Activation of Fc Receptor-Mediated Phagocytosis by Mouse Peritoneal Macrophages Following the Intraperitoneal Administration of Liposomes

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The effects of liposomes on the phagocytic activity of mouse peritoneal macrophages were investigated using IgG-opsonized sheep red blood cells (SRBC). The highest ingestion index of opsonized SRBC via Fc receptors of macrophages from BALB/c mice was observed for macrophages harvested on day 4 following the intraperitoneal injection of liposomes (2.27 μmol lipid/mouse). An increase in the ingestion index was observed irrespective of liposomal charge. Binding parameters of Fc receptors of macrophages from liposome- or saline-injected mice were determined using horseradish peroxidase-conjugated IgG, and an increase in the number of binding sites with the same binding constant was observed in macrophages from liposome-injected mice. The activation mechanism of mouse peritoneal macrophages by liposomes differed from that by lipopolysaccharides. Liposomes thus appear to contribute to the activation of immune response.

KEY WORDS: liposome; macrophage; phagocytosis; Fc receptor.

INTRODUCTION

Liposomes have been artificially made into membraneous vesicles composed essentially of naturally occurring phospholipids and have been found to serve as an immunological adjuvant or carrier of drugs (1). After being intravenously injected, they are quickly removed from the blood circulation and trapped by Kupffer cells of the liver and macrophages of spleen, through phagocytosis with or without receptors (2–4). Liposomes have been used to study the effector phase of immune response and examined for their potential to serve as an adjuvant and as a carrier for antigens (5,6).

Macrophages have many unique functions, such as enhancing phagocytosis, protease secretion, increasing the production of interleukin-1, and cellular cytotoxicity, when activated by various naturally occurring synthetic agents (7). Changes caused by liposomes in the immune system have not been studied in detail, particularly those in the functions of macrophages. This paper reports the activating effects of liposomes on phagocytosis of opsonized red blood cells via Fc receptors of mouse peritoneal macrophages following the intraperitoneal injection of liposomes.

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MATERIALS AND METHODS

Experimental Animals. Inbred BALB/c and C3H/HeJ male mice 6–9 weeks old were purchased from Japan SLC, Inc. (Shizuoka, Japan).

Chemicals and Reagents. Dipalmytoylphosphatidylcholine (DPPC) and phosphatidylserine (PS) were purchased from Nippon Oil and Fat Co., Ltd. (Tokyo), and Nisshin Seiyu Co., Ltd. (Tokyo), respectively. Phosphatidic acid (PA), dicetylphosphate (DCP) and stearylamine (SA) were from Sigma Chemical Co. (St. Louis, MO). Cholesterol (Chol) was obtained from Wako Pure Chem. Ind. Ltd. (Osaka, Japan). γ-Globulin-free fetal calf serum (FCS) and lipopolysaccharides (LPS) from *Salmonella typhimurium* (3125-25-5) were from GIBCO Laboratories (Grand Island, NY) and Difco Laboratories (Detroit, MI), respectively. Sheep red blood cells (SRBC) were purchased from Japan Bio-Test (Tokyo). IgG of rabbit anti-SRBC was from Inter-Cell Technologies Inc. (Hopewell, NJ). Horseradish peroxidase-conjugated mouse IgG and its $F(ab')_2$ fragment were from Organon Teknica Co. (Wilson Drive, PA) and Rockland (Gilbertsville, PA), respectively.

Liposome Preparation. Multilamellar liposomes of typically DPPC:PS:Chol = 3.2:2:4.8 (molar ratio) were prepared as reported previously (8) and filtered through a membrane filter (0.45 μm, Corning, NY). Liposomal ζ potentials were estimated by the rotating prism method of microelectrophoresis (Laser Zee Model 501, Pen Kem Inc, Bedford Hills, NY) in 10 m*M* Tris/HCL-buffered saline (pH 7.4). LPS contamination in the liposome preparation was assessed by a *Limulus* amebocyte lysate assay (Wako Pure Chem. Ind. Ltd.).

Macrophage Preparation and Ingestion of Opsonized SRBC. Liposomes (2.27 μmol lipid/mouse) or LPS (20 μg/mouse) were injected into BALB/c or C3H/HeJ mice intraperitoneally. Physiological saline for medical use (Otsuka Pharmaceutical Co., Ltd., Tokyo) was injected into age-matched sham control mice. The peritoneal macrophage monolayer was prepared according to Ngwenya and Yamamoto (9). The identification of macrophages in peritoneal adherent cells was carried out by phagocytosis of latex particle and Giemsa staining, and more than 95% of adherent cells were macrophages.

Phagocytosis of IgG-opsonized SRBC by peritoneal macrophages were assessed according to the method of Bianco *et al.* (10), and the ingestion index was expressed as the product of the percentage of macrophages which ingest SRBC and the average number of SRBC ingested by a macrophage following the counting of 200 macrophages on each coverslip at random.

Analysis of Fc Receptors. Binding parameters of Fc receptors of macrophages were determined according to the modified method of Yagawa *et al.* (11). Peritoneal macrophages suspended in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (1×10^5 cells/0.2 mL) were incubated with various amounts (0.06–1 μg/0.3 mL) of horseradish peroxidase conjugated to either mouse IgG (peroxidase-IgG) or its $F(ab')_2$ fragment [peroxidase-$F(ab')_2$] at 4°C for 5 hr in a centrifuge tube (Corning) preblocked with 3% skim milk to prevent the nonspecific binding of antibodies.

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After being washed three times with PBS, 0.3 mL of 0.05% H_2O_2 containing 0.27 m*M* 2,2'-azino-bis-(methylbenzothiazoline sulfolic acid) dissolved in 100 m*M* citrate buffer (pH 4) was added and incubated at 37°C for 20 min. The amounts of IgG or $F(ab')_2$ bound to macrophages were determined based on absorbance at 405 nm.

RESULTS

Fc Receptor-Mediated Phagocytosis of Opsonized SRBC. The effects of liposomes on Fc receptor-mediated phagocytosis of IgG-coated SRBC were investigated using peritoneal macrophages of BALB/c mice. The ingestion index increased as early as day 1 and continued to do so up to day 4 following liposome intraperitoneal injection (Fig. 1A). No increase in saline-injected control mice could be detected throughout the experimental period. The ingestion index of macrophages from liposome-administered mice was maintained at control levels when using nonopsonized SRBC as target cells.

The effects of the liposomal dose on the ingestion index were examined using peritoneal macrophages harvested on day 4 following liposome injection. As shown in Fig. 1B, this index increased with liposomal dose and reached a plateau at 2.27 μmol lipid/mouse.

To determine the effects of liposomal charge on macrophage activation, neutral, negatively and positively charged liposomes were prepared. Table I summarizes the ζ potential of liposomes and ingestion index of macrophages induced by liposomes of various lipid composition. Values of the latter parameter for macrophages treated with five kinds of liposomes were virtually the same, indicating that the kinds of liposomal lipids and ζ potential did not contribute directly to macrophage activation.

Estimation of Binding Parameters. To clarify in detail the mechanism for the increase in the ingestion index, binding parameters of Fc receptors of macrophages were determined. IgG bound to macrophages from liposome- and saline-injected mice increased with the amount of peroxidase-IgG (Fig. 2, inset) added. Nonspecific binding of IgG estimated from peroxidase-$F(ab')_2$ binding was negligible. Schatchard plot analysis showed straight lines, and binding constants (K) were 3.6×10^8 and 3.0×10^8 M^{-1} for macrophages from liposome- and saline-injected mice, respectively. The number of binding sites (n) of liposome-treated macrophages (3.2×10^5/cell) was 4.6 times that in the case of saline treatment (0.7×10^5/cell) (Fig. 2).

Fc Receptor-Mediated Phagocytosis of Macrophages from C3H/HeJ. To examine the contamination of LPS in the liposomal preparation, C3H/HeJ, an LPS-nonresponding mouse strain, was used. As shown in Fig. 3, peritoneal macrophages from liposome-injected C3H/HeJ mice showed essentially the same extent of increase in the Fc receptor-mediated ingestion index as macrophages from BALB/c mice. LPS per se did not elevate the ingestion index and showed the same levels as those of saline-treated macrophages. The *Limulus* test showed the absence of contamination of LPS in the liposomal preparation.

DISCUSSION

Liposomes are taken up by macrophages (4,12,13) and act as immunological adjuvants (14,15). The mechanism of macrophage activation by liposomes is still unclear. Fc receptors on the surface of macrophages participate in regulating the activity of macrophages, such as through phagocytosis of the immune complex, antibody-dependent cellular cytotoxicity, production of active oxygens, and release of prostaglandins (16).

The activation of mouse peritoneal macrophages by intraperitoneal injection of empty liposomes was assessed in this study based primarily on the extent of Fc receptor-mediated phagocytosis of opsonized SRBC. Following the intraperitoneal injection of liposomes into BALB/c mice, the activation of peritoneal macrophages was highest on day 4 (Fig. 1A) at a lipid concentration of 2.27 μmol lipid/mouse (Fig. 1B). Phagocytosis did not increase in nonopsonized SRBC (Fig. 1A), indicating that Fc receptors of macro-

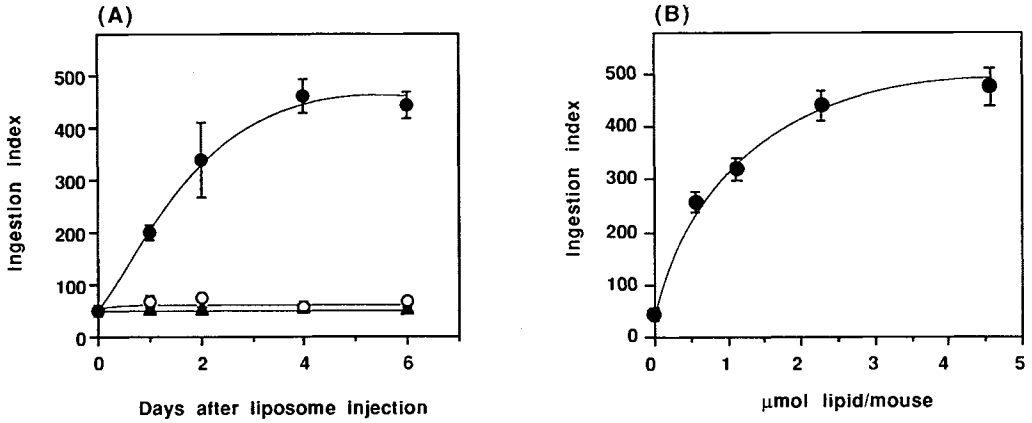

Fig. 1. Effects of liposomes on Fc receptor-mediated phagocytosis of opsonized SRBC by peritoneal macrophages. (A) Macrophages of BALB/c mice were harvested on the indicated days after intraperitoneal injection of liposomes (2.27 μmol lipid/mouse; filled symbols) or saline (open symbols) to BALB/c mice. SRBC opsonized with a subagglutinating level (ca. 40 μg protein/mL) of IgG (circles) or nonopsonized SRBC (triangles) were used. (B) Macrophages from BALB/c mice were harvested on day 4 after intraperitoneal injection of various doses of liposomes (0–4.55 μmol lipid/mouse). The ingestion indexes are the means ± SD of triplicate samples for five experiments.

Table I. Effect of Lipid Composition of Liposomes on Fc Receptor-Mediated Phagocytosis of Peritoneal Macrophages

Lipid composition	ζ potential (mV)[a]	Ingestion index[b]
Control (saline)	—	44.5 ± 3.2
DPPC:Chol = 5.2:4.8	−2.6 ± 0.8	357.0 ± 24.6
DPPC:PS:Chol = 3.2:2:4.8	−32.3 ± 1.3	461.9 ± 33.2
DPPC:PA:Chol = 3.2:2:4.8	−38.1 ± 1.4	422.8 ± 27.2
DPPC:DCP:Chol = 3.2:2:4.8	−33.9 ± 2.1	324.0 ± 23.6
DPPC:SA:Chol = 3.2:2:4.8	+41.5 ± 0.7	406.8 ± 5.5

[a] Each value is the mean ± SD of triplicate experiments.
[b] Each value is the mean ± SD of five experiments.

phages are a possible factor in the increase in phagocytosis induced by liposome administration.

Receptors for negatively charged phospholipids, such as PS, are distributed on a macrophage surface, and negatively charged liposomes are taken up preferentially by macrophages (4,17,18). Liposomes composed of PS may thus be taken up by macrophages, activate them, and enhance Fc receptor-mediated phagocytosis of opsonized SRBC. However, relatively high ingestion index values were observed irrespective of liposomal lipid or charge (Table I). Liposomal uptake would thus appear not to be correlated to the activation of macrophages. Fc receptor-mediated phagocytosis of IgG-coated SRBC did not occur in macrophages treated directly with liposomes *in vitro*, and B lymphocytes were found to be required to activate Fc receptor-mediated phagocytosis (19). It thus appears that a factor secreted from or a serum factor modified by liposome-treated B lymphocytes contributes to macrophage activation.

The activation of Fc receptor-mediated phagocytosis by macrophages from liposome-injected mice may occur in the

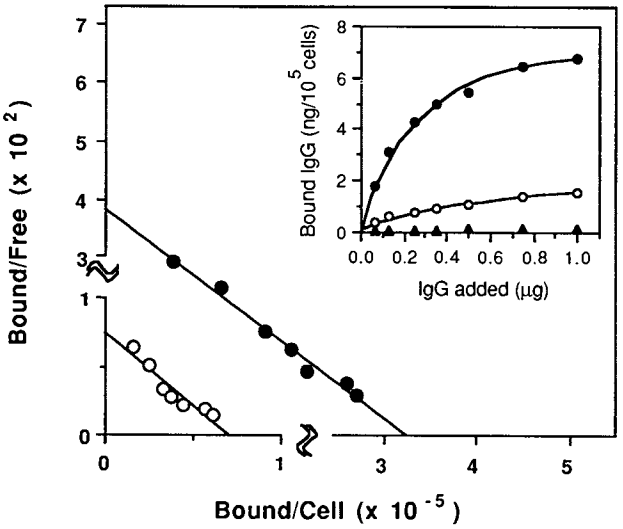

Fig. 2. Schatchard plot of the binding of IgG to macrophages. Macrophages of BALB/c mice were harvested on day 4 after intraperitoneal injection of liposomes (2.27 μmol lipid/mouse; filled circles) or saline (open circles). The inset shows the direct plot of the binding of IgG to macrophages. Filled triangles represent the binding of $F(ab')_2$ to macrophages. The points are the means of triplicate samples.

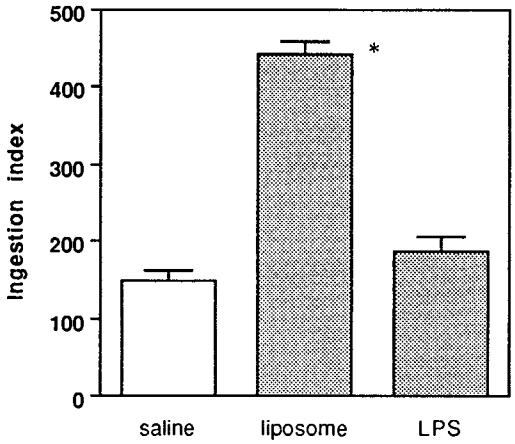

Fig. 3. Fc receptor-mediated phagocytosis of macrophages from C3H/HeJ mice. Macrophages of C3H/HeJ mice were harvested on day 4 after intraperitoneal injection of liposomes (2.27 μmol lipid/mouse) or LPS (20 μg/mouse) and ingested opsonized SRBC. Ingestion indexes are the means ± SD of triplicate samples for five experiments. (*) Statistically significant difference from saline, $P < 0.01$.

following two ways: an increase in the number of Fc receptors and an enhancement of the activity of preexisting Fc receptors. Thus, binding parameters were determined by Schatchard plot analysis. The number of Fc receptors on macrophages (n) from liposome-injected mice was increased prominently, but the binding constant (K) was essentially the same for both macrophages (Fig. 2). The activation of Fc receptor-mediated phagocytosis by macrophages may thus quite likely be due to the increase in the number of Fc receptors on macrophages, but not in the activity of preexisting Fc receptors.

Macrophages are activated by LPS (20,21) and thus the effects of LPS on liposomal effects were investigated in greater detail using macrophages from C3H/HeJ mice, as an LPS-nonresponder strain. The ingestion index of Fc receptor-mediated phagocytosis after LPS injection remained at control levels but increased following liposome injection (Fig. 3). These effects thus arise from liposomes themselves, not from contamination of LPS in the liposomal preparation. Liposomal effects on peritoneal macrophages are the same, regardless of the mouse strain.

Macrophage activation and immune adjuvant effects of liposomes are induced by liposomes taken up by macrophages (1,22). However, the activation of Fc receptor-mediated phagocytosis of opsonized SRBC by liposomes may not be induced by liposomes taken up by macrophages, and it is suggested that some soluble factor(s) contributes to the macrophage activation following liposome injection. The mechanism for the activation of macrophage functions by liposomes, particularly Fc receptor-mediated phagocytosis, should be clarified for evaluating the bactericidal or cytotoxic activity of macrophages.

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