 7.4) were inoculated intraperitoneally (i.p.) in 6-week-old nude mice. After 7 days, the mice were injected i.p. with liposome-entrapped or free DNA at a dose of 20 μg of DNA (≈8.9 μmol of lipid) per mouse. Twenty-four hours later, 8-Br-cAMP (1 mg/100 g of body weight) and iBuMeXan (0.5 mg/100 g) were injected i.p. After 5 hr, ascites fluid was harvested from the peritoneal cavity by five 2-ml injections of phosphate-buffered saline. The ascites fluid, which contained RDM-4 cells and macrophages, was incubated in a glass Petri dish for 6 hr. The nonadherent RDM-4 cells were harvested from the supernatant, and adherent macrophages were harvested from the glass with a cell scraper. After centrifugation, cell pellets were resuspended in 0.25 mM Tris·HCl (pH 7.4) for CAT activity assay. The blood, hearts, spleens, lungs, stomachs, kidneys, and livers of the mice were also collected and homogenized. An aliquot of each homogenized organ was dissolved in Protosol (New England Nuclear) for measurement of ^{3}H by liquid scintillation counting. After centrifugation, the organ extracts were assayed for CAT activity. Two mice per group were used. Variations of data between experiments were less than 15%.

CAT Assay. CAT activity was determined by a modification of the method of Gorman *et al.* (14). Ascites cells were suspended in 125 μl of 0.25 mM Tris·HCl (pH 7.4) and frozen at −20°C until assayed. The cells were thawed, homogenized by sonication, and centrifuged. Protein concentration in the supernatant was measured by the method of Lowry *et al.* (15). [*dichloroacetyl*-1,2-^{14}C]Chloramphenicol (0.5 μCi; 1 μCi = 37 kBq) and 20 μl of 4 mM acetyl coenzyme A were added per 125 μl of cell or organ extract (3 mg of protein). The reaction was allowed to proceed at 37°C for 90 min. Chloramphenicol and its acetylated derivatives were extracted with ethyl acetate, separated by silica gel thin-layer chromatography, and visualized by autoradiography. Regions of the chromatogram containing the acetylated or nonacetylated chloramphenicol were then scraped from the plates and ^{14}C cpm were determined by liquid scintillation counting.

RESULTS

Characteristics of Liposomes. Two different lipid compositions were used for liposome preparation. Liposomes composed of [Ole$_2$]PtdEtn, cholesterol, and oleic acid (4:4:2 molar ratio) are pH-sensitive and become destabilized at pH below 6.0 (5). These liposomes were unilamellar and 0.29 ± 0.08 μm in diameter as determined by negative-stain electron microscopy. They entrapped 16.5% of the input DNA and contained ≈106 antibody molecules per liposome. Liposomes composed of [Ole$_2$]PtdCho, cholesterol, and oleic acid (4:4:2 molar ratio) are pH-insensitive (5). They were also unilamellar, were 0.18 ± 0.06 μm in diameter, contained ≈48 antibody molecules per liposome, and entrapped 14.4% of the input DNA. These two types of liposome, both filter-extruded, although different in size and pH-sensitivity, had the same antibody/lipid ratio.

Distribution of Liposomes in BALB/c Nude Mice. The distribution of liposomes was monitored by the radioactivity of [^{3}H]CE in several organs. This radiolabeled lipid marker has been shown to be a faithful marker for liposomes, and it is neither exchangeable with cellular lipids nor metabolizable by cells (12). Table 1 shows the distribution of liposomes in the mice. The recovery of [^{3}H]CE in these organs and cells was in the range of 60–70% of input ^{3}H cpm. Five different treatments were performed: (*i*) mice bearing RDM-4 cells were injected with pH-insensitive immunoliposomes that had been extruded through a 0.2-μm filter, (*ii*) mice bearing RDM-4 cells were injected with unextruded pH-sensitive immunoliposomes, (*iii*) mice bearing RDM-4 cells were injected with extruded pH-sensitive immunoliposomes, (*iv*) mice not bearing RDM-4 cells were injected with extruded pH-sensitive immunoliposomes, and (*v*) mice bearing RDM-4 cells were injected with antibody-free, pH-sensitive liposomes.

Distributions of radioactivity in spleens were markedly different in different groups. The non-tumor-bearing mice injected with immunoliposomes and the mice bearing RDM-4 cells but injected with antibody-free liposomes showed a very high degree of liposome accumulation in the spleen. In mice bearing RDM-4 cells and injected with immunoliposomes, the spleen accumulation was decreased by a factor of 3–4, no matter if the immunoliposomes were pH-sensitive or not, or whether they were extruded or not. Concomitantly, there was a 3- to 5-fold increase of liposome accumulation in the heart. Liposome uptake in nonadherent cells, the majority of which were RDM-4 cells, increased about 2-fold (≈20% of injected dose) when compared with uptake by nonadherent cells from mice treated with antibody-free, pH-sensitive liposomes. There was no significant change of ^{3}H distribution in livers, lungs, stomachs, kidneys, blood, and adherent cells of the ascites fluid.

Expression of CAT Gene in Ascites Cells. CAT gene expression was monitored by CAT enzyme activity, which is not found in eukaryotic cells. CAT activity was found in the RDM-4 cells (nonadherent cells in ascites fluid) of the mice injected with pH-sensitive immunoliposomes; however, the expression of the activity was dependent on cAMP stimulation (8-Br-cAMP plus iBuMeXan) and on the presence of antibody in the liposomes (Fig. 1). CAT activity was not found in extracts of the nonadherent cells without stimulation by 8-Br-cAMP plus iBuMeXan. CAT activity (443 pmol per mg per hr) in the nonadherent cells of the mice injected with pH-sensitive immunoliposomes was much higher than that

Table 1. Distribution of liposomes in BALB/c nude mice

Compartment	Distribution, %*				
	PIIL, ext.	PSIL, unext.	PSIL, ext.	PSIL, ext. (−tumor)†	PSL, ext.
Organs					
Liver	3.6 ± 0.0 (4.5 ± 0.0)	2.5 ± 0.3 (2.5 ± 0.5)	1.6 ± 0.7 (2.1 ± 0.9)	4.5 ± 1.4 (4.5 ± 0.4)	2.2 ± 0.9 (1.5 ± 0.3)
Heart	33.0 ± 1.9 (3.1 ± 0.3)	40.0 ± 7.9 (4.1 ± 0.2)	30.7 ± 12.2 (3.3 ± 1.3)	7.4 ± 2.7 (0.8 ± 0.1)	11.5 ± 3.2 (1.5 ± 0.7)
Spleen	40.0 ± 5.9 (8.1 ± 0.2)	43.8 ± 7.6 (4.0 ± 0.2)	30.5 ± 4.2 (5.1 ± 1.5)	145.0 ± 27.4 (22.2 ± 3.8)	144.4 ± 24.8 (29.5 ± 4.5)
Lung	13.5 ± 0.9 (1.5 ± 0.3)	8.8 ± 1.1 (1.0 ± 0.1)	13.0 ± 2.8 (2.0 ± 0.8)	6.5 ± 1.3 (0.5 ± 0.2)	6.2 ± 1.7 (1.1 ± 0.6)
Stomach	13.0 ± 3.1 (11.0 ± 4.4)	17.8 ± 2.3 (13.4 ± 6.8)	24.4 ± 3.3 (14.8 ± 6.5)	20.7 ± 1.3 (6.4 ± 1.9)	12.1 ± 4.2 (9.8 ± 6.6)
Kidney	3.9 ± 1.1 (1.3 ± 0.0)	9.6 ± 1.3 (2.5 ± 0.7)	7.0 ± 0.7 (2.4 ± 0.4)	2.1 ± 1.1 (1.1 ± 0.5)	3.0 ± 0.7 (1.5 ± 0.9)
Blood	1.0 ± 0.7 (1.2 ± 0.3)	0.7 ± 0.3 (0.1 ± 0.0)	0.6 ± 0.1 (0.1 ± 0.0)	0.6 ± 0.2 (0.1 ± 0.0)	0.4 ± 0.0 (0.2 ± 0.1)
Ascites cells					
Adherent	45.0 ± 3.9 (13.3 ± 0.6)	35.7 ± 11.4 (9.8 ± 4.2)	33.8 ± 7.5 (9.6 ± 1.9)	45.0 ± 3.9 (14.4 ± 0.9)	35.5 ± 4.5 (10.5 ± 0.2)
Nonadherent	49.5 ± 11.0 (16.6 ± 2.3)	37.1 ± 12.3 (18.9 ± 2.5)	57.3 ± 14.1 (20.5 ± 3.1)	—	24.5 ± 2.9 (10.1 ± 0.8)

PIIL, pH-insensitive immunoliposomes; PSIL, pH-sensitive immunoliposomes; PSL, pH-sensitive liposomes (no antibody); ext., extruded through 0.2-μm filter; unext., unextruded.
*Data (mean ± variation of two mice) are expressed as % of liposomes recovered per g of organ weight, per ml of blood, or per mg of ascites-cell protein; numbers in parentheses are the % of total injected liposomes.
†PSIL were injected into mice not carrying RDM-4 lymphoma cells.

(73 pmol per mg per hr) in the nonadherent cells of the mice injected with antibody-free liposomes (Table 2). Mice injected with free DNA showed no CAT activity in the cells. In this experiment, there was no detectable CAT activity in the adherent cells (containing macrophages) no matter what type of liposome was used. RDM-4 cells grown in tissue culture and not treated with liposomes also showed no activity.

The expression of CAT activity in the ascites cells seemed dependent on the type of liposomes used. Table 3 shows the data of an experiment in which three different kinds of liposomes were used. It is clear that filter-extruded pH-sensitive immunoliposomes were about 4 times more effective than filter-extruded pH-insensitive immunoliposomes for gene expression in the nonadherent cells (RDM-4 cells).

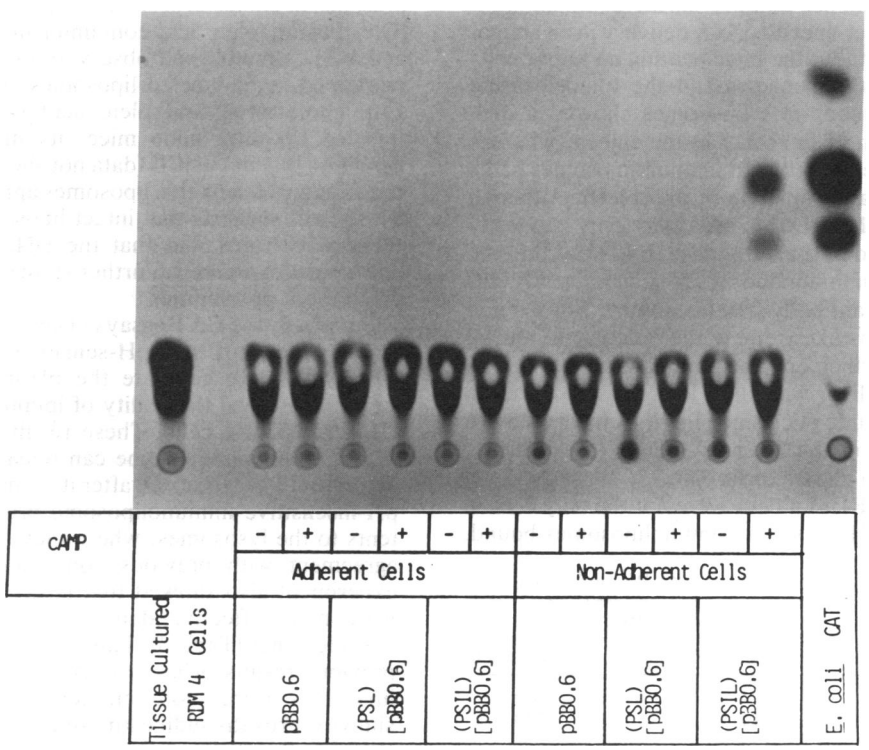

FIG. 1. CAT activity in ascites cells. High-mobility spots are acetylated [^{14}C]chloramphenicols. Low-mobility spot is the unmodified [^{14}C]chloramphenicol. [pBBO.6] stands for entrapped DNA, and pBBO.6 stands for naked DNA. PSL represents pH-sensitive liposomes and PSIL represents pH-sensitive immunoliposomes. Both liposomes and immunoliposomes had been extruded through 0.2-μm filters.

Table 2. CAT activity in ascites cells and organs of nude mice

pBBO.6-CAT DNA	"cAMP" injection†	CAT activity*							
		Nonadherent cells	Adherent cells	Kidney	Liver	Heart	Spleen	Lung	Stomach
In PSIL	+	443	0	0	35	0	80	0	216
	−	0	0	4	73	0	86	0	134
In PSL	+	73	0	9	0	0	0	0	0
	−	0	0	0	0	0	19	0	120
Free	+	0	0	0	0	0	0	0	90
	−	0	0	0	0	0	0	0	60

PSIL, pH-sensitive immunoliposomes; PSL, pH-sensitive liposomes. Both PSIL and PSL had been extruded through 0.2-μm filters.
*Expressed as pmol of acetyl groups transferred per mg of protein per hr.
†Injection with 8-Br-cAMP and iBuMeXan.

Immunoliposomes that had not been filter-extruded, and thus were larger in size, seemed more efficient than those that had been extruded. In all cases, gene expression in the adherent cells (macrophages) was fairly low.

CAT Activity in the Organs of Nude Mice. To examine whether the foreign gene was expressed in the organs of nude mice, CAT activity in the organ extracts was also assayed. Extracts from kidney, heart, and lung showed no detectable CAT activity with any type of liposome treatment (Table 2). However, spleens and livers of the tumor-bearing mice treated with pH-sensitive immunoliposomes, but not with pH-insensitive immunoliposomes or free DNA, showed low levels of CAT activity. Extracts from stomachs of the treated mice showed variable amounts of CAT activity. A separate examination of untreated normal nude mouse showed similar CAT activity in the stomach. Since the food residues in the stomach were not removed before the extract was prepared, the CAT activity in the stomach extract was probably due to the microorganisms in the food residues.

DISCUSSION

This paper provides evidence that pH-sensitive immunoliposomes can mediate target-specific DNA delivery in an animal model. In the current study, the mice bearing no tumor cells but treated with immunoliposomes and the tumor-bearing mice treated with antibody-free liposomes showed a high degree of accumulation of liposome in the spleen, whereas the tumor-bearing mice treated with immunoliposomes had a much lower liposome accumulation in the spleen. Although the accumulation in the RDM-4 cells was only modestly (about 2-fold) increased in the latter case, it is clear that the biodistribution of the immunoliposomes is significantly different from that of the antibody-free liposomes. Nonspecific uptake by spleen cells is likely due to the reticuloendothelial cells (macrophages), which are also abundant in liver. However, accumulation in liver was fairly low. This observation may be unique to the nude mice since i.p. injected liposomes normally accumulate in the liver of mouse (16). Further studies are required to clarify this point.

Although the target RDM-4 cells were not the only cells in the mouse to which pH-sensitive immunoliposomes bound, the RDM-4 cells were the only ones that expressed significant CAT activity in response to 8-Br-cAMP plus iBuMeXan. The adherent cells (macrophages) isolated from the same peritoneal cavity did not express significant amounts of enzyme activity despite the fact that they accumulated some liposomes. Furthermore, the spleen cells in the non-tumor-bearing mice, which took up a large amount of the pH-sensitive immunoliposomes, showed only a very low level of gene expression. There are several possible explanations for this observation. It is possible that the liposomes were unstable in the mouse peritoneal cavity, resulting in separation of DNA from liposomes. The liposome remnants might have then entered the circulation via the thoracic duct and been taken up by the macrophages in the spleen. Other possibilities are that DNA was degraded in the macrophages or that the intact DNA was delivered but could not be efficiently expressed in macrophages because of the lack of cAMP regulatory factor(s). Wynshaw-Boris *et al.* (9) reported that such factor(s) may be tissue-specific. When liposomes containing [^{125}I]iodotyraminyl-inulin were intravenously injected into mice, the [Ole_2]PtdEtn/oleic acid liposomes were very leaky in the presence of plasma (17). However, the presence of cholesterol significantly increased the stability of [Ole_2]PtdEtn/oleic acid-containing liposomes (B. E. Tsusaki and L.H., unpublished observation). When [^{14}C]inulin was entrapped in ^{3}H-labeled liposomes composed of [Ole_2]PtdEtn, cholesterol, and oleic acid (4:4:2 molar ratio) and injected i.p. into nude mice, its distribution profile was similar to that of [^{3}H]CE (data not shown). Thus, cholesterol-containing pH-sensitive liposomes appear to be stable *in vivo*. This result suggests that intact liposomes were delivered to the macrophages, but that the DNA was either degraded and/or not expressed. Further studies are required to distinguish these possibilities.

Data from the CAT assays (Table 3) indicated that replacing the [Ole_2]PtdEtn of pH-sensitive immunoliposomes with [Ole_2]PtdCho to generate the pH-insensitive immunoliposomes decreased the ability of immunoliposomes to deliver DNA to RDM-4 cells. These results indicate that the pH-sensitive immunoliposome can release its contents into the cytoplasm of a target cell after it is endocytosed, whereas the pH-insensitive immunoliposome primarily delivers its contents to the lysosomes, where they are degraded. This is in agreement with previous conclusions (5, 18). The same experiment also showed that larger, unextruded liposomes were more effective delivery vehicles than the smaller, extruded ones (Table 3). This result is not consistent with our previous results of *in vitro* experiments in which extruded liposomes were more efficient in delivering the herpes simplex virus thymidine kinase gene to mouse Ltk^{-} cells (6). Whether this discrepancy is due to a difference in cell types or growth conditions (*in vitro* vs. *in vivo*) is not known.

Nicolau *et al.* (19) reported gene expression in the livers of rats that received intravenous injection of the preproinsulin

Table 3. CAT activity in ascites cells of BALB/c nude mice

Liposome	CAT activity	
	Nonadherent cells	Adherent cells
PSIL, extruded	502	88
PSIL, unextruded	3119	114
PIIL, extruded	122	220

All mice were injected with 8-Br-cAMP plus iBuMeXan. PSIL, pH-sensitive immunoliposomes; PIIL, pH-insensitive immunoliposomes; extruded, liposomes were extruded through a 0.2-μm filter.

I gene encapsulated in antibody-free, conventional, pH-insensitive liposomes. This work clearly demonstrated the gene-delivery potential of liposomes *in vivo*. Nicolau's group (20) also showed that liposomes bearing galactosyl groups were targeted to hepatocytes. However, our work differs from their work in several important ways. Our previous work (6) and the work described here indicate that pH-sensitive immunoliposomes give more efficient cytoplasmic delivery than pH-insensitive immunoliposomes. The current study utilized an antibody instead of galactosyl groups to direct the liposomes to the target cells. Although the uptake of liposomes by reticuloendothelial cell-rich tissues such as spleen could not be avoided, our immunoliposome system exhibited target specificity in gene delivery.

The implications of our results for cancer therapy cannot be overlooked. DNA coding for cytotoxins such as diphtheria toxin (21) could be delivered to the target tumor cells. This type of chemotherapy is potentially superior to the immunoliposome-mediated delivery of conventional cytotoxic drugs such as methotrexate in several ways. First, the action of a gene should be much more potent than the action of a drug, due to a large degree of amplification through transcription and translation. Second, if DNA leaks out of the liposomes it should not be toxic because of rapid digestion by extracellular nuclease, whereas free cytotoxic drugs are toxic to normal tissues. Third, our results indicate that although the host organs took up liposomes, they did not significantly express the exogenous gene. This added level of specificity in cancer therapy may be very important, because by selecting appropriate control mechanisms, the delivered toxic gene may be expressed only in the tumor cells.

Our work also has implications for gene therapy. Two potential advantages of the antibody-coated liposomes in delivering DNA are their high transfer efficiency and safety. Successful human gene therapy will require efficient gene transfer and proper expression of the delivered gene in appropriate target cells (22). Retroviral vectors have been used as successful gene carriers (for review, see ref. 23). In many cases, the transferred genes were expressed in several hematopoietic cell types *in vitro* and in murine bone marrow stem cells. Although the retrovirus system has a much higher transformation efficiency than the conventional liposomes do, the viral oncogene and the random insertion of the retroviral genome into the host genome are undesirable side effects. The pH-sensitive immunoliposomes may be a good compromise in this regard. Furthermore, the fact that expression of the liposome-delivered gene can be properly controlled by an external signal, such as cAMP in the present study, is also an important advantage in human gene therapy.

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