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Adjuvant Effects of Liposomes Containing Lipid A: Enhancement of Liposomal Antigen Presentation and Recruitment of Macrophages

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Liposomes containing lipid A induced potent humoral immune responses in mice against an encapsulated malaria antigen (R32NS1) containing NANP epitopes. The immune response was not enhanced by lipid A alone or by empty liposomes containing lipid A. Experiments to investigate the adjuvant mechanisms of liposomes and lipid A revealed that liposome-encapsulated R32NS1 was actively presented by bone marrow-derived macrophages to NANP-specific cloned T cells. The degree of presentation was related to the amount of liposomal antigen added per macrophage in the culture medium. At high cell densities, poor presentation occurred when liposomes lacked lipid A but excellent presentation occurred when the liposomes contained lipid A. Liposomes containing lipid A and encapsulated antigen also activated gamma interferon-treated macrophages to produce nitric oxide. Macrophage activation and antigen presentation occurred with liposomes that could not be detected by the *Limulus* amebocyte lysis assay. Intraperitoneal injection of liposomal lipid A caused a marked increase in the recruitment of immature (peroxidase-positive) macrophages to the peritoneum. On the basis of these experiments, we propose that the mechanism of the adjuvant action of liposomal lipid A is partly due to increased antigen presentation by macrophages and partly due to recruitment of an increased number of macrophages serving as antigen-presenting cells.

In recent years, there has been a growing interest in the use of liposomes as carriers of antigens (Ag) for immunization (1, 2, 17, 28). Liposomes are delivered avidly and efficiently to macrophages, and because of this, it has been presumed that macrophages could serve as Ag-presenting cells for liposomal Ag (1, 28). Support for this hypothesis has been provided by in vitro model systems that have demonstrated that liposomal Ag are immunologically presented to T lymphocytes by cultured macrophages (9, 18).

One of the theoretical advantages of liposomes is that they can carry adjuvants in addition to Ag, and one adjuvant that has been successfully employed is lipid A (LA), the endotoxic moiety of gram-negative bacterial lipopolysaccharide (LPS) (2, 3, 4, 22, 23). Although liposomal LA has been extensively utilized as an adjuvant, the mechanism of its adjuvant activity is not yet fully understood. Liposomal LA can be a potent mitogen for stimulation of murine B lymphocytes (5), and this might contribute to its adjuvant activity in mice. It has also been reported elsewhere that liposomal LPS activates macrophages to develop tumoricidal activity (13, 14). However, the macrophage-activating properties of liposomal LPS are controversial, and it has also been reported that when compared with LPS or LA alone, liposomal LPS or liposomal LA has a markedly diminished ability to activate macrophages to induce tumoricidal activity (8, 11) and also has a diminished capacity to cause secretion of interleukin-1 by macrophages (12).

In the present study, we investigated possible cellular mechanisms underlying the ability of liposomal LA to stimulate enhanced humoral immunity to a poorly immunogenic Ag in mice. In vitro experiments demonstrated that liposomal LA caused marked enhancement of immunological presentation of liposomal R32NS1 by cultured bone marrow-derived macrophages. We also found that intraperitoneal injection of liposomal LA resulted in vigorous recruitment of peritoneal inflammatory macrophages.

MATERIALS AND METHODS

Lipids. Lipids were purchased from the following sources: dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol, Avanti Polar Lipids, Inc., Alabaster, Ala.; cholesterol, Calbiochem-Behring, La Jolla, Calif.; and LA (isolated from *Salmonella minnesota* R595), List Biological Laboratories, Campbell, Calif.

Ag and antibodies. The Ag used in this study, R32NS1 and R32LR, were kindly supplied by SmithKline Beecham Pharmaceuticals, Swedeland, Pa. R32 consists of 30 repeats of the tetrapeptide NANP interspersed with two tetrapeptide NVDP repeats from the immunodominant repeat region of the circumsporozoite (CS) protein of *Plasmodium falciparum*. In the case of R32NS1, R32 is linked to 81 amino acids from the nonstructural protein of influenza virus (NS1) (25). In the case of R32LR, R32 is linked to the first two amino acids, leucine and arginine, from a nonsense reading of the tetracycline resistance gene of the vector (19). A monoclonal antibody against CS protein, Pf 1B2.2 (31), was the primary antibody used in the enzyme-linked immunosorbent assay (ELISA) described below.

Preparation of liposomes. Detailed procedures for preparation of liposomes were described previously (6, 23, 24, 29). Liposomes were composed of dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, cholesterol, and LA in molar ratios of 1.8:0.2:1.5:0.04 (*Limulus* positive) or 1.8:0.2:1.5:0.005 (*Limulus* negative), in which the molarity of LA is expressed as LA phosphate. *Limulus*-positive and *Limulus*-negative liposomes either caused coagulation or failed to cause coagulation, respectively, in the *Limulus* amebocyte lysate (LAL) assay, as described previously (24).

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LA contains approximately 1.1 μg of LA per nmol of LA phosphate. Except in the experiments in which *Limulus*-negative liposomes were identified as having been used, all experiments utilized *Limulus*-positive liposomes. For the purpose of following the uptake of liposomes by macrophages, a trace amount of cholesterol [^{14}C]oleate (58 mCi/mmol; E. I. du Pont de Nemours, NEN Research Products, Boston, Mass.) was included in the lipid bilayer as a nonexchangeable lipid marker.

Multilamellar liposomes were prepared by dispersion of lyophilized mixtures of lipids in Dulbecco's phosphate-buffered saline (PBS; GIBCO, Grand Island, N.Y.) containing or lacking R32NS1, such that the liposomal phospholipid concentration was 10 mM with respect to the buffer. The liposomes were washed twice with 0.15 M NaCl at 27,000 × *g* for 10 min at 20°C to remove unencapsulated Ag. The resulting liposomes were suspended in 0.15 M NaCl to give a final phospholipid concentration of 10 mM and stored at 4°C until used. Liposome-encapsulated malaria Ag was estimated to be approximately 10 ng/nmol of liposomal phospholipid.

Phagocytosis of liposomes by bone marrow-derived macrophages. Macrophages derived from the bone marrow of C3H/HeN mice (Frederick Cancer Research Facility, Frederick, Md.) were cultured in 24-well culture plates (Costar, Cambridge, Mass.) for 6 days, as described previously (7, 29). Phagocytosis of liposomes by macrophages was determined as previously described (30). Briefly, macrophage cultures were washed three times with RPMI 1640 medium and incubated with liposome-encapsulated Ag (0.6 μg of R32NS1 and/or 50 nmol of liposomal phospholipid per ml of RPMI 1640 per 10^6 cells) at 37°C with 5% CO_2 and 95% relative humidity for the indicated time periods. At the end of the incubation, the cultures were washed three times with 1 ml of PBS to remove noningested Ag, covered with 1 ml of PBS, and placed on ice. Cells were harvested by repeated washing and scraping of culture wells with ice-chilled PBS. The amounts of liposomal Ag phagocytosed by macrophages were estimated by measuring uptake of cholesterol [1-^{14}C] oleate-labelled liposomes. When metabolic inhibitors were used to distinguish between phagocytosis and nonspecific binding, the cultures were preincubated for 10 min with antimycin A and NaF, inhibitors of mitochondrial respiration and glycolysis, respectively. During subsequent incubation of the macrophage cultures with liposomes, the inhibitors were present in the medium.

Immunizations. C3H/HeN mice were used for the immunization protocols. Five mice per group were injected with each immunogen. Animals were injected intraperitoneally with 7 μg per dose of free Ag, free LA, liposomal Ag [L(Ag)], liposomes containing Ag and LA [L(Ag + LA)], or various combinations of these. Free LA was dissolved in 0.5% triethylamine, diluted in 0.154 M NaCl, and injected at doses similar to those of liposomal LA. Control groups consisted of animals immunized by direct injection of Ag into the peritoneal cavity. Blood was collected by tail vein bleeding on days 5, 10, and 15 following immunization. Sera were stored at −70°C until used.

ELISA. Solid-phase ELISAs were performed to measure the levels of immunoglobulin G (IgG) antibody against a capture Ag (R32LR) containing the same repeating units as the Ag that was used for immunization (29). Assays were performed at room temperature (27°C) in 96-well, U-bottom Immulon-2 polystyrene plates (Dynatech Laboratories, Alexandria, Va.). Ag (R32LR) in a solution of PBS containing 0.0004% boiled casein was added to the wells to give a concentration of 10 ng of Ag per well in a volume of 50 μl.

Blocking buffer was prepared by dissolving 5 g of bovine technical grade milk casein in 100 ml of 0.1 M NaOH by boiling. The volume was brought to 1 liter with PBS after the casein was dissolved, the pH was adjusted to 7.4 by using HCl, and then 0.1 g of thimerosal and 0.2 g of phenol red were added. All constituents of the blocking buffer were purchased from Sigma Chemical Co., St. Louis, Mo.

Control wells used for creating a standard curve contained R32LR Ag in concentrations ranging from 0.15 to 10 ng, and an anti-CS protein monoclonal antibody was used as the primary antibody. The plates were covered and stored overnight. The wells were emptied by aspiration, filled with blocking buffer, and allowed to stand for 2 h. Blocking buffer was removed and, except for control wells which received blocking buffer alone, 50 μl of mouse sera diluted 1:50 in blocking buffer was added to each well. The primary antibody for standard curve was anti-CS protein Pf 1B2.2 (31), which was added in a concentration of 100 ng/50 μl of blocking buffer per well. The plates were covered and incubated for 2 h. The well contents were aspirated, and the wells were washed three times with PBS containing 0.05% Tween 20 (J. T. Baker, Phillipsburg, N.J.). Fifty μl of peroxidase-conjugated goat anti-mouse IgG (heavy plus light chains) (Bio-Rad, Richmond, Calif.) diluted 1:500 in blocking buffer was added to each well and incubated for 1 h. The wells were washed three times with PBS containing 0.05% Tween 20. ABTS peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was added, and A_{405} was determined after 1 h with a Vmax Kinetic Microplate Reader (Molecular Devices, Palo Alto, Calif.).

Ag presentation assay. Bone marrow-derived macrophages were used as Ag-presenting cells in T-cell proliferative assays. Marrows from femurs of 8- to 10-week-old C57BL/10 (*H-2*^{b}) mice (Jackson Laboratory, Bar Harbor, Maine) were isolated and cultured for 10 days in 96-well Costar 3590 plates in macrophage growth medium (RPMI 1640 containing 10% fetal bovine serum, L-glutamine [8 mM], penicillin [100 U/ml], streptomycin [100 μg/ml], and 10% L-929 cell-conditioned medium [7, 29]). Macrophages were seeded at densities ranging from 5 × 10^4 to 1 × 10^6 cells per well. On day 9, macrophage cultures were supplemented with 5 U of murine gamma interferon (IFN-γ) per ml (a gift from Carol Nacy, Walter Reed Army Institute of Research). Macrophage growth medium supplemented with fetal bovine serum was replaced with RPMI 1640 medium before Ag addition. Different concentrations of liposomal Ag in a volume of 200 μl of RPMI 1640 were added to the macrophage cultures as indicated. The cultures were incubated for 90 min, which was found to be the optimum length of time in other experiments (data not shown), at 37°C, with 5% CO_2 and 95% relative humidity. At the end of the incubation period, noningested Ag were removed by aspirating the medium, cells were washed three times with 200 μl of RPMI 1640 maintained at 37°C, and 200 μl of the same medium per well was added to the cultures. Macrophage cultures were exposed to 3,000 R irradiation from a cobalt source (Gammacell 220, cobalt 60 irradiator; Atomic Energy of Canada Ltd.). The media were aspirated from the wells and replaced with 100 μl of T-cell growth medium (RPMI 1640 containing EHAA [1:1 ratio; Whittaker M. A. Bioproducts, Walkersville, Md.], 100 U of penicillin per ml, 100 μg of streptomycin per ml, 8 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 μM 2-mercaptoethanol, and 5% fetal bovine serum [HyClone, Logan, Utah]).

An $(NANP)_{40}$-specific T-cell clone (a gift from G. Corradin, University of Lausanne, Switzerland) was maintained in our laboratory as described previously (27). At 14 to 21 days after restimulation with the Ag in the presence of syngeneic splenic cells, viable T cells were collected after removal of dead cells by centrifugation over Ficoll-Metrizoate and were adjusted to 10^5 cells per ml in T-cell growth medium. A 100-μl volume of the T-cell clone (10^4 cells) in complete RPMI 1640 was added to each well containing Ag-pulsed macrophages. The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere for 72 h. During the last 16 h of incubation, 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mM [NEN]) was added to each well. At the end of the culture period, cells were harvested and processed for scintillation spectroscopy (LKB betaplate).

Activation of macrophages to produce nitric oxide. Nitric oxide produced by macrophages treated with murine INF-γ and liposomal LA was measured by a modification of previously described methods (16). Cultured peritoneal macrophages from C57BL/6 mice (1.2×10^5 cells per well) were incubated for 2 h with Dulbecco's modified Eagle medium (DMEM) containing 5% fetal calf serum (FCS) (16). The cells were washed and incubated for 90 min with 100 μl of DMEM medium containing 5% FCS, 10 units of IFN-γ per ml, and either R32NS1 *Limulus*-negative or R32NS1 *Limulus*-positive liposomes. The cells were washed twice and incubated with DMEM containing 5% FCS. After 72 h, 50 μl of the medium was removed and assayed for nitric oxide by the Griess reaction (15). Briefly, 50 μl of the medium was incubated with 50 μl of 1% sulfanilamide and 50 μl of 1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4 (Sigma Chemical Co.) at room temperature for 5 min. Optical density was measured at 543 nm, and nitrite was quantified by comparison with $Na(NO)_2$ as standard.

Peritoneal cell collection. Seven-week-old C3H/HeN male mice were injected intraperitoneally with 0.1-ml volumes of (i) sterile PBS, (ii) 100 μg of liposome-encapsulated R32NS1 malaria Ag [L(Ag)], or (iii) 100 μg of liposome-encapsulated R32NS1 in which the liposomes also contained 20 μg of LA as an adjuvant [L(Ag + LA)]. Five mice for each group were injected. Cells were collected after 6 and 24 h by peritoneal lavage with 10 ml of PBS. Peritoneal fluid was withdrawn through the anterior abdominal wall with an 18-gauge needle and collected in 50-ml tubes.

Peritoneal cells were counted in a hemocytometer. Differential counts were made on Diff-Quick-stained cell smears prepared by centrifugation of 0.2 ml of the cell suspension in a Cytospin centrifuge (Shandon Southern Instruments, Ltd., Camberley, United Kingdom) at 10,000 rpm for 7 min. The peritoneal cell suspensions were centrifuged at $400 \times g$ for 10 min at 4°C and adjusted to 10^6 cells per ml in PBS. The spleens were removed from the animals and weighed.

Identification and quantitation of mature and immature macrophages. Cell smears were prepared on glass slides after cytocentrifugation as described above. The slides were fixed in an ethanol-formaldehyde (9:1) solution for 1 min, washed in tap water, and air dried. Peroxidase staining was performed by Kaplow's method (20). Briefly, 20 mg of diaminobenzidine hydrochloride (Sigma) was dissolved in 40 ml of 0.1 M Tris-HCl buffer (pH 7.4), and 0.2 ml of 3% H_2O_2 was added to the solution. The cell preparations were incubated in this mixture for 2 h in a water bath at 37°C, with intermittent shaking. The slides were then counterstained in either acid hematoxylin solution or Giemsa stain for 10 min, washed in tap water, and mounted with Permount. Peroxidase-positive cells characterized by pink-to-light-purple granules in the cytoplasm were counted.

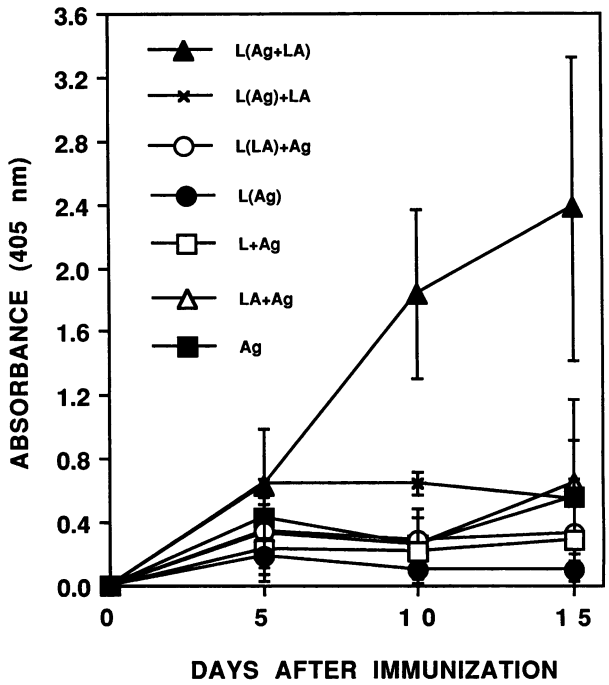

FIG. 1. Immune response of mice to different combinations of liposomal malaria Ag and LA. The IgG antibody response of C3H/HeN mice injected intraperitoneally with R32NS1 alone or in various liposomal formulations was measured by binding to R32LR in a solid-phase ELISA. When free Ag or free LA was used, it was injected in amounts comparable to those of liposomal Ag [L(Ag)] or liposomal LA [L(LA)]. The sera obtained were tested by ELISA for IgG activity against R32NS1 antigen. Each absorbance value was corrected by subtracting the absorbance value obtained for the corresponding preimmune serum. The data shown represent the means ± standard deviations of triplicate observations. At 10 and 15 days, all L(Ag + LA) values were very significantly different from those of L(Ag) and L(Ag) + LA ($P < 0.01$), and the values of L(Ag) + LA compared with those of L(Ag) were also significantly different ($P < 0.03$; two-tailed Student's t test).

RESULTS

Immune response to liposomal Ag. Liposomes, malaria Ag (R32NS1), and LA, either alone or in different combinations, were injected into the mice in order to induce serum antibodies (Fig. 1). A potent immune response was obtained after injection of liposomes containing R32NS1 Ag and LA [i.e., L(Ag + LA)]. In contrast, a relatively low level of antibody response was obtained after injection of each of the other formulations, including liposomes containing Ag but lacking LA [L(Ag)], Ag alone, Ag mixed with LA (LA + Ag), empty (Ag-free) liposomes containing LA [L(LA) + Ag], or empty liposomes lacking LA (L + Ag), and liposomes containing Ag followed by injection of LA [i.e., L(Ag) + LA] (Fig. 1). The observations presented in Fig. 1 are consistent both with the well-known poor immunogenicity of nonencapsulated R32 Ag in rabbits and monkeys (22) and with previous observations demonstrating excellent immunogenicity of R32 repeat epitopes encapsulated in liposomes containing LA (2, 22). The data suggest that LA was the key element responsible for the enhanced immune response in

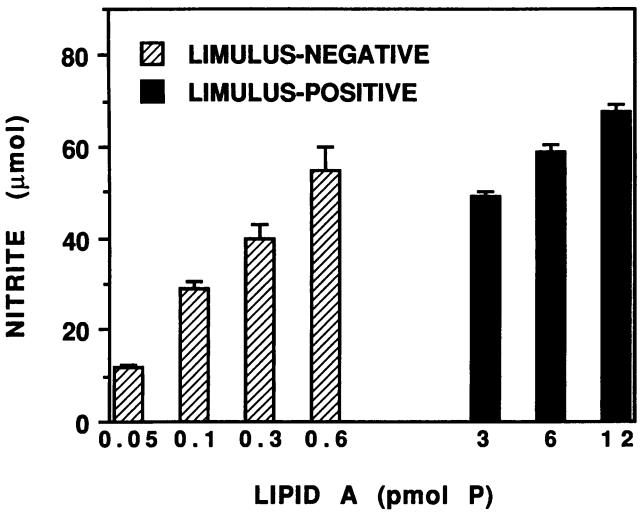

FIG. 2. Effect of liposomal LA on macrophage activation. Liposomes containing encapsulated antigen R32NS1 and different concentrations of LA (*Limulus*-negative and *Limulus*-positive liposomes) were incubated for 90 min with macrophage cultures (1.2×10^5 cells per well) that had been pretreated with murine IFN-γ. The cultures were incubated for 72 h at 37°C with 5% CO_2 and 95% humidity. Culture supernatants were then collected for nitric oxide measurements as described in Materials and Methods. The data are the means ± standard deviations of triplicate observations.

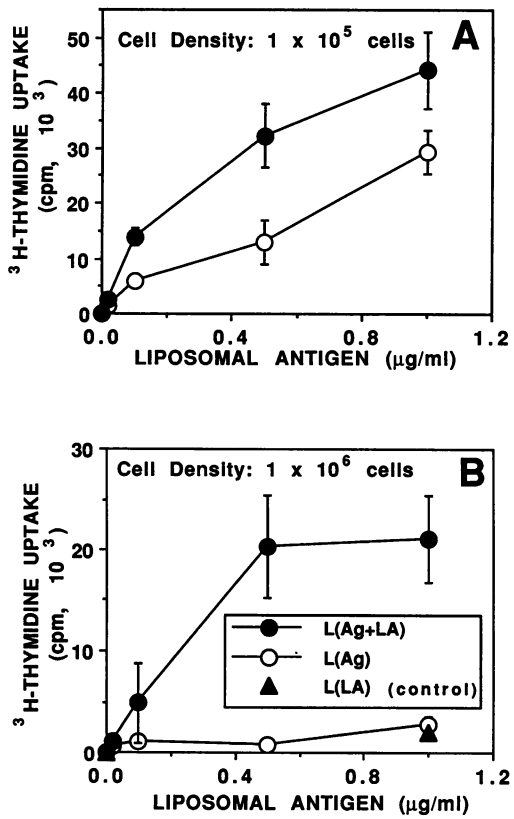

FIG. 3. Effects of cell density and liposomal Ag concentration on T-cell proliferation. C57BL/10 bone marrow-derived macrophages were seeded at 10^5 (A) or 10^6 (B) cells per well. Macrophages were pulsed with different concentrations of L(Ag) or L(Ag + LA) for 90 min prior to the addition of an $(NANP)_{40}$-specific T-cell clone (10^4 cells per well). During the last 16 h of the 72-h culture period, 1 μCi of [^{3}H]thymidine was added. At the end of the incubation period, the cells were processed for scintillation spectroscopy. Data are expressed as mean counts per minute of [^{3}H]thymidine uptake ± standard deviations of triplicate cultures.

vivo and that the simultaneous presence of Ag and LA in the liposomes was required for expression of adjuvant activity.

Effects of LA on phagocytosis of liposomes by macrophages. A series of experiments was devised to try to elucidate the cellular mechanisms by which LA exerts its effects. Cholesterol [^{14}C]oleate-labelled liposomes (50 nmol of liposomal phospholipid in [L(Ag)] or [L(Ag + LA)]) were incubated with macrophage cultures (10^6 cells per well) for 6 h at 37°C with 5% CO_2 and 95% humidity. Separate macrophage cultures were preincubated for 10 min with the metabolic inhibitors NaF and antimycin A in a final concentration of 10 mM and 1 μg/ml, respectively, before addition of liposomes. At the end of 6 h, cells were harvested by washing with cold PBS and liposome uptake was determined by measurement of radioactivity. Ingestion of liposome-encapsulated Ag by cultured macrophages resulted in continuously increasing levels of liposome uptake over a period of 24 h (data not shown). There was no evidence of increased phagocytosis induced by LA. A slight but not significant reduction in the rate of phagocytosis of [L(Ag + LA)] compared with that of L(Ag) was observed. Phagocytosis was distinguished from nonspecific binding of liposomes to macrophage surfaces by separate control cultures incubated with metabolic inhibitors (antimycin A and NaF). The liposome uptake of L(Ag) (in nanomoles of phospholipid per 10^6 macrophages ± standard deviations of triplicate observations) was 16.94 ± 0.98 without inhibitors and 0.83 ± 0.17 with inhibitors. For L(Ag + LA), the uptake was 12.87 ± 0.80 without inhibitors and 0.72 ± 0.16 with inhibitors.

Effects of LA on activation of macrophages. Induction of nitric oxide production by cultured macrophages with liposomes containing Ag and LA in the presence of IFN-γ was examined (Fig. 2). Two different types of liposomes with either a very low epitope density of liposomal LA (*Limulus* negative) or a very high epitope density of liposomal LA (*Limulus* positive) were employed. The total amount of LA added to the cells (as shown on the abscissa of Fig. 2) was adjusted by adding different dilutions of either *Limulus*-negative or *Limulus*-positive liposomes. The data indicate that nitrite production by stimulated macrophages was dependent on the total amount of LA added to the cells but was not dependent on the epitope density of LA in the liposomes (Fig. 2).

It should be pointed out that our phagocytosis model is limited in sensitivity at low levels of liposomes. Because of this and because of the great disparity in the total amount of LA that is present in equal numbers of *Limulus*-negative and *Limulus*-positive liposomes, it was not technically possible to compare extremely small amounts of *Limulus*-positive and large amounts of *Limulus*-negative liposomes in order to maintain identical total amounts of LA (Fig. 2). However, Fig. 2 suggests that this limitation in the assay system may not be important, because despite differences in total LA there was considerable overlap in the total amount of nitrite generated by *Limulus*-negative and *Limulus*-positive liposomes.

Effects of LA on Ag presentation by macrophages. Proliferative activities of an $(NANP)_{40}$-reactive T-cell clone were assayed in cultures seeded with 10^5 or 10^6 bone marrow (BM) precursor cells per well (Fig. 3). To prevent the inherent

TABLE 1. Proliferation of an $(NANP)_{40}$-specific T-cell clone expressed as a function of bone-marrow derived macrophage cell density[a]

No. of BM cells seeded on day 0	Mean [^{3}H]thymidine uptake (cpm [10^3] ± SD)		Fold increase induced by LA
	L(Ag)	L(Ag + LA)	
5×10^4	32.39 ± 5.17	34.52 ± 6.04	1
5×10^5	5.86 ± 0.94	21.98 ± 5.70	4
1×10^6	0.85 ± 0.27	17.56 ± 1.61	21

[a] C57Bl/10 BM cells seeded at different cell densities on day 0 were pulsed for 90 min on day 10 with 0.05 μg of liposomal R32NS1 per well. The cells were then washed and irradiated prior to the addition of the $(NANP)_{40}$-specific T-cell clone (10^4 cells per well). The cultures were incubated for 72 h at 37°C with 5% CO_2 and 95% humidity. During the last 16 h of incubation, 1 μCi of [^{3}H]thymidine was added, and the cells were then harvested for scintillation spectroscopy. The data are the mean counts per minute of [^{3}H]thymidine uptake ± standard deviations of triplicate cultures and represent at least three experiments.

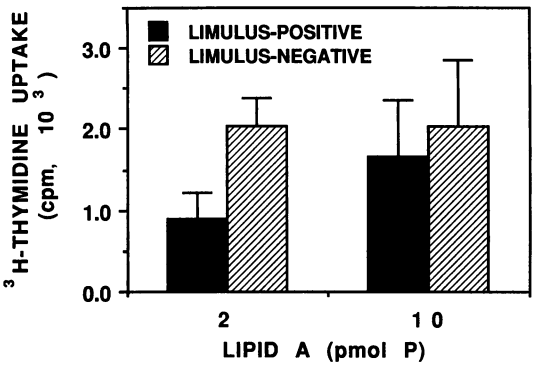

FIG. 4. Effect of liposomal LA concentration on Ag presentation. An $(NANP)_{40}$-specific T-cell clone (10^4 cells per well) was cultured for 72 h with syngeneic C57BL/10 bone marrow-derived macrophages (5×10^4 cells per well) which had been pulsed for 90 min with liposomes containing R32NS1 and the concentrations of LA indicated (*Limulus*-positive or *Limulus*-negative liposomes). The cells were processed as described in the legend to Fig. 3.

variability between different T-cell proliferation assays, the data presented in Fig. 3A and B were derived from the same experiment. At an original cell density of 10^5 cells per well (Fig. 3A), liposomes containing Ag and LA caused enhanced T-cell proliferation when compared with liposomes lacking LA. This enhanced Ag presentation was more pronounced at a higher cell density (10^6 cells per well) (Fig. 3B). In the latter case, liposomes lacking LA essentially lacked activity. Liposomes containing LA but lacking the Ag [L(LA)] did not cause nonspecific proliferation of T cells. The data suggest that cell density and liposome concentration each play a role in affecting Ag presentation by liposomal LA.

The factors affecting liposomal Ag presentation by bone marrow-derived macrophages are further highlighted by expressing the data as a function of BM cell number (Table 1). When the number of BM cells seeded was increased, liposomal LA caused an enhancement of up to 21-fold in Ag presentation (Table 1). At lower cell densities, the liposomal Ag was probably being presented at a maximum efficiency and inclusion of liposomal LA therefore did not cause any increase in Ag presentation. The data suggest that LA had a marked stimulatory effect on Ag presentation, especially at high cell densities.

The role of liposomal LA epitope density on the stimulation of Ag presentation by macrophages was examined by comparing the effects of R32NS1 *Limulus*-negative and R32NS1 *Limulus*-positive liposomes (Fig. 4). In each case, the total amount of LA added to the macrophages (shown on the abscissa) was controlled by adjusting the total amounts of liposomes added. *Limulus*-negative and *Limulus*-positive liposomes were each highly effective in stimulating Ag presentation.

Effects of liposomal LA on recruitment of inflammatory cells. Differential cell counts were performed on inflammatory exudates that were obtained at 6 and 24 h following intraperitoneal injection of liposomes. Inclusion of LA in the liposomes resulted in an increase in spleen weights of the mice and also in a significant increase (threefold) in the numbers of intraperitoneal polymorphonuclear leukocytes and promonocytes (Table 2). Peroxidase-positive macrophages, which characteristically migrate to sites of inflammation, were also significantly increased after injection of L(Ag + LA) when compared with injection of L(Ag). All of these effects were more pronounced at 6 than at 24 h after injection.

DISCUSSION

This study demonstrates that liposomes containing large amounts of LA exert a strong adjuvant activity for a poorly immunogenic liposomal Ag in mice (Fig. 1). Although similar findings were obtained with the same or related Ag in rabbits (22), monkeys (23), and humans (4), a unique aspect of the current study is the demonstration that neither LA nor liposomal LA had the capability by itself to serve as a potent adjuvant. Liposomal LA by itself did not stimulate the immune response to free Ag [L(LA) + Ag], and free LA did not stimulate significantly the immune response either to free Ag (LA + Ag) or even to liposomal Ag [LA + L(Ag)]. Adjuvant activity occurred only when the LA and the Ag were both present in the liposomes [L(Ag + LA)]. These results are compatible with a previous immunization study with a liposomal hapten (dinitrophenyl-aminocaproyl-phosphatidylethanolamine) in which an adjuvant effect of LA was observed when LA was in the same liposomes as the hapten but not when LA was in different liposomes (10). It appears that liposomal LA exerts highly focused in vivo adjuvant effects that are effective only for liposomal Ag.

The mechanism underlying the adjuvant effect of liposomal LA, has not yet been completely elucidated. In this study, we have shown that liposomal LA can cause marked enhancement of Ag-specific in vitro presentation of a liposomal Ag by bone marrow-derived macrophages (Fig. 3). Enhancement of presentation by macrophages was not associated with increased phagocytosis of liposomes containing LA (see paragraph on effects of LA on phagocytosis in Results). On the basis of these results, we propose that enhanced presentation induced by liposomal LA was at least partially responsible for the enhanced in vivo humoral immunity to R32NS1 that was observed after injection of liposomes containing both R32NS1 and LA.

It has been recognized for some time that the adjuvanticity of LPS is related to stimulation of interactions between macrophages and T lymphocytes (21). We have shown in this study that high cell densities are correlated with decreased Ag presentation to T lymphocytes (Fig. 3B). We have also shown that under conditions of high cell density, the stimulatory effect of liposomal LA was most apparent (Fig. 3 and Table 1).

In addition to stimulating Ag presentation by macro-

TABLE 2. Recruitment of cells after intraperitoneal injection of liposomes containing or lacking LA[a]

Amt (μg)		Spleen wt (mg)	% of polymorphonuclear leukocytes/total leukocytes		% of peroxidase-positive macrophages/total macrophages		% of peroxidase-positive cells/total leukocytes	
Liposomal LA	Liposomal Ag		6 h	24 h	6 h	24 h	6 h	24 h
0	0	54.8 ± 7.6	0.7 ± 0.6	3.0 ± 2.0	4.0 ± 0.8	0	0.1 ± 0.1	0
0	100	60.6 ± 5.6	17.0 ± 4.0	4.0 ± 4.0	11.0 ± 6.0	4.0 ± 1.0	0.8 ± 0.2	0.1 ± 0.1
20	100	79.3 ± 4.6	60.0 ± 14.0	56.0 ± 1.0	55.0 ± 6.0	11.0 ± 5.0	7.0 ± 0.2	0.4 ± 0.1

[a] Five C3H/HeN mice per group were injected intraperitoneally with 0.1-ml volumes of PBS, liposomes containing R32NS1 [L(Ag)], and liposomes containing R32NS1 and lipid A [L(Ag + LA)], as described in Materials and Methods. Data are means ± standard deviations. All values for the three groups were significantly different at 6 h ($P < 0.05$; two-tailed Student's t test).

phages, intraperitoneal injection of liposomes containing LA caused marked recruitment of migratory inflammatory cells, including polymorphonuclear leukocytes and immature macrophages, into the peritoneum (Table 2). The disappearance of macrophages that was observed between 6 and 24 h is compatible with the macrophage disappearance reaction, in which macrophages are depleted from fluids in the peritoneal cavity because of their acquisition of an adhesive property (26).

Recruitment of large numbers of potential Ag-presenting cells might be expected to result in increased Ag presentation. However, the in vitro results obtained in this study demonstrated that high densities of BM-derived macrophages resulted in decreased Ag presentation that was partially overcome by the inclusion of liposomal LA. On the basis of our in vivo and in vitro experiments, we propose that the adjuvant effect of liposomal LA is at least twofold, including (i) recruitment of Ag-presenting cells and (ii) enhancement of Ag presentation at high BM-derived cell density.

From a theoretical standpoint, activation of macrophages could be initiated either through recognition and binding of liposomal LA on the surface of the macrophage or through an intracellular mechanism. To test whether recognition of LA fatty acids by macrophage surface receptors played a role, we employed *Limulus*-positive and *Limulus*-negative liposomes. It has been shown that sequestration of LA fatty acids can be modulated by changing the epitope density of LA in the liposomes and sequestration can be monitored by the LAL coagulation assay (24). Detection of liposomal LA by the LAL assay is strongly dependent on the epitope density of LA in the liposomes (24). It was previously shown that liposomal LPS and liposomal LA that lacked the ability to activate macrophages to induce interleukin-1 secretion also lacked LAL activity (12). Dijkstra et al. proposed that the sequestered liposomal LA was prevented from interacting with the macrophage and that LA exerted its activity on macrophages only by interacting with the surface of the macrophage plasma membrane (12). Our data demonstrate that activation of cultured macrophages to produce nitric oxide and to enhance Ag presentation can be achieved readily with *Limulus*-negative or *Limulus*-positive liposomes. Therefore, these results are more compatible with a model of an intracellular mechanism of activation than with one of interaction of liposomal LA with the cell surface.

From this study, it is evident that either high or low epitope densities of liposomal LA exert potent abilities to enhance liposomal Ag presentation by macrophages. Although liposomes containing high epitope densities of LA are readily detected by LAL assay, they need not be pyrogenic in vivo (4, 22, 23). This therefore lends support to an immunization strategy for vaccines containing liposomal LA that utilizes high levels of liposomal LA epitope densities in order to stimulate Ag presentation by macrophages.

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