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Phagocytosis of liposomes by macrophages: intracellular fate of liposomal malaria antigen

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Liposomes containing a synthetic recombinant protein were phagocytosed by macrophages, and the internalized protein was recycled to the cell surfaces where it was detected by enzyme-linked immunosorbent assay. The transit time of the liposome-encapsulated protein from initial phagocytosis of liposomes to appearance of protein on the surfaces of macrophages was determined by pulse-chase experiments. The macrophages were pulsed with liposomes containing protein and chased with empty liposomes, and vice versa. The amount and rate of protein antigen expression at the cell surfaces depended on the quantity of encapsulated protein ingested by the macrophages. Although liposomes were rapidly taken up by macrophages, the liposome-encapsulated protein was antigenically expressed for a prolonged period (at least 24 h) on the cell surface. Liposomes were visualized inside vacuoles in the macrophages by immunogold electron microscopy. The liposomes accumulated along the peripheries of the vacuoles and many of them apparently remained intact for a long time (> 6 h). However, nonliposomal free protein was also detected in the cytoplasm surrounding these vacuoles, and it was concluded that the free protein in the cytoplasm was probably en route to the macrophage surface. Exposure of the cells to ammonium chloride did not inhibit the appearance of liposomal antigenic epitopes on the cell surface, and this suggests that expression of the liposomal antigenic epitopes at the surface was not a pH-sensitive phenomenon. There was no significant effect of a liposomal adjuvant, lipid A, on the rate or extent of surface expression of the liposomal protein.

Introduction

The purpose of this study was to investigate the cell biology, intracellular location, and fate of liposomes and liposome-encapsulated protein after phagocytosis by macrophages. Based on data derived from several different types of approaches an apparent consensus has evolved that liposomes become concentrated in, and are gradually degraded in, lysosomal vacuoles within phagocytic cells [1–14]. Although liposomes are strongly lysosomotropic, several studies have also demonstrated that liposomes or liposomal contents apparently can escape to a certain extent from vacuoles directly into the cytosol [15–17]. Occasional reports have suggested that liposomes can travel to intracellular organelles other than lysosomes, including mitochondria and the nucleus [18,19]. Liposome-encapsulated detection probes have been developed by different laboratories for localizing intracellular liposomes, and these have included radioactive tracers [2,7,9–14], heavy metals [7,15], dyes and fluorescent markers [10,12,14,18,19], and enzymes [4,10,12,13,20]. Certain types of detection probes have been of particular interest because of potential practical applications. For example, liposomes have been proposed as lysoso-

Abbreviations: ABTS, 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid); Ag, antigen; Chol, cholesterol; CS, circumsporozoite; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DPBS, Dulbecco's phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; LA, lipid A; L, liposomes; L(Ag), liposomes containing antigen; L(Ag + LA), liposomes containing both antigen and lipid A; MAb, monoclonal antibody.

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motropic carriers for enzyme replacement therapy [1,4,20].

From a theoretical standpoint, liposomes continue to be useful as models for understanding the disposition patterns of materials ingested by macrophages. The fate of liposome-encapsulated protein in macrophages is of particular interest in immunology. Liposomes are highly effective in the stimulation of immune responses to encapsulated protein antigens [21–23]. It is widely believed that the process of immunological presentation of protein antigen to cells in the immune system involves initial processing of the antigen by specialized antigen presenting cells, among which are included macrophages, B lymphocytes, and dendritic cells [24]. It is presumed that the ability of liposomes to enhance immune responses is due to natural targeting of liposomes to macrophages [25,26].

In the present study we have developed enzyme-linked immunosorbent assays and immunogold electron microscopic methods to detect the presence of cell surface-associated and intracellular liposome-associated protein antigen. By using these techniques we have detected concentrations of liposomes and liposomal antigenic epitopes in cytoplasmic vacuoles, and we have also observed nonliposomal free antigenic epitopes in the cytosol after phagocytosis of liposomes. We have discovered that phagocytosed liposomal antigenic epitopes rapidly appear on the surface of the macrophage, and we have measured the kinetics of transfer of liposomal antigenic epitopes to the cell surface.

Materials and Methods

Lipids

Lipids were purchased from the following sources: dimyristoylphosphatidylcholine (DMPC), Sigma Chemical Co., St. Louis, MO; dimyristoylphosphatidylglycerol (DMPG), Avanti Polar Lipids, Inc., Birmingham, AL; cholesterol (Chol), Calbiochem-Behring, La Jolla, CA; lipid A (isolated from *Salmonella minnesota* R595), List Biological Laboratories, Campbell, CA.

Antigen

The antigen ($R32NS1_{81}$, also known as R32NS1) was kindly supplied by SmithKline Beecham Pharmaceuticals, Swedeland, PA. The amino acid sequence of the recombinant antigen ($M_r = 22347$), containing epitopes from the immunodominant repeat region of the CS protein of *Plasmodium falciparum*, is shown in Fig. 1. It consists of 30 repeats of the tetrapeptide (Asn-Ala-Asn-Pro) interspersed with two tetrapeptide (Asn-Val-Asp-Pro) repeats of the CS protein, linked to an 81 amino acid nonstructural protein of influenza virus.

Preparation of liposomes

Multilamellar liposomes were prepared by minor modifications of the method reported by Richards et al. [27]. Lyophilized mixtures of lipids were dispersed in Dulbecco's phosphate-buffered saline (DPBS) with or without R32NS1 antigen. The resulting liposomes were washed twice with 0.15 M NaCl at $27000 \times g$ for 10 min at 20°C. The liposomes were suspended in 0.15 M NaCl to give a final phospholipid concentration of 10 mM and stored at 4°C.

Cholesteryl [1-^{14}C]oleate (Du Pont-NEN Research Products, Boston, MA) was included in the liposomes as a lipid marker to follow the uptake of liposomes by macrophages [28]. Liposomes lacking LA (L or L(Ag)) contained DMPC/DMPG/Chol in molar ratios of 1.8:0.2:1.5; and liposomes containing LA (L(LA) or L(Ag + LA)) contained DMPC/DMPG/Chol/LA in molar ratios of 1.8:0.2:1.5:0.04. Phospholipid concentration of the liposomes was measured by phosphate assay [29].

Analysis of liposome-encapsulated protein

$R32NS1_{81}$ is an extremely hydrophobic protein. In preliminary experiments, attempts to quantitate the amount of liposomal $R32NS1_{81}$ by a modified Lowry assay [30] were unsuccessful because encapsulated antigen bound tightly to the liposomal lipids and was inaccessible to the assay reagents.

The amount of protein encapsulated in liposomes was determined by amino acid analysis after saponification of the liposomes. Liposomes containing encapsulated protein (10–20 μg of protein present in 100 μl of liposomes) were added to a teflon-lined screw cap tube and 300 μl of 40% KOH in methanol were added. The mixture was heated to 65°C for 30 min, allowed to come to room temperature then neutralized by addition of conc. HCl. Approximately 1–5 μg of protein were reduced to dryness using a Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, NY), in a

1- M D P (N A N P)$_{15}$ N V D P (N A N P)$_{15}$ N V D P N T V S S F Q V D

141- C F L W H V R K R V A D D E L Q D A P F L D R L R R D Q K S

171- L R G R G S T L G L D I E T A T R A G K Q I V E R I L K E E

201- S D E A L K M T M L V N

Fig. 1. Protein sequence of $R32NS1_{81}$ as predicted from its gene sequence. Amino acids are represented with standard one letter abbreviations. These are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and W, Trp (structure courtesy of SmithKline Beecham Pharmaceuticals).

16 × 125 mm ignition tube. An excess (300–500 μl) of boiling 6 M HCl, containing 0.5% phenol and 0.5% 2-mercaptoethanol, was then added. The tubes were sealed under vacuum and heated for 16 h at 110°C in a Reacti-Therm Heating Block (Pierce Chemical Co., Rockford, IL) to hydrolyze the protein. This method of hydrolysis degrades tryptophan and cysteine residues. The tube was opened and the contents dried in the Speed-Vac. The sample was dissolved in citrate sample buffer (Beckman Instrument Co., Columbia, MD) containing 1 nmol of norleucine as an internal standard.

The sample was analyzed on a Beckman 6300 amino acid analyzer using standard Beckman citrate buffer and visualizing with ninhydrin reagent at 440 and 570 nm. Peak sizes were measured with a Hewlett-Packard 3390A integrator and converted directly to nmol of amino acid residue by comparison to standards. Norleucine was added to standards as an internal standard.

Macrophage cultures

Bone-marrow derived macrophages from C3H/HeN mice (Frederick Cancer Research Facility, Frederick, MD) were cultured according to the methods of Belosevic et al. [31]. Macrophage culture medium was prepared by supplementing RPMI 1640 medium (GIBCO, Grand Island, NY) with 10% heat-inactivated FBS (GIBCO), 50 μg/ml of Gentamycin (Schering Corp., Kenilworth, NJ) and 10% L cell conditioned medium (one week culture supernate of L-929 cells used as a source of CSF-1). Marrows from femurs of 6–10-week-old mice were flushed with Ca^{2+}-free, Mg^{2+}-free DPBS. Cell suspensions were sedimented at 200 × *g* for 10 min at 4°C and resuspended in macrophage culture medium to give densities of $1 \cdot 10^6$ cells/ml.

Bone-marrow cells were distributed in 96 well Costar® 3590 plates ($1 \cdot 10^5$ cells/well) covered with Costar® 3096 lids (Costar, Cambridge, MA) and incubated at 37°C with 5% CO_2 and 95% relative humidity. The cultures were grown for a total of 6 days and were supplemented with 50 μl and 75 μl of macrophage growth medium on the third and fifth days, respectively. Falcon® 1006 bacterological culture petri dishes (Falcon, Oxnard, CA) were used instead of Costar® 3590 plates in experiments involving phagocytosis of liposomes.

Phagocytosis of liposomes

Cholesteryl [1-^{14}C]oleate-labelled liposomes (200 nmol of liposomal phospholipid in 2 ml of incomplete RPMI (RPMI 1640 without FBS)) were added to each culture dish containing macrophages ($2 \cdot 10^6$ cells) and incubated for varying time periods at 37°C with 5% CO_2 and 95% relative humidity. Metabolic inhibitors NaF and antimycin A were employed to distinguish phagocytosis from nonspecific adherence of liposomes to macrophages [32]. At the end of the incubation the medium was removed and the cultures washed three times with 2 ml of 37°C saline and placed on an ice-cold surface. Cells were harvested by washing the plates thoroughly with 1 ml of ice-chilled saline. Aliquots (0.5 ml) of the cell suspension were digested with 0.5 ml of tissue solubilizer (NCS) in scintillation vials. 10 ml of Hydrofluor liquid scintillation cocktail containing enough glacial acetic acid to neutralize NCS were added and radioactivity was counted. In experiments with metabolic inhibitors, cultures were incubated for 10 min with 10 mM NaF and 1 μg/ml of antimycin A before addition of liposomes. The inhibitors remained present in the cultures during incubation with liposomes.

Electron microscopy

Bone marrow derived macrophages were incubated with liposomal malaria antigen for 6 h. Macrophage cultures were washed with DPBS, fixed for 20 min at 4°C in a mixture of 1% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and washed with 0.1 M phosphate buffer. The cells were dehydrated with ethanol at progressively lower temperatures between 4°C and −20°C and infiltrated with LR Gold resin (London Resin Company) containing 0.75% benzoin methyl ether as an initiator (33]. The cells were polymerized for 48 h at −20°C under an ultraviolet light source and sectioned with a diamond knife. Ultrathin sections were mounted on unsupported nickel grids and blocked for 15 min in a solution of 5% nonfat dry milk, 0.9% NaCl, 0.01% Tween-20 in 0.1 M phosphate buffer (pH 7.4) (PBS-Milk-Tween). Sections were then immersed in primary anti-CS protein antibody (Pf 1B2.2) [34] diluted to a final concentration of 1 μg/ml with PBS-Milk-Tween and incubated for 2 h at room temperature. Grids were jet washed with a solution of 1% bovine serum albumin (Fraction V, ICN Immunobiologicals), 0.9% NaCl, 0.01% Tween-20 in 0.1 M phosphate buffer (pH 7.4) (PBS-BSA-Tween) and incubated for 1 h at room temperature in a rabbit anti-mouse IgG secondary antibody (ICN Immunobiologicals) diluted to approximately 50 μg/ml with PBS-Milk-Tween. Grids were jet washed again and incubated for 1 h at room temperature in goat anti-rabbit IgG immunoglobulin-gold (Janssen) diluted 1/20 with PBS-Milk-Tween. Grids were jet washed with PBS-BSA-Tween and 0.1 M phosphate buffer and fixed for 15 min with 2.5% gluteraldehyde in 0.1 M buffer to stabilize the gold particles, rinsed with distilled water and dried. The sections were stained with 2% uranyl acetate in 50% methanol, contrasted with Reynold's lead citrate, carbon coated in a vacuum evaporator, and examined with a JEOL 100CX electron microscope.

Controls for nonspecific gold labelling were performed by incubating sections with secondary antibody

and gold or with gold alone. Antibody specificity was confirmed by incubating untreated macrophages with primary antibody, secondary antibody, and immunoglobulin gold. As noted in the results, extensive and diffuse cytoplasmic labelling with gold was observed, and two representative electron micrographs were selected for illustration.

ELISA for protein detection on macrophage surfaces

6-day-old cultures of bone-marrow macrophages grown on 96-well Costar® 3590 plates were used. It is pertinent to mention that this plate was suitable both for cell culture and for efficient antigen binding to the wells for ELISA.

Growth medium was removed from culture wells, and liposome-encapsulated protein preparations (L(Ag) or L (Ag + LA)) in incomplete RPMI were added to the wells (50 μl containing 5 nmol liposomal phospholipid/$5 \cdot 10^4$ cells). The cultures were incubated for varying time periods at 37°C with 5% CO_2 and 95% humidity. At the end of the incubation period non-ingested liposomes were aspirated and wells were washed with 50 μl of incomplete RPMI. Cells were fixed by addition of 50 μl of a solution of 3% paraformaldehyde to each well for 20 min at room temperature and the cells were washed twice with 0.1 M glycine (100 μl/well). DPBS was added to the experimental wells and CS protein was added to the wells designated for the standard curve (1.5–100 pg R32NS1 in 100 μl of DPBS/well). The plates were covered with lids and allowed to stand at room temperature overnight. The contents of the wells were aspirated, filled with blocking buffer, and incubated at room temperature for 1 h. Blocking buffer was aspirated and peroxidase-conjugated anti-CS protein Pf 1B2.2 [34] was added (100 ng/100 μl of blocking buffer/well), and the plates were covered and incubated at room temperature for 1 h. Contents of the wells were aspirated and wells were washed three times with DPBS. Peroxidase substrate (200 μl) was added and absorbance at 405 nm was determined after color development.

'Pulse-chase' experiments

Bone marrow macrophages cultured for 6 days in 96 well Costar® 3590 ELISA plates were 'pulsed' by adding L(Ag) (5 nmol of phospholipid/$5 \cdot 10^4$ cells). Following a 3 h incubation period for the pulse, a chase was initiated by replacement of L(Ag) with L. In another set of experiments, cultures were incubated with L for 3 h, then L was replaced with L(Ag) for 24 h. Controls consisted of cultures in which antigen-containing-liposomes L(Ag) were not removed and the 'pulse' was continued for 27 h. At the end of pulse or chase periods cells were fixed with paraformaldehyde and malaria antigen on the macrophage surface was quantitated by ELISA as detailed above.

To study the effect of ammonium chloride on the surface expression of phagocytosed liposomal antigen by macrophages, bone marrow macrophage cultures were preincubated for 50 min with or without 10 mM NH_4Cl in incomplete RPMI 1640 at 37°C with 5% CO_2 and 95% humidity. Ammonium chloride concentration of 10 mM was maintained throughout the duration of both pulse and chase.

Results

Phagocytosis of liposomes containing encapsulated protein

Bone marrow-derived macrophages grown on culture plates were allowed to phagocytose radiolabelled liposomes [L(Ag) or L(Ag + LA)] in the presence or absence of metabolic inhibitors for varying time periods up to 6 h (Fig. 2). Phagocytosis occurred continuously over the entire observation period, and saturation of liposome uptake by the macrophages was not observed. As expected with nonopsonized liposomes, the rate and extent of phagocytosis was less than that previously described with phagocytosis of complement-opsonized liposomes [35].

Treatment with metabolic inhibitors (Fig. 2, bottom

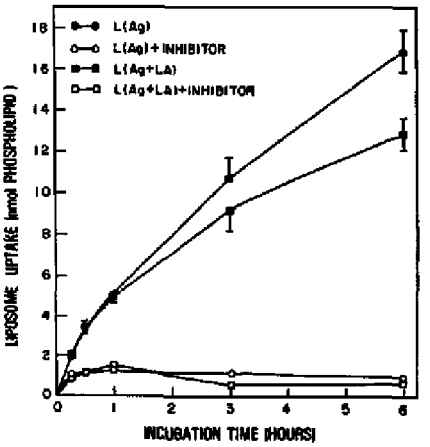

Fig. 2. Phagocytosis of liposomal malaria antigen by bone marrow-derived macrophages and effects of metabolic inhibitors on phagocytosis. Cholesteryl [1-^{14}C]oleate-labelled liposomes (200 nmol of liposomal phospholipids in L(Ag) or L(Ag+LA), as indicated) were added to macrophage cultures ($2 \cdot 10^6$ cells). The cultures were incubated for up to 6 h at 37°C with 5% CO_2 and 95% humidity. In inhibition experiments (bottom two curves) cells were also incubated with 10 mM NaF and 1 μg/ml of antimycin A. The inhibitors were added 10 min prior to the liposomes and were present throughout the incubation period. Cells were harvested by washing with chilled PBS and liposomal Ag uptake was quantitated by measurement of radioactivity. The data represent the means ± S.D. of triplicate observations.

two curves) revealed that only a small fraction of the total amount of liposomes added to the assay was adsorbed nonspecifically to the macrophage surface at the beginning of the incubation. The level of nonspecific adsorption remained unchanged throughout the incubation.

Internalization of liposomal protein into vacuolar compartments of macrophages

After incubation of macrophages with L(Ag), vacuoles appeared in the macrophage cytoplasm, and the vacuoles contained numerous liposomes that were densely labelled by antibody to malarial antigen (Fig. 3). The ability to detect the liposomal antigen with specific antibodies is consistent with the hydrophobic nature of the antigen, and also with the likelihood that the antigen was at least partially inserted in the lipid bilayer of the liposomes as a transmembrane protein having some of its antigenic epitopes exposed on the liposome surface. Many of the protein-containing liposomes apparently remained intact with a considerable amount of liposome-associated antigen for at least as long as 6 h after incubation. Liposomes were mainly located at the peripheries of the vacuoles and often seemed to adhere to the vacuolar membranes (Fig. 4). Antigenic epitopes detected by the antibody were often observed in the cytoplasm next to the vacuoles (Figs. 3 and 4). The original antigen consisted largely of repeats of Asn-Ala-Asn-Pro, and it is possible that fragments containing Asn-Ala-Asn-Pro were recognized by the antibody. However, liposomes were invariably present only within the vacuoles, and liposome-like structures were rarely, if ever, present in the cytosol or associated with any other subcellular organelles.

Appearance and antigenic expression of phagocytosed protein on the surface of macrophages

Shortly after phagocytosis of liposomes containing encapsulated protein was initiated, expression of the ingested protein on the macrophage surface was observed by ELISA using a monoclonal antibody (Pf 1B2.2) (Fig. 5). Detectable amounts of the Pf 1B2.2-specific epitope were present on macrophage surfaces at the earliest time examined (15 min) following incubation of macrophages with liposomal antigen. Incorporation of lipid A into liposomes (L(Ag + LA)) did not have any significant influence on antigenic expression when compared with liposomes lacking this adjuvant (L(Ag)) (Fig. 5). Antigenic expression on the surface of the macrophages continued to increase over 5 h with increasing incubation time. Antigenic expression decreased after 5 h for cells incubated with L(Ag +

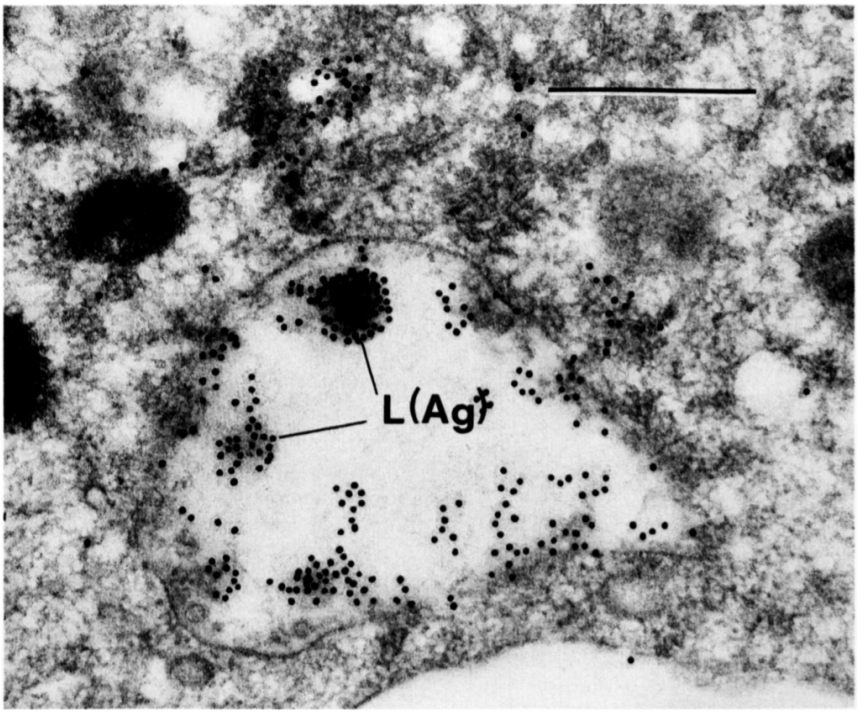

Fig. 3. Immunogold electron microscopy of macrophages after phagocytosis of liposomes containing malaria antigen. The macrophages were fixed 6 h after incubation with liposomes containing malaria antigen. The malaria antigen was detected by a specific monoclonal antibody (Pf 1B2.2) to the antigen followed by treatment with gold-labelled second antibody. See Materials and Methods for further details. Bar = 0.5 μm.

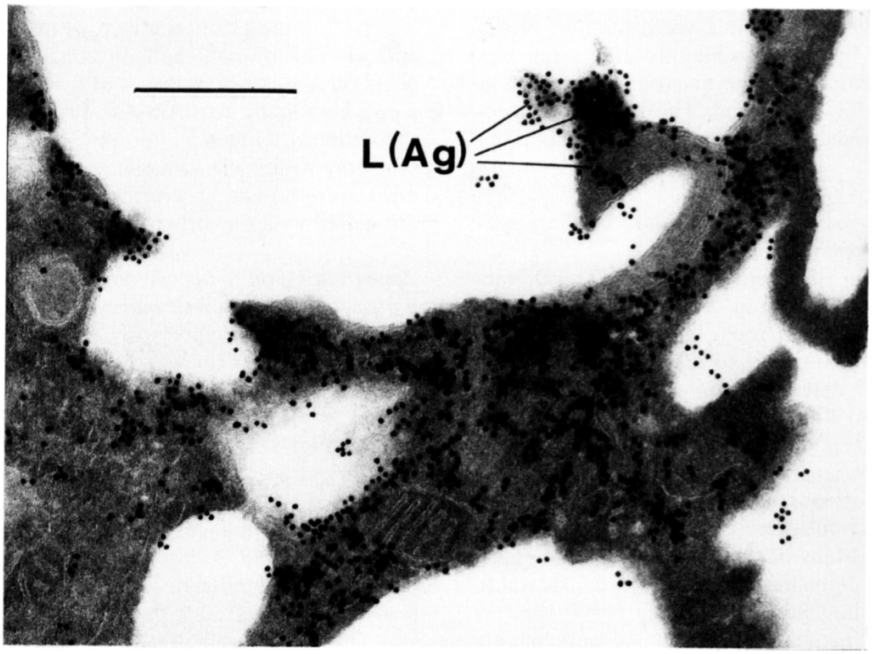

Fig. 4. Immunogold electron microscopy of macrophages after phagocytosis of liposomes containing malaria antigen. See legend to Fig. 3 for details. Bar = 0.5 μm.

LA). Calculations showed that at any time during the incubation the antigen expressed on the macrophage surface represented less than 1% of the total intracellular protein accumulated by phagocytosis of liposomes (calculated from data of Figs. 2 and 5 and the phospholipid : antigen ratios of the liposomes). However, as will be shown below (see descriptions of Figs. 6A and 6B), pulse-chase experiments demonstrated that turnover of expressed antigen did occur, and it is likely that much more than 1% of the total accumulated antigen eventually appeared on the surfaces of the macrophages.

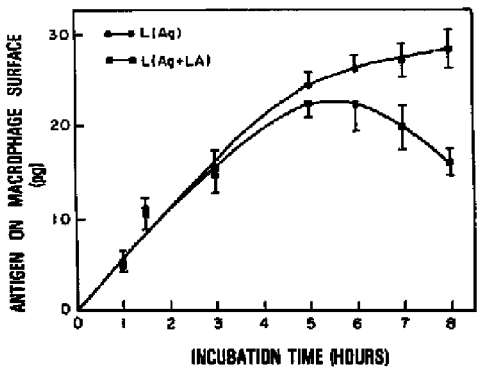

Fig. 5. Time course of antigen expression on macrophage surfaces after incubation with liposome-encapsulated protein. Liposomes containing antigen (5 nmol of liposomal phospholipids in L(Ag) or L(Ag+LA), as indicated) were incubated with macrophages ($5 \cdot 10^4$ cells) for the time periods shown. Cells were then fixed with paraformaldehyde and antigen expression on the macrophage surface was detected by ELISA using a specific monoclonal antibody, Pf 1B2.2. The data represent the means ± S.D. of triplicate experiments.

Kinetics of intracellular liposomal antigen processing by macrophages

'Pulse-chase' experiments were performed in order to analyze and study the kinetics of intracellular processing of protein after ingestion of liposomes. Macrophage cultures were 'pulsed' for 3 h with liposomes containing encapsulated antigen (L(Ag)) followed by a 24 h 'chase' in which the liposomes containing encapsulated antigen were replaced by 'empty' liposomes (Fig. 6A). Control cells were incubated with liposomes containing antigen throughout the 27 h incubation period. As shown in Fig. 6A, surface expression of antigen which had been increasing for 3 h rapidly stopped increasing when the macrophages were incubated with empty liposomes during the 'chase' interval. During the 24 h chase period the surface expression of antigen slowly diminished.

A parallel experiment was also performed in which the macrophages were first incubated with 'empty' liposomes (L) followed by liposomes containing antigen (L(Ag)) (Fig. 6B). Upon examining macrophages that had been first incubated for 3 h with empty liposomes, significant amounts of antigen were detected on the macrophage surfaces within an hour after adding the

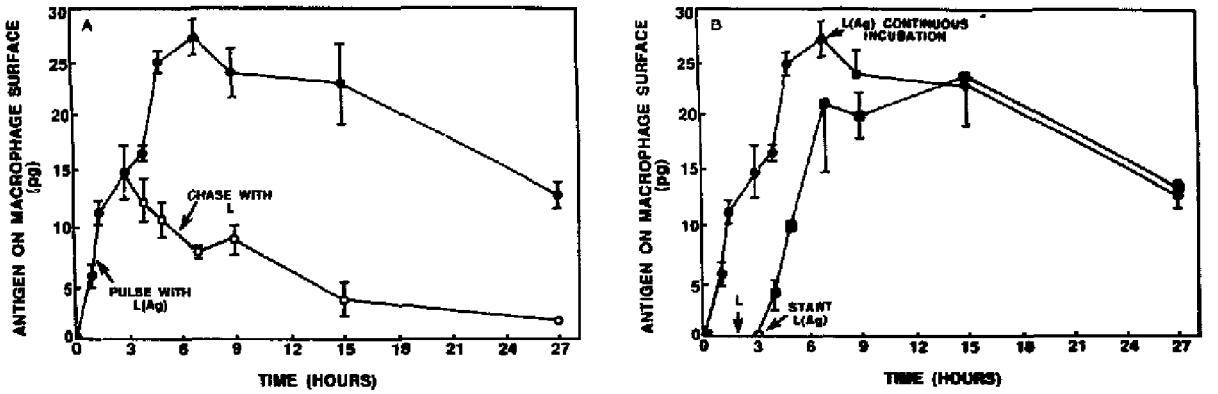

Fig. 6. Kinetics of antigen expression on the macrophage surfaces as determined by pulse-chase experiments. (A) Macrophage cultures ($5 \cdot 10^4$ cells/well) were incubated (pulsed) with L(Ag) for 3 h, followed by a 24 h chase in which L(Ag) was replaced by L. Control cultures were incubated continuously for 27 h with L(Ag). (B) Macrophage cultures ($5 \cdot 10^4$ cells/well) were incubated with L for 3 h, followed by a 24 h incubation in which L was replaced by L(Ag). Control cultures were incubated continuously for 27 h with L(Ag). Protein expression on the macrophage surface was determined by ELISA as described in the legend to Fig. 5. The data represent the means ± S.D. of triplicate observations.

antigen-containing liposomes, and the amount of surface expression rapidly increased during the subsequent 12 h period (Fig. 6B). However, surface expression of antigen did not attain the same maximum level reached by control cultures that had been incubated continuously with antigen-containing liposomes.

Role of lysosomes in surface expression of liposome-encapsulated protein

Ammonium chloride, an inhibitor of lysosomal activity that increases intralysosomal pH, was utilized to investigate the role of lysosomes on the appearance of protein antigen on the macrophage surface. The patterns of surface expression of liposomal antigen were only slightly influenced, if at all, by the presence of NH_4Cl when compared to controls (Fig. 7).

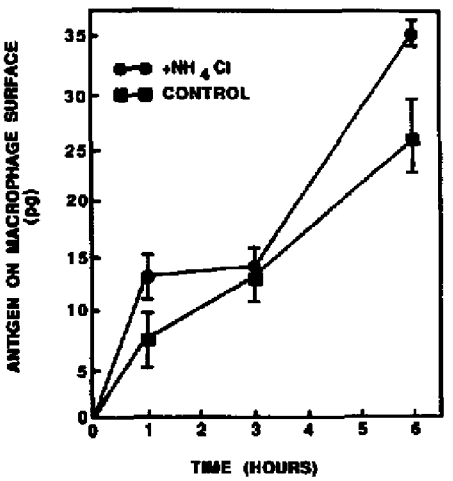

Fig. 7. Effects of ammonium chloride on antigen expression on macrophage surfaces. Appearance of antigen on macrophage surfaces was determined, as described in the legend to Fig. 5, in the presence of 10 mM of ammonium chloride in the medium during incubation of macrophages with antigen-containing liposomes. The data represent the means ± S.D. of triplicate values.

Discussion

In this study we followed the fate of liposome-encapsulated protein antigen that was incubated with cultured bone marrow-derived macrophages. The liposomes containing the antigen were phagocytosed by the macrophages. Electron microscopy revealed that after phagocytosis the ingested liposomes were concentrated in large intracellular vacuoles that were probably formed by coalescence of smaller vacuoles. Liposomes appeared to be closely associated with the vacuolar membranes. Although gradual liposomal degradation may have occurred in the vacuoles as described before [10,11,13,14], large numbers of apparently intact liposomes were observed after six hours. Liposomes were not observed, or were found only rarely, in the cytoplasm.

In contrast with liposomes per se, the antigenic protein epitopes encapsulated within the liposomes readily escaped from the vacuoles. Antigenic epitopes were observed in the cytoplasm as determined by immunogold electron microscopy and they even appeared on the surfaces of the macrophages where they were detected by ELISA. The antigenic epitopes in the cytoplasm were not randomly distributed in the cytoplasm and, as in Fig. 3 they were often located in clusters that gave the appearance that they might be emerging from the vacuoles. As shown at the bottom of Fig. 4, the epitopes were essentially excluded from mitochondria.

The experiments described in this study suggest that there was a continuous directed flow of intracellular liposomal antigen. This flow resulted in translocation of phagocytosed liposomal antigenic epitopes from intracellular sites to the macrophage surface. The kinetics of turnover of epitopes derived from liposome-encapsulated protein detected at the macrophage surface, as studied by 'pulse-chase' experiments, revealed that the antigen appeared on the macrophage surface within 15 min after initial incubation, and continued to be expressed, albeit at a markedly diminished level, even 24 h after initiation of chase with empty liposomes. It should be noted that the data of Fig. 6A differ from classical pulse-chase experiments in that the slowness of phagocytosis of liposomes did not permit a sufficient amount of antigen to be introduced into the cell to allow the pulse to be stopped before cell surface expression begin. In the companion experiment to the pulse-chase experiment (Fig. 6B) we controlled for potential nonspecific binding of liposomes to macrophages by preincubating the macrophages with empty liposomes for 3 h. This preincubation did not cause any delay in the onset of antigen expression on the macrophage surface after exposure to liposomes containing antigen and, after an appropriate (and expected) delay, the antigen expression by preincubated and nonpreincubated cultures became identical. These data are compatible with the active processing of liposomes by macrophages, and the kinetics of cell surface expression of antigen in Figs. 6A and 6B correlated very closely with the kinetics of phagocytosis of liposomes (Fig. 2). The results from these experiments are compatible with a previous report that showed that breakdown products of liposomal albumin were released from Kupffer cells, but approximately half of the total amount of liposome-encapsulated ^{125}I-labelled albumin that had been ingested remained associated with the cells after 4 h [13].

As noted in the results, less than 1% of the ingested liposomal antigen was detected on the macrophage surface at each measured time interval. In contrast, it was also noted (Fig. 2) that much more than 1% of the radioactive cholesterol oleate label was nonspecifically bound to macrophages. However, it is unlikely that the ELISA signal that we have interpreted as recycling of antigen actually represents nonspecific binding of liposomes to macrophages. The kinetics of phagocytosis (Fig. 2) correlated very well with antigen expression (Figs. 5, 6A, and 6B). In contrast, the kinetics of nonspecific binding of radioactive liposomal cholesterol oleate in the presence of inhibitors in the phagocytosis experiment (Fig. 2) correlated very poorly with the kinetics of antigen expression detected by ELISA on the macrophages. Nonspecific binding of radioactivity did not increase with time during incubation between hours 1 and 6 (Fig. 2); in fact, a measurable and significant decrease of nonspecific binding was observed. In contrast, expression of antigen on the surface of macrophages was a dynamically increasing phenomenon during the same time frame (Fig. 5) and the expression correlated well with the process of phagocytosis.

It is well-known from the literature on the cell biology of liposomes that endocytosed or phagocytosed liposomes appear in phagocytic vacuoles, 'dense body' lysosomes, and in poorly characterized large vacuoles [1–13,28]. In the present study, most of the liposomes that were ingested by macrophages were concentrated in large and relatively clear vacuoles and these results therefore are consistent with previous observations. Our studies with immunogold electron microscopy demonstrating the appearance of antigenic epitopes in the cytoplasm are also in agreement with experiments from other laboratories in which liposome-encapsulated proteins were noted as being present in the cytosol after internalization of liposomes by cells [15–17].

The appearance of liposomal antigenic protein epitopes on the surface of the macrophages that we observed by using monoclonal antibodies specific for the protein epitopes could be consistent with results from certain types of immunologic studies involving liposomes. Previous reports have demonstrated immunological presentation of liposome-encapsulated protein by macrophages to T lymphocytes in a process that presumably involves recognition of major histocompatibility gene complex molecules [36,38]. In support of this, we found in separate experiments, to be reported elsewhere, that the same methods described in this paper for incubating liposomes with macrophages did result in immunological presentation of antigen to T lymphocytes. However, in the present study liposomal antigenic epitopes were located and identified only by antigen-antibody reactions using specific monoclonal antibodies, and presentation of antigen to T cells was not addressed.

As noted above, immunogold electron microscopy showed that liposomes containing antigen accumulated in large vacuoles in the macrophages and the antigen apparently was released in large amounts into the cytoplasm. It is our belief that this observation is consistent with a cytoplasmic route by which antigen could have travelled from the vacuoles to the surface of the cell. Previous studies have demonstrated that degradation of liposomal contents can be inhibited by raising the intracellular pH with a lysosomotropic agent [7,9,13,38]. The inability to block the appearance of antigenic epitopes on the cell surface with ammonium chloride, as found in our experiments, suggests that the liposomal antigenic epitopes that followed our proposed cytoplasmic pathway, were not degraded by lysosomal enzymes sensitive to ammonium chloride. Even

if partial degradation of the intact protein did occur, sufficiently large antigenic epitopes containing Asn-Ala-Asn-Pro remained that reacted with the monoclonal antibody and allowed detection by immunogold electron microscopy. These observations are therefore further evidence that we are describing a novel intracellular pathway for transit of liposomal antigenic epitopes from the liposomes to the cell surface.

In a recent study, chloroquine-treatment of macrophages completely inhibited immunologic processing and presentation to T hybridoma cells of low concentrations of an immunogenic liposomal peptide at the macrophage surface [38]. However, in the same study, at high liposomal peptide concentrations substantial immunologic presentation was observed. We are currently examining the potential influence, if any, of pH-sensitive degradation of antigen on immunologic presentation of phagocytosed liposome-encapsulated R32NS1 to antigen-specific cloned T helper lymphocytes.

We conclude that liposome-encapsulated protein that is phagocytosed by macrophages can enter an intracellular compartment in which at least some of the antigenic epitopes are not degraded by lysosomal enzymes. Furthermore, although liposomes do enter large vacuoles, at least some of the antigenic epitopes, as detected by a monoclonal antibody, can escape into the cytoplasm. These experiments therefore provide evidence to support the possible existence of a pathway in which liposomal contents can bypass lysosomal degradation. The occurrence of an intracellular pathway for endocytosed or phagocytosed materials that avoids lysosomes has been previously hypothesized [16], and such a pathway therefore might be used as a basis for delivery of nondegraded liposomal antigenic protein epitopes to the cytosol and to the cell surface.

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