Quantitative Analysis of Liposome–Cell Interactions in Vitro: Rate Constants of Binding and Endocytosis with Suspension and Adherent J774 Cells and Human Monocytes†

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ABSTRACT: We have characterized the parameters describing the total association (uptake) of liposomes with murine macrophage-like cell line J774 cells and human peripheral blood monocytes at 4 °C and at 37 °C with or without inhibitors of endocytosis. The uptake of neutral liposomes composed of phosphatidylcholine (PC)/cholesterol (Chol) (2:1 mole ratio) is about 10-fold lower than that of negatively charged liposomes composed of phosphatidylserine (PS)/PC/Chol (1:1:1). However, the rate of uptake of PC/Chol liposomes at 37 °C is still 10-fold higher than that by fluid-phase pinocytosis. The uptake of liposomes, which is mediated by high-affinity binding to the cell surface binding sites and subsequent endocytosis, could be simulated and predicted by model calculations employing mass action kinetics. The number of binding sites, affinity constants of binding at 37 °C and 4 °C, on- and off-rate constants of binding, and endocytic rate constants for both types of liposomes were determined. The number of binding sites and the binding constants for PS/PC/Chol liposomes binding to J774 cells is severalfold to an order of magnitude higher than that for PC/Chol liposomes, but the rate constants at which they are endocytosed following binding to the cells are similar for both liposome types. The binding of liposomes, especially PS/PC/Chol, to J774 cells and monocytes is greatly enhanced by adherence of cells to plastic substratum and is also increased by maturation/differentiation in the case of monocytes. Our quantitative analysis indicates that the binding and endocytosis of liposomes, especially PS-containing liposomes, is mediated by binding sites that have strong affinity, comparable to or about an order of magnitude smaller than other known particle–cell interactions with specific receptors such as virus and lipoproteins binding to cells.

Lipid vesicles (liposomes) have been used as model membranes to study the structure and behavior of lipid bilayers and lipid–protein interactions (Margolis, 1984; McConnell, 1983; Papahadjopoulos et al., 1990; Hong et al., 1991) as well as carriers for macromolecules to cells in vitro and in vivo (Gregoriadis, 1988; Ostro, 1987). Recent advances in liposome formulation have shown a much improved pharmaceutical efficacy over free drugs against certain tumor models in mice (Papahadjopoulos et al., 1991), but detailed knowledge of the mechanism of liposome uptake by cells and consequent clearance from blood is lacking. Understanding the interaction between liposomes and cells is one of the key issues in designing suitable liposome-carrier systems.

Many studies both in vitro and in vivo have indicated that the uptake (total association: binding and subsequent endocytosis) of liposomes is modulated to a great extent by rather specific factors. Several in vitro studies have shown different rates of liposome uptake by different cell types (Heath et al., 1985; Dijkstra et al., 1984; Straubinger et al., 1990; Daleke et al., 1990; Lee et al., 1992). In vivo localization studies of intravenously injected liposomes have shown that liposomes are preferentially taken up by certain cells. In general, mononuclear phagocytic cells in the reticuloendothelial system (RES),[1] such as Kupffer cells in liver, show an avid uptake of liposomes (Gregoriadis & Ryman, 1972; Fidler et al., 1980; Poste et al., 1982). It has been shown that negatively charged liposomes containing phosphatidylserine (PS), phosphatidylglycerol (PG), or phosphatidic acid (PA) are generally taken up faster and to a larger extent than neutral liposomes by phagocytic (or endocytic) cells in vivo or in vitro (Allen et al., 1991; Lee et al., 1992; Gabizon & Papahadjopoulos, 1992; Senior, 1987).

The uptake of liposomes is generally believed to be mediated by nonspecific association (or adsorption) of liposomes onto the cell surface and subsequent endocytosis. Although the binding sites for liposomes have not been identified, there is evidence indicating the involvement of a membrane protein(s) which may require Ca^{2+} for the binding (Pagano & Takeichi, 1977; Dijkstra et al., 1985). It has also been shown that the liposomes bound to the cell surface are internalized through coated pit-mediated endocytosis in some cells (Straubinger et al., 1983; Chin et al., 1989). It is still not clear what the determining factors are that define the uptake by various cells, although the different levels of binding for a given

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[1] Abbreviations: PS, bovine brain phosphatidylserine; PC, egg yolk phosphatidylcholine; Chol, cholesterol; PG, phosphatidylglycerol; PA, phosphatidic acid; RES, reticuloendothelial system; HPTS, pyranine (1-hydroxypyrene-3,6,8-trisulfonate); LY, Lucifer Yellow; Rho-PE, rhodamine phosphatidylethanolamine; PBS, phosphate-buffered saline containing 0.2 g/L KCl, 8.0 g/L NaCl, 0.1 g/L $CaCl_2$, and 0.1 g/L $MgCl_2$; DMEM, Dulbecco's-Modified Eagle's Medium containing 4.5 g/L glucose, 0.58 g/L glutamine, 3.7 g/L $NaHCO_3$, 0.1 g/L $CaCl_2$, and 0.1 g/L $MgCl_2$.

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liposome composition by two types of cells suggest that the binding itself maybe the controlling step and is not governed by a simple electrostatic interaction between liposome and cell membranes (Lee et al., 1992). In our previous report, we have demonstrated the importance of lipid specificity and cell type in the rate of liposome uptake. The intriguing cell type dependency shown by the comparison between J774 and CV1 cells as well as the correlation between the extent of binding and the level of uptake suggested that the step of liposome binding to the cell surface determines the overall uptake rate and thus regulates the behavior of each cell type (Lee et al., 1992).

In the current report, we characterize in detail the parameters of liposome binding and endocytosis by J774 cells, a murine macrophage-like cell line, using two types of liposomes: neutral liposomes composed of phosphatidylcholine (PC)/cholesterol (Chol) (2:1) and negatively charged liposomes composed of PS/PC/Chol (1:1:1). In terms of uptake rate by J774 cells, these two lipid compositions are the two extreme cases, and the negatively charged liposomes containing PG, PA, or phosphatidylinositol instead of PS behave similarly to PS/PC/Chol liposomes (Lee et al., 1992). We also demonstrate that cells cultured in the adherent state exhibit more binding and uptake of PS-containing liposomes than cells cultured in suspension, an observation applying both to J774 cells and to human peripheral monocytes. We have also obtained, for the first time, the equilibrium binding affinity and the kinetic rate constants of liposome–cell interactions as well as the endocytic rate constants. The analysis of the binding data has included Scatchard plots as well as determination of the on- and off-rate constants of liposome binding both at 4 °C and at 37 °C under conditions inhibiting endocytosis. We have employed a formalism based on mass action kinetics (Nir et al., 1986) that views the overall association of liposomes with cells as a two-step process: a binding step described by a second-order reaction followed by an endocytosis step described by a first-order reaction.

MATERIALS AND METHODS

Bovine brain PS, egg PC, and Rhodamine-labeled phosphatidylethanolamine (Rho-PE) were purchased from Avanti Polar Lipids (Brimingham, AL), and Chol was from Calbiochem (San Diego, CA). Lucifer Yellow (LY), for measurement of pinocytosis, and pyranine (1-hydroxypyrene-3,6,8-trisulfonate (HPTS)) were purchased from Molecular Probes (Junction City, OR).

Liposomes. The concentration of the phospholipids was determined according to Bartlett (1959). The liposomes were made by reverse-phase evaporation followed by extrusions according to the method of Szoka et al. (1980). The extrusion was through membranes of pore size 0.05 μm (Nuclepore, Pleasanton, CA) four times under high pressure argon (~100 psi). The diameters of the liposomes ranged between 80 and 110 nm as estimated by dynamic light scattering (Malvern Instruments, U.K.). The liposomes were labeled with Rho-PE at 0.2 mol % of total phospholipid. HPTS was encapsulated inside the liposomes as described previously (Straubinger et al., 1990), and the unencapsulated HPTS was removed by a gel filtration column (Sephadex G-75).

Cell Culture and Incubation with Liposomes. The J774 cells were cultured in suspension in roller bottles with Dulbecco's-Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum. For the experiments with J774 cells in monolayer, the suspension cells were plated at ~2×10^6 cells per 60-mm Petri dish two days before the incubation with liposomes. In all cases, cells were washed with serum-free DMEM before the incubation with liposomes. For all the binding experiments at 4 °C, Petri dishes (for monolayer cells) and polypropylene tubes (for suspension cells) containing the mixture of cells and liposomes were continuously shaken on ice at 100 rpm by a rotary shaker. After a given period of incubation with liposomes, the cells were washed with cold phosphate-buffered saline (PBS), and the fluorescence associated with 10^6 cells was measured in a Spex Fluorolog 2 fluorometer. The washing procedure for the suspension cells involved spinning the cells at 1000 rpm in Beckman centrifuge (200*g*) for 5 min and resuspending them in cold PBS (repeated twice). For the monolayer cells, the incubation medium was aspirated and 3 mL of cold PBS was added (repeated three times). The washed monolayer cells were suspended by treatment with PBS containing 0.2% (w/v) EDTA.

For the inhibition of endocytosis at 37 °C, the cells were preincubated for 30–60 min with the following metabolic inhibitors: (1) 1 μg/mL antimycin A (from Calbiochem, San Diego, CA) and 10 mM NaF (Dijkstra et al., 1984), (2) 0.1% sodium azide (Blumenthal et al., 1987), or (3) combination of both (1) and (2). The antimycin A was solubilized in ethanol and was added to the cells to a final ethanol concentration of 1%.

Isolation of Human Peripheral Blood Monocytes. Human peripheral blood monocytes were isolated from normal adult donors as described in Boyum (1968). Cells from human buffy coat were layered over Ficoll-Hypaque (density 1.077 g/mL, Histopaque from Sigma) and centrifuged at room temperature for 0.5 h at 400*g* to obtain the mononuclear cell band. Cells were washed, suspended in RPMI 1640 containing 10% fetal bovine serum, and left to adhere to the plastic culture dish for 1 h at 37 °C. Nonadherent cells (lymphocytes) and adherent cells (monocytes) were separated and cultured for 5–11 days. For monocyte culture, the adherent cells were suspended by treatment with cold PBS containing 0.2% EDTA and 5% fetal bovine serum. Half of the monocytes from each donor were suspended on the first day and cultured in a Teflon container for suspension culture. The other half were cultured in monolayer state (plastic culture dish) and later suspended using the same method after the indicated period of monolayer culture. In all cases, the viability of the cells was checked to be ≥95% using the dye Tryphan Blue.

Rate of Pinocytic Uptake of Solutes in Solution and in Liposomes. The fluid-phase pinocytosis by cells was measured using the fluorescent dye HPTS and was compared with the accumulation of LY, a widely used fluorescent marker of fluid-phase pinocytosis, as described previously (Swanson et al., 1985, 1987; Scieszka & Cho, 1988). After a 1-h incubation at 37 °C, the cells were washed three times with cold PBS, and the amount of solutes taken up per 10^6 cells was measured.

Mass Action Kinetic Model for Liposome Binding and Endocytosis. The process of liposome uptake mediated by endocytosis was viewed as a sequence of two steps: (i) association of liposomes with the cell surface and (ii) endocytosis itself, which involves several first-order steps. Dissociation of surface-bound liposomes can occur and was explicitly taken into account. The procedure described here follows closely that described in Nir et al. (1986) for virus binding to and fusion with cells. Notations used are as follows: The molar concentration of cells which have I surface-bound liposomes and J internalized liposomes is denoted by $A(I,J)$. The molar concentration of free liposomes is denoted by L, or $L(t)$. Initially, at time $t = 0$, $L(t) = L_0$. Mass

conservation of liposomes is expressed by

$$L_0 = L + \sum^{N}\sum^{N_E} A(I,J)\,(I+J) \quad (1)$$

in which N is the largest number of liposomes that can bind to a single cell and N_E is the largest number of liposomes that can be endocytosed per cell. Equation 1 assumes that the liposomes degraded in the cell following endocytosis are not released back into the medium or, alternatively, that the degraded liposomes are accounted for. The molar concentration of free cells is denoted by $G = A(0,0)$. Initially, $G(t) = G_0$. Mass conservation for the cells gives

$$G_0 = \sum\sum A(I,J) \quad (2)$$

In formulating and solving the set of nonlinear differential equations that describe binding and endocytosis of liposomes, we have employed only three parameters: C ($M^{-1}s^{-1}$), the rate constant of association of a liposome to the cell surface; ϵ (s^{-1}), the rate constant of endocytosis of a surface-bound liposome; and D (s^{-1}), the dissociation rate constant. The kinetics of association, dissociation, and endocytosis are described by the following equations:

$$\begin{aligned} dA(I,J)/dt = {} & CL\,A(I-1,J)\,(N+1-I)/N \\ & - CL\,A(I,J)(N-I)/N + D\,A(I+1,J)(I+1) \\ & + \epsilon\,A(I+1,J-1)(I+1) - (\epsilon + D)\,A(I,J)\,I \end{aligned} \quad (3)$$

$$dL/dt = -CL\sum\sum A(I,J)\,(N-I)/N + D\sum\sum A(I,J)\,I \quad (4)$$

The solution of these nonlinear differential equations is based on Taylor series expansion as described in Nir et al. (1986).

Forms of Scatchard Plots. Upon setting $\epsilon = 0$, eqs 3 and 4 reduce to equations describing kinetics and equilibrium of liposome binding to cells, e.g., cell association at 4 °C or at 37 °C in the presence of inhibitors of endocytosis. By defining

$$C/D = K \quad (5)$$

and setting $\epsilon = 0$, eqs 3–5 yield

$$r = (L_0 - L)/G_0 = KL/(1 + KL/N) \quad (6)$$

in which r is the number of liposomes bound per cell. This equation may be rearranged to a form of a Scatchard plot:

$$r/L = K - rK/N \quad (7)$$

whose intercept and slope are K and $-K/N$, respectively. In certain publications (e.g., Ellens et al. (1990)) a somewhat different form is employed:

$$r/L = kN - rk \quad (8)$$

Hence, $kN = K$, which will be considered in making comparisons.

Kinetics of Binding. Having determined N from a Scatchard plot at equilibrium and assuming equal rate constants for all association and dissociation processes enables simulation and prediction of binding kinetics by employing just two parameters, C and D. However, since K in eq 5 is also known from a Scatchard plot, only one parameter is needed. An estimate for the parameter C can be readily obtained for early times and small liposome concentrations from the relation (Nir et al., 1986)

$$L(t) = L_0 \exp(-G_0 Ct) \quad (9)$$

The advantage of this expression is that the parameter C can be determined independently of N and K. Indeed, for $NG_0 \gg L_0$ the binding of the liposomes does not affect the availability of binding sites for additional liposomes, so that the actual value of N does not make a difference. As in all aggregation phenomena (Nir et al., 1983), initially the deaggregation processes are of little importance; hence, the initial binding kinetics is independent of D values. Thus, fixing C from eq 9 yields the parameter D from eq 5. In the actual simulations of the kinetics of binding we employed an analytical expression (eq II 6 in Bentz et al. (1988)).

Determination of ϵ, the Rate Constant of Endocytosis. In principle, it is possible to determine all the parameters from several experiments focusing on the early time points, by determining first the parameter C from eq 9 and then the parameters ϵ and D from eqs 3 and 4. The application of inhibitors of endocytosis enables us to determine first the parameters N, K, and C and then determine the parameter ϵ from the cell association results in the absence of inhibitors.

Measurement of Endocytic Rate by HPTS. HPTS has been previously used as an aqueous content marker for the liposome uptake and endocytosis (Straubinger et al., 1990; Daleke et al., 1990). Because of spectral sensitivity to pH, this probe can report the acidification of the compartment in which liposomes are located along the endocytic pathway. After incubation of liposomes containing HPTS with cells at 37 °C for different durations, the excitation spectrum (λ_{ex} 390–460 nm; λ_{em} 510 nm) of the cell-associated liposomal HPTS was measured to determine the total amount of cell-associated liposomes and the ratio γ of $I(\lambda_{ex}$ at 450 nm$)/I(\lambda_{ex}$ at 413 nm). The fraction of liposomes inside the cells (endocytosed) out of the total cell-associated liposomes was calculated from the measured γ according to the following equation in Daleke et al. (1990):

$$\text{fraction inside} = (\gamma_{pH7.4} - \gamma_{measured})/(\gamma_{pH7.4} - \gamma_{pH6.5}) \quad (10)$$

Thus, the total cell-associated liposomes were decomposed into two populations: L(surface), liposomes bound to the cell surface, and L(inside), liposomes internalized inside the cells. The kinetic equation

$$d[L(\text{inside})]/dt = \epsilon L(\text{surface}) \quad (11)$$

provided another means to calculate ϵ, the rate constant of endocytosis, as has been described previously for other systems (e.g., Wiley and Cunningham (1982)). If L(surface) stays relatively constant over time, eq 11 yields $\epsilon\Delta t = \Delta[L(\text{inside})]/L(\text{surface})$.

RESULTS AND DISCUSSION

Role of Fluid-Phase Pinocytosis in Liposome Uptake. In our recent report on the interaction of liposomes with cells in vitro (Lee et al., 1992), we showed that the uptake by J774 cells of neutral liposomes composed of PC/Chol (2:1) was very low compared with the uptake of negatively charged liposomes composed of PS/PC/Chol (1:1:1). It has been demonstrated that the inclusion of PS in the lipid bilayer of neutral liposomes PC/Chol induced increased binding and consequently a high level of total uptake of the liposomes (Nishikawa et al., 1990; Lee et al., 1992). However, it is not clear whether the low level uptake of PC/Chol liposomes is mediated by random entrapment of liposomes in the pinocytic vesicles. To address this question, we compared the uptake of aqueous contents of liposomes with the fluid-phase uptake of solutes mediated by pinocytosis.

The pinocytic uptake of free HPTS in solution was measured as a function of the total amount of HPTS added in the incubation medium. The amount of free HPTS taken up per cell was linearly dependent on the concentration of HPTS in

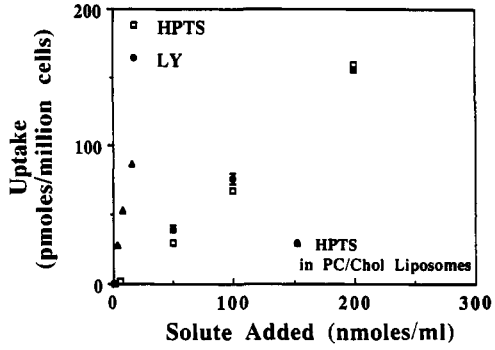

FIGURE 1: Rate of uptake by fluid-phase pinocytosis vs liposome uptake. The rate of fluid-phase pinocytosis in J774 cells is compared with the liposome (PC/Chol) mediated uptake of solutes by adherent J774 cells. The J774 cells in monolayer culture were washed with serum-free DMEM and incubated with varying concentrations of HPTS or Lucifer Yellow. After a 1-h incubation at 37 °C, the cells were washed with cold PBS three times and solubilized in 0.2% Triton X-100 for the measurement of the amount of dye taken up via fluid-phase pinocytosis. The ▲ represents the uptake of HPTS encapsulated in PC/Chol liposomes added at lipid concentrations of 25, 50, 100 nmol/mL, which corresponds to an uptake rate of 7.4 pmol/10^6 cells per 1 h per 1 nmol of HPTS added in 1 mL. When HPTS was in PS/PC/Chol liposomes at 25 nmol of phospholipid in 1 mL, the uptake was 505 pmol/10^6 cells per 1 h, which is beyond the scale of the graph.

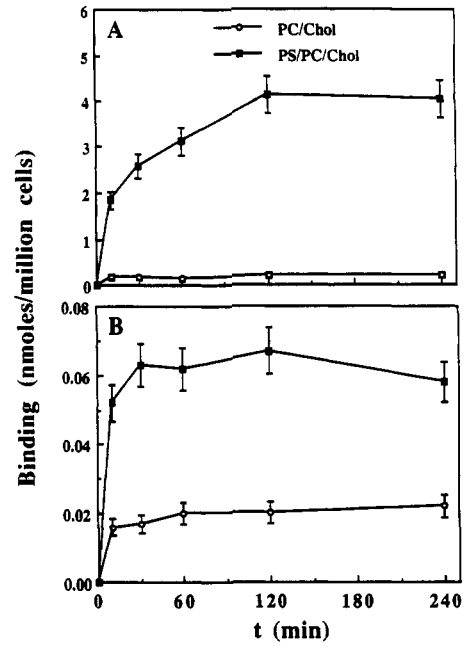

FIGURE 2: Kinetics of liposome binding to J774 cells, adherent and in suspension. J774 cells were grown in suspension in a roller bottle in DMEM containing 10% fetal bovine serum. Half of the cells were plated for adherence two days before the binding study (A), and the other half were kept in suspension (B). Liposomes composed of PS/PC/Chol (1:1:1) or PC/Chol (2:1) were labeled with Rho-PE (0.2 mol %). Both types of liposomes were added at 50 nmol/mL phospholipid concentration to the cells (1×10^7 cells/mL in suspension, and $\sim 3 \times 10^6$ cells per Petri dish) and incubated for different periods of time at 4 °C. The cell association of liposomes is expressed as nanomoles of total phospholipid bound per million cells.

bulk solution (Figure 1), which is one of the criteria that the solute is taken up via fluid-phase pinocytosis. On average, 72 pmol of HPTS was taken up by 10^6 J774 cells in monolayer culture per 1 h per 100 nmol of HPTS added in 1 mL at 37 °C. The pinocytic rate obtained using HPTS was confirmed by similar results obtained with another fluorescent marker, Lucifer Yellow (LY) (see Figure 1), which has been used extensively as a marker for pinocytosis previously. The pinocytic uptake rate of LY in J774 cell has been reported to be 2-fold higher than that by thioglycollate-elicited mouse peritoneal macrophages (Swanson et al., 1985, 1987), and that corresponds to ~64 pmol/10^6 J774 cells per 1 h at 100 nmol of LY in 1 mL using the conversion of 0.23 mg of protein/10^6 J774 cells. Our pinocytic uptake rate obtained by HPTS or LY is in good agreement with their value.

When HPTS was encapsulated inside PC/Chol liposomes and incubated with J774 cells, a 10.4-fold enhanced uptake of HPTS was observed when compared with free HPTS uptake via pinocytosis. When 25 nmol of PC/Chol liposomes was added per 1 mL of medium, it corresponded to 3.8 nmol of HPTS/mL. Therefore, the uptake rate of HPTS encapsulated in PC/Chol liposomes was 7.4 pmol/10^6 cells per hour when added at 1 nmol of HPTS in 1 mL (Figure 1). Thus, the uptake of solutes encapsulated in PC/Chol liposomes was an order of magnitude higher than the uptake of solutes mediated by fluid phase pinocytosis, 0.72 pmol/10^6 cells per hour per nanomole of HPTS in 1 mL (Figure 1). Likewise, the uptake of PS/PC/Chol liposomes was 187-fold higher than the pinocytosis-mediated uptake of solutes by these cells. There exists a possibility that the efflux of solutes after fluid-phase pinocytic uptake is larger than the efflux of the same solutes in the liposomes. However, the efflux rate of LY, especially in J774 cells, during the initial 1 h is small, and the total influx rate of solutes through fluid-phase pinocytosis is not larger than twice the net uptake (influx minus efflux) rate (Swanson et al., 1987). Therefore, the uptake of liposomes is significantly faster than the uptake of solutes by fluid-phase pinocytosis even after we account for the possibility that the processing of solutes after uptake may be altered when the solutes are presented in a different form. This comparison of the uptake of PC/Chol liposomes or PS/PC/Chol liposomes with the pinocytic rate demonstrates that the uptake of liposomes via fluid-phase pinocytosis contributes only a small portion to the total uptake and the majority of liposome uptake is mediated by a mechanism different from the fluid phase pinocytosis.

Binding to Adherent vs Suspension Cells. We have shown previously that the binding is an important step in the overall uptake of liposomes (defined as binding followed by endocytosis) in J774 cells cultured in monolayer state. Figure 2A shows the binding kinetics of liposomes, PC/Chol and PS/PC/Chol, to monolayer cells at 4 °C. The binding of PS-containing liposomes to J774 cells proceeded very rapidly during the initial 10–30 min and then increased steadily up to 2 h of incubation time, reaching seemingly an equilibrium. In contrast, the binding of PC/Chol liposomes seemed to have reached an equilibrium within the initial 30 min.

We next studied the binding of these liposomes to J774 cells grown in suspension. The increased bining of PS-containing liposomes over PC/Chol liposomes shown above with adherent J774 cells was not as pronounced in suspension J774 cells (Figure 2B). There was ~20-fold enhanced binding of PS/PC/Chol liposomes over the binding of PC/Chol liposomes to adherent J774 cells, but there was only ~2–6-fold enhanced binding of PS over PC liposomes to suspension J774 cells. The overall binding of either liposomes to the suspension cells was dramatically less than to the adherent cells. The adherent cells showed ~50-fold more binding of PS-containing liposomes than the suspension cells and ~10-fold more of PC/Chol liposomes. Therefore, a higher level of liposome binding seemed to be induced by letting J774 cells adhere to plastic Petri dishes, but this induction was 5 times more for the liposomes containing PS. It suggests that the number of high-affinity binding sites for PS-containing

Table I: Uptake of PS/PC/Chol Liposomes vs PC/Chol Liposomes by J774 Cells and Human Peripheral Blood Monocytes Cultured in Suspension and in Adherence[a]

cells	culture condition (incubation °C)	ratio of cell-associated liposomes per 10^6 cells (PS/PC/Chol over PC/Chol)
J774 cells	suspension[b] (4 °C)	1.9
	adherent[b] (4 °C)	3.6
	suspension (37 °C)	2.2
	adherent (37 °C)	6.9
donor A	7 days	
monocytes	suspension (37 °C)	9.3
	adherent (37 °C)	23.8
lymphocytes	suspension (37 °C)	8.1
donor B	9 days	
monocytes	suspension (4 °C)	10.4
	suspension (37 °C)	8.6
	adherent (4 °C)	35.6
	adherent (37 °C)	43.1
lymphocytes	suspension (4 °C)	9.6
	suspension (37 °C)	12.8
donor C	5 days	
monocytes	suspension[c] (4 °C)	4.1
	suspension (37 °C)	3.6
	adherent (4 °C)	7.0
	adherent (37 °C)	16.2
	11 days	
monocytes	suspension[d] (4 °C)	24.8
	suspension (37 °C)	21.2
	adherent (4 °C)	30.7
	adherent[e] (37 °C)	20.0
lymphocytes	suspension (4 °C)	9.0
	suspension (37 °C)	20.6

[a] After being cultured in a roller bottle, half of the J774 cells were transferred to a Teflon container and grown as suspension cells for two days and the other half were adhered to a plastic culture dish and resuspended after two days by 2 mM EDTA containing cold PBS, and EDTA was removed by washing with incubation medium. The amount of cell-associated liposomes after 3 h of incubation at 50 nmol/mL phospholipid concentration both at 4 °C and at 37 °C was measured, and the ratio of cell-associated PS/PC/Chol liposomes over PC/Chol liposomes was calculated. The ratios were measured both at 4 °C and at 37 °C. In all cases, 2×10^6 cells were incubated in suspension, regardless of whether they were cultured in suspension or in adherence, in 1 mL of medium (DMEM for J774 cells and RPMI 1640 for monocytes and lymphocytes) in polypropylene tubes. Each experiment was done in duplicate, and the percent error within the duplicate was <15% of the mean. [b] The mean uptake of PC/Chol liposomes by J774 cells in suspension was ~0.04 nmol/10^6 cells. [c,d,e] The mean uptake of PC/Chol liposomes by monocytes was ~0.03 nmol/10^6 cells. The lowest uptake of PC/Chol liposomes was 0.01 nmol/10^6 cells (*c*) and ~0.01 nmol/10^6 cells (*d*), and the highest was 0.04 nmol/10^6 cells (*e*).

liposomes may be preferentially expressed in J774 cells in monolayer during the culture for two days in the adherent state.

In order to investigate the possibility that the binding of PS-containing liposomes is regulated by culturing the cells differently, and also to eliminate the possibility that this difference in the binding may be due to the difference in incubation and washing conditions, we adopted a procedure to prepare J774 cells in suspension with a different history of culture. First, the J774 cells grown in roller bottles were split into two: half were cultured in Teflon containers as suspension cells, and the other half were plated to plastic culture dishes. After two days, the cells grown in plastic dishes were suspended by treatment with cold PBS containing EDTA. The induction of enhanced binding of PS-containing liposomes is best demonstrated by plotting the ratio PS/PC/Chol liposomes bound per cell over PC/Chol liposomes bound per cell. Table I shows that this ratio for the cells cultured in monolayer is twice that for the cells grown in suspension when the incubation was at 4 °C. The absolute value of liposome binding may vary depending on the cell surface area when the culture condition is changed. It could also depend on daily variations in the cells. However, when the binding of PS-containing liposomes is expressed relative to the binding of PC liposomes, the change in that ratio reflects a specific regulation of binding sites on cell surface.

Binding of Liposomes to Human Peripheral Monocytes/Macrophages. The regulation of liposome binding by the culture conditions, as implied from the results in the preceding section with adherent and suspended J774 cells, was assessed with monocytes isolated from human peripheral blood cells. The level of binding of the two types of liposomes to human peripheral blood monocytes was measured and compared with the results from J774 cells. Monocytes cultured for 5–11 days in different states, one in suspension and the other in adherent state, showed different levels of binding and uptake of PS/PC/Chol liposomes relative to PC/Chol liposomes. Differences in binding depended on the number of days in culture as well as on the condition of culture. Table I shows that the monocytes cultured in adherent state always exhibited a higher binding ratio of PS/PC/Chol to PC/Chol liposomes than the monocytes cultured in suspension. However, this ratio depended on the possible maturation/differentiation of monocytes in culture since the ratio increases from 5 to ~30–40 as a function of days of culture, irrespectively of whether the cells were cultured in suspension or as adherent. In contrast, the lymphocytes did not show this dependency on the culture duration as this ratio in lymphocytes stayed ~10. Overall, the extent of liposome binding by lymphocytes was 2–3-fold less per cell than the extent by monocytes. Also, the extent of liposome binding by monocytes in suspension culture was at a level similar to that by J774 cells grown in suspension.

On the basis of these data, we conclude the following: The binding and uptake of PS-containing liposomes by monocytes in suspension increases ~5-fold as the peripheral monocytes mature and differentiate into macrophages whereas the binding and uptake of PC liposomes stays relatively constant during this process. The rate of this maturation/differentiation and the increase in the binding and uptake of PS-containing liposomes depend on the culture condition; they are shown to be facilitated by the attachment of monocytes to plastic, which bears similarities to the maturation of circulating blood monocytes into fixed tissue macrophages of RES and also to the extravasation into inflammation sites and activation. Lymphocytes are in general less active in binding and taking up liposomes and also do not show much change in their ability of liposome uptake over 10 days of culture.

The above findings might give us a better explanation of the fate of liposomes in vivo upon intravenous injection. When liposomes are introduced into the blood stream, one of the important factors that determines the site of clearance may well be the competition for liposome binding among the cells that the liposomes encounter. As it has been shown that the mononuclear phagocyte system plays a major role in the clearance of liposomes, the most important factor in the determination of the site of liposome clearance may be the competition between the resident macrophages in liver and spleen and the circulating cells in the blood, especially peripheral blood monocytes. The contribution from lymphocytes is small, as we have shown that they exhibit less binding and uptake of liposomes than monocytes. The binding of liposomes to the mature and differentiated resident macrophages fixed in the sinusoids of RES could be at least severalfold larger than the binding to the circulating peripheral

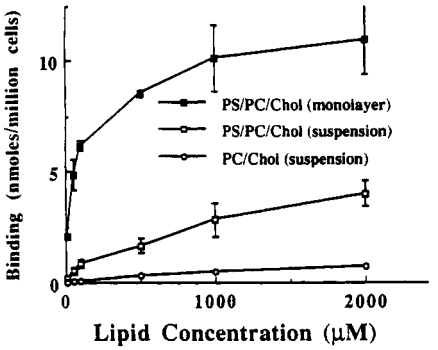

FIGURE 3: Equilibrium binding of liposomes as a function of liposome concentration. The amount of liposomes bound per 10^6 J774 cells (expressed as nanomoles of total phospholipid bound per million cells), both in suspension and in monolayer, after 3 h of incubation at 4 °C was measured as a function of liposome concentration. The curve for the binding of PC/Chol liposomes to cells in monolayer was indistinguishable from that to cells in suspension in this scale (not shown). The liposomes were labeled with Rho-PE.

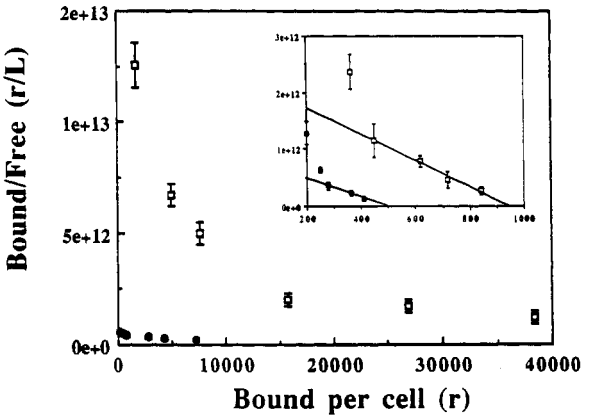

FIGURE 4: Scatchard plots for the binding of liposomes to J774 cells in suspension at 4 °C. Liposomes, PS/PC/Chol (square), and PC/Chol (circle), were incubated with 5×10^6 J774 cells in 1 mL of DMEM medium for 3 h at 4 °C at lipid concentrations between 10 and 2000 μM, and the results were analyzed by Scatchard plots (see Materials and Methods section for details). The inset shows approximately linear Scatchard plots with lower liposome/cell ratios for lipid concentration between 20 and 200 μM incubated with 10^7 J774 cells/mL.

blood monocytes. As will be shown later in the case of J774 cells, the endocytic rate of liposomes in adherent cells is severalfold larger than that in suspension cells, which makes the total uptake (binding plus endocytosis) by the fixed macrophages significantly higher than the uptake by the monocytes. This is in accord with the observation that most liposomes end up in the lysosomes of resident macrophages of the liver and spleen rather than being taken up by the peripheral blood monocytes. The PS-containing liposomes may bind to the adherent resident tissue macrophages ~30-fold more than the neutral PC/Chol liposomes; therefore, the clearance rate of liposomes containing PS is as expected very fast in comparison with that of neutral liposomes.

This preferential increase in the binding of PS-containing liposomes over PC liposomes by monocytes as well as by J774 cells bears similarity to the adherence-induced selective expression of various proteins in monocytes, such as several monokines and protooncogenes (Haskill et al., 1988; Kaplan & Gaudernack, 1982), as well as the expression of a number of cell-surface receptors including transferrin receptors (Taetle & Honeysett, 1988) upon monocyte maturation and differentiation. Also, an enhanced recognition of tumor cells by activated human blood monocytes has been shown to have a correlation to an increased expression of PS on the tumor cell surface (Utsugi et al., 1991). Moreover, it has been proposed previously that macrophages recognize and remove senescent erythrocytes via the increased expression of PS on the outer leaflet of erythrocyte membranes (Schroit et al., 1985; Allen et al., 1988; Connor et al., 1989). Thus, the increased capacity of monocytes to bind PS-containing liposomes upon adherence or maturation/differentiation is certainly supporting the possibility that the binding sites of PS-containing liposomes, like other known cellular proteins, are composed of cell surface proteins which are modulated by the processes of monocyte activation and maturation/differentiation.

Equilibrium Binding to J774 Cells at 4 °C. Figure 3 shows the binding of liposomes of both lipid compositions as a function of concentration of liposomes. The amount of liposomes bound at equilibrium per 10^6 cells in the adherent state and in suspension was monitored after 4 h of incubation at 4 °C (Figure 3). The binding of PS-containing liposomes to the monolayer cells exhibited a classical saturation curve reminiscent of specific binding. At saturation, ~11 nmol of phospholipids was bound per 10^6 cells, which corresponds to 77 000 liposomes bound per cell. This calculation was based on the assumption that the average diameter of unilamellar liposomes was 85 nm with an area per phospholipid in the bilayer of 0.6 nm^2. The binding of PS-containing liposomes to suspension cells showed a linear increase as the liposome concentration was increased up to 100 nmol/mL. Beyond that concentration, the binding seemed to be approaching saturation, but it never showed a complete plateau. The slow increase in binding at high concentration of liposomes may be due to rather nonspecific adsorption of the liposomes or binding to the lower affinity binding sites. By comparison, the highest number of PS/PC/Chol liposomes bound per suspension cell was ~28 000. In contrast, approximately ~5000 or less PC/Chol liposomes were observed to be bound either to a suspension or to a monolayer cell with the highest concentration of liposomes we investigated; the difference between adherent and suspension cells is minimal in terms of PC/Chol liposome binding when high concentrations of PC/Chol liposomes were used, whereas adherent J774 cells bound severalfold more PC/Chol liposomes than suspension cells at low liposome concentrations (≤~200 μM phospholipid concentration). Thus, the total number of binding sites for PC/Chol liposomes remained approximately the same when cells changed from suspension to adherent state, while the number of binding sites for PS-containing liposomes increased about 3-fold.

Equilibrium and Kinetic Parameters of Liposome Binding and Uptake. We have determined the affinity constant (K) of PC/Chol and PS/PC/Chol liposomes for their binding to J774 cells in suspension as well as both the on- and off-rates (C and D) of the binding. First, the equilibrium binding, such as the results in Figure 3, was analyzed by Scatchard plots, and the initial estimates of the affinity constant K and the number of binding sites N were obtained from these analyses to be utilized for the calculation of C and D by a mass action model. Representative Scatchard plots for PS/PC/Chol and PC/Chol liposomes with J774 cells in suspension are shown in Figure 4. The inset of Figure 4 shows approximately linear portions of Scatchard plots when the lipid concentrations were between 20 and 200 nmol/mL. These conditions were used for the detailed study of binding in the following sections (see Table II for a summary of different experiments). However, the Scatchard plots for the wide range of lipid concentrations (between 5 and 2000 nmol/mL) exhibited deviations from linearity. At high liposome concentration (≥400 nmol/mL),

Table II: Parameters Describing Liposome Binding to and Endocytosis by J774 Cells[a]

experiment no.	temp (°C)	ϵ(s^{-1}), endocytic rate constant	C (M^{-1} s^{-1}), on-rate constant	D (s^{-1}), off-rate constant	K (M^{-1}), affinity constant	N, no. of binding sites
PS/PC/Chol						
1	4		3.7×10^9	0.001	3.7×10^{12}	3200
2			1×10^9	0.001	1×10^{12}	1800
3			6×10^8	0.0006	1×10^{12}	3000
4					2.2×10^{12}	1000
5					1.4×10^{13}	11000
2	37	0.0025	1.3×10^9	0.0004	3.2×10^{12}	3000
3[b]		0.001	2.3×10^9	0.0009	2.6×10^{12}	3300
6[c]		0.00024				
7[c,d]		0.0014				
PC/Chol						
1	4		1.5×10^8	2.3×10^{-4}	6.5×10^{11}	300
2			3.5×10^8	7×10^{-4}	5×10^{11}	300
3			2.5×10^8	2.5×10^{-4}	1×10^{12}	350
4					8×10^{11}	500
5					5.7×10^{11}	2900
2	37	0.002	4×10^8	8×10^{-4}	5×10^{11}	300
3[b]		0.001	1.5×10^8	0.0011	1.4×10^{11}	350

[a] The cells used were J774 cells in suspension at the concentration of 10^7/mL unless otherwise stated. The label for the liposomes was Rho-PE, except for the cases specified if HPTS was used. The estimated uncertainties in the parameters N, C, D, and ϵ are 30%, 10%, 50%, and 50%, respectively. [b] The details of the calculations for these cases are shown in Table IV. [c] The endocytic rate constants for these cases were obtained experimentally using HPTS-containing liposomes (see Figure 7). [d] The endocytic rate constant for this case was for adherent J774 cells using HPTS-containing liposomes.

the slope ($-k = -K/N$) of the Scatchard plot decreased, implying a correspondingly lower affinity of liposome binding under the condition of a high number of liposomes bound per cell. This behavior may be possibly due to the steric hindrance from the already bound vesicles on the cell surface, thus reducing the access of liposomes to the binding sites, as has been suggested previously for binding of antibodies to multivalent antigens (Cowan & Underwood, 1988). It is also conceivable that there may exist at least two different liposome binding sites, one with a high-affinity and the other with a low-affinity constant. A nonlinear Scatchard plot has been shown experimentally in the other systems with heterogeneous receptors (e.g., Eldar et al. (1990)), and a computer simulation of binding of liposomes to the cells with two affinity binding sites indeed demonstrates a curved Scatchard plot (S. Nir, R. Peled, and K.-D. Lee, unpublished results). Similarly, the Scatchard plots with adherent J774 cells for both types of liposomes also exhibited biphasic curvilinear behaviors (data not shown). For PS/PC/Chol liposomes, it is estimated that an adherent cell has 24 000 high-affinity binding sites ($k = 3 \times 10^9$ M^{-1}) and 39 000 low-affinity binding sites ($k = 3 \times 10^8$ M^{-1}), which yields K equal to 7.1×10^{13} M^{-1} and 1.2×10^{13} M^{-1}, respectively. For PC/Chol liposomes, an adherent J774 cell has 500 high-affinity binding sites ($k = 3 \times 10^9$ M^{-1}) and 3600 low-affinity binding sites ($k = 4.4 \times 10^7$ M^{-1}). We note, however, that in most cases, especially with suspension cells, the corresponding numbers of binding sites are severalfold lower (see Table II).

With the estimation of K and N from the Scatchard analysis, we further characterized the parameters of liposome binding to suspension J774 cells in detail using a mass action kinetic model. In Figure 5, the binding data at 4 °C are shown for several concentrations of liposomes as a function of incubation time. These data were fitted to the mass action model as described in the Materials and Methods section. We have first determined the parameters K and N from Scatchard plots. Then, an initial guess was made for the parameter C from the binding results with the most dilute vesicle concentrations at the earliest time points (see eq 9). Refinements were done by changing the parameters, although in most cases the values of parameters that achieve the best fit for the kinetics

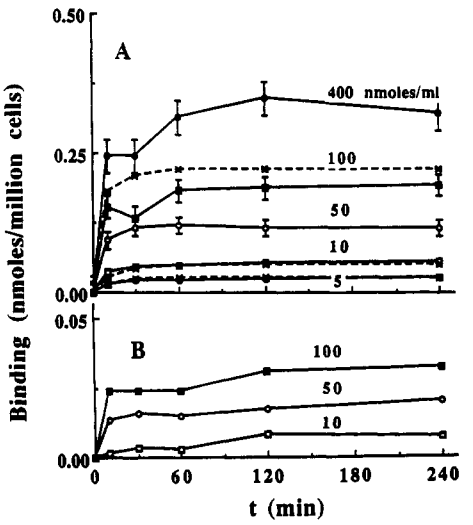

FIGURE 5: Kinetics of liposome binding to J774 cells in suspension. The amount of liposomes (labeled with Rho-PE) bound to 10^6 cells in suspension is given as a function of time of incubation at 4 °C with varying concentrations of liposomes for (A) PS/PC/Chol and (B) PC/Chol liposomes. In (A), the dotted lines show the simulated kinetic curves of liposome binding obtained from the calculations employing the mass action kinetic model. The parameters for the fit shown here are $C = 3.7 \times 10^9$ M^{-1} s^{-1}, $D = 0.001$, and $N = 3200$. The estimated uncertainties in N, C, and D are 30%, 10%, and 50%, respectively.

of binding were close to the initial estimation. The parameter D was obtained from eq 5. Representative examples of the calculated kinetics of liposome binding are shown in Figure 5A, and the determined parameters of binding are summarized in Table II.

The strength of the binding between a liposome and a cell is expressed by the equilibrium constant K. The affinity constant K between a liposome and a cell is equal to the product of k, the affinity of binding between a liposome and a binding site which might involve several receptors, and N, the number of these binding sites in a cell; thus $K = kN$ as in eq 8. Table II indicates that at 4 °C the values of K $(0.1–1.4) \times 10^{13}$ M^{-1}, for liposomes composed of PS/PC/Chol are between 2- and 14-fold larger than those found for PC/Chol liposomes. At

37 °C, we found by the use of inhibitors of endocytosis K values around 3×10^{12} M^{-1} for PS/PC/Chol liposomes, and the corresponding values for PC/Chol liposomes are 6–18-fold smaller (see the next section). For comparison, Sendai virus binding to erythrocyte ghosts (at 37 °C) yields $K = (0.6–3) \times 10^{12}$ M^{-1} (Nir et al., 1986), whereas its binding to the larger HL-60 cells yields $K = 10^{12}–10^{13}$ M^{-1} (Pedroso de Lima et al., 1991). For influenza virus binding to the latter cells at neutral pH, the value of K is larger than 10^{13} M^{-1} (Düzgünes et al., 1992). The binding of large glycophorin-bearing liposomes to fibroblast cells expressing the influenza hemagglutinin gave $k = 7 \times 10^{10}$ M^{-1} (Ellens et al., 1990), which according to eq 8 and $N = 1700$ yields $K = 1.2 \times 10^{14}$ M^{-1}. Hence, the strength of binding of PS-containing liposomes to cells is comparable to the binding of certain viruses to cells but is about an order of magnitude below the high-affinity binding of glycophorin to influenza hemagglutinin. The number of binding sites N for virus has been estimated in the range of several thousands (Düzgünes et al., 1992); hence, it is generally comparable to that found here for liposome binding. It is also interesting to note that the affinity constant of liposomes binding to the cells is comparable to, although severalfold to an order of magnitude lower than, that of lipoproteins binding to their cell-surface receptors. The affinity constants K for the interaction of low density lipoproteins and apo-E high density lipoproteins with human fibroblasts can be calculated to be $\sim 3.6 \times 10^{13}$ and 2.3×10^{14} M^{-1}, respectively, from the k and N values of Goldstein and Brown (1977) and Pitas et al. (1979). The affinity constants K of acetylated low density lipoproteins to scavenger receptors in smooth muscle cells and murine macrophages range from 8×10^{12} to 2×10^{13} M^{-1} (R. E. Pitas, personal communication). The rate constant C of association of PS-containing liposomes to J774 cells is 2–10-fold larger than for neutral PC/Chol liposomes. Yet, it is 1–2 orders of magnitude below the values found for virus binding to cells (Nir et al., 1990; Pedroso de Lima et al., 1991; Düzgünes et al., 1992), although it is close to the association rate of lipoprotein particles to their cell-surface receptors (Pitas et al., 1979).

Binding vs Total Uptake. The kinetics of total association (uptake) of liposomes with cells was significantly faster at 37 °C than at 4 °C (Figure 6). This uptake at 37 °C was dramatically reduced by metabolic inhibitors strongly suggesting that endocytosis following binding at 37 °C accelerates the total uptake under this condition. Figure 6 and Table III demonstrate that during a 1-h incubation the total cell association at 37 °C in the presence of inhibitors was similar to the total cell association at 4 °C, and it is probably the liposomes bound to the cell surface without being endocytosed.

The effectiveness of the inhibition of endocytosis was investigated by using HPTS-containing liposomes. As described in the Materials and Methods section, we can monitor the acidification of liposomal aqueous content from the dependence of HPTS excitation spectrum on the pH and thus calculate the extent of endocytosis (Straubinger et al., 1990; Daleke et al., 1990). Table III shows the fraction endocytosed out of total cell-associated liposomes under different conditions of incubation as calculated by this method. At 37 °C, the total uptake of liposomes was enhanced as a significant fraction of the cell-associated liposomes was endocytosed and acidified (Table III). Lowering the incubation temperature to 4 °C blocks endocytosis completely as the average pH of the liposomal contents remains neutral. Antimycin A plus NaF with and without sodium azide also inhibited 100% of the endocytosis. Sodium azide alone blocked the endocytosis of

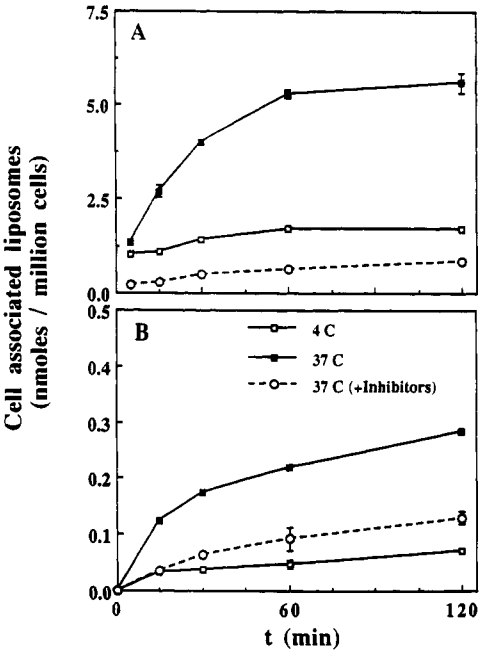

FIGURE 6: Kinetics of cell association of liposomes at 37 °C and at 4 °C. J774 cells in monolayer (A) and in suspension (B) were incubated with PS/PC/Chol liposomes labeled with Rho-PE (50 nmol/mL) both at 4 °C (open squares) and at 37 °C (closed squares), and the amount of liposomes bound to 10^6 cells was monitored as a function of time and expressed as nanomoles of total phospholipid bound per million cells. The dotted curve (circles) shows the binding at 37 °C with metabolic inhibitors (antimycin A plus NaF).

Table III: Effect of Metabolic Inhibition on the Uptake of Liposomes[a]

	30-min incubation		60-min incubation	
incubation condition	cell-associated liposomes[b]	% endocytosed[c]	cell-associated liposomes[b]	% endocytosed[c]
37 °C	192 (±8)	59	265 (±5)	75
4 °C	75 (±11)	0	66 (±3)	0
37 °C with antimycin + NaF	61 (±6)	0	84 (±4)	0
antimycin + NaF + Azide	62 (±3)	0	88 (±10)	0
azide	91 (±2)	20	130 (±5)	25

[a] Liposomes containing HPTS (PS/PC/Chol, 50 nmol/mL) were incubated with 10^7 cells/mL at 4 °C, at 37 °C with antimycin A plus NaF, at 37 °C with sodium azide (0.1%), and at 37 °C with antimycin A plus NaF and azide for 30 min and 1 h, and the amount of cell-associated liposomes for each case was compared with that at 37 °C without any metabolic inhibitors. [b] The amount of cell association is expressed as picomoles of total phospholipid taken up per 10^6 cells. [c] The column % endocytosed represents the percent of liposomes internalized at the end of each incubation as calculated from HPTS excitation spectra.

liposomes incompletely as 20–25% of the cell-associated liposomes was calculated to be endocytosed. Therefore, the combination of antimycin A and NaF was employed to calculate the parameters of binding at 37 °C under the condition of no endocytosis.

As will be seen in Tables III and IV and Figure 7, the number of liposomes on the cell surface at 37 °C reaches a steady-state value which is similar to the equilibrium binding value at 4 °C or at 37 °C without endocytosis. This implies that the number of binding sites on the cell surface remains relatively constant despite the internalization of the liposome-binding site complex, most likely due to a fast recycling of the binding sites back to the cell surface after endocytosis.

Table IV: Experimental and Calculated Kinetics of Liposome Binding and Endocytosis at 37 °C[a]

liposome composition and concentration (nmol/mL)	inhibitors[b]	time of incubation (min)	% of liposomes cell-associated exptl	% of liposomes cell-associated calcd[c]	% of liposomes at cell surface calcd[c]	% of liposomes endocytosed calcd[c]
PS/PC/Chol						
10	–	15	3.7 (±0.1)	2.2	1.4	0.8
		30	4.6 (±0.6)	3.7	1.5	2.2
		60	5.8 (±0.1)	6.4	1.5	4.9
	+	15	1.9 (±0.4)	1.5	1.5	
		30	2.0 (±0.9)	2.1	2.1	
		60	2.5 (±0.1)	2.4	2.4	
50	–	15	2.4 (±0.2)	1.9	1.2	0.7
		30	3.5 (±0.1)	3.2	1.3	1.9
		60	4.5 (±0.3)	5.7	1.3	4.4
	+	15	0.8 (±0.2)	1.3	1.3	
		30	1.2 (±0.2)	1.7	1.7	
		60	2.2 (±0.7)	1.9	1.9	
100	+	15	0.9 (±0.1)	1.2	1.2	
		30	1.3 (±0.2)	1.4	1.4	
		60	1.6 (±0.1)	1.5	1.5	
PC/Chol						
50	–	15	0.22 (±0.1)	0.15	0.09	0.06
		30	0.32 (±0.1)	0.25	0.09	0.16
		60	0.43 (±0.1)	0.43	0.1	0.33
	+	15	0.11 (±0.1)	0.13	0.13	
		30	0.15 (±0.1)	0.16	0.16	
		60	0.19 (±0.1)	0.17	0.17	
100	–	15	0.09 (±0.02)	0.13	0.08	0.05
		30	0.21 (±0.03)	0.22	0.09	0.13
		60	0.31 (±0.02)	0.38	0.09	0.29
	+	15	0.06 (±0.03)	0.11	0.11	
		30	0.09 (±0.02)	0.13	0.13	
		60	0.14 (±0.01)	0.14	0.14	

[a] The concentration of J774 cells was 10^7/mL, which corresponds to 1.67×10^{-14} M. The liposomes were labeled with Rho-PE. A lipid concentration of 50 nmol/mL corresponds to a vesicle concentration of 8×10^{-10} M. [b] The inhibitors used were a combination of antimycin A + NaF + NaN_3 as described in the Materials and Methods section. [c] The parameters for the calculation shown in this case were $\epsilon = 0.001$ (s^{-1}), i.e., same rate constant of endocytosis for both PS/PC/Chol and PC/Chol liposomes; $C = 1.5 \times 10^8$ M^{-1} s^{-1} and $D = 0.0011$ s^{-1} and $N = 350$ for PC/Chol liposomes; $C = 2.3 \times 10^9$ M^{-1} s^{-1} and $D = 0.0009$ s^{-1} and $N = 3300$ for PS/PC/Chol liposomes. The uncertainty in the parameters is below 50%.

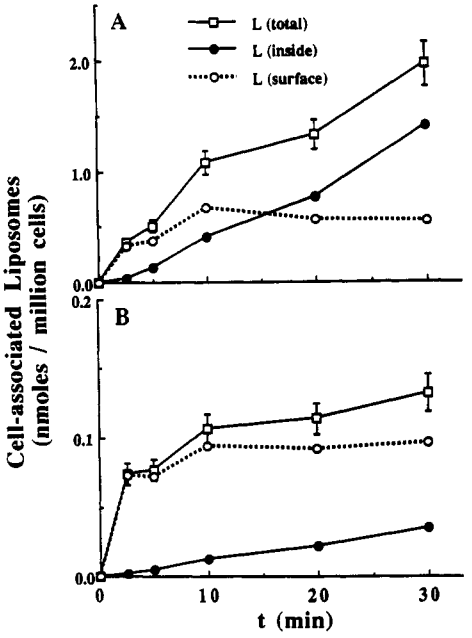

FIGURE 7: Kinetics of liposome endocytosis monitored by HPTS liposomes. Liposomes containing HPTS (PS/PC/Chol, 50 nmol/mL) were incubated with 5×10^6 J774 cells in monolayer (A) and in suspension (B). From the excitation spectra of cell-associated HPTS, the total cell-associated liposomes L(total) were decomposed into two populations: liposomes at cell surface, L(surface), and liposomes endocytosed, L(inside).

Calculation of Endocytic Rate by a Mass Action Kinetic Model. The kinetic data of total cell-associated liposomes at 37 °C with and without metabolic inhibitors were analyzed by a mass action kinetic model as described in the Materials and Methods section. The results in Table IV illustrate that the model described in eqs 1–4 can explain and predict the composite process of liposome binding to the cells followed by endocytosis. We have limited the analysis up to incubation times of 1 h since the results of Dijkstra et al. (1984) indicated that at later times a competing process of liposome degradation and regurgitation occurs. In this study, we first determined at 37 °C the parameters C, D (on- and off-rates of a liposome to a cell), and N (number of binding sites per cell) from binding results in the presence of inhibitors of endocytosis. Then, utilizing these values, the rate constant of endocytosis ϵ was determined from the results at 37 °C without inhibitors.

The rate constant of endocytosis varies by an order of magnitude depending on the day of experiment (see Table II), although when the rate constant ϵ was determined by Rho-PE label, its variation was merely from 0.001 to 0.003 s^{-1}. A remarkable result is that within the same experiment the rate constant of endocytosis of liposomes by J774 cells in suspension is independent of the composition of liposomes. Hence, the large variation in the total uptake of liposomes by cells is due entirely to the differences in the binding characteristics, whereas the rate constant of endocytosis is a property of the cell under the given conditions.

The trend demonstrated by the calculations employing the mass action kinetic model using the data obtained with Rho-PE-labeled liposomes (Table IV) indicates that the amount of cell-surface bound liposomes at 37 °C reaches a constant value after less than 30 min. It is noteworthy that this trend

as well as the actual value of endocytic rate constant ϵ obtained from these calculations agrees quite well with the ϵ obtained experimentally using HPTS-containing liposomes as discussed below. When inhibitors of endocytosis are employed, the time of equilibration of binding is prolonged. Moreover, endocytosis results in a reduction in the amount of liposomes bound to the cell surface at steady state. More importantly, the calculations in Table IV illustrate that the total uptake of PS/PC/Chol liposomes is more than 10-fold faster than that of PC/Chol liposomes, despite using the same endocytic rate constant in both cases. Therefore, the difference in the overall rate of liposome uptake via endocytosis between different liposome compositions appears to be solely due to the differences in binding capacity.

Measurement of Endocytic Rate Using HPTS-Containing Liposomes. The endocytic rate at 37 °C (without inhibitors) was also determined experimentally by an independent method by the use of liposome-encapsulated HPTS (Figure 7) and employing eq 11. Figure 7 shows the increase of total cell-associated liposomes, L(total), during the initial 30-min incubation of PS/PC/Chol liposomes at 37 °C with J774 cells in both the adherent (Figure 7A) and suspension (Figure 7B) states. The cell-surface-bound liposomes, L(surface), were distinguished from the liposomes internalized, L(inside), by following the spectral changes of the fluorescence dye HPTS as an aqueous content marker of liposomes. As shown for the J774 cells in the adherent state in Figure 7A, L(surface) reached a steady-state value of 0.5 nmol/10^6 cells within several minutes. This corresponds to ~3700 liposomes at the cell surface in the steady state. For the J774 cells in suspension (Figure 7B), the steady-state value of L(surface) was ~0.1 nmol/10^6 cells, which corresponds to ~700 liposomes at the cell surface. When L(inside)/L(surface) was plotted as a function of time, it showed a linear increase over time with the slope ϵ. The values of ϵ for the endocytosis of PS-containing liposomes by adherent and suspension cells were 0.086/min and 0.012/min (or 0.0014 and 0.00024 s^{-1}), respectively (also see Table II). Therefore, the J774 cells in the adherent state showed a higher endocytic rate of PS/PC/Chol liposomes as well as a 7-fold higher binding at the cell surface than the J774 cells in suspension. An estimation of endocytic rate of PS/PC/Chol liposomes by adherent J774 cells by the use of metabolic inhibitors also supports the above observation obtained by the use of HPTS (data not shown). If this is true for the monocyte/macrophages in vivo, then the uptake of PS-containing liposomes by the resident macrophages in the liver and spleen would be much faster than that by the circulating monocytes not only because of the increased expression of binding sites per cell but also because of a higher rate of endocytosis as well.

The value of ϵ determined for the adherent J774 cells indicates that $t_{1/2}$, the time required for half of the prebound liposomes to be endocytosed, is ~8 min on the basis of the equation $\epsilon t_{1/2} = \ln 2$. The endocytic rate constant of liposomes is at least 1 order of magnitude smaller than the rate of endocytosis of formaldehyde-treated serum albumin via scavenger receptors (Eskild et al., 1989), severalfold smaller than that of epidermal growth factor receptors (Carpenter & Cohen, 1976), transferrin receptors (Ciechanover et al., 1983), and low density lipoprotein receptors (Glodstein & Brown, 1977), but comparable to that of galactose receptors in hepatocytes (Weigel & Oka, 1982).

The creation of an endosome involves a fusion process. In this context, it is of interest to note that fusion rate constants determined in virus fusion with several suspension cells at 37 °C are in the range 0.001–0.1 s^{-1} (Nir et al., 1990; Pedroso de Lima et al., 1991; Düzgünes et al., 1992), which is in general larger than the values found here for endocytosis. Hence, it might be indicative that fusion is not the rate-limiting step in determining the rate of endocytosis.

CONCLUSIONS

The main mechanism of cell association of liposomes by J774 cells and human peripheral blood monocytes at 37 °C is through binding to cell-surface binding sites followed by endocytosis. We have used model calculations employing mass action kinetics to simulate the experimental data of binding and endocytosis of liposomes and determined affinity constants of liposome binding at 37 °C and 4 °C, on- and off-rate constants of the binding, and endocytic rate constants. Our detailed study with J774 cells demonstrates that the overall rate of uptake is dictated by the binding stage. We have also demonstrated that the binding of PS-containing liposomes is modulated by cell–substrate interactions and maturation/differentiation of monocytes into macrophages. The order of magnitude excess in total uptake of PS/PC/Chol liposomes over PC/Chol liposomes is due to the difference in binding, since both liposomes exhibited the same rate constant of endocytosis. The conclusion that the amount of PS/PC/Chol liposomes bound to J774 cells exceeds significantly that of PC/Chol liposomes was based on the results of binding experiments at 4 °C and at 37 °C in the presence of inhibitors of endocytosis. We have established experimentally that no endocytosis occurred at 4 °C or at 37 °C in the presence of the inhibitors employed. Our model of liposome binding to cells followed by endocytosis is capable of yielding predictions for the kinetics of binding as well as total uptake. Even for PC/Chol liposomes, whose uptake is extremely slow, the route of uptake via binding and endocytosis is an order of magnitude faster than that via fluid-phase pinocytosis.

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