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EFFECTS OF NEGATIVELY CHARGED LIPIDS ON PHAGOCYTOSIS OF LIPOSOMES OPSONIZED BY COMPLEMENT

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Ingestion of liposomes opsonized by specific antibody plus complement was investigated in vitro. Although the antibodies alone (IgM) did not have an opsonizing effect, in the presence of such antibodies uptake and ingestion of liposomes by mouse peritoneal macrophages was enhanced 5- to 10-fold by addition of complement. Phagocytosis of complement-opsonized liposomes was strongly dependent on the charge of the liposomal lipids. The presence of a negatively charged (i.e., acidic) lipid profoundly suppressed the uptake of the liposomes. Each of three acidic liposomal lipids, phosphatidylserine, phosphatidylinositol and dicetyl phosphate, suppressed liposome uptake. We conclude that opsonization of liposomes with complement greatly stimulates ingestion of liposomes by murine macrophages. However, most of the opsonic enhancement conferred by complement can be prevented by the presence of negatively charged membrane lipids.

Introduction

In recent years there has been great interest in the interactions of liposomes with phagocytic cells. Much of this interest has been caused by the observation that injection of liposomes into animals leads to massive liposome uptake by the reticuloendothelial system [1,2]. This has been used in vivo as the basis for delivering drugs to treat parasitic infections in macrophages [3] and for intracellular delivery of macrophage-activating substances [4]. Numerous reports have also demonstrated that liposomes can be ingested by phagocytic cells in tissue culture [5].

Liposomes can be opsonized by attachment of IgG antibodies [6–13]. Except for two preliminary reports from our laboratory [14,15], complement opsonization of liposomes has never been examined. Despite the tremendous potential importance of opsonization for phagocytosis, the effect of negative membrane charge on phagocytosis of opsonized particles has not been extensively investigated. The few studies that have dealt with the issue of membrane charge of opsonized particles have produced inconclusive results (reviewed in Ref. 16). The surface charge of liposomal model membranes can be easily altered, but to date effects of surface charge on opsonized liposomes have not been examined. All of the reported studies on opsonized liposomes have utilized liposomes containing one or more negatively charged lipids [6–15]. The purpose of the present study was to investigate whether negatively charged liposomal lipids could suppress the phagocytosis of complement-opsonized liposomes. In this study, we show that negatively charged membrane lipids could strongly suppress, and even completely reversed, the opsonization effects induced by complement.

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Abbreviations: PI, phosphatidylinositol; PS, phosphatidylserine; DMPC, dimyristoylphosphatidylcholine.

Materials and Methods

Lipids and radiochemicals

Lipids were obtained from the following sources: dimyristoylphosphatidylcholine (DMPC) and cholesterol, Calbiochem-Behring, La Jolla, CA; dicetyl phosphate, K & K Laboratories, Plainview, NY; bovine galactosyl ceramide, Supelco Inc., Bellefonte, PA; plant phosphatidylinositol (PI) and beef phosphatidylserine (PS), Applied Science Labs, Inc., State College, PA; and cholesteryl[1-^{14}C]oleate (58 mCi/mmol), New England Nuclear, Boston, MA.

Preparation and opsonization of liposomes

Liposomes were prepared from DMPC, cholesterol and dicetyl phosphate in a molar ratio of 2 : 1.5 : 0.22 (see Ref. 17 for detailed descriptions of methods for preparing liposomes and for sensitizing them with antiserum and complement). Per μmol of DMPC, 150 μg of galactosyl ceramide were added to the lipid mixture. When PI or PS was included in the liposomes, its molar ratio to DMPC was 1 : 1 so that the molar phospholipid concentration in the liposomes remained constant. Lipids were dried in vacuo and dispersed in a small volume of 0.154 M NaCl with the aid of a few acid-washed 0.5-mm glass beads. The final phospholipid concentration in the aqueous dispersion was 10 mM in all experiments. Trace amounts of cholesteryl[1-^{14}C]oleate were incorporated in the liposomes to monitor the uptake of the vesicles by the macrophages. The liposomes have a wide size distribution, and this is analyzed elsewhere [18]. It is difficult to know whether diameter, surface area or volume is the most important variable, but it is clear from electron micrographs (not shown) that every type of complement-opsonized liposome, from the smallest to the largest, is ingested by macrophages.

For opsonization, liposomes (0.1–0.5 ml, having 1–5 μmol of phospholipid) were incubated with anti-galactosyl ceramide serum (100–500 μl) and 0.5–1.0 ml of guinea-pig serum as a complement source, for 30 min at room temperature. Anti-galactosyl ceramide serum was obtained from rabbits 4 or 5 weeks after immunization [19]. Antiserum was decomplemented routinely at 56°C for 1 h after dialyzing against 0.154 M NaCl. Anti-galactosyl ceramide activity was demonstrated by estimating the complement-dependent release of trapped glucose from liposomes, as described elsewhere [17]. Briefly, glucose release assays contained 0.5 ml of glucose assay reagent, 5 μl of liposomes, increasing volumes of rabbit antisera, 120 μl of guinea-pig serum as complement source, and sufficient 0.154 M NaCl to give a total volume of 1 ml. Glucose release from the liposomes was measured as increased absorbance at 340 nm after incubation at room temperature for 30 min. All data have been corrected for any glucose release in the absence of rabbit serum. After incubation of liposomes with anti-galactosyl ceramide serum and complement, 10 ml 0.154 M NaCl were added, and the vesicles were washed twice by centrifugation at $10000 \times g$ for 10 min. Liposomes were resuspended in 0.154 M NaCl in a final concentration of 5 mM phospholipid.

Anti-galactosyl ceramide serum contained almost exclusively IgM antibodies at 4–5 weeks [19], and because of this, when used in the absence of complement, there was little or no enhancement of liposome uptake (not shown). However, in the presence of complement, opsonization occurred, and phagocytosis was greatly stimulated (see Results). Because of the potential for agglutination of liposomes that have been sensitized only with antibodies [6,20] routine control experiments for complement-opsonized liposomes were performed with liposomes lacking both antibodies and complement rather than with liposomes having antibody but lacking complement. The presence of complement inhibits the agglutination of liposomes that have been coated with antibodies [20].

Cell Culture

Resident peritoneal cells from male C_3HeB/FeJ mice (Jackson Laboratories, Bar Harbor, ME) were collected after intraperitoneal injection of 8–10 ml of medium 1640 (G.I.) supplemented with 2 mM L-glutamine (Gibco), 50 μg/ml of gentamycin (Microbiological Associates) and 10% heat-inactivated fetal bovine serum. Peritoneal fluid was withdrawn through the anterior abdominal wall with a 20-gauge needle and collected in 40 ml Falcon tubes.

The total number of peritoneal cells was counted in a hemocytometer. Differential counts were made

on Diff-Quick stained cell smears prepared by centrifugation of 200 μl of the cell suspension in a Cytospin centrifuge (Shandon Southern Instruments, Ltd., Camberley, U.K.) at 10000 rpm for 7 min. Viability of the cells was tested by exclusion of Trypan blue, and was always approx. 90%.

The peritoneal cell suspension was centrifuged at $400 \times g$ for 10 min and adjusted to $1 \cdot 10^6$ macrophages/ml in RPMI 1640 with 10% fetal bovine serum. Subsequently the cells ($5 \cdot 10^5$ unless indicated otherwise) were added to 16-mm culture wells (Costar tissue culture plates) in a total volume of 0.5 ml, and allowed to adhere at 37°C in 5% CO_2 and 95% relative humidity. After 4 h, non-adherent cells were removed by washing the monolayer with medium, and the macrophages were allowed to stay overnight before incubation with liposomes.

Incubation with liposomes

Cholesteryl[1-^{14}C]oleate-labelled liposomes (approximately 50 nmol phospholipid/opsonized liposome preparation) were added to each culture ($5 \cdot 10^5$ cells) in Medium 1640 without fetal bovine serum in a final volume of 300 μl and incubated at 37°C in 5% CO_2 and 95% relative humidity. At the end of the incubation period, medium with liposomes was removed, and the cells were washed three times with 0.5 ml of ice-cold phosphate-buffered saline. Subsequently, 0.5 ml of 0.1 M NaOH were added to the monolayer and the cells were scraped from the bottom of the well with a Teflon-coated policeman. The cells were allowed to digest for 1–2 h at room temperature. For determination of cell-associated radioactivity, 200-μl samples were taken and dissolved in 1 ml tissue solubilizer (NCS). Subsequently, the cell digest was neutralized with glacial acetic acid, and 10 ml of a Liquifluor toluene scintillation cocktail was added. Radioactivity was counted on a Searle model 6880 Mark III liquid scintillation counter. All determinations were performed in triplicate, and the results shown are the averages of at least two similar and representative experiments.

Results

Phagocytosis of liposomes

Uptake of liposomal vesicles, either non-opsonized or opsonized, is shown as a function of the number of the cells in Fig. 1. At concentrations higher than $7.5 \cdot 10^5$, the cells tended to detach from the bottom of the culture wells. Therefore, in all the experiments reported below, $5 \cdot 10^5$ macrophages per culture were used for incubation with liposomes.

Opsonization of the liposomes with anti-galactosyl ceramide serum and complement resulted in an enhancement of the uptake of the vesicles by the macrophages. This occurred regardless of whether the uptake was assayed as a function of cell concentration (Fig. 1) or as a function of incubation time (Fig. 2). Even at the earliest time tested (at 10 min in Fig. 2), uptake of opsonized liposomes was much higher than that of non-opsonized ones, reaching a plateau approximately after 2 h of incubation (Fig. 3). At the maximum level, uptake of antibody-complement opsonized liposomes was approx. 6-fold higher than uptake of non-opsonized ones (Fig. 3).

Uptake of liposome vesicles, whether opsonized or not, was blocked to a great extent by the use of metabolic inhibitors (Fig. 3). Cells were preincubated for 10 min with sodium fluoride and antimycin-A, inhibitors of glycolysis and mitochondrial respiration, respectively. During subsequent incubation of the cells with liposomes the inhibitors were still present in the medium. Fig. 3 shows that this treatment resulted in 80–85% reduction of the cell-associated radioactivity as compared to control cells to which no inhibitors were added. This demonstrates that the uptake process was energy-dependent, and therefore most of the uptake consisted of phagocytosis. Presumably, any uptake of liposomes that was not blocked by inhibitors represented binding of liposomes to the surface of the cells.

Fig. 4A shows that the total amount of cell-associated liposome radioactivity, representing both binding to and ingestion by the cell, increased as a function of liposome concentration in the medium. By subtracting the amounts of cell-associated radioactivity found in the presence of inhibitors from those amounts in their absence, a curve could be drawn that represented the amount due to actual ingestion of the liposomes (Fig. 4B).

Uptake of the liposomes by the cells was also followed by light, or phase contrast, microscopy.

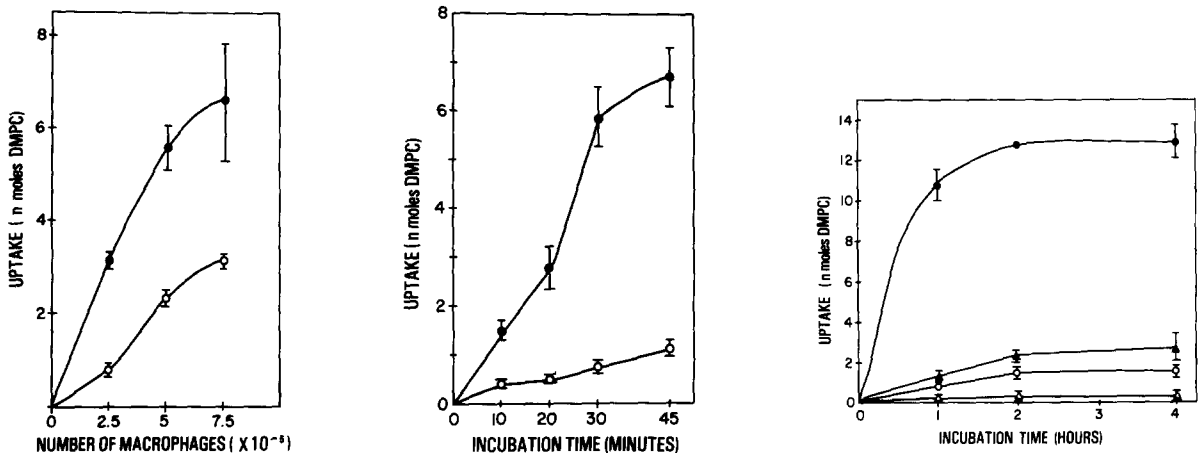

Fig. 1. Effect of cell density on the uptake of liposomes. Cholesteryl[^{14}C]oleate-labelled liposomes were added to an increasing number of resident mouse peritoneal macrophages in monolayer culture. Uptake of the liposomes is expressed as nmol DMPC, based on estimating cell-associated radioactivity. ○, non-opsonized liposomes; ●, liposomes opsonized with anti-galactosyl ceramide serum and complement.

Fig. 2. Kinetics of uptake of liposomes by cultured macrophages. Cholesteryl[^{14}C]oleate-labelled liposomes were incubated with $5 \cdot 10^5$ macrophages for various periods of time. Symbols as in Fig. 1.

Fig. 3. Effect of metabolic inhibitors on the uptake of liposomes. Cultured macrophages ($5 \cdot 10^5$) were preincubated for 10 min with NaF and antimycin-A in final concentrations of 10 mM and 1 μg/ml, respectively. During subsequent incubation of the cells with liposomes, the inhibitors were still present in the medium. Liposomes opsonized with anti-galactosylceramide serum and complement: ●, without inhibitor; ○, with inhibitor. Non-opsonized liposomes: ▲, without inhibitor; △, with inhibitor.

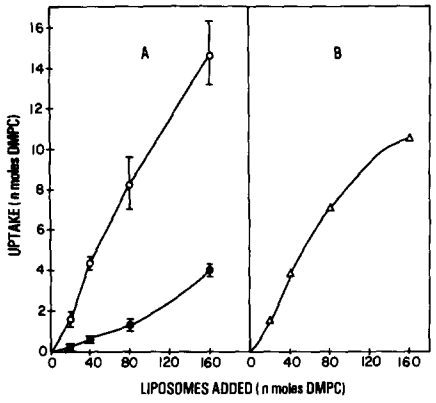

Fig. 4. Uptake of opsonized liposomes by cultured macrophages after exposure to increasing amounts of vesicles. Effect of metabolic inhibitors. (A) $5 \cdot 10^5$ macrophages were incubated for 30 min with vesicles in the presence (●——●, binding alone) or absence (○——○; binding plus ingestion) of NaF and antimycin-A). (B) Curve (ingestion alone) obtained by subtracting the amounts of cell-associated radioactivity found in the presence of metabolic inhibitors from the amounts estimated in the absence of inhibitors as plotted in A.

After ingestion of large amounts of opsonized liposomes, the macrophages developed a striking granular appearance due to ingestion of liposomal vesicles (see photograph in Ref. 14). As a result, the cells became rounded and had a knobby appearance, but they remained viable by Trypan blue analysis.

Effects of phospholipid composition

Effects of phosphatidylinositol (PI) and phosphatidylserine (PS) on phagocytosis are shown in Fig. 5. In each case, compared to liposomes containing DMPC as the only phospholipid, phagocytosis of liposomes containing PI or PS in addition to DMPC was suppressed (Fig. 5). Fig. 6 shows that the suppressing effect of PI on the uptake of opsonized liposomes was dose-dependent. In the presence of equimolar amounts of PI and DMPC, approx. 75% suppression was observed compared to liposomes lacking PI (Fig. 6).

The question arose as to whether the suppressing effects of PI and PS on phagocytosis were due

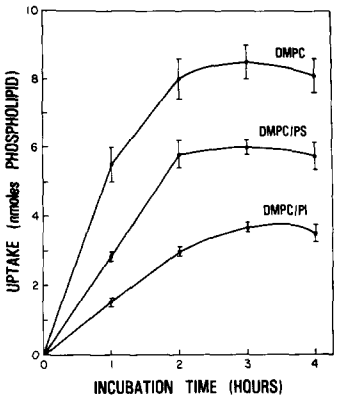

Fig. 5. Effect of phospholipid composition on the uptake of liposomes by cultured macrophages. $5 \cdot 10^5$ macrophages were incubated for up to 4 h with cholesteryl[^{14}C]oleate-labelled opsonized liposomes (phospholipid; cholesterol; galactosyl ceramide). The liposomes contained 40 nmol of phospholipid, but lacked dicetyl phosphate. The liposomal phospholipid consisted of DMPC, DMPC/PS (1 : 1) or DMPC/PI (1 : 1). Radioactivity was determined in cells digested after extensive washing.

to inhibition of the opsonization process itself. Perhaps liposomes containing PS or PI did not permit attachment of specific antibodies to the antigen (galactosyl ceramide) on the liposomes, and thereby did not permit complement fixation by the liposomes, though it should be pointed out that excess antiserum and complement were always used for opsonization. To exclude this possibility, the antiserum was adsorbed with liposomes containing either PS or PI, and the adsorbed antisera were tested for residual antibody activities. As shown in Fig. 7, under the conditions used, each of the liposome preparations tested removed virtually all of the anti-galactosyl ceramide antibodies from the antiserum. It should be pointed out that although antibody binding did occur, complement activation by negatively charged liposomes having bound antibody, conceivably might have been impaired. However, separate experiments testing complement-damage to negatively charged liposomes of similar composition to those used in Fig. 7 did not show significant differences in complement activation leading to differences of glucose release between standard test liposomes and negatively charged ones.

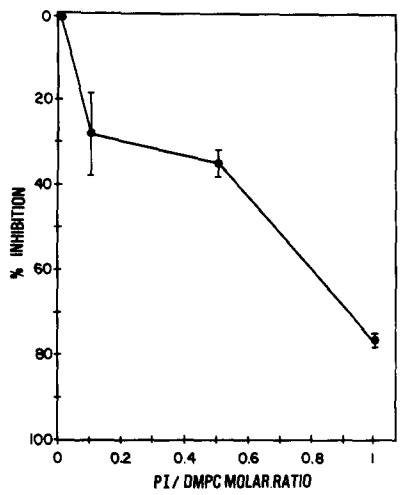

Fig. 6. Effect of increasing amounts of exogenous PI on the uptake of opsonized liposomes by cultured macrophages. $5 \cdot 10^5$ macrophages were incubated for 1 h with opsonized liposomes containing increasing amounts of PI. Suppression of phagocytosis is expressed as the percentage of inhibition of the uptake of the vesicles as compared to control liposomes (no PI, 0% inhibition).

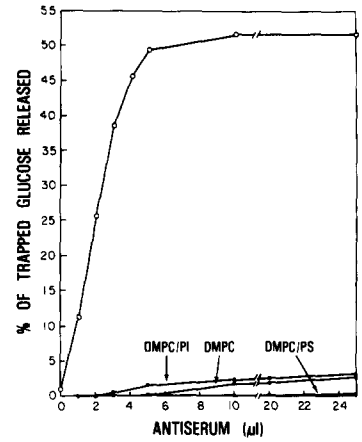

Fig. 7. Immunologic activities of anti-galactosyl ceramide serum against liposomes containing galactosyl ceramide. The activity of the antiserum was removed by absorption with liposomes containing as phospholipid DMPC, DMPC/PS (1 : 1), or DMPC/PI (1 : 1) (lines as indicated). Antiserum (0.5 ml) was incubated with liposomes (0.1 ml) swollen in 0.154 M NaCl. After 30 min at room temperature, the liposomes were removed by centrifugation at $27000 \times g$ for 10 min, and the absorbed antisera tested by complement-dependent glucose release from test liposomes (DMPC; cholesterol; dicetyl phosphate; galactosyl ceramide); O——O, unabsorbed. The results were corrected for the dilution due to the volume of saline in the liposome suspension.

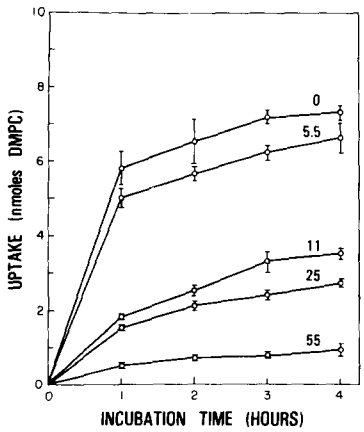

Fig. 8. Effect of increasing amounts of dicetyl phosphate (percentages indicated on the curves) in the opsonized liposomes on their uptake by macrophages. $5 \cdot 10^5$ macrophages were incubated with 10 μl of each liposome preparation, with a total of 40 nmol of phospholipid per culture (dicetyl phosphate was not included in the calculation of phospholipid).

Effect of liposomal charge

Each of the phosphatides (PS and PI) that suppressed phagocytosis (Fig. 5) was an acidic molecule having a strong negative charge. Were the suppressive effects of PS and PI unique properties of these phosphatides, or were the suppressive effects of PI and PS due to the negative charges carried by the phosphatides? To determine whether a negatively charged non-phosphatide lipid could be suppressive, we examined uptake of liposomes containing different concentrations of dicetyl phosphate. Fig. 8 demonstrates that decreased uptake of opsonized liposomes was related to increased concentration of dicetyl phosphate in the liposomes. We conclude that suppression of phagocytosis of antibody-complement opsonized liposomes was at least partly related to the presence of a negative surface charge.

Discussion

Opsonization with complement is a powerful means of enhancing the uptake of numerous types of particle by phagocytic cells (reviewed in Ref. 16). In this study we have examined whether surface charge can play a role in the phagocytosis of opsonized liposomes. We have regulated the liposomal surface charge by changing the phosphatide (DMPC, PI or PS) or by introducing a negatively charged lipid that is not a phosphatide, dicetyl phosphate. Each of these types of lipids has been used in the past to change the charge (zeta potential) of the liposomal membrane [21,22]. We have discovered that a negative liposomal surface charge can have a strong suppressive effect on ingestion of liposomes when the liposomes are opsonized with complement. It appears that complement-opsonization and negative surface charge can be opposing forces that work against each other. Under the conditions employed in this study, when these two opposing forces were compared, it was evident that the suppressive effects of negative surface charge could overcome the enhancing effects of complement-opsonization.

It is possible that under different experimental conditions, opsonization could play a dominant role. Indeed, at lower levels of negative surface charge, enhancing effects of opsonization were clearly observed (Figs. 1 and 2), and the suppressing effect of negative charge occurred only in a graded fashion (Fig. 8).

To our knowledge, all previously reported studies on the phagocytosis of opsonized liposomes have utilized liposomes having at least one negatively charged lipid [6–15]. Our data would indicate that the negative charge that was present might have played an unrecognized role in the results in certain studies. The present finding of suppressive effects of negatively charged lipids might represent a potential theoretical drawback to the use of dinitrophenylated lipids as antigens in certain types of study on opsonized liposomes.

It should be pointed out that nearly all of the antibodies in our system were IgM [19], and because of this the opsonization process that we examined in this study was almost exclusively due to the attachment of complement components. Except in rare instances, IgM antibodies are non-opsonic and IgM binding does not promote phagocytosis [16]. In independent experiments, using highly purified IgM antibodies, we have confirmed that this also holds true for phagocytosis of liposomes. It is possible that the effects of negative charge seen in this study may not occur, or may be qualitatively or quantitatively different, in other opsonic models in which complement is not present, and in which IgG antibodies, or even

lectins [6], serve as the opsonic stimulus. To our knowledge, the present system is the first in vitro model in which opsonization of liposomes by complement fixation has been reported. From a practical standpoint, if liposomes (or other materials) were subjected either to IgG or IgM immunological attack in vivo, under conditions in which complement would be likely to be activated, then opsonization by complement fixation might be an important consideration. Under these circumstances, it might be expected that negatively charged lipids could be a factor that would partially modulate the degree of complement-dependent phagocytosis.

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