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Phagocytosis of carbohydrate-modified phospholipid vesicles by macrophage

(liposomes/perturbed angular correlation spectroscopy/cell-surface receptor/drug-delivery systems)

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ABSTRACT **Modification of the surface of distearoyl phosphatidylcholine vesicles with synthetic glycolipids dramatically affects the rate of uptake of these vesicles by mouse peritoneal macrophage. The high rate of uptake of 6-aminomannose-modified vesicles is effectively inhibited by cytochalasin B and chloroquine but not by colchicine, indicating that the mechanism of vesicle uptake is phagocytosis. Other modified vesicles appear to have some effect on the rate of uptake of 6-aminomannose-modified vesicles suggesting that the various vesicle types compete for the same initial binding sites. Analysis of 6-aminomannose-modified vesicles by γ-ray perturbed angular correlation spectroscopy shows that the rotational correlation time of the encapsulated $^{111}In^{3+}$ does not change when the vesicles associate with macrophage. This result is consistent with transmission electron microscopy, which indicates that the aminomannose-modified vesicles remain intact after phagocytosis as aggregates of fused and intact vesicles surrounded by a single bilayer membrane structure.**

There is a growing body of evidence that carbohydrate cell-surface determinants play a significant role in intercellular recognition processes. Examples of mammalian carbohydrate recognition systems include (*i*) the receptor for galactose-terminated glycoproteins found in hepatocytes (1, 2), (*ii*) the receptor for 6-phosphomannosyl-containing glycoproteins found in fibroblasts (3, 4), and (*iii*) the receptor for mannose-terminated glycoproteins found in macrophage and polymorphonuclear leukocytes of the reticuloendothelial system (5–7). The existence of these receptors suggests that it should be possible to control the tissue distribution and cellular uptake of phospholipid vesicles by attaching appropriate carbohydrate determinants to the vesicle surface (8–11).

The modification of the surface of distearoyl phosphatidylcholine vesicles with particular synthetic glycolipids has been shown to affect dramatically the tissue distribution and stability of these vesicles in mice (12, 13). The use of vesicles loaded with ^{111}In enables the structural integrity of the vesicles to be determined *in vivo* by γ-ray perturbed angular correlation (PAC) spectroscopy and the tissue distribution to be determined by standard gamma-counting techniques (14–16). After intravenous injection, vesicles modified with 6-aminomannose derivatives of cholesterol produced initial retention of high levels of intact vesicles in the lung, followed by concentration of intact vesicles in the liver and spleen. These modified vesicles concentrated in the axillary spaces associated with aggregates of polymorphonuclear leukocytes and macrophage when administered subcutaneously.

The unusual tissue distributions and long lifetimes observed *in vivo* for aminomannose-modified vesicles must depend in a complex way on interactions of these vesicles with serum components and specific cell types. To establish a better basis for understanding the remarkably stereospecific mechanisms for recognition and transport of carbohydrate-modified phospholipid vesicles *in vivo*, it is essential to study the interaction of such modified vesicles with various isolated cell types. In this paper we report measurements of the kinetics of uptake of surface-modified vesicles by mouse peritoneal macrophage.

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MATERIALS AND METHODS

L-α-Distearoyl phosphatidylcholine (Ste_2-PtdCho) from Calbiochem and cholesterol from Sigma were used without further purification. Fucosyl, glucosyl, galactosyl, xylosyl, mannosyl, aminomannosyl, and glucuronic acid derivatives of cholesterol* were gifts from Merck Sharp & Dohme (10). Dicetyl phosphate and stearylamine were purchased from Sigma, the trisodium salt of nitrilotriacetic acid was from Aldrich and ultrapure $InCl_3$ was from Ventron (Danvers, MA). [*oleate*-1-^{14}C]Cholesteryl oleate with specific activity of 51 Ci/mol (1 Ci = 3.7×10^{10} becquerels) was purchased from New England Nuclear. Carrier-free $^{111}InCl_3$ was purchased from Medi+Physics (Glendale, CA) and purified according to the method of Hwang and Mauk (14). The ionophore A_{23187} was a gift from Eli Lilly.

Newborn calf serum, medium-199, and penicillin/streptomycin were purchased from Microbiological Associates (Los Angeles, CA). Cytochalasin B, colchicine, and chloroquine were purchased from Sigma. Ferritin (cationized) also was purchased from Sigma.

Preparation of Liposomes. Small unilamellar vesicles were prepared by the method of Mauk and coworkers (14–16). For most of the *in vitro* phagocytosis studies, [^{14}C]cholesteryl oleate was included in the lipid mixture as a marker. Total phospholipid was determined by phosphorus assay as described (17). The effect of temperature on the permeability of vesicles containing $^{111}In^{3+}$ was monitored by PAC spectroscopy (14–16).

Cultivation of Mouse Peritoneal Mononuclear Phagocytes. Cells from the peritoneal cavity of unstimulated male Swiss–Webster mice (25–30 g) were harvested by the method of Steinman and Cohn (18). A suspension of 4.2×10^6 cells [$\approx$42 μg of protein by microassay (19)] was plated to a plastic petri dish, and the adhered monolayer of macrophage ($1.3–1.6 \times 10^6$ cells) free of lymphocytes was maintained for 24 hr before

Abbreviations: PAC, γ-ray perturbed angular correlation; Ste_2-PtdCho, L-α-distearoyl phosphatidylcholine; Pam_2-PtdCho, L-α-dipalmitoyl phosphatidylcholine.

* 6-(5-cholesten-3β-yloxy)hexyl 1-thio-β-D-glucopyranoside; 6-(5-cholesten-3β-yloxy)hexyl 1-thio-β-L-fucopyranoside; 6-(5-cholesten-3β-yloxy)hexyl 1-thio-β-D-galactopyranoside; 6-(5-cholesten-3β-yloxy)hexyl 1-thio-α-D-mannopyranoside; 6-(5-cholesten-3β-yloxy)hexyl 1-thio-β-D-xylopyranoside; [6-(5-cholesten-3β-yloxy)hexyl 1-thio-β-D-glucopyranoid]uronic acid; 6-(5-cholesten-3β-yloxy)hexyl 6-amino-6-deoxy-1-thio-α-D-mannopyranoside.

use with 0.2 ml of medium 199 containing 20% (vol/vol) newborn calf serum and penicillin/streptomycin (500 units/ml).

Measurement of Phagocytosis. Freshly prepared liposomes were added to the petri dish culture to an activity of about 15,000 cpm (≈30 μl or ≈16.5 μg of phosphorus), and phagocytosis was permitted to proceed at 37°C or at temperatures as otherwise specified. At selected time intervals, the tissue culture medium was aspirated, and monolayers were rinsed with saline three times to remove unattached liposomes. Then 0.5 ml of 0.1% trypsin in saline was added to the monolayer petri dish culture and incubated at 37°C for 10 min. Cells of the monolayer suspension were scraped off the petri dish carefully with a rubber policeman and pipetted into a scintillation counting vial to a final volume of 1.5 ml with the addition of two 0.5-ml washes. The viability of the macrophage, which was determined by 0.05% trypan blue, was greater than 96%. The counting vials were briefly sonicated in a bath sonicator (Laboratory Supply, Hicksville, NY) before the addition of 20 ml of Aquasol. A Beckman LS-350 liquid scintillation counter with an average counting efficiency of 65% was used to assay for ^{14}C radioactivity.

Inhibition Assay. This assay was performed by the method of Axile and Reaven (20). Cytochalasin B freshly prepared as a stock solution of 1 mM in dimethyl sulfoxide was added to a petri dish containing tissue culture medium at the concentrations specified in *Results*. Macrophage cells were incubated with liposomes for 2 hr in this modified medium. The tissue culture medium was then aspirated, and monolayers were rinsed three times with saline. Phagocytosis was measured as described above. Stock solutions of 1 mM colchicine and chloroquine were freshly prepared in 0.9% NaCl/5 mM sodium phosphate, pH 7.4. Colchicine solution was used the same way as cytochalasin B. Chloroquine was added 15 min before the addition of vesicles.

Competition Assay. ^{14}C-Labeled liposomes were first mixed with cold (unlabeled) liposomes and were then added to a petri dish containing culture medium and macrophage. After 2 hr of incubation at 37°C, phagocytosis was measured as described above. The % (wt/wt) of ^{14}C-labeled and unlabeled liposomes was obtained by phosphorus assay of both labeled and cold liposomes.

Electron Microscopic Study. After *in vitro* cultivation, both untreated macrophage and macrophage that had been exposed to aminomannose-substituted liposomes were prepared for electron microscopy. In some cases, ferritin particles were encapsulated in the aminomannose liposomes during sonication before they were added to the macrophage culture. Macrophage monolayers were rinsed three times with saline and then were fixed for 1 hr in 2.5% (vol/vol) glutaraldehyde containing 0.1 M cacodylate buffer (pH 7.4 at room temperature). Monolayers were rinsed with saline and postfixed again with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C. Monolayers were dehydrated in graded alcohol and embedded in Epon-812. Sections were cut with LKB Ultrotome III and picked up on an uncoated grid. The transmission electron microscopy was carried out by D. A. Golde and S. Poolsawat at the UCLA School of Medicine.

RESULTS

Vesicle Integrity. Because phospholipid vesicles with various surface modifications have potentially very important applications as vehicles for the delivery of pharmacologically active agents, it is important to understand what factors influence their integrity in the presence of serum. Leakage of entrapped ^{111}In from vesicles in the presence of serum can be monitored by the PAC technique because complexation of $^{111}In^{3+}$ with high molecular weight serum components results in a decrease in the rotational correlation time of the $^{111}In^{3+}$. The percentage of ^{111}In which remained encapsulated at various fixed temperatures in the presence of serum is shown in Fig. 1 for Ste_2-PtdCho vesicle compositions that differ in cholesterol content. Both the transition temperature for ^{111}In leakage and the width of the transition region depended on cholesterol composition. The width of the transition region for ^{111}In leakage appeared to be influenced by the presence of cholesterol substituted with aminomannose.

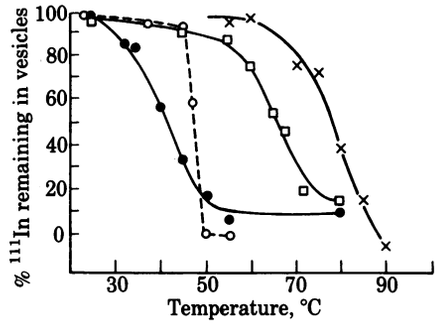

FIG. 1. Effect of cholesterol content on transition temperature for permeability of vesicles to $^{111}In^{3+}$. The percentage of $^{111}In^{3+}$ originally encapsulated that remains after incubation for 0.5 hr in the presence of serum as determined by the PAC method is plotted vs. the incubation temperature for various mole ratios of Ste_2-PtdCho:cholesterol:aminomannose. ×, (2.0:0.5:0.5); □, (2.1:0.4:0.5); ●, (2.25:0.25:0.5); ○, (2.5:0.0:0.5).

The effect of phospholipid composition on the leakage of ^{111}In from vesicles is shown in Fig. 2. For vesicles containing only Ste_2-PtdCho and cholesterol, the temperature at which half of the ^{111}In remains encapsulated by the PAC criterion was about 80°C. Because the entire transition region is well above physiological temperature, this vesicle composition was used for *in vivo* studies in mice (12, 13) and for the macrophage-uptake studies reported here. The transition temperature for ^{111}In leakage was strongly dependent on the phospholipid composition (Fig. 2). The transition range for ^{111}In leakage could be adjusted to physiological temperatures by mixing various amounts of L-α-dipalmitoyl phosphatidylcholine (Pam_2-PtdCho) with Ste_2-PtdCho and cholesterol. Regulation of the release of vesicle contents is an important element in any strategy for the use of vesicle systems for the delivery of pharmacologically-active agents (21).

Interaction of Vesicles with Mouse Peritoneal Macrophage. The uptake of Ste_2-PtdCho by mouse peritoneal macrophage

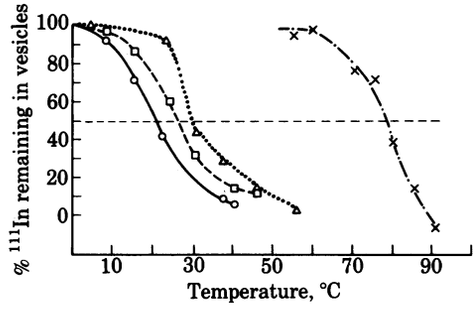

FIG. 2. Effect of phospholipid composition on transition temperature for permeability of vesicles to $^{111}In^{3+}$. The percentage of $^{111}In^{3+}$ originally encapsulated that remains after incubation for 0.5 hr in the presence of serum as determined by the PAC method is plotted vs. the incubation temperature for various mole ratios of Pam_2-PtdCho: Ste_2-PtdCho:cholesterol:aminomannose. ×, (0.0:2.0:0.5:0.5); △, (0.5:1.5:0.5:0.5); □, (1.0:1.0:0.5:0.5); ○, (1.5:0.5:0.5:0.5).

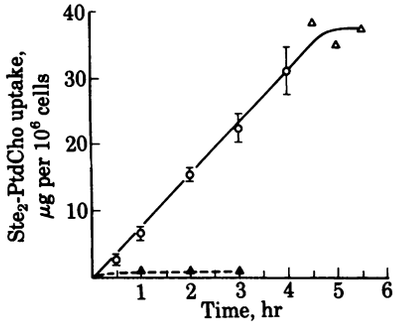

FIG. 3. Uptake of various modified phospholipid vesicles by mouse peritoneal macrophage at 37°C as a function of incubation time. ○ and △, Aminomannose-modified vesicles; ▲, stearylamine-, dicetylphosphate-, glucose-, glucuronic acid-, galactose-, xylose-, fucose-, and mannose-modified vesicles and unmodified Ste_2-PtdCho/cholesterol vesicles.

for various vesicle compositions as measured by the ^{14}C activity remaining with the cells is shown in Fig. 3. Aminomannose-modified vesicles showed a substantial rate of uptake (7.7 μg per 10^6 cells) for about 4 hr. The rate of uptake for the other vesicle systems studied was too small to determine with the same concentration of ^{14}C label used for the aminomannose system. The rate of uptake of aminomannose-modified vesicles was linear in the cell population. Analysis of aminomannose-modified vesicles by the PAC method indicated that the rotational correlation time of encapsulated $^{111}In^{3+}$ does not change when the vesicles associate with macrophage. The temperature dependence of the rate of uptake of aminomannose-modified vesicles yielded an Arrhenius plot giving an activation energy of 12.6 Kcal/mol above a transition temperature of 17°C.

Inhibition of Vesicle Uptake. The rate of vesicle uptake by macrophage was reduced by using cytochalasin B, colchicine, and chloroquine (Fig. 4). Cytochalasin B was clearly the most effective inhibitor, whereas chloroquine and colchicine had less effect at comparable concentrations.

The rate of uptake of aminomannose-modified vesicles was also affected by competition with various other vesicle systems (Fig. 5). The competition of unlabeled aminomannose vesicles with ^{14}C-labeled vesicles gave a line of unit slope, as would be expected. Other modified vesicles (freshly prepared) appeared to have some effect on the rate of aminomannose vesicle uptake,

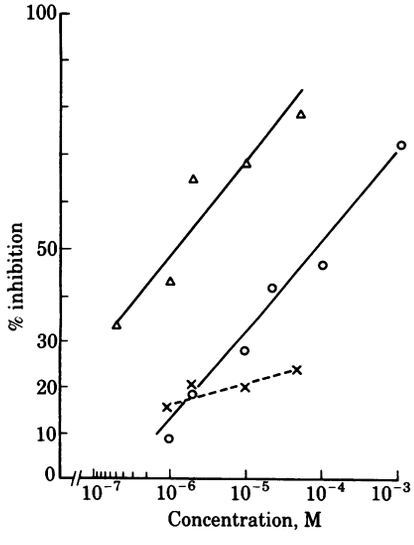

FIG. 4. Effect of various inhibitors on the rate of uptake of aminomannose-modified phospholipid vesicles by mouse peritoneal macrophage at 37°C. △, Cytochalasin B; ×, colchicine; ○, chloroquine.

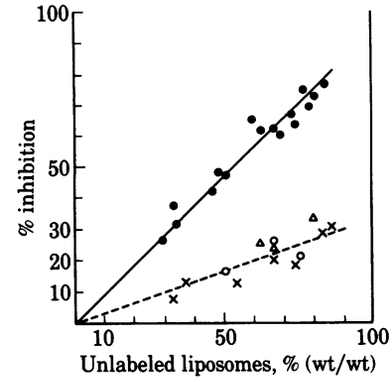

FIG. 5. Effect of various unlabeled (cold) vesicle types on the rate of uptake of ^{14}C-labeled aminomannose-modified phospholipid vesicles by mouse peritoneal macrophage at 37°C. ●, Unlabeled aminomannose-modified vesicles; ×, unlabeled mannose-modified vesicles; △, unlabeled stearylamine-modified vesicles; ○, unlabeled, unmodified Ste_2-PtdCho/cholesterol vesicles.

suggesting that the various vesicle types compete for the same binding sites. Yeast mannan dissolved in the medium at concentrations from 1 to 3 mg/ml inhibited the rate of aminomannose vesicle uptake by 8–12%.

Transmission Electron Microscopy. Transmission electron micrographs showing the interaction of aminomannose-modified vesicles with macrophage are seen in Fig. 6. An unactivated macrophage cell is shown in Fig. 6*a* while a similar cell after a 4-hr incubation with aminomannose-modified vesicles is shown in Fig. 6*b*. There is evident formation of vacuoles and aggregates at higher magnification, which appear to consist of fused and intact vesicles and broken membrane fragments surrounded by a single bilayer membrane structure as shown in Fig. 6*c*. The encapsulated ferritin particles can also be seen inside individual vacuoles.

DISCUSSION

The permeability of phospholipid vesicles to $^{111}In^{3+}$ is clearly strongly dependent on vesicle composition (Figs. 1 and 2). Reducing the cholesterol content or replacing Ste_2-PtdCho with Pam_2-PtdCho reduces the transition temperature determined by the PAC method. It is important that the vesicle permeability is measured in the presence of serum in this assay to provide a better approximation to the conditions expected *in vivo*. A variety of serum components have been shown to bind to vesicle membranes (22), and the transition temperature determined by the PAC method is significantly reduced for vesicles in the presence of serum.

The rate of uptake of small unilamellar vesicles composed of Ste_2-PtdCho and cholesterol modified with aminomannose is much greater than the rate of uptake for any of the other vesicle systems studied (Fig. 3). It has been proposed that negatively-charged vesicles composed of phospholipids that are fluid at 37°C are taken up by cells by fusion, whereas negatively-charged solid vesicles and neutral fluid and solid vesicles are taken up predominantly by endocytosis (23). It also has been suggested that positively-charged fluid vesicles are taken up by endocytosis (24). The observation that cytochalasin B effectively inhibits aminomannose-modified vesicle uptake indicates that the uptake mechanism for these vesicles is endocytosis (20).

Compounds such as colchicine generally are not expected to inhibit endocytosis (25, 26). Thus, the results for colchicine (Fig. 3) are also consistent with the conclusion that endocytosis is the predominant mechanism of aminomannose-modified vesicle uptake. It has been suggested that chloroquine inhibits receptor-mediated ligand uptake by macrophage and fibroblasts (27,

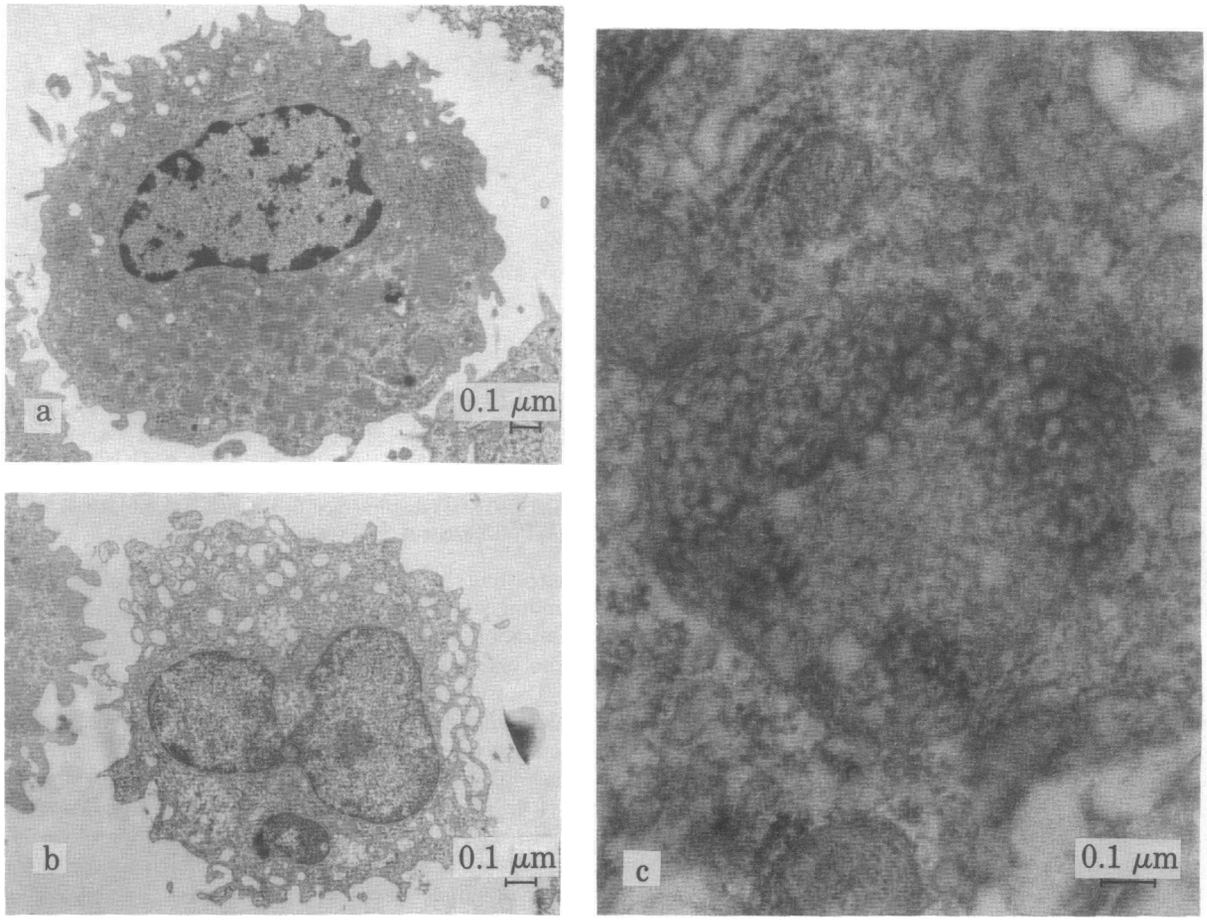

FIG. 6. Transmission electron micrographs showing the interaction of mouse peritoneal macrophage with aminomannose-modified phospholipid vesicles containing lead-coupled ferritin as a marker. (*a*) Unactivated macrophage cell in the absence of vesicles. (*b*) Macrophage cell after incubation for 4 hr in the presence of aminomannose-modified vesicles. (*c*) Higher-magnification image of an aggregate of vesicles internalized in the macrophage cell.

28). The observation that chloroquine also inhibits aminomannose-modified vesicle uptake indicates that endocytosis may occur through a receptor-mediated process.

The temperature dependence of the rate of uptake of aminomannose-modified vesicles by macrophage is also consistent with endocytosis. Silverstein *et al.* (29) have argued that phagocytosis does not proceed unless the temperature of incubation exceeds some critical threshold of about 18–21°C. For pinocytosis, there appears to be no critical thermal transition, and an activation energy of 17.6 kcal/mol is observed for solute uptake in mouse fibroblasts (30).

The extraordinarily high rate of uptake of aminomannose-modified vesicles is apparently not simply due to charge effects because neither positively-charged vesicles (modified with stearylamine) nor negatively-charged vesicles (modified with dicetyl phosphate or glucuronic acid) show measurable uptake under the conditions of these experiments. The observation that unmodified vesicles and vesicles modified with mannose or stearylamine are about equally effective in competing with the uptake of ^{14}C-labeled aminomannose-modified vesicles indicates that the initial binding steps must not depend on the stereochemistry of aminomannose. This is also consistent with the observation that mannan weakly inhibits aminomannose vesicle uptake. However, the rates of reaction steps in the endocytosis mechanism that follow the initial binding of vesicles to macrophage could be very sensitive to the binding constant for the aminomannose determinant with receptors on the macrophage cell surface. The mechanics of the endocytosis process will certainly depend on the (*i*) magnitude of binding constants for specific determinants with receptors, (*ii*) concentration of determinants and receptors per unit area of vesicle and cell surface, (*iii*) mobility of determinants and receptors in the vesicle cell membranes, (*iv*) the flexibility of the vesicle and cell membrane, and (*v*) the size of the vesicle.

The existence of receptor activity for D-mannose has been demonstrated for rat and mouse alveolar macrophage (31, 32) and for human polymorphonuclear leukocytes (33). However, the rate of uptake by mouse peritoneal macrophage of aminomannose vesicles is much greater than the rate of uptake of mannose vesicles. Ryser and Shen (34) have shown that methotrexate conjugated to polylysine (which contains a large number of amino groups) is rapidly taken up by tumor cells in culture. Although the mechanism of this process has not yet been established, it is possible that the rapid endocytosis observed for aminomannose-modified vesicles is simply dependent on the presence of large numbers of amino groups per unit area extending well beyond the vesicle surface region.

PAC studies of the uptake by macrophage of aminomannose-modified vesicles containing $^{111}In^{3+}$ provide the interesting result that the vesicles remain intact after endocytosis. This experimental result may be related to our previous observation that aminomannose-modified vesicles remain intact for about 600 hr *in vivo* in mice after subcutaneous injection (12). It is important to understand why the integrity of the aminomannose-labeled vesicles is not destroyed by the lysosomal machinery of the macrophage. This may be simply related to the robust vesicle composition used in these experiments or to the effective pH of the vesicle surface after endocytosis.

In the absence of serum factors, mouse alveolar macrophage are still capable of binding yeast cells (32). Mannose is the major soluble component of the yeast cell wall. When serum is omitted from the incubator medium, mouse peritoneal macrophage retain 75% of the uptake activity for aminomannose vesicles observed when serum is present in the incubation medium. This result indicates that serum is not a critical factor for endocytosis of these vesicles.

The exchange of cell membrane-bound cholesterol with exogenous synthetic glycolipids (35) and with cholesterol contained in lipid vesicles (36) has been reported. Because there was no measurable uptake of ^{14}C activity from freshly-prepared vesicles composed of Ste_2-PtdCho and cholesterol except when the vesicles were modified with aminomannose, it seems unlikely that exchange of the ^{14}C label is a serious complication in these measurements. For the Ste_2-PtdCho/cholesterol vesicles used in these experiments, the exchange of [^{14}C]cholesterol oleate with macrophage membrane-bound cholesterol is probably negligible during the 1–2 hr incubation periods that usually were used.

The unusual effects of vesicle surface modification with aminomannose provide potentially powerful approaches to a better understanding of the details of carbohydrate-determinant recognition and the mechanisms of endocytosis, lysis, and receptor-protein recycling in phagocytic cells. However, the remarkably large rate of endocytosis of aminomannose-modified vesicles by macrophage also suggests some interesting applications. For example, Fidler (37) has shown that phospholipid vesicles can be used to deliver macrophage activation factor to macrophage *in vivo* and that macrophage thus activated are effective in reducing metastic tumors. The use of aminomannose-modified vesicles might substantially improve this approach. Other examples of potentially interesting applications include treatment of leishmaniasis (38, 39) and Gaucher disease (40) and enhancement of the production of interferon (41, 42).

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