Vesicle Targeting: Timed Release and Specificity for Leukocytes in Mice by Subcutaneous Injection

Abstract. *When unilamellar vesicles were administered subcutaneously in mice, the half-time for the destruction of the vesicles varied from 12 to 600 hours, depending on their composition. The vesicles tested consisted of distearoyl phosphatidylcholine, cholesterol, and certain sugar and amino-sugar derivatives of cholesterol. Vesicles with amino-sugar derivatives showed the greatest longevity and became localized with high specificity in aggregates of polymorphonuclear leukocytes. A substantial delay between the time that the vesicles broke open and the time that labels contained in the vesicles were excreted suggests that the vesicles undergo endocytosis before destruction.*

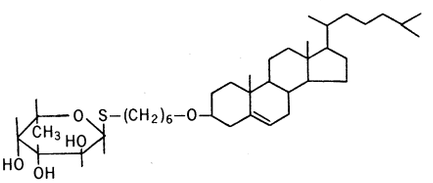
Liposomes are potential vehicles for transporting therapeutic and diagnostic agents from the site of administration to specific targets within the body (*1*). To be effective, the liposomes must not only reach the appropriate tissue but must also release their contents at the desired time. Since it had been shown previously that subcutaneously administered liposomes remain largely near the site of injection (*2, 3*), we tested the effects of particular surface modifications on lipid vesicle stability after we injected them subcutaneously. We assessed the structural integrity of lipid vesicles in vivo by perturbed angular correlation spectroscopy (PAC), utilizing $^{111}In^{3+}$ as a γ-ray probe (*3, 4*). The data we obtained may prove useful in developing liposome systems capable of providing controlled release of therapeutic agents.

Unilamellar vesicles of distearoyl phosphatidylcholine (DSPC) and cholesterol (Chol) (molar ratio, 2:1), which entrap the weak chelator nitrilotriacetic acid (NTA), were prepared by probe sonication (*3*). The $^{111}In^{3+}$ was loaded into the vesicles by using the ionophore A23187 as described previously (*5*). In some preparations the composition was altered by replacing up to one half of the molar amount of cholesterol with various cholesterol derivatives or hydrocarbon compounds with charged hydrophilic head groups as shown in Table 1.

When vesicles are disrupted in vivo, ^{111}In-NTA complex is released, and $^{111}In^{3+}$ is available to bind to nearby macromolecules (*3, 4*). Binding of $^{111}In^{3+}$ to a macromolecule causes a large change in the effective molecular rotational correlation time at the site of the indium nucleus, and consequently there is a decrease in the time-integrated angular correlation factor, $\langle G_{22}(\infty)\rangle$ (*6*). The $\langle G_{22}(\infty)\rangle$ value can thus be used to monitor the extent of breakup of vesicles; its value ranges from ~ 0.62 for ^{111}In-NTA contained in intact vesicles to 0.18 for the ion bound to serum proteins. The stability and permeability of the vesicles containing ^{111}In-NTA complex were also checked by the PAC technique (*3*). For all systems in Table 1 (*7*), the $\langle G_{22}(\infty)\rangle$ values remained high after incubation at 37°C for 0.5 hour in the presence of heat-inactivated calf serum. On disruption of the vesicles by a small addition of isopropanol, the $\langle G_{22}(\infty)\rangle$ dropped to the value expected for $^{111}In^{3+}$ binding to serum proteins (*8*).

For the studies in vivo, vesicles containing ^{111}In-NTA were injected subcutaneously into Swiss-Webster mice (18 to 22 g) near the midline of the back at the level of the scapulae. At appropriate times the mice were killed and PAC measurements were immediately made on the portions of skin tissue that showed significant radioactivity. The amounts of radionuclide in all organs and tissues were determined with a well-type gamma counter.

The stability of subcutaneously administered vesicles was affected by surface charge (Fig. 1a and Table 1). Negatively charged vesicles (dicetyl phosphate) exhibited a shorter half-time than the control DSPC:Chol vesicles, whereas positively charged vesicles (stearylamine) showed an enhanced half-time. The presence of neutral sugars on the vesicle surface increased the lifetime in vivo to an even greater extent (*9*). Table 1 shows the increased stability observed for fucose, galactose, mannose, and acetamido-galactose derivatives of cholesterol with the structure:



Changes in the half-time of vesicles in vivo resulting from the presence of amino-sugar derivatives of cholesterol were impressive. The half-times for vesicles bearing the aminogalactose and aminomannose derivatives were substantially longer—100 and 600 hours, respectively (Table 1 and Fig. 1b)—than the half-times for other derivatives. Examination of the inside surface of the skin revealed localized swelling within the axillary space. These soft, ovoid structures, averaging 3 by 1.5 by 1.0 mm, were observed within 1 hour and persisted up to 8 hours after subcutaneous injection of vesicles bearing the aminomannose and aminogalactose derivatives. The areas of localized swelling contained intact vesicles and accounted for essentially all of the radioactivity associated with the skin. For all other vesicle systems examined, no comparable

Table 1. Effect of composition on the vesicle lifetime (*7*). Each subcutaneous injection of lipid vesicles typically contained 1.0 mg of total lipid, 20 μCi of $^{111}In^{3+}$ bound to 1 m*M* NTA inside, and phosphate-buffered saline (0.9 percent NaCl, 5 m*M* sodium phosphate, *p*H 7.4) inside and outside of the vesicles. The total injection volume was 200 μl. The data are based on PAC measurements of the skin. The 100 percent intact level corresponds to the $\langle G_{22}(\infty)\rangle$ value of the vesicles prior to injection; 0 percent intact corresponds to the $\langle G_{22}(\infty)\rangle$ of $^{111}In^{3+}$ bound to serum proteins. Numbers in parentheses are the percentages of injected $^{111}In^{3+}$ recovered in the skin.

Composition (molar ratio)	Nodule formation	Time (hours) that given percentages of vesicles are intact		
		80 per cent	50 per cent	20 per cent
DSPC : Chol (2:1)	−	9 (81)	21 (71)	43 (70)
DSPC : Chol : DPC (2:0.5:0.5)	−	3 (74)	12 (63)	25 (59)
DSPC : Chol : SA (2:0.5:0.5)	−	24 (81)	41 (80)	78 (75)
DSPC : Chol : Fuc (2:0.5:0.5)	−	13 (76)	41 (64)	120 (64)
DSPC : Chol : Gal (2:0.5:0.5)	−	28 (73)	57 (68)	117 (70)
DSPC : Chol : Man (2:0.5:0.5)	−	42 (74)	64 (68)	170 (65)
DSPC : Chol : AcAmGal (2:0.5:0.5)	−	43 (79)	84 (75)	170* (74)
DSPC : Chol : NH_2Gal (2:0.5:0.5)	+	35 (67)	100 (62)	210 (49)
DSPC : Chol : NH_2Man (2:0.5:0.5)	+	200 (79)	600* (79)	
DSPC : Chol : NH_2Man (2:0.75:0.25)	+	55 (72)	95 (56)	250 (65)
DSPC : Chol : NH_2Man (2:0.9:0.1)	−	30 (70)	55 (71)	120 (65)
DSPC : Chol : Man : SA (2:0.5:0.25:0.25)	−	26 (76)	70 (70)	200* (67)

*Extrapolated value.

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bleb formation was observed and the vesicles remained largely associated with the skin at the site of injection.

The effects observed with the aminomannose derivative of cholesterol were dose-dependent (Table 1 and Fig. 1b). Decreasing the proportion of aminomannose derivative reduced the lifetime in vivo and decreased the extent of bleb formation. For vesicles of DSPC:Chol: NH_2Man in the molar ratios 2:0.9:0.1, localized swelling was not apparent. Combining the mannose derivative of cholesterol with stearylamine did not produce the longevity or the concentration in the axillae that was observed with the aminomannose derivative (Table 1). Therefore, the specificity observed with the amino-sugar derivatives was a result of the stereochemistry of the surface groups and was not a simple additive effect of sugar plus charge.

Segal *et al.* (*10*) reported enlargement and increased radioactivity in draining lymph nodes after intratesticular injection of liposomes in rats. An affinity of neutral and positively charged liposomes for lymphoid tissue is indicated by enhanced uptake of such liposomes by the spleen (*11*). Ryman *et al.* (*12*) also reported that neutral and cationic liposomes are more satisfactory than anionic liposomes for visualizing regional lymph nodes when the liposomes are injected in the footpad of rats. Our data show that an NH_2 group positioned on a mannose or galactose residue confers substantial specificity for polymorphonuclear leukocytes when vesicles are administered subcutaneously.

In developing vesicle systems for controlled delivery of therapeutic agents an important factor to be considered is the accessibility of the released material to a given cellular or subcellular site, in particular, whether the entrapped material is released intra- or extracellularly. We therefore examined the effects in vivo of the indium complexes of ethylenediaminetetraacetic acid [$(^{111}$In-EDTA$)^{-1}$] and diethylenetriaminepentaacetic acid [$(^{111}$In-DTPA$)^{-2}$]. These indium complexes are very stable (*13*) and, when administered subcutaneously, are rapidly cleared from circulation by the kidneys. In fact, greater than 90 percent of the $(^{111}$In-EDTA$)^{-1}$ and $(^{111}$In-DTPA$)^{-2}$ administered in this way is excreted within 2.4 and 1.4 hours, respectively (Fig. 1c). Lipid vesicles containing these complexes were prepared by the loading procedure described previously (*3*) except that EDTA or DTPA was substituted for NTA. Figure 1c shows the effect of vesicle encapsulation on the rate of excretion of the complexes. For DSPC : Chol vesicles, the elimination of $(^{111}$In-EDTA$)^{-1}$ lagged by about 8 hours the destruction of the vesicles as measured by the PAC technique on preparations containing ^{111}In-NTA. This delayed excretion suggests that the vesicles may be taken up within a cell or compartment and then degraded. The prolonged delay in excretion of $(^{111}$In-DTPA$)^{-2}$ is consistent with this hypothesis, since membrane permeability should decrease with increasing net charge on the complex. Because of the extended lifetimes of vesicles bearing amino-sugar surface groups, it is difficult to determine by this method if they too release their contents intracellularly.

For all systems studied a delay is observed between the time of injection and the onset of vesicle destruction (Fig. 1, a and b). Investigations of vesicle-cell interactions, including the role of glycolipids and glycoproteins in vesicle-cell adhesion (*14*), indicate that this delay may arise from a stable adsorption of vesicles onto a cell surface. Therefore, we suggest the following mechanism for vesicles remaining at the site of injection: (i) When injected subcutaneously, the vesicles bind initially to intercellular com-

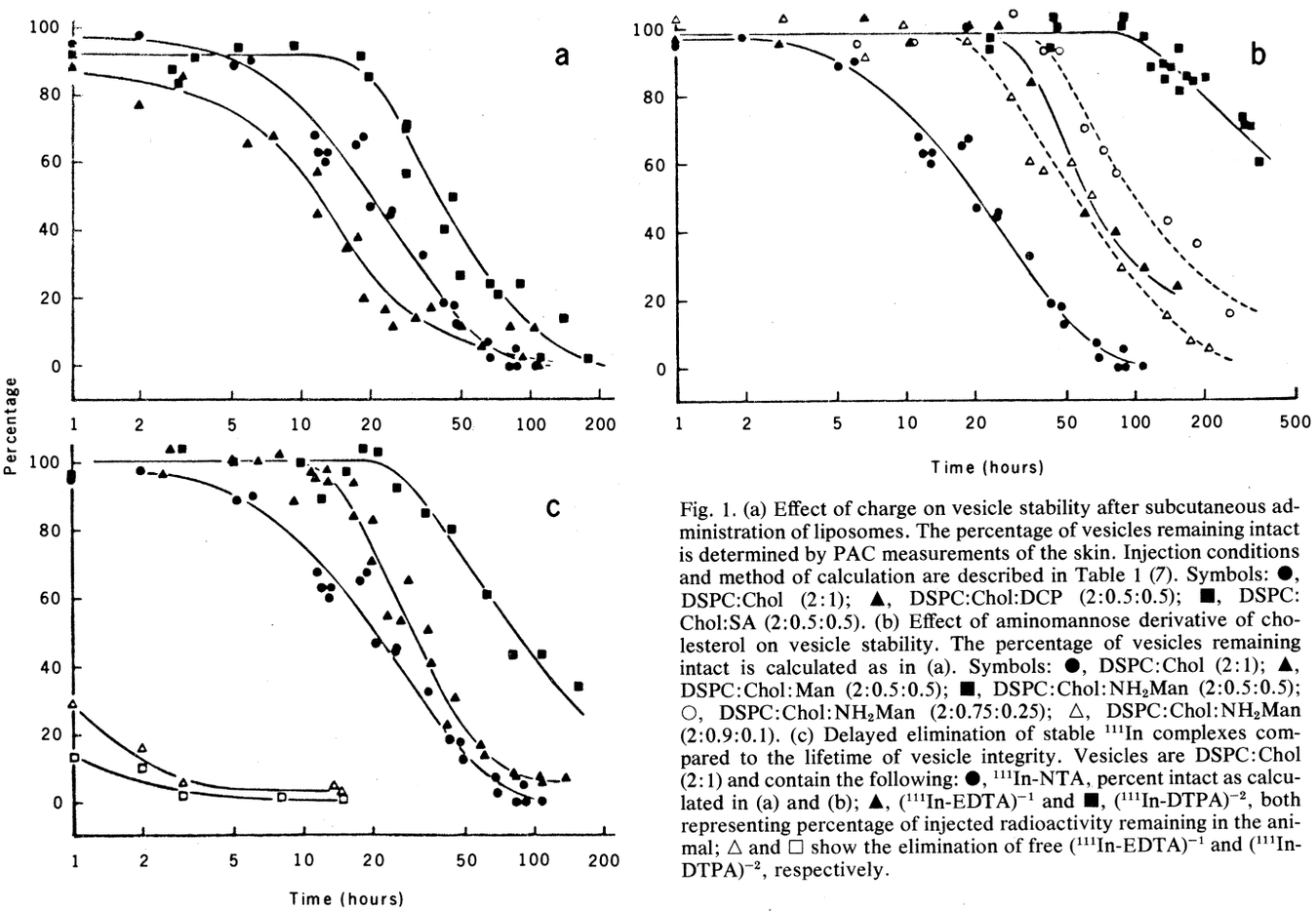

Fig. 1. (a) Effect of charge on vesicle stability after subcutaneous administration of liposomes. The percentage of vesicles remaining intact is determined by PAC measurements of the skin. Injection conditions and method of calculation are described in Table 1 (*7*). Symbols: ●, DSPC:Chol (2:1); ▲, DSPC:Chol:DCP (2:0.5:0.5); ■, DSPC:Chol:SA (2:0.5:0.5). (b) Effect of aminomannose derivative of cholesterol on vesicle stability. The percentage of vesicles remaining intact is calculated as in (a). Symbols: ●, DSPC:Chol (2:1); ▲, DSPC:Chol:Man (2:0.5:0.5); ■, DSPC:Chol:NH_2Man (2:0.5:0.5); ○, DSPC:Chol:NH_2Man (2:0.75:0.25); △, DSPC:Chol:NH_2Man (2:0.9:0.1). (c) Delayed elimination of stable ^{111}In complexes compared to the lifetime of vesicle integrity. Vesicles are DSPC:Chol (2:1) and contain the following: ●, ^{111}In-NTA, percent intact as calculated in (a) and (b); ▲, $(^{111}$In-EDTA$)^{-1}$ and ■, $(^{111}$In-DTPA$)^{-2}$, both representing percentage of injected radioactivity remaining in the animal; △ and □ show the elimination of free $(^{111}$In-EDTA$)^{-1}$ and $(^{111}$In-DTPA$)^{-2}$, respectively.

ponents, for example, collagen, or to nearby cell surfaces and remain intact for an extended period of time; (ii) later, the vesicles undergo endocytosis and are degraded; (iii) if the chelator is NTA, the indium ion is quickly released and binds to a macromolecule, and therefore the radioactivity remains within the cell; or if the chelator is EDTA or DTPA, the complex slowly crosses the cell membrane and the radioactivity is subsequently excreted. Vesicles bearing amino-sugar derivatives of cholesterol could operate by a similar mechanism, but with substantially greater binding constants for polymorphonuclear leukocytes that may account for their transport to the axillary region.

The half-time of vesicle destruction in vivo can be varied over a 50-fold range by the inclusion in the lipid bilayer of various hydrocarbon compounds with charged head groups or sugar and amino-sugar derivatives of cholesterol. This can provide means for controlled deposition and release of therapeutic agents by subcutaneous injection. In combination with the timed release feature, localization of vesicles in tissues may allow visualization or treatment of tumors and metastases of various systems. In addition, observations made with the PAC technique together with studies of the tissue distribution of vesicles containing stable indium complexes may provide considerable insight into the mechanisms of vesicle metabolism. The highly stereospecific effect of the sugar derivatives in targeting these phospholipid vesicles suggests potentially powerful approaches for studying the critical structural features of cell surface receptor molecules.

MARCIA R. MAUK
RONALD C. GAMBLE
JOHN D. BALDESCHWIELER
Arthur Amos Noyes Laboratory of Chemical Physics, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena 91125

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7. Abbreviations: DCP, dicetyl phosphate; SA, stearylamine; Fuc, 6-(1-thio-1-deoxy-β-L-fucopyranosyl)-1-(cholest-5-en-3β-yloxy)hexane; Gal, 6-(1-thio-1-deoxy-β-D-galactopyranosyl)-1-(cholest-5-en-3β-yloxy)hexane; Man, 6-(5-cholesten-3β-yloxy)hexyl-1-thio-α-D-mannopyranoside; AcAmGal, 6-(5-cholesten-3β-yloxy)-hexyl-2-acetamido-2-deoxy-1-thio-β-D-galactopyranoside; NH_2Gal, 6-(5-cholesten-3β-yloxy)-hexyl-6-amino-6-deoxy-1-thio-β-D-galactopyranoside; NH_2Man, 6-(5-cholesten-3β-yloxy)-hexyl-6-amino-6-deoxy-1-thio-α-D-mannopyranoside.
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Asbestos-Induced Epithelial Changes in Organ Cultures of Hamster Trachea: Inhibition by Retinyl Methyl Ether

Abstract. *The epithelium of the hamster trachea in organ culture undergoes hyperplasia and squamous metaplasia after exposure to the amphibole types of asbestos, crocidolite and amosite. These changes are inhibited when the synthetic vitamin A analog, retinyl methyl ether, is incorporated into the culture medium. These findings suggest a possible use for retinoids in the prevention and treatment of respiratory tract disease associated with environmental exposure to asbestos.*

Occupational exposure to asbestos increases the risk of bronchogenic carcinoma, particularly in long-term cigarette smokers (*1*). The basis for this effect is not known. The amphibole types of asbestos, crocidolite and amosite, induce hyperplastic lesions of basal cells and squamous metaplasia in the differentiated epithelium of organ cultures of hamster trachea (*2*). These cytologic alterations may constitute preneoplastic events.

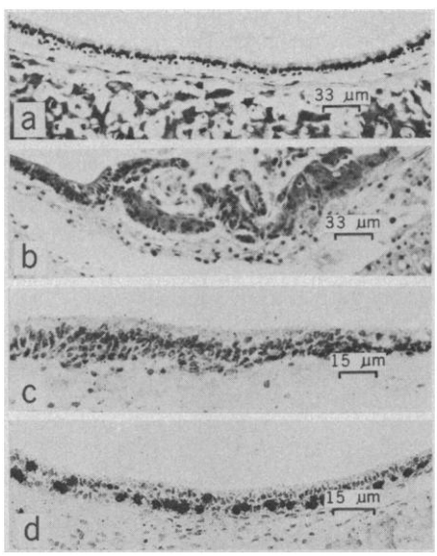

Fig. 1. Epithelium of normal tracheal organ cultures (a and c) and cultures exposed to asbestos (b and d). Note the squamous metaplasia in (b). Autoradiographs of normal and asbestos-treated tracheal epithelia are shown in (c) and (d), respectively. All tissues were maintained in the medium for 3 weeks without retinyl methyl ether.

Synthetic analogs of vitamin A (retinoids) prevent the development of squamous metaplasia and carcinoma in the bladder (*3*), mammary gland (*4*), and respiratory tract (*5*) of animals exposed to chemical carcinogens. Retinoids also reverse the epithelial hyperplasia and squamous metaplasia induced by polycyclic aromatic hydrocarbons in organ cultures of prostatic (*6*) and tracheal tissues (*7*). We now report a similar effect on the changes induced by asbestos in the tracheal epithelium.

Organ cultures are used in our laboratory to evaluate the effects of particulates and fibers, alone and in combination with polycyclic aromatic hydrocarbons, on the differentiated respiratory mucosa (*2*). We then graft tissues into syngeneic hamsters to assess the carcinogenicity of these materials (*8*).

In the experiments reported here, organ cultures were prepared from the tracheas of 60 female golden Syrian hamsters (age, 6 weeks) (15.16 BIO strain, TELACO, Bar Harbor, Maine) (*9*). Tissue from one animal yielded about 15 to 16 individual cultures, each having a mu-

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