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Receptor-mediated endocytosis of poly(acrylic acid)-conjugated liposomes by macrophages

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Abstract

The uptake characteristics of negatively-charged liposomes made by conjugation of poly(acrylic acid) (PAA) were studied with respect to cultured RAW macrophages. The PAA-conjugated liposomes were internalized and digested in an acidic compartment at a much faster rate than the unmodified phosphatidylcholine (PC) liposomes. After incubation for 18 h, an over 5-fold increase in the uptake of PC liposomes was obtained by PAA conjugation. Subsequently, part of the aqueous phase of the internalized liposomes was exocytosed. Recognition of PAA by the macrophages seems to be responsible for the enhanced uptake of PAA-conjugated liposomes. Cross-competition experiments showed that PAA-conjugated liposomes inhibited the uptake of acetylated-low density lipoprotein (acetyl-LDL) by the macrophages and vice versa. The uptake of PAA-conjugated liposomes was also inhibited by dextran sulfate and maleylated-bovine serum albumin (maleyl-BSA), which are also known to bind to scavenger receptors. Poly(C) and BSA, which are not ligands for the scavenger receptor, competed poorly with the uptake of PAA-conjugated liposomes. Enhanced uptake of PAA-conjugated liposomes by CHO cells with low scavenger receptor expression was not observed. Unexpectedly, LDL, which is not a ligand for scavenger receptor, also partially inhibited the uptake of PAA-conjugated liposomes. The interaction of PAA-conjugated liposomes with macrophages is complex, and the endocytosis of PAA-conjugated liposomes most likely involves multiple receptors and/or pathways. The data obtained suggest that the high affinity binding of PAA-conjugated liposomes to macrophages may be due to recognition of the negative charges of PAA by cell surface receptors, including the scavenger receptor.

Keywords: Liposome; Poly(acrylic acid); Endocytosis; Macrophage; Scavenger receptor

1. Introduction

Liposomes are effective carriers for the introduction of various agents into cells. Lipid components and compounds entrapped in the aqueous compartment of the vesicles can be transferred into cells by various mechanisms. The mechanism of liposome–cell interaction is an important factor in liposomal drug delivery. Liposomes interact with cells by such mechanisms as fusion with the cell membrane, phagocytosis, and receptor-mediated endocytosis. The mechanism of uptake is dependent on the particular liposome formulation used. The efficacy of liposomes as intracellular delivery vehicles can be improved by enhancing the uptake and specificity of liposome interaction with cells. It has been shown that cells capable of phagocytosis, such as macrophages, can endocytose liposomes [1]. Liposomes containing negatively-charged lipids, such as phosphatidylserine (PS), are more efficiently endocytosed than liposomes containing only neutral lipids [2]. It is believed that scavenger receptor recognition may be responsible for the improved uptake of the negatively-charged liposomes by macrophages [3].

PAA is a synthetic polymer which is negatively-charged at physiological pH and is known to exhibit biological

Abbreviations: PAA, poly(acrylic acid); PC, phosphatidylcholine; LDL, low density lipoprotein; BSA, bovine serum albumin; PS, phosphatidylserine; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid; SUV, small unilamellar vesicles; PBS, phosphate-buffered saline; MPB-PE, *N*-(4-(*p*-maleimidophenyl)butyryl)dimyristoyl phosphatidylethanolamine; cholether, cholesteryl hexadecyl ether; AET, 2-aminoethanethiol; DCC, dicyclohexylcarbodiimide; 4-pp, 4-pyrrolidinopyridine; PL, phospholipid.

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activities, such as the induction of interferons [4]. PAA also belongs to a family of poly(carboxylic acid)s, which are known to render liposomes sensitive to pH by destabilizing the liposomes at acidic pH [5–7]. Negatively-charged liposomes were made by covalently conjugating PAA to egg PC liposomes, and their interactions with RAW cell line of mouse macrophages and Chinese hamster ovary (CHO) cells were compared to that of unmodified liposomes. The extent of endocytosis and exocytosis was measured by the amount of radiolabeled-liposomes incorporated into and released from the macrophages. Liposomes labeled with a pH-sensitive fluorescent probe (HPTS) were used to study the fate of liposomes inside the cell. Competition and cross-competition studies were carried out with various reagents to characterize the nature of the receptors responsible for the uptake of PAA-conjugated liposomes.

2. Materials and methods

2.1. Materials

Egg PC, bovine brain PS, and *N*-(4-(*p*-maleimidophenyl)butyryl)dimyristoylphosphatidylethanolamine (MPB-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). The purity of the lipids was checked by thin-layer chromatography, and the lipids were used without further purification. [^{3}H]Cholesteryl hexadecyl ether ([^{3}H]cholether) was obtained from NEN Research Products (Boston, MA). [^{3}H]Inulin (M_r 5600) and Na^{125}I were obtained from Amersham (Arlington Heights, IL). Maleic anhydride, sodium pyruvate, acridine orange, ethidium bromide, LDL, BSA, heparin, and dextran sulfate (M_r 500 000) were obtained from Sigma (St. Louis, MO). 2-Aminoethanethiol (AET) and dicyclohexylcarbodiimide (DCC) were obtained from Aldrich (Milwaukee, WI). 4-Pyrrolidinopyridine (4-pp) was obtained from Fluka (Ronkonkoma, NY). Poly(C) was obtained from Calbiochem (San Diego, CA). 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was obtained from Molecular Probes (Eugene, OR). Dimethylformamide was vacuum distilled. All other reagents used were reagent grade or better. Distilled deionized water was used in the experiments.

2.2. Liposome preparation

A lipid solution in chloroform was dried under N_2 and further under vacuum overnight. The lipid film was hydrated with PBS (pH 7.4) or by Tris buffer (50 mM Tris, 10 mM NaCl, pH 7.4). SUV were made by probe sonication of the aqueous lipid solution under N_2. Ti particles and large liposomes were removed by centrifuging the SUV solution for 5 min at 12 000 × *g* in a Beckman Microfuge 11. All liposome solutions were sterilized by passing through a 0.2 μm filter prior to incubation with macrophages. The phosphate content was determined by Böttcher modification of Bartlett assay [8].

2.3. PAA conjugation

Acenaphthalene-labeled PAA was synthesized, thiolated, and conjugated to egg PC-SUV [5,9]. Briefly, acenaphthalene-labeled PAA (M_r 40 000) was obtained by radical polymerization of acrylic acid with acenaphthalene [9]. Acenaphthalene-labeled PAA was allowed to react with AET in the presence of 4-pp and DCC in dimethylformamide for 24 h at room temperature. The reaction mixture was filtered and precipitated into ethyl acetate. 4% of the monomers in PAA was modified by AET as determined by thiol analysis [10]. AET-modified PAA (10 mg/ml) was reduced with dithiothreitol under N_2 for 15 min and passed through Sephadex G-50 (Sigma) equilibrated with 0.9% saline. Polymer fractions collected were immediately allowed to react for 4 h with 10 mg egg PC-SUV containing 5% MPB-PE in 1 ml buffer. Unreacted polymer was separated from liposomes using a Bio-Gel A-5m column (Bio-Rad, Richmond, CA) equilibrated with Tris buffer.

2.4. Cell cultures

RAW264.7 macrophages (ATCC, Rockville, MD) were cultured in RPMI1640 media containing glutamine (Hyclone, Logan, UT) supplemented with 1 mM pyruvate, Fungi-Bact solution (Irvine Scientific, Santa Ana, CA), and 10% fetal bovine serum (Hyclone). CHO cells (ATCC) were cultured in Ham's F-12 media (Irvine Scientific) supplemented with 10% fetal bovine serum. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Cell concentration was determined using a hemocytometer.

2.5. Endocytosis assay

Liposomes were labeled with [^{3}H]cholether or with [^{3}H]inulin. [^{3}H]Cholether labeled liposomes were made as follows. [^{3}H]Cholether (4.5 μCi) in toluene was added to a lipid solution (10 mg) in chloroform, and SUV were made as described above. For experiments designed to follow the fate of the liposomal aqueous phase, [^{3}H]inulin (25 mCi) in buffer (1 ml) was added to dry lipid (20 mg) during the hydration step. The solution was passed through five freeze-thaw cycles to maximize inulin encapsulation prior to sonication [11]. Excess [^{3}H]inulin was removed after sonication by passing through a Bio-Gel A-5m column equilibrated with Tris buffer. The liposomes were incubated with cells at 37°C or at 4°C in a 6-well tissue culture plate in a 5% CO_2 atmosphere for the desired incubation times. For the time-dependent assays, the amount of liposomes containing 51 nmol of phospholipids was added to

each well containing $1 \cdot 10^6$ cells/ml media. After varying time intervals, the cells were washed twice with cold PBS, scraped off the plates, and centrifuged at $3500 \times g$ for 3 min in the Beckman Microfuge 11. The pellets were dissolved in 0.5M NaOH/0.5% SDS solution prior to scintillation counting. Samples were tested in triplicate for all experiments, and the error bars in the figures indicate standard deviation (S.D.).

2.6. Exocytosis assay

Radiolabeled liposomes were incubated with macrophages as described above for 24 h. The cells were washed twice with cold PBS to remove free liposomes and further incubated in media for varying periods of time. The cells were scraped off the plates, and both the pellets and supernatants were collected by centrifugation and counted for radioactivity.

2.7. HPTS assay

HPTS assay was carried out as described previously [1]. Briefly, HPTS (35 mM) in Tris buffer was added to dry lipid during the hydration step, and egg PC-SUV were prepared as described for [^{3}H]inulin liposomes. After incubation with HPTS liposomes, cells were washed twice with cold PBS and incubated further for 5 min at 37°C. Cells were dislodged, and excitation spectra were recorded at 510 nm emission with 8 nm bandwidth using a SLM spectrofluorometer. The fraction of HPTS endocytosed in acidic compartment was calculated as follows,

$$\text{fraction endocytosed} = \frac{R_{7.4} - R_{\text{measured}}}{R_{7.4} - R_{\text{low}}}$$

where $R_{7.4}$, R_{measured}, and R_{low} are the 460/413 nm ratio of HPTS-liposomes at pH 7.4, liposome treated cells, and HPTS pinocytosed, respectively.

2.8. Competitive assays

LDL was radioiodinated with Na^{125}I using the iodine monochloride method [12] and acetylated using acetic anhydride [13]. Amine analysis [14] indicated that over 90% of the amine groups in LDL was acetylated. Maleyl-BSA was prepared as described previously [15]. Over 95% of the amino groups in BSA was maleylated. The uptake of [^{3}H]cholether labeled PAA-conjugated liposomes (51 nmol/10^6 cells) was measured after 12 h incubation with various concentrations of the following reagents: egg PC-SUV, free PAA, acetyl-LDL, LDL, maleyl-BSA, BSA, poly C, heparin, and dextran sulfate. For cross-competition experiments, the extent of [^{125}I]acetyl-LDL (38 μg protein) and [^{3}H]cholether labeled PS-SUV (25 nmol PL) uptake was measured after 12 h incubation with PAA-conjugated liposomes.

2.9. Cytotoxicity assay

Cells were exposed to varied concentrations of PAA-conjugated liposomes (0, 3.2, 6.4, 32, 64 nmol PL) and free PAA (0, 50, 100, 500 μg) in tissue culture plates. The cell viability after 24 h was determined using an acridine orange/ethidium bromide assay [16]. Cells were incubated with 1 ppm solution of acridine orange and ethidium bromide (15 μl/well) for a few minutes and were examined under a fluorescence microscope. Viable cells were stained green and nonviable cells appeared orange. The total cell proteins of cells incubated with PAA-conjugated liposomes were compared to that of cells grown without

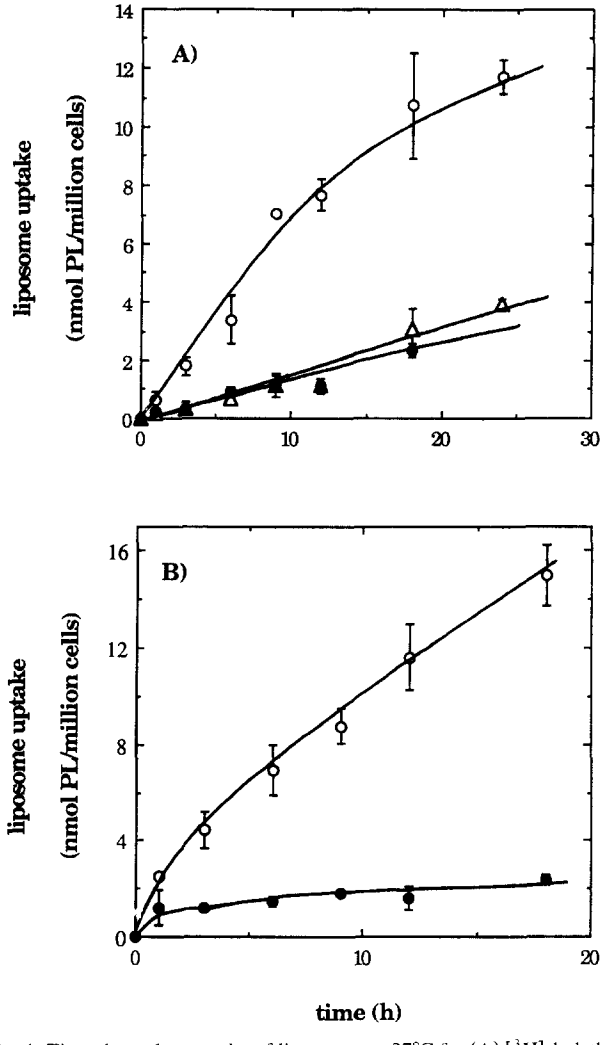

Fig. 1. Time-dependent uptake of liposomes at 37°C for (A) [^{3}H]cholether labeled liposomes and (B) [^{3}H]inulin labeled liposomes. Liposomes corresponding to 51 nmol PL were added to 10^6 cells in 1 ml of culture media. PAA-conjugated SUV (○), egg PC-SUV (△), and egg PC-SUV in the presence of equivalent amount of free PAA as PAA-conjugated liposomes (●) were compared. Error bars indicate standard deviation.

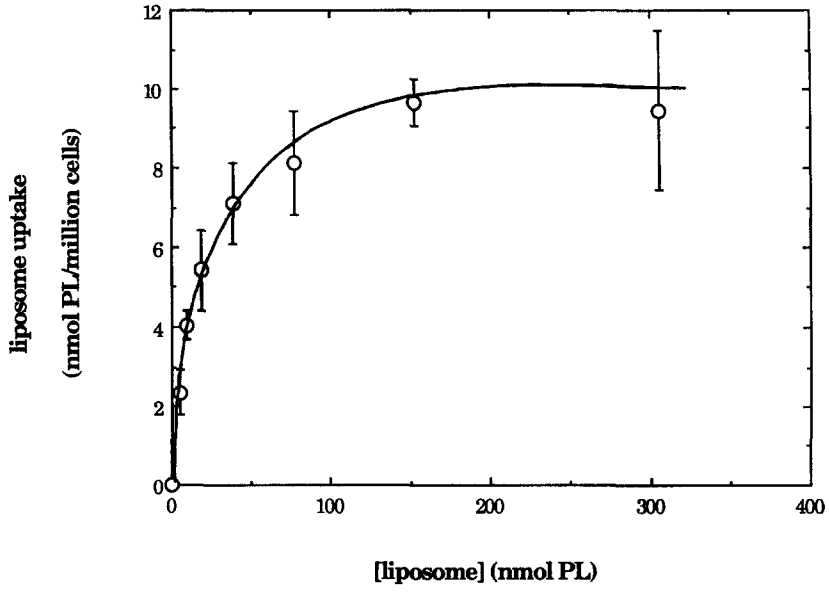

Fig. 2. Concentration-dependent uptake of PAA-conjugated liposomes. The uptake of [^{3}H]cholether labeled liposomes was determined after incubation with 10^6 cells for 12 h at 37°C.

PAA-conjugated liposomes. The cell protein concentration was estimated by the Peterson's modified Lowry assay using BSA as a standard [17].

3. Results

3.1. PAA-conjugated liposome preparation

The particle sizes of unmodified liposomes and PAA-conjugated liposomes were determined using laser light scattering. The mean liposome size was 28.8 nm ± 7.5 (S.D., $n = 10$) and increased slightly to 42.2 nm ± 9.7 (S.D., $n = 10$) after PAA conjugation. The extent of polymer conjugation to liposomes was determined by measuring the fluorescence emission intensity of acenaphthalene-labeled PAA at 340 nm with 295 nm excitation using a standard curve. It was calculated that approx. 150 polymer strands were conjugated to the SUV (170 mol phospholipid/mol polymer) using 30 nm as the vesicle diameter and assuming 60% of phospholipid molecules reside on the outer monolayer.

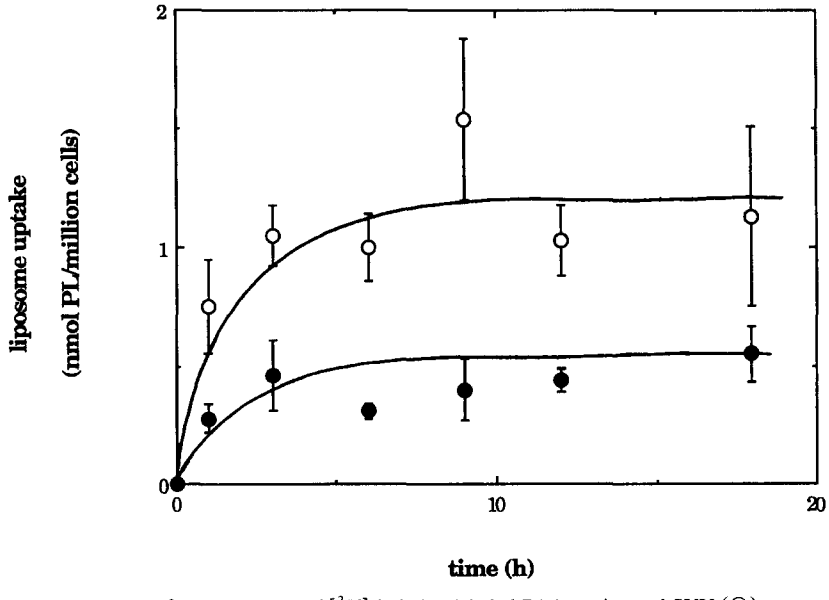

Fig. 3. Time-dependent uptake of liposomes at 4°C. The uptake of [^{3}H]cholether labeled PAA-conjugated SUV (○) was compared to egg PC-SUV in the presence of free PAA (●). Liposomes corresponding to 51 nmol PL were added to 10^6 cells in 1 ml of culture media.

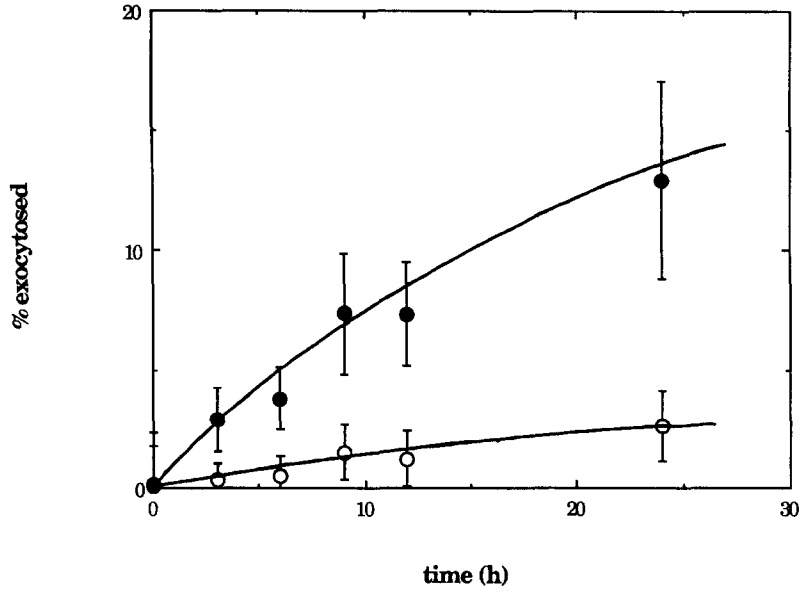

Fig. 4. Time-dependent exocytosis of PAA-conjugated SUV. Exocytosis of [^{3}H]cholether (○) and [^{3}H]inulin (●) was measured at 37°C after liposomes (51 nmol PL) were incubated with 10^6 cells for 24 h.

3.2. Exdocytosis by RAW cells

Uptake of PAA-conjugated liposomes by the macrophages was studied and compared to egg PC-SUV uptake (Fig. 1). [^{3}H]Inulin, a nondegradable aqueous marker, and [^{3}H]cholether, a lipid marker, were used to quantitate the uptake of aqueous and lipid components, respectively. The uptake of both the aqueous and lipid phase markers of PAA-conjugated liposomes by macrophages was much higher compared to that of unmodified liposomes. Increased uptake was not observed when free PAA was added to egg PC-SUV indicating that PAA conjugation to the liposomes is necessary for improved uptake by the macrophages. Egg PC-SUV containing 5% MPB-PE behaved similarly to pure egg PC-SUV (data for egg PC-SUV/MPB-PE are not shown due to the similarity of the data for egg PC-SUV shown in Fig. 1). Uptake processes showed saturation at high vesicle concentrations with a maximum uptake value of 10 nmol PL/10^6 cells, corresponding to about $6.2 \cdot 10^5$ liposomes/cell. (Fig. 2).

The extent of liposome binding to cell surface receptors, as opposed to endocytosis, was estimated by incubat-

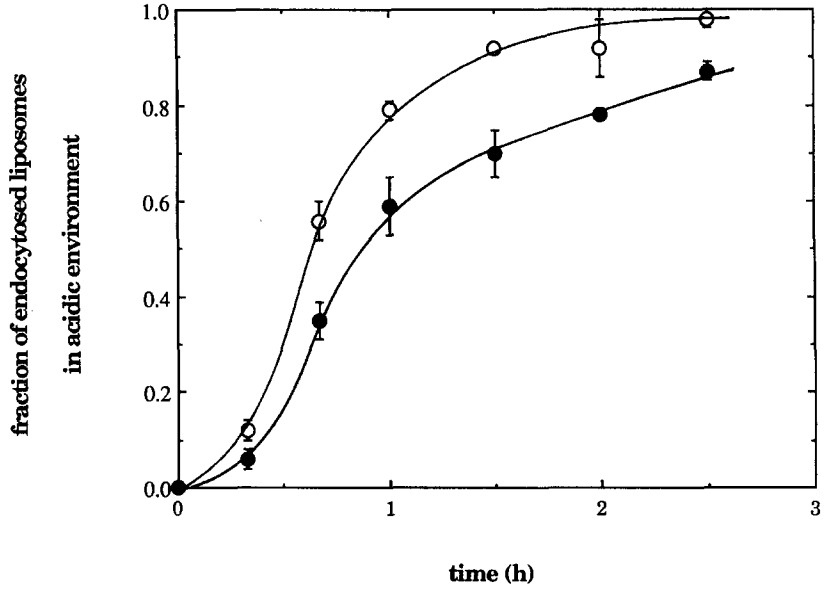

Fig. 5. Fraction of liposomes endocytosed into acidic compartments. The fraction of PAA-conjugated SUV(○) and egg PC-SUV (●) found in acidic environment of the macrophages was measured by HPTS.

ing the cells with liposomes at 4°C, where endocytosis is inhibited. At 4°C ligands bind to cell surface receptors, but internalization of ligands is prevented. The extent of binding of PAA-conjugated liposomes to macrophage receptors was larger compared to that of unmodified liposomes (Fig. 3).

3.3. Exocytosis by RAW cells

The extent of release of PAA-conjugated liposomes associated with macrophages was measured with time (Fig. 4). After 12 h, approx. 13% of [^{3}H]inulin and only 3% of [^{3}H]cholether that were originally associated with the macrophages were found in the supernatant. The greater release of the aqueous probe from cells compared to the lipid probe is similar to the exocytosis behavior observed with Kupffer cells [18].

3.4. HPTS

The intracellular fate of endocytosed liposomal contents in macrophages was studied using HPTS (Fig. 5). Over 95% of the endocytosed PAA-conjugated liposomes was found in an acidic environment as opposed to 88% for egg PC liposomes in the first 3 h. The fraction of liposomes found in acidic environment is higher than the previously published values [1]. This could be attributed to different cell line and liposome composition used.

3.5. Competitive assay

To characterize the nature of receptors responsible for the uptake of PAA-conjugated liposomes, the extent of inhibition of liposome uptake by various reagents was determined (Fig. 6). Poly(C) and heparin did not compete effectively with the uptake of PAA-conjugated liposomes while dextran sulfate competed efficiently with PAA-conjugated liposome uptake. BSA did not compete with the uptake of PAA-conjugated liposomes while maleyl-BSA partially inhibited the uptake of PAA-conjugated liposomes. Both LDL and acetyl-LDL partially inhibited the uptake of PAA-conjugated liposomes, with acetyl-LDL competing more effectively than LDL at lower concentrations. Free PAA (50 μg) inhibited the uptake of 51 nmol PAA-conjugated liposomes by 46% after incubation for 12 h (data not shown). Egg PC-SUV (25 nmol) inhibited the uptake of an equimolar amount of PAA-conjugated liposomes by only 20% after 12 h incubation (data not shown). In a cross-competition experiment, PAA-conjugated liposomes competed efficiently with the uptake of acetyl-LDL by the macrophages (Fig. 7). PAA-conjugated liposomes (25 nmol) also reduced the uptake of an equimolar amount of PS-SUV by 58% after 12 h incubation (data not shown). To make sure that these inhibitors were acting by binding to the cells and not by binding to PAA-conjugated liposomes, cells were incubated with 0.35 mg acetyl-LDL at 4°C for 8 h prior to incubation with PAA-conjugated liposomes for 12 h. Subsequent binding of PAA-conjugated liposomes to the macrophages in the absence of acetyl-LDL was inhibited by 80%, suggesting that the inhibitors are acting by binding to the cells and not to the liposomes.

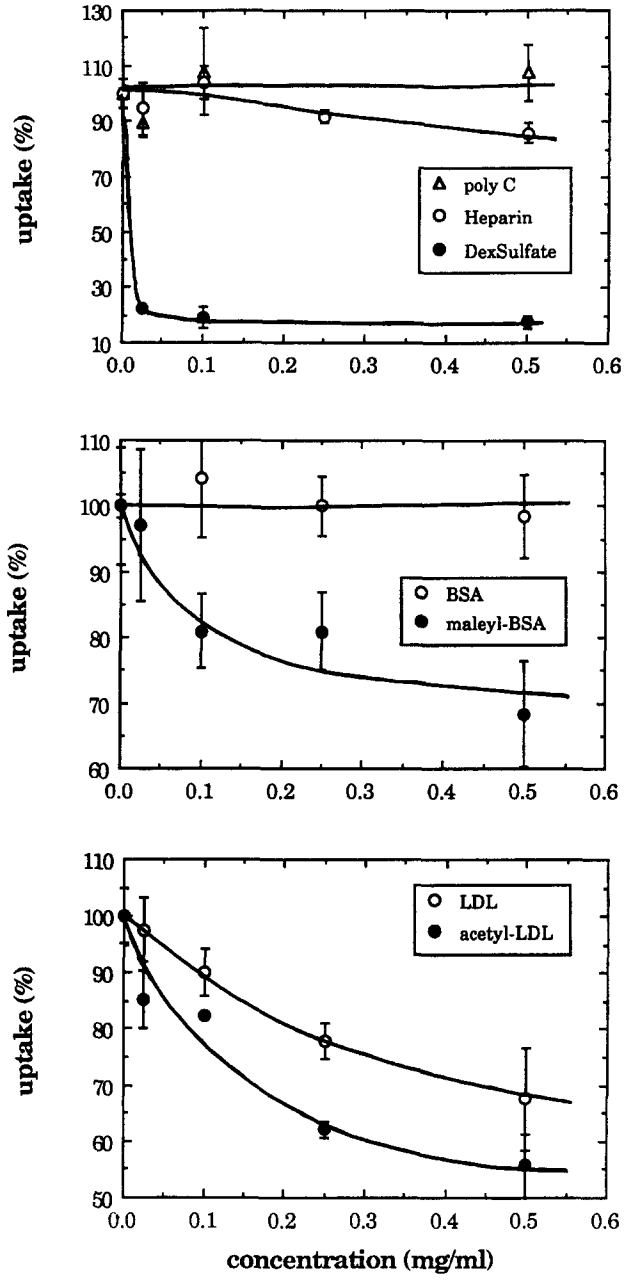

Fig. 6. Extent of inhibition of PAA-conjugated SUV uptake by various reagents. The uptake of [^{3}H]cholether labeled PAA-conjugated SUV (51 nmol PL/10^6 cells) was measured after 12 h incubation with following reagents at concentrations ranging from 0.025 to 0.5 mg/ml: acetyl-LDL, LDL, maleyl-BSA, BSA, poly C, heparin, and dextran sulfate. The concentrations of acetyl-LDL and LDL were determined by the weight of protein.

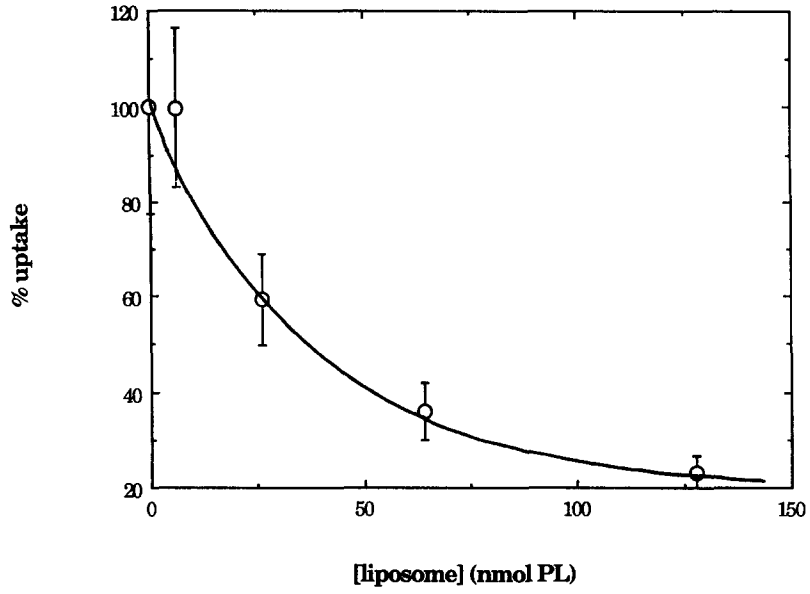

Fig. 7. Extent of inhibition of acetyl-LDL by PAA-conjugated SUV. The uptake of ^{125}I-acetyl-LDL was measured after 12 h incubation with various concentrations of PAA-conjugated SUV.

3.6. Endocytosis by CHO cells

The extent of uptake of PAA-conjugated liposomes and unmodified liposomes by CHO cells was investigated (Fig. 8). The extent of uptake of PAA-conjugated liposomes was similar to that of the unmodified liposomes. For both types of liposomes, the extent of uptake by CHO cells was much less compared to the uptake by RAW cells. About 1.1 nmol of PAA-conjugated liposomes was taken up by CHO cells after incubation with 51 nmol liposomes for 24 h. This is similar to the previous result obtained for PS liposomes showing 2 nmol uptake when 100 nmol liposomes were incubated for 24 h with CHO cells [19].

3.7. Cell cytotoxicity

The effect of PAA on cell survival was investigated to ensure that PAA did not kill the cells. Cells incubated both with and without PAA-conjugated liposomes showed greater than 98% viability after 24 h. The total cell concentration determined by protein content of the cells was the sa ıe within experimental error for both cells with and

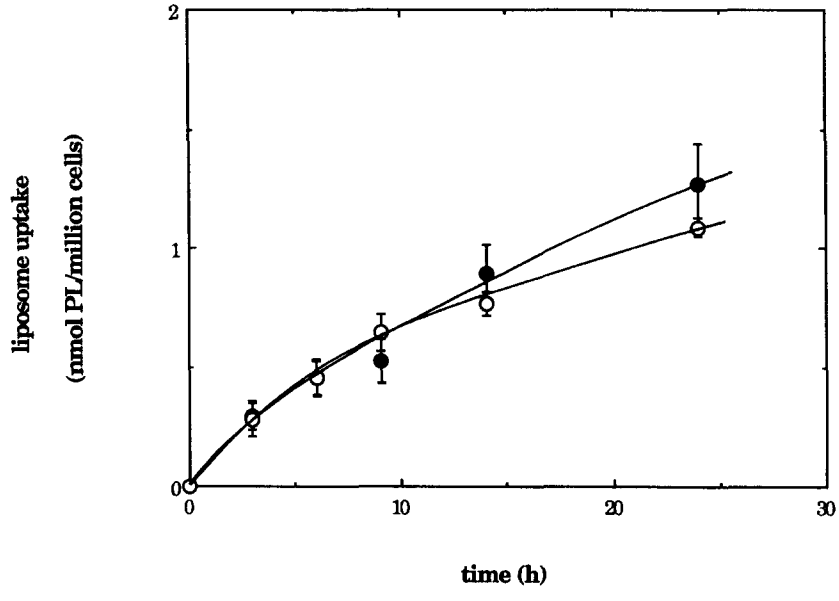

Fig. 8. Time-dependent uptake of liposomes at 37°C by CHO cells. The extent of uptake of PAA-conjugated SUV(○) and egg PC-SUV (●) was compared. Liposomes corresponding to 51 nmol PL were added to 10^6 cells in 1 ml of culture media.

without PAA-conjugated liposome incubation (data not shown).

4. Discussion

Negatively-charged liposomes were made by conjugation of negatively-charged polymer, PAA, to the liposome surface. PAA-conjugated liposomes are rapidly endocytosed by the macrophages and digested in an acidic compartment. Subsequently, part of their aqueous component is exocytosed. PAA conjugation seems to be responsible for the improved uptake of the liposomes since free PAA inhibited the uptake of PAA-conjugated liposomes but did not alter the uptake of unmodified liposomes. The improved recognition of PAA-conjugated liposomes by the macrophages is probably due to the negative charges on the polymer which are recognized by certain receptors on the macrophages. Some negatively-charged macromolecules are recognized and readily endocytosed by macrophages via scavenger receptor [20]. Acetyl-LDL, poly(I), maleyl-BSA, and sulfated polysaccharides (dextran sulfate and fucoidan) are effective ligands for scavenger receptor whereas native LDL, BSA, heparin, and poly(C) are not [20]. The scavenger receptor is involved in important biological functions of macrophages, such as the development of foam cells through uptake of acetyl-LDL [21]. The expression of scavenger receptor is limited to macrophages, monocytes, and endothelial cells [22].

Competitive assays showed that BSA, heparin, and poly(C) competed poorly while dextran sulfate competed efficiently for the uptake of PAA-conjugated liposomes by the macrophages. Acetyl-LDL and maleyl-BSA partially inhibited the uptake of PAA-conjugated liposomes. Enhanced uptake of PAA-conjugated liposomes by CHO cells, where the expression of scavenger receptors is low [19], was not observed. These data seem to suggest that one of the endocytic pathways of PAA-conjugated liposomes may be through internalization by the scavenger receptor. Unexpectedly, LDL partially inhibited the uptake of PAA-conjugated liposomes. In addition, the observed differences among the ligands in their ability to compete for the uptake of PAA-conjugated liposomes suggest that other receptors are involved in the uptake of PAA-conjugated liposomes. Previously published results indicate the complexity of the interactions of negatively-charged macromolecules with cells, and PAA seems to be no exception. Maleyl-BSA is also recognized by a second receptor distinct from the scavenger receptor [23]. Results have been published which suggest that the receptor involved in PS liposome uptake may not be the scavenger receptor as previously believed from cross-competition experiments [19]. Therefore, it is not surprising that more that one receptor and/or pathway may be involved in the endocytosis of PAA-conjugated liposomes. The observation that multiple pathways seems to be involved in the uptake of PAA-conjugated liposomes could be attributed to the range of distribution obtained in the extent of PAA conjugation. Liposomes with low conjugation of PAA may behave more like the unmodified liposomes and thus recognized by receptors other than the scavenger receptors. This may also explain the slight inhibition of PAA-conjugated liposome uptake by the unmodified liposomes. The mechanisms for the partial inhibition of PAA-conjugated liposomes by LDL are not clear. Possible explanations include competition from LDL which could have suffered oxidative damage and thus became a substrate for the scavenger receptors, interaction of LDL with PAA-conjugated liposomes, and inhibition of some of the uptake pathways of PAA-conjugated liposomes by internalized LDL imparting its effect intracellularly.

Negative charges and their presentation are important in the binding interaction of ligands with cell surface receptors. Flexibility of the polymer chain and high density of negative charges on the polymer probably contribute to the ease of recognition of PAA by the macrophages. Compared to PS liposomes, the presentation of negative charges by PAA-conjugated liposomes is expected to be different due to the random conformation of the polymer chain compared to the uniform distribution of charges on PS liposomes. The effect of spacer groups, which situate the negative charges away from the liposome surface, is believed to be also important in the recognition of liposomes by cells. A spacer group between liposomes and conjugated ligands was necessary to obtain cell association of folate-conjugated liposomes, and the extent of uptake depended on the length of the spacer group [24]. The flexible, long polymer chains probably are effective spacer groups in providing improved recognition of PAA-conjugated liposomes by the macrophages.

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References

[1] Daleke, D.L., Hong, K. and Papahadjopoulos, D. (1990) Biochim. Biophys. Acta 1024, 352–366.
[2] Lee, K.-D., Hong, K. and Papahadjopoulos, D. (1992) Biochim. Biophys. Acta 1103, 185–197.
[3] Nishikawa, K., Arai, H. and Inoue, K.J. (1990) J. Biol. Chem. 265, 5226–5231.
[4] Finter, N.B. (1973) Interferons and Interferon Inducers, p. 324, Elsevier, New York.
[5] Maeda, M., Kumano A, and Tirrell, D.A. (1988) J. Am. Chem. Soc. 110, 7455–7459.

[6] Seki, K. and Tirrell, D.A. (1984) Macromolecules 17, 1692–1698.
[7] Fujiwara, M., Grubbs, R.H. and Baldeschwieler, J.D. (1991) Polym. Prepr. 32 (1), 275.
[8] Böttcher, C.J.F., Van Gent, C.M. and Fries, C. (1961) Anal. Chim. Acta 24, 203–204.
[9] Heyward, J.J. and Ghiggino, K.P. (1989) Macromolecules 22, 1159–1165.
[10] Habeeb, A.F.S.A. (1972) Methods Enzymol. 25, 457–464.
[11] Mayer, L.D., Hope, M.J., Cullis, P.R. and Janoff, A.S. (1985) Biochim. Biophys. Acta 817, 193.
[12] Goldstein, J.L., Basu, S.K. and Brown, M.S. (1983) Methods Enzymol. 98, 241–260.
[13] Basu, S.K. et al. (1976) Proc. Natl. Acad. Sci. 73, 3178–3182.
[14] Lai, C.Y. (1977) Methods Enzymol. 47, 236–243.
[15] Takata, K., Horiudi, S. and Morino, Y. (1989) Biochim. Biophys. Acta 984, 273–280.
[16] Mishell, B. and Shiigi, S. (1980) Selected Methods in Cellular Immunology, W.H. Freeman & Co., San Francisco.
[17] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[18] Derksen, J.T.P., Morselt, H.W.M. and Scherphof, G.L. (1987) Biochim. Biophys. Acta 931, 33–40.
[19] Lee, K.-D., Pitas, R.E. and Papahadjopoulos, D. (1992) Biochim. Biophys. Acta 1111, 1–6.
[20] Krieger, M. (1992) Trends Biochem. Sci. 17, 141–146.
[21] Goldstein, J.L. et al. (1979) Proc. Natl. Acad. Sci. USA 76, 333–337.
[22] Pitas, R.E. (1990) J. Biol. Chem. 265, 12722–12727.
[23] Haberland, M.E. et al. (1989) J. Immunol. 142, 855–862.
[24] Lee, R.J. and Low, P.S. (1994) J. Biol. Chem. 269, 3198–3204.