Biochemical, Morphological, and Ultrastructural Studies on the Uptake of Liposomes by Murine Macrophages

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ABSTRACT

The interaction of multilamellar liposomes with mouse peritoneal macrophages cultured in vitro has been examined. The principal mechanism of liposome uptake by these cells is by phagocytic engulfment. Studies with radiolabeled liposomes demonstrated that they are incorporated into macrophages as intact structures and that treatment of macrophages with inhibitors of phagocytosis prevents liposome uptake. Incubation of macrophages with liposomes containing encapsulated fluorescein-labeled bovine serum albumin resulted in localization of fluorescence within discrete cytoplasmic vacuoles. Ultrastructural observations confirmed that liposomes were internalized and were enclosed within phagosomes. Electron microscopy also revealed that, by 24 hr following phagocytosis, adjacent phagosomes containing liposomes prepared from bovine brain phosphatidylserine, egg phosphatidylcholine, and lysolecithin (mol ratio, 4.95/4.95/0.1) fused within the cytoplasm. In contrast, phagosomes containing neutral liposomes consisting solely of egg phosphatidylcholine did not fuse and remained as discrete single structures. Negatively charged bovine brain phosphatidylserine/egg phosphatidylcholine/lysolecithin liposomes were phagocytosed at a much faster rate (12 times faster) than were neutral egg phosphatidylcholine liposomes.

INTRODUCTION

We have shown recently that liposomes containing the lymphokine MAF* can activate peritoneal (17, 18) and alveolar (26) macrophages in vitro and render them cytotoxic to tumor cells. Little is known, however, about the mechanisms involved in the uptake of liposomes by macrophages. This information is not only pertinent to understanding how liposome-encapsulated MAF leads to macrophage activation but would also be of potential value in developing liposomes with high affinities for macrophages which could be used as carriers for delivery of agents to macrophages in vivo. Most of the available information on liposome-cell interactions has come from studies with nonphagocytic fibroblasts, epithelia, and lymphoid cells (for reviews, see Refs. 10, 13, 16, and 28). Since these cells do not possess the specialized phagocytic abilities of macrophages, it is by no means certain that the pattern(s) of liposome uptake observed in nonphagocytic cells will apply to macrophages.

In this study, we have examined the mechanism of uptake of MLV liposomes by murine PEC cultured in vitro. MLV liposomes were chosen for study because this type of liposome has been found to localize not only in the liver and spleen but also in the lung (5). Data presented here indicate that the predominant pathway for uptake of liposomes by macrophages is by phagocytosis and that there are major differences in the rate of phagocytic uptake of neutral and negatively charged liposomes. The behavior of internalized liposomes also is influenced by their lipid composition, and phagosomes containing negatively charged, but not neutral, MLV undergo extensive intracellular fusion. These differences in the uptake and intracellular behavior of neutral and charged liposomes may contribute to differences in the kinetics of macrophage activation, as described in the accompanying paper (6), in which activation occurs significantly faster when MAF is encapsulated in negatively charged MLV.

MATERIALS AND METHODS

Animals. Specific-pathogen-free C57BL/6N × C3H* F1 (hereafter called B6C3F1) mice, 6 to 8 weeks old, were obtained from the Animal Production Area of the Frederick Cancer Research Center.

Media. The components of the CMEM were obtained from Flow Laboratories, Rockville, Md. Gentamicin (Schering Corporation, Kenilworth, N. J.) was added routinely to the media at a final concentration of 50 μg/ml. PBS and HBSS were obtained from Grand Island Biological Co., Grand Island, N. Y. Endotoxin-free fetal calf serum (Limbulus lysate test) was obtained from Microbiological Associates, Bethesda, Md.

Collection and Cultivation of Peritoneal Macrophages. Thioglycollate-stimulated peritoneal macrophages were collected by peritoneal lavage from mice given i.p. injections of 2 ml thioglycollate broth (Baltimore Biological Laboratories, Cockeysville, Md.) 5 days before harvest, as described previously (4, 18). The PEC were centrifuged at 250 × g for 10 min, resuspended in serum-free minimal essential medium, and plated into tissue culture dishes with a 16-mm diameter well (No. 3524; Costar, Cambridge, Mass.) (for radioactivity measurements) or onto 25-mm-diameter coverslip glass placed in Falcon tissue culture dishes (for microscope visualization). After incubation for 40 min at 37°C, the culture vessels were rinsed two times with HBSS to remove nonadhering cells, and fresh CMEM was added. The remaining adherent macrophages were cultivated for 24 hr at 37°C in CMEM containing 5% 

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fetal calf serum in an incubator (5% CO₂/air) before further studies. Quadruplicate samples were used in each experiment. BSA. BSA, Fraction V, was purchased from Sigma Chemical Co. (St. Louis, Mo.) and labeled with carrier-free Na¹²⁵I (Amersham/Searle Corp., Arlington Heights, Ill.), as described previously (7). ¹²⁵I-labeled BSA was dialyzed against PBS at 4 °C until no radioactivity was detected in the dialysate. The specific activity of ¹²⁵I-labeled BSA preparations ranged from 5 to 10 x 10⁶ cpm/mg protein. Preparations of ¹²⁵I-labeled BSA (specific activity, 45 Ci/mmol) also were purchased from New England Nuclear, Boston, Mass. BSA was labeled with fluorescein isothiocyanate and separated from excess fluorescein isothiocyanate by dialysis against PBS for 72 hr at 4 °C, as described previously (21).

**Liposomes.** MLV liposomes were prepared from PC or a mixture of PS, PC, and LL [4.95/4.95/0.1 (mol ratio)] using chromatographically pure lipids, as described in the accompanying paper (6). Encapsulation of ¹²⁵I-labeled BSA and FITC-BSA within liposomes was achieved as described previously (18). Leakage of encapsulated ¹²⁵I-labeled BSA from liposomes incubated in CMEM was measured by dialysis, as described elsewhere (20). The average amount of free ¹²⁵I-labeled BSA diffusing from the dialysis sac over a 4-hr period (calculated from 4 separate preparations) expressed as a percentage of the total ¹²⁵I-BSA diffusing per hr at 37 °C was PC liposomes, 6.5 and PS/PC/LL liposomes, 11.6.

**Macrophage-Liposome Interactions.** For morphological studies on cellular uptake of liposomes containing FITC-BSA, coverslip cultures of macrophages were washed twice with prewarmed PBS and then incubated at 37 °C with liposomes (100 nmol phospholipid per 10⁶ macrophages) in CMEM containing 5% fetal calf serum for the periods indicated in the text. The cells then were washed twice with PBS, fixed with 2% formaldehyde in PBS (pH 7.2) for 30 min at 24 °C, processed for fluorescence microscopy as described elsewhere (23), examined, and photographed with a Zeiss photomicroscope with a fluorescence attachment.

For studies on the uptake of liposomes containing ¹²⁵I-labeled BSA, macrophages were incubated in CMEM with the same dose of liposomes for up to 24 hr. At predetermined intervals, the cultures were washed 3 times with PBS, the macrophage monolayers were dissolved in 1 ml of 0.1 N NaOH, and their radioactivity content was measured in a gamma spectrometer. The reduction in radioactivity detected in wells containing only CMEM served as a background control to monitor nonspecific adsorption of liposomal ¹²⁵I-labeled BSA and free ¹²⁵I-labeled BSA to serum proteins deposited on the plastic substrate. Additional controls were done in which macrophages were incubated at 37 °C in CMEM containing an amount of free ¹²⁵I-labeled BSA identical to that encapsulated in liposomes plus liposomes containing HBSS. The amount of macrophage-associated radioactivity then was measured at several intervals to determine uptake of free ¹²⁵I-labeled BSA by macrophages in the presence of liposomes.

**Transmission Electron Microscopy.** Coverslip cultures of macrophages were fixed in 2.5% cacodylate-buffered glutaraldehyde, pH 7.3, for 1 hr at room temperature and then washed with cacodylate buffer. The samples then were processed in the cold by fixing with a 1% saturated aqueous solution of thiocarbohydrazide for 10 min, washing 5 times with distilled water, and treating with osmium vapors for 10 min.

Samples then were washed with distilled water, stained en bloc with 1% aqueous uranyl acetate for 30 min, and dehydrated with a graded series of ethanol. Samples were transferred to absolute ethanol at room temperature, infiltrated, and embedded in Spurr's low-viscosity medium. The blocks were polymerized at 70 °C overnight, and the coverslips were removed from the block by dissolving the glass in hydrofluoric acid. The blocks were rinsed well with distilled water and placed back in the oven to dry. Thin sections were cut with glass knives in a LKB Ultratome III, stained with Reynold's lead citrate, and examined in a Hitachi HU-12A electron microscope at an operating voltage of 75 kV.

**RESULTS**

**Uptake of MLV Liposomes by Macrophages.** The interaction of neutral (PC) and negatively charged (PS/PC/LL) MLV liposomes containing ¹²⁵I-labeled BSA with monolayer cultures of mouse peritoneal macrophages is shown in Chart 1. The results indicate that cell-associated ¹²⁵I-BSA accumulated considerably faster with PS/PC/LL liposomes compared with PC liposomes. The amount of liposome-derived ¹²⁵I-labeled BSA associated with macrophages following incubation with PS/PC/LL liposomes increased rapidly during the first hr and reached a maximum after 4 hr (Chart 1). Thereafter, the amount of cell-associated radioactivity declined, presumably due to intracellular degradation of BSA and efflux of free ¹²⁵I at a rate faster than uptake of liposomal ¹²⁵I-labeled BSA. In contrast, introduction of ¹²⁵I-labeled BSA to macrophages by PC liposomes was much less efficient, with slow but gradual accumulation taking place over the entire 24-hr incubation period (Chart 1).

Most of the radioactivity recovered in association with macrophages (Chart 1) is attributable to intracellular ¹²⁵I-labeled BSA derived from internalized liposomes. Very little activity, if any, is attributable to ¹²⁵I-labeled BSA entrapped in liposomes absorbed to the cell surface or to uptake of free ¹²⁵I-
labeled BSA leaking out of liposomes. This conclusion is based on the following evidence. (a) Control macrophages incubated in CMEM containing a similar amount of free $^{125}$I-labeled BSA together with liposomes containing HBSS do not incorporate any significant radioactivity (not shown). This finding excludes the possibility that $^{125}$I-labeled BSA leaks out of liposomes and is subsequently incorporated into macrophages via liposome-induced changes in plasma membrane permeability or via "piggyback endocytosis" during endocytosis of liposomes. (b) In control experiments, exposure of liposome-treated macrophages to 0.2% PBS (diluted in water) for 30 sec to induce elution-lysing of liposomes and release of $^{125}$I-labeled BSA from extracellular liposomes failed to reduce the levels of cell-associated radioactivity (not shown). Since this treatment induces the release of >90% of $^{125}$I-labeled BSA associated with liposomes, the results indicate that adsorbed liposomes do not account for any significant fraction of the cell-associated radioactivity and that the standard washing procedure is sufficient to elute and lyse liposomes adsorbed to macrophage surface.

MLV liposomes are incorporated into macrophages as intact structures without detectable loss of encapsulated $^{125}$I-labeled BSA. This was demonstrated by showing that, in macrophages treated with MLV, liposomes containing $^{125}$I-labeled BSA and a radiolabeled phospholipid ($^3$H)dipalmitoylphosphatidylcholine incorporated into the liposome membrane, the ratio of 2 radiolabels recovered in association with macrophages is similar to that in liposomes incubated in CMEM without macrophages (Table 1). Alteration in the ratio of the radiolabels, indicating loss of $^{125}$I-labeled BSA, was detected, however, after incubation of macrophages with liposomes for several hr (Table 1). This presumably results from release of encapsulated $^{125}$I-labeled BSA and/or its degradation to free $^{125}$I that escapes from within the cell.

Collectively, the above experiments establish that the cell-associated radioactivity shown in Chart 1 represents intracellular radioactivity associated with internalized liposomes. The marked differences in the rate and amount of $^{125}$I-labeled BSA internalized from PC and PS/PC/LL liposomes suggest either that the mechanism of liposome uptake is different or that PS/PC/LL liposomes are internalized much more efficiently.

Previous studies on the interaction of liposomes with non-phagocytic cells have shown that uptake of intact liposomes can occur either by endocytosis or by fusion of liposomes with the plasma membrane (for reviews, see Refs. 13 to 16 and 28). To examine the role of these mechanisms in liposome uptake by macrophages, we incubated monolayer cultures of macrophages with MLV incubated with or without mouse macrophages containing FITC-BSA and examined them by fluorescence microscopy to determine the intracellular distribution of FITC-BSA. As shown in Fig. 1, liposome-derived FITC-BSA was concentrated in discrete cytoplasmic foci in a pattern similar to that seen in endocytic uptake of fluorescent dyes into phagosomes and phagolysosomes (for review, see Ref. 1). The distribution of fluorescence was similar with both PC and PS/PC/LL liposomes, but cell-associated FITC-BSA was much higher with PC/PS/LL liposomes (Fig. 1). This is consistent with the data shown in Chart 1. No significant fluorescence was detected at the surface of treated cells, suggesting that very few, if any, liposomes remain adsorbed after washing with PBS. Moreover, diffuse distribution of FITC-BSA within the cytoplasm was not seen at any time. This indicates that fusion of liposomes with the plasma membrane with release of FITC-BSA directly into the cytoplasm is not occurring on any appreciable scale. The absence of diffuse cytoplasmic fluorescence also indicates that extensive transfer of FITC-BSA to the cytosol from endocytic vacuoles is not occurring. These observations suggest that uptake of liposomes by macrophages occurs primarily via phagocytosis, with resulting localization of liposomes within phagosomes or phagolysosomes.

Examination of macrophages incubated for 24 hr with PS/PC/LL liposomes containing FITC-BSA revealed that more than 90% of cells showed detectable fluorescence (Fig. 1). A similar labeling efficiency was observed in macrophages incubated with PC liposomes for 24 hr but, since the majority of these cells contained only a few fluorescent foci, the fluorescence intensity was too weak to be recorded photographically.

Further support for the conclusion that phagocytosis is the major mechanism of liposome uptake by macrophages was provided by experiments in which pretreatment of macrophages with metabolic inhibitors that block phagocytosis markedly reduced their ability to incorporate MLV (Table 2). This treatment of macrophages also produced a similar reduction in phagocytosis of opsonized sheep RBC (Table 2).

**Ultrastuctural Studies of Liposome Uptake by Macrophages.** Transmission electron microscopy of thin sections of macrophages incubated with PS/PC/LL liposomes for 1 hr revealed numerous MLV profiles situated within the cytoplasm, each enclosed within a phagosome (Fig. 2a). In contrast, examination of sections from 100 individual macrophages incubated with PC liposomes for the same period failed to reveal any liposomes (Fig. 2a). Uptake of PC liposomes was detected, however, after longer incubation times (Fig. 2b). Following incubation with liposomes for 4 or 24 hr, internalized liposomes occupy a substantial proportion of the macrophage cytoplasm (Fig. 2, b' and c'). Liposome adsorption to the plasma membrane was not observed.

An interesting phenomenon was detected in macrophages treated with PS/PC/LL MLV in which phagosomes containing liposomes were found to fuse with each other within the cytoplasm. This process, shown in Fig. 3, did not occur with internalized PC liposomes. We interpret Fig. 3 as showing that, after the initial engulfment of MLV within a phagosome (Fig. 3a), adjacent phagosomes containing MLV become apposed closely (Fig. 3b) and fuse (Fig. 3, c and d) to create a complex

<table>
<thead>
<tr>
<th>Liposome composition</th>
<th>Macrophages</th>
<th>0</th>
<th>1 hr</th>
<th>4 hr</th>
<th>8 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>0.85</td>
<td>0.71</td>
</tr>
<tr>
<td>PS</td>
<td></td>
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<td>0.96</td>
<td>0.62</td>
<td>0.58</td>
</tr>
<tr>
<td>PS/PC/LL</td>
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<td>0.91</td>
<td>0.77</td>
<td>0.54</td>
</tr>
<tr>
<td>PS/PC/LL +</td>
<td></td>
<td>1.0</td>
<td>0.89</td>
<td>0.74</td>
<td>0.42</td>
</tr>
</tbody>
</table>

*a* Mol ratio, 4.95/4.95/0.1.
and incubated for a further 2 hr at 37°. The cultures then were washed 3 times, indicated composition (100 nmol phospholipid per 10⁵ cells) containing I²5I-BSA suspension of opsonized Na₅lCrO₄-labeled sheep RBC or MLV liposomes of the

Treatment

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Sheep RBC</th>
<th>PC</th>
<th>PS/PC/LL$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, control macrophages</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2-Deoxyglucose (5 x 10⁻⁵ M) and sodium azide (5 x 10⁻³ M)</td>
<td>16</td>
<td>16</td>
<td>18</td>
</tr>
</tbody>
</table>

$^*$ Mol ratio, 4.95/4.95/0.1.

bifurcated MLV enclosed within a common membrane (Fig. 3, e and f). Some degree of mixing of individual lipid bilayers from the 2 original liposomes may occur within such newly fused structures (Fig. 3d). Serial sectioning through fused phagosomes revealed that the observation was not due to a sectioning artifact. Moreover, in all cells that contained 3 or more negatively charged liposomes, intracellular fusion of phagosomes occurred by 24 hr (observations on 82 cells). This type of intracellular fusion also might explain that very large fluorescent cytoplasmic vesicles observed in macrophages incubated with PS/PC/LL liposomes containing FITC-BSA (Fig. 1, d' and e') that are considerably larger than the fluorescent vesicles seen in macrophages incubated with PC liposomes (Fig. 1, d and e).

DISCUSSION

The present results, together with those in the accompanying paper (6), provide a detailed analysis of the interaction of liposomes with macrophages. The evidence presented here indicates that phagocytosis is the principal mechanism for the uptake of MLV liposomes by macrophages. This conclusion is based on several lines of evidence. (a) Experiments with liposomes containing both encapsulated and membrane markers established that liposomes are incorporated as intact structures without any detectable loss of encapsulated material. (b) Fluorescence microscopic observations of macrophages treated with liposomes containing FITC-BSA revealed that the fluorescent label was consistently confined to cytoplasmic vacuoles. This suggests that fusion of liposomes with the plasma membrane does not occur to any significant extent since this would result in diffuse labeling of the cytoplasm. (c) Inhibition of phagocytosis by metabolic inhibitors markedly reduced liposome uptake. (d) Ultrastructural examination of MLV internalized by macrophages indicates that they were surrounded by a phagosome membrane.

Both neutral and negatively charged MLV are incorporated into macrophages by phagocytosis, although the latter are phagocytosed at a significantly faster rate. This differs from the behavior of the same liposomes when interacting with cells that are not "professional" phagocytes, such as fibroblasts or lymphocytes. Not only is endocytic uptake of liposomes limited in such cells with the majority of cell-associated liposomes merely being absorbed to the cell surface without internalization but also the limited endocytosis that occurs is restricted to neutral liposomes and charged liposomes composed of lipids that are below their gel-to-liquid crystalline transition temperature (16). Charged liposomes composed of lipids that are above their transition temperature (i.e., fluid), such as the PS/PC/LL liposomes used here, do not evoke significant endocytosis in nonphagocytic cell populations and, instead, fuse with the plasma membrane (16, 27). Efficient uptake of liposomes by phagocytes of the smooth dogfish (Mustelus canis) requires incorporation of recognition molecules into the liposome bilayer (29). Such is not the case with murine phagocytic macrophages. The PS/PC/LL liposomes used here provided a highly effective phagocytic stimulus, and uptake of these liposomes at the end of a 1-hr incubation period was 12 times greater than that of neutral PC liposomes.

Collectively, these findings indicate that the specialized phagocytic activity of murine macrophages is the dominant factor in determining the outcome of their interaction with liposomes. This contrasts with the interaction of liposomes with fibroblasts, lymphocytes, and other cells lacking highly specialized phagocytic activities in which highly variable and complex combinations of liposome fusion, endocytosis, and adsorption, as well as selective and reciprocal exchange of membrane components between host cells and liposomes, all occur (13, 16, 24, 28).

However, the data presented here and in the accompanying paper (6) have only addressed the issue of endocytic internalization of liposomes. The question of whether other complex events are also occurring, such as liposome-mediated alterations in the topography of plasma membrane components, alterations in plasma membrane function, and/or recycling of internalized membranes (± liposomal components) back to the cell surface has still to be investigated.

Electron micrographs of internalized liposomes have been published in several studies of liposome-cell interactions in vitro (3, 12, 30) and in vivo (2, 22, 25). However, the fusion of phagosomes containing liposomes has not been described previously. This phenomenon occurred with high frequency following endocytosis of negatively charged PS/PC/LL liposomes, but did not occur with neutral PC liposomes. The mechanism responsible for this phenomenon is not known. It is of interest to note that PC liposomes are able to fuse with other liposomes (9, 11, 14) or with natural membranes (8, 19) but will do so when small amounts of PS or other acidic phospholipids are included in the liposome membrane (14, 15).

In spite of the marked differences in the rate of phagocytosis of PC and PS/PC/LL liposomes, results presented in the accompanying paper (6) indicate that both types of liposomes can serve as carriers for MAF and induce similar levels of macrophage tumoricidal activity. The more rapid uptake of PS/PC/LL liposomes demonstrated here is consistent with data reported in the accompanying paper (6) showing that liposomes of this composition containing MAF induce macrophages activation significantly faster than do MAF encapsulated in PC liposomes.

REFERENCES

Macrophage-Liposome Interactions


Fig. 1. Fluorescence micrographs of mouse peritoneal macrophages incubated at 37° for different times with MLV liposomes containing encapsulated FITC-BSA showing internalization of liposomes and localization within the cytoplasm. a to e, = PC liposomes; a' to e', PS/PC/LL (mol ratio, 4.95/4.95/0.1). a, a', 1 hr; b, b', 2 hr; c, c', 4 hr; d, d', 8 hr; e, e', 24 hr. x 300.
Fig. 2. Transmission electron micrographs of mouse peritoneal macrophages incubated at 37° for different times with MLV liposomes prepared from PC (a to c) or PS/PC/LL (mol ratio, 4.95/4.95/0.1) (a' to c') showing internalized liposomes within phagosomes in the cytoplasm. a, a', 1 hr; b, b', 4 hr; c, c', 24 hr.
Fig. 3. Transmission electron micrographs of MLV liposomes (PS/PC/LL; mol ratio, 4.95/4.95/0.1) internalized by mouse peritoneal macrophages showing the possible sequence of events in intracellular fusion of adjacent phagosomes within the cytoplasm. Following initial phagocytic engulfment of liposomes (a), adjacent phagosomes containing liposomes establish close contact within the cytoplasm (b, c arrows) and then undergo fusion (d arrow) to create complex bifurcated structures (d to f). Following fusion, the 2 original liposomes are enclosed within a common limiting membrane (d), and some degree of intermixing of lamellae from the 2 liposomes is evident (d). Complex profiles suggestive of fusion between several liposomes also are seen (e arrow and f). The electron micrographs were made at the following times after treatment with liposomes: a, 1 hr; b, 4 hr; c, 4 hr; d to f, 24 hr.
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