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Liposome-mediated cytosolic delivery of macromolecules and its possible use in vaccine development

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In the majority of bacterial and viral infections the generation of cytotoxic T cells is of particular interest because such pathogens are able to escape the host defence mechanisms by surviving intracellularly within the phagocytic cells. To generate a $CD8^+$ T lymphocyte response against exogenous antigens, the prerequisite is their delivery into the cytosol followed by processing and presentation along with class I major histocompatibility complex (MHC-I) molecules. In the present study we describe the method of liposome-based delivery of antigens and other macromolecules into the cytosol of target cells. To develop safe and effective methods for generating $CD8^+$ T lymphocytes, we exploited the fusogenic character of lipids derived from lower organisms, that is baker's yeast (*Saccharomyces cerevisiae*). The degree of fusion with model membrane systems using yeast lipid liposomes varied from 40–70%, as opposed to 1–8% observed with egg PtdCho liposomes, depending on the assay system used. The fusion of yeast lipid liposomes with macrophages resulted in effective delivery of the entrapped solutes into the cytoplasmic compartment. This was further supported by the inhibition of cellular protein synthesis in J774 A1 cells by ricin A, encapsulated in the yeast lipid liposomes. Interestingly, the model antigen ovalbumin, when entrapped in the yeast lipid liposomes, successfully elicited antigen reactive $CD8^+$ T cell responses. It may be concluded that the liposomes made of lipids derived from *S. cerevisiae* can spontaneously fuse with macrophages, delivering a significant portion of their contents into the cytoplasmic compartment of the cells.

Keywords: $CD8^+$ T lymphocyte; cytosolic delivery; fusogenic lipids; liposomes; yeast.

$CD8^+$ cytotoxic T cells play an important role in the protection against a variety of intracellular infections. The prompt development of cytotoxic T cell responses has been shown to be crucial for protecting the host against diseases caused by *Trypanosoma cruzi* [1,2], *Toxoplasma gondii* [3], *Mycobacterium tuberculosis* [4], *Leishmania donovani* [5] *Listeria monocytogenes* [6,7] and viruses [8]. To mount an effective immunity against these diseases, it is therefore, important to develop vaccines that generate a strong $CD8^+$ T cell response.

Normally, the protein antigens delivered to antigen presenting cells (APCs) are taken up by endocytosis. The antigen is then processed within the endosomes to produce the peptides that generally bind to class II major histocompatibility complex (MHC-II) molecules. This results in the activation of $CD4^+$ T cells [9]. $CD4^+$ T-helper (Th) cells provide aid to cytotoxic T cells (CTL) and B cell in their various immunological responses.

Cytotoxic T cells represent the other effector arm of the cell-mediated immune responses. These cells are considered to be of strategic importance in the killing of cells infected with intracellular pathogens. The $CD8^+$ T cell phenotype can considerably influence the outcome of infection by intracellular pathogens. However, for eliciting the $CD8^+$ T cell responses, the antigen is required to be processed and presented by the MHC I antigen presentation pathway [10,11]. In order to generate the $CD8^+$ T lymphocyte response, it is obligatory that antigen should be in the cytosol of the antigen presenting cells.

Exogenous antigen can, however, enter the class I processing pathway if it is delivered in a vehicle that is able to undergo fusion with either the plasma membrane under normal physiological conditions or the endosomal membrane at low pH. Earlier investigators have attempted to use pH sensitive liposomes [12,13] or virosomes [14,15] for protein delivery into the cytosol. Such vehicles are likely to prove toxic to the host, however, and involve cumbersome methods for their preparation. These limitations restrict their use as vehicles for delivering the newer generation of vaccines against intracellular infections.

In lower organisms the plasma membrane is mainly composed of amino-phospholipids along with cardiolipin and phosphatidyl glycerol. The eukaryotic plasma membrane lipid composition is different from that of lower organisms and earlier work from our laboratory revealed the composition and distribution of phospholipids in eukaryotic cells [16]. They contain all classes of phospholipids, distributed in a set fashion in the two leaflets of the bilayer. The amino-phospholipids are mainly confined to the inner leaflet and play a major role in exocytosis, which involves membrane–membrane fusion. This

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Abbreviations: ANTS, l-aminonapthaline-3,6,8-trisulphonic acid; APC, antigen presenting cell; chol, cholesterol; CTL, cytotoxic T cell; DPX, *N*,*N*′-p-xylylenebis (pyridinium bromide); DRVs, dried-reconstitued vesicles; phosphatidylcholine, PtdCho; FITC, fluorescein isothiocyanate; FITC-dextran, FITC-labeled dextran; FITC-PHA, FITC-labeled lectin from phaseolus vulgaris; HBSS, Hanks' balanced salt solution; IL, interleukin; LUVs, large unilamellar vesicles; MHC-I, class I major histocompatibility complex; MHC-II, class II major histocompatibility complex; NBD-PE, L-α-phosphatidylethanolamine-*N*-(4-nitrobenzo-2-oxa-1,3diazole); RET, resonance energy transfer; Rh-PE, *N*-(lissamine rhodamine B sulphonyl) phosphatidyl ethanolamine.

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type of architectural difference in lipid distribution certainly implies an evolutionary trend of biological significance. We recently demonstrated that asymmetric distribution of phospholipids is also maintained in yeast cells [17,18]. However, similar to other lower organisms, yeast cells undergo rapid cell duplication, in which the two opposite sites of the inner leaflet come close to each other and fuse under physiological conditions. *Saccharomyces cerevisiae*, a representative yeast, has been generally regarded to be safe, and is easy to cultivate. We therefore considered it of interest to utilize the fusogenic properties of the vesicles formed from yeast lipids to deliver antigen to APCs.

In the present study, we have analyzed the fusogenic properties of yeast lipid liposomes. The liposomes displayed a high tendency to undergo spontaneous membrane fusion under physiological conditions with both model membrane systems as well as intact cells. We have demonstrated that the range of macromolecules encapsulated in the yeast lipid vesicles could be readily introduced into the cytoplasmic compartment of target cells. In addition, we have also shown that ovalbumin entrapped in the vesicles could generate a strong ovalbumin-specific $CD8^+$ T cell response.

MATERIALS AND METHODS

Animals

Male Balb/c mice (8–10-week-old; 20 ± 2 gm weight) were obtained from the Institute's Animal House Facility (Institute of Microbiol Technology, Chandigargh, India).

Chemicals and reagents

Egg PtdCho (PtdCho) was prepared using the standard method [19]. Cholesterol was purchased from Centron Research Laboratory, Bombay, India, and crystallized three times from methanol prior to use. Calcein (Alfa Division of Ventron Corp., Denver, MA) was a kind gift from G. C. Varshney (Institute of Microbiol Technology, Chandigargh, India). Peptone and yeast extract were from Hi Media Laboratories, Bombay, while dextrose was from S. D. Fine Chemicals, Boisar, India. DMEM, Hanks' balanced salt solution (HBSS) and fetal bovine serum were obtained from Life Technologies (Grand Island, New York, USA). Fluorescein isothiocyanate (FITC)-labeled lectin from *Phaseolus vulgaris* (FITC-PHA), FITC-dextran (69 kDa), ovalbumin, lysozyme (chicken egg white), deglycosylated ricin A, imidazole, percoll, Sephadex G-50, Sepharose 6B, and EDTA were from Sigma Chemical Company (St Louis, MO, USA). l-Aminonapthaline-3,6,8-trisulphonic acid (ANTS) and *N*,*N*′-p-xylylenebis (pyridinium bromide) (DPX) were bought from Molecular Probes, Inc. L-α-phosphatidylethanolamine-*N*-(4-nitrobenzo-2-oxa-1,3-diazole) (NBD-PE) and *N*-(lissamine rhodamine B sulphonyl) phosphatidyl ethanolamine (Rh-PE) (Avanti polar lipids) were a kind gift from Dr Anu Puri (NIH, Frederick, MD). Anti-IA^d, anti-Mac 2 and anti-L3T4 were gifts from Dr Gyan Mishra (IMT, Chandigarh, India). ^{125}I-labeled Sodium iodide (carrier free), [^{35}S]L-methionine and [^{51}Cr]sodium chromate were bought from Bhabha Atomic Research Center, Trombay, India. Lysozyme was radio iodinated using the published method [20]. The amount of protein entrapped in the liposomes was determined using the BCA protein assay (Pierce Chemical Company). J 774 A1, a macrophage cell line, was procured from American Type Culture Collection (Rockville, MD, USA) and was grown in DMEM (pH 7.2) containing L-glutamine (4 mM), sodium pyruvate (110 mg·L^{-1}), penicillin (100 U·mL^{-1}), streptomycin sulfate (100 μg·mL^{-1}) and sodium bicarbonate (3.7 g·L^{-1}) in 75 mL plastic bottles (Costar, MA, USA) at 37 °C under 7.5% CO_2.

Yeast lipids

S. cerevisiae (NCYC 366) was cultured in YPD media (1% yeast extract, 2% peptone and 2% dextrose pH 6.0). The cells were harvested from mid-log phase (14–16 h) and pelleted at 2000 ***g*** for 10 min. The cell pellet was suspended in methanol and sonicated for 60 min in a bath-type sonicator (0–4 °C) and finally lipids were extracted following the published procedure [21].

Yeast lipid composition

Total phospholipid (53.6 ± 1.2%, w/w), total glycolipid (7.8 ± 1.3%, w/w) and total sterol (14.7 ± 1.1%, w/w) contents in the yeast lipid extract were determined following the published procedures [22]. The total phospholipid composition (PtdCho, 48.1 ± 0.7%; phosphatidyl-ethanolamine, 23.1 ± 1.7%; phosphatidylinositol, 16.1 ± 0.7%; phosphatidylserine, 6.0 ± 0.6%; phosphatidylglycerol, 3.9 ± 0.8%; cardiolipin, 1.6 ± 0.3%) in the mixture was analyzed as described earlier [21].

Lipid vesicles

Large unilamellar vesicles (LUVs) were prepared essentially by the freeze-thaw method [23]. The outer diameter of these vesicles was about 140 + 50 nm. LUVs for resonance energy transfer (RET) experiments were formed using yeast lipids (or egg PtdCho), NBD-PE and Rh-PE in a molar ratio of 98.4 : 1 : 0.6. For the aqueous content mixing experiments, LUVs were prepared by hydrating yeast lipids or egg PtdCho (15 μmol lipid P) with 1.5 mL of 10 mM Tris-HCI (pH 7.5), containing ANTS (25 mM) and NaCl (40 mM) or DPX (90 mM). The entrapped ANTS (or DPX) from free ANTS (or DPX) was separated by gel filtration using a Sephadex G-50 column.

Dried reconstituted vesicles (DRVs) were formed using the known method [24]. Finally, free-flowing, lyophillized, dried powder was rehydrated with distilled water and reconstituted in $NaCl/P_i$. The preparation was centrifuged at 14 000 ***g*** and the pellet was washed at least three times with $NaCl/P_i$ to remove traces of the unentrapped solute.

Erythrocyte membrane vesicles

Erythrocytes from fresh human blood were isolated by removing the plasma and buffy coat. The cells were lysed and then resealed after washing three times with the lysis buffer [25]. The erythrocyte membrane vesicles thus prepared were pelleted at 1200 ***g*** (15 min, 4 °C). After washing several times, the preparation was resuspended in $NaCl/P_i$ for further use.

RET assay

The lipid mixing between the NBD/rhodamine-labeled yeast lipids (or egg PtdCho) LUVs (about 600 nmol, lipid P) and unlabeled yeast lipid (or egg PtdCho) LUVs or erythrocyte membrane vesicles (about 3.5 μg protein) was followed by monitoring RET [26] between NBD (absorption, 470 nm; emission, 520 nm) and rhodamine (absorption, 536 nm; emission, 585 nm). The excitation wavelength was chosen to be 20 nm below the absorption maxima of NBD to allow a

better resolution between the scattered light peak and the NBD emission peak, and also to minimize the direct excitation of rhodamine. The efficiency (E) of RET was calculated as follows:

$$E = 1 - F/F\mathrm{t}$$

where F is the NBD fluorescence in the presence of rhodamine, and Ft is the NBD fluorescence at the maximal dequenching, which was measured after disrupting the vesicles with Triton X-100 (1% final concentration).

Aqueous content mixing assay

Quenching of the ANTS fluorescence by DPX was monitored to follow the mixing of the aqueous contents of the vesicles undergoing fusion [27]. The ANTS-containing LUVs were mixed with an excess (10-fold) of the DPX-containing LUVs in a total volume of 3 mL. The mixture was incubated at 37 °C and the ANTS fluorescence was measured at varying periods of time. The ANTS fluorescence observed at zero minutes was taken as 100% fluorescence while the fluorescence values observed after lysing a mixture of ANTS-containing and DPX-containing LUVs with Triton X-100 (1% final concentration) were taken as 0% fluorescence. The excitation and emission wavelengths used were 380 nm and 540 nm, respectively.

Macrophage–yeast lipid DRV interaction

The interaction of the macrophage with the yeast lipids DRVs was monitored by observing the transfer of fluorescently labeled water-soluble as well as membrane markers from the vesicles to the macrophages.

Transfer of fluorescent membrane markers. Membranes of DRVs were fluorescently labeled by incorporating NBD-PE (5 mol percentage) in egg PtdCho/cholesterol or yeast lipids. The J774 A1 cells (1×10^6 per well) were cultured overnight in a 24-well plate. The cells were washed with DMEM, and incubated with NBD-labeled DRVs (600 μmol lipid P per well) for different time periods. The incubated cells were washed once with DMEM and three times with NaCl/P_i. The washed cells were fixed, and scrapped off the culture plates using Versene solution (DMEM supplemented with 3 mM EDTA and 1% fetal bovine serum). The cells were washed three times with NaCl/P_i and were analyzed for NBD fluorescence (excitation: 470 nm, emission: 520 nm), using log amplifiers on a Lysis II software of FACS-analyzer (Becton Dickinson, Mountain View, CA, USA). Ten-thousand cells (events) were acquired on Lysis II software after 'live' gating on the log forward scatter/NaCl/Cit parameter, and the fluorescence was measured on gated cells only. The analysis of mean fluorescence intensity was done on histograms, where abscissa and ordinate denote log NBD fluorescence and relative cell counts, respectively.

Transfer of water soluble fluorescent markers. Three water soluble fluorescent markers (calcein, FITC-dextran and FITC-PHA) were used in this study. The J774 A1 (1×10^6 cells) were cultured overnight on a sterile cover slip in DMEM containing 10% fetal bovine serum. The cells were washed with DMEM and incubated at 4 °C for 2 h. After washing once with fetal bovine serum-free DMEM, the cells were pulsed with calcein, FITC-dextran or FITC-PHA containing DRVs (600 μmol lipid) for 60 min in fetal bovine serum-free DMEM at 37 °C. After washing, the fixed macrophages were observed under Leitz fluorescence microscope at 100×, using an I 3 filter.

Intracellular localization of lysozyme

DRVs loaded with ^{125}I-labeled lysozyme (about 4×10^5 c.p.m.·mg^{-1} protein) were used in these experiments. J774 A1 cells (5×10^6 cells per well) were cultured in six-well plates, incubated for 1 h at 37 °C with lipid vesicles. The cells were washed with NaCl/P_i followed by fixation with 1% paraformaldehyde solution. The cells were detached from the culture plates using chilled Versene solution. The cells were broken following the published procedure by the nitrogen cavitation method using Parr bomb and pelleted by centrifuging at 750 ***g*** for 15 min [28]. The supernatant was further centrifuged at 10^5 ***g*** for 1 h at 4 °C using a Beckman L8–55 M ultracentrifuge. The pellet was suspended in 3.0 mL of imidazole buffer (3 mM imidazole, 0.25 M sucrose; pH 7.4), and then fractionated into plasma membrane/nucleus, cytosol, endosome/lysosome, and other organelles (on the basis of enzyme marker assay), as described earlier [28].

Inhibition of protein synthesis in the macrophages by ricin A

J774 A1 cells (1×10^6 cells per well) were cultured in a 24-well plate overnight at 37 °C. Next day, the cells were incubated separately with free ricin A, or encapsulated in egg PtdCho/chol DRVs and yeast lipid DRVs at 37 °C for 1 h. The cells were washed, and then pulsed with L-[^{35}S]methionine (1 μCi per well) in a total volume of 200 μL per well for 6 h at 37 °C. The cells were washed two times with DMEM and were treated with 7 M guanidine (50 μL per well). The final volume was made up to 200 μL per well with NaCl/P_i. The suspension was vortexed, and a small aliquot (20 μL) from the cell lysate, was withdrawn in Eppendorff tubes. The lysate was then treated with trichloroacetic acid (25%, 100 μL) and bovine serum albumin (BSA; 1%, 50 μL), and centrifuged. The precipitate was washed once with 10% tricholoroacetic acid and counted for β-emission in a RackBeta Scintillation Counter, after suspending in 10 mL of Scintillation fluid.

$CD8^+$ T lymphocyte response

Effector cells. Different groups of Balb/c mice were injected separately with three different doses of free ovalbumin, yeast lipid DRVs with no ovalbumin (sham liposomes), free ovalbumin mixed with sham liposomes, ovalbumin encapsulated in egg PtdCho/chol or yeast lipid DRVs (100 μg per animal per week) for 3 weeks. On day 21, animals were sacrificed and spleens were removed aseptically. The splenic tissue was macerated, and the supernatant separated by simple decantation. The erythrocytes were removed by treatment with Gey's solution (3 mL per spleen) for 10 min on ice [29]. The cells were washed three times with HBSS, and then allowed to adhere at 37 °C for 1 h to get rid of the macrophages. The remaining cells were passed through a nylon wool column to remove B lymphocytes. The residual macrophages and B cells together with $CD4^+$ T lymphocytes were finally removed by treating the above cell suspension with a cocktail of anti-Mac 2 Ab (2 μg per spleen), anti-L3T4 Ab (2 μg per spleen) and anti-IA^d Ab (3 μg per spleen) at 4 °C for 30–45 min, followed by treatment with baby rabbit complement at 37 °C for 30 min. The dead cells were removed by the Ficoll–Hypaque density gradient method. The remaining cells, rich in $CD8^+$ T cells, were washed with cold HBSS, counted, and used in the

experiments. The enriched population stained with anti-CD8 Ab, was >98% pure, as evaluated by FACScan.

Target cells. Balb/c mice were injected with thioglycollate broth. On day 4, peritoneal macrophges were harvested and washed three times with HBSS. The cells (about 2×10^7 cells per mL) were incubated separately with ovalbumin encapsulated in DRVs and free ovalbumin, at 37 °C for 1–2 h. The cells were washed three times to remove the free antigen. This was followed by incubation with ^{51}Cr (100 μCi, ^{51}Cr for 2×10^7 cells) for 45–60 min at 37 °C. The cells were finally washed with RPMI solution and were used as target cells.

Cytotoxicity assay. The ^{51}Cr-labeled macrophages (1×10^6 per 500 μL) were incubated with $CD8^+$ T cells (1×10^7 per 500 μL) at 37 °C for 6 h. The cells were pelleted at 3000 *g* (15 min, 5 °C), and the amount of ^{51}Cr released was determined by measuring the radioactivity in the supernatant. In another set of experiments, the fixed number of target cells was incubated with effector cells at different target : effector cell ratios and again target cell lysis was determined by measuring ^{51}Cr release. Total ^{51}Cr release was calculated by treating an aliquot of the cell suspension with Triton X-100 (10% final concentration). Auto release of ^{51}Cr was determined by incubating the ^{51}Cr labeled macrophages for 6 h, and then measuring the radioactivity released in the supernatant. The amount of auto release was substracted from the total release to determine the extent of macrophage lysis. In most experiments, the auto (spontaneous) release was less than 25%. The percentage specific release was calculated as the (mean sample c.p.m. − mean spontaneous c.p.m. / mean maximum c.p.m. − mean spontaneous c.p.m.) × 100% [13].

RESULTS

Fusion of yeast lipid LUVs with model membrane systems

LUV–LUV fusion. The LUV–LUV fusion was assessed by monitoring the mixing of both the membrane lipids and the

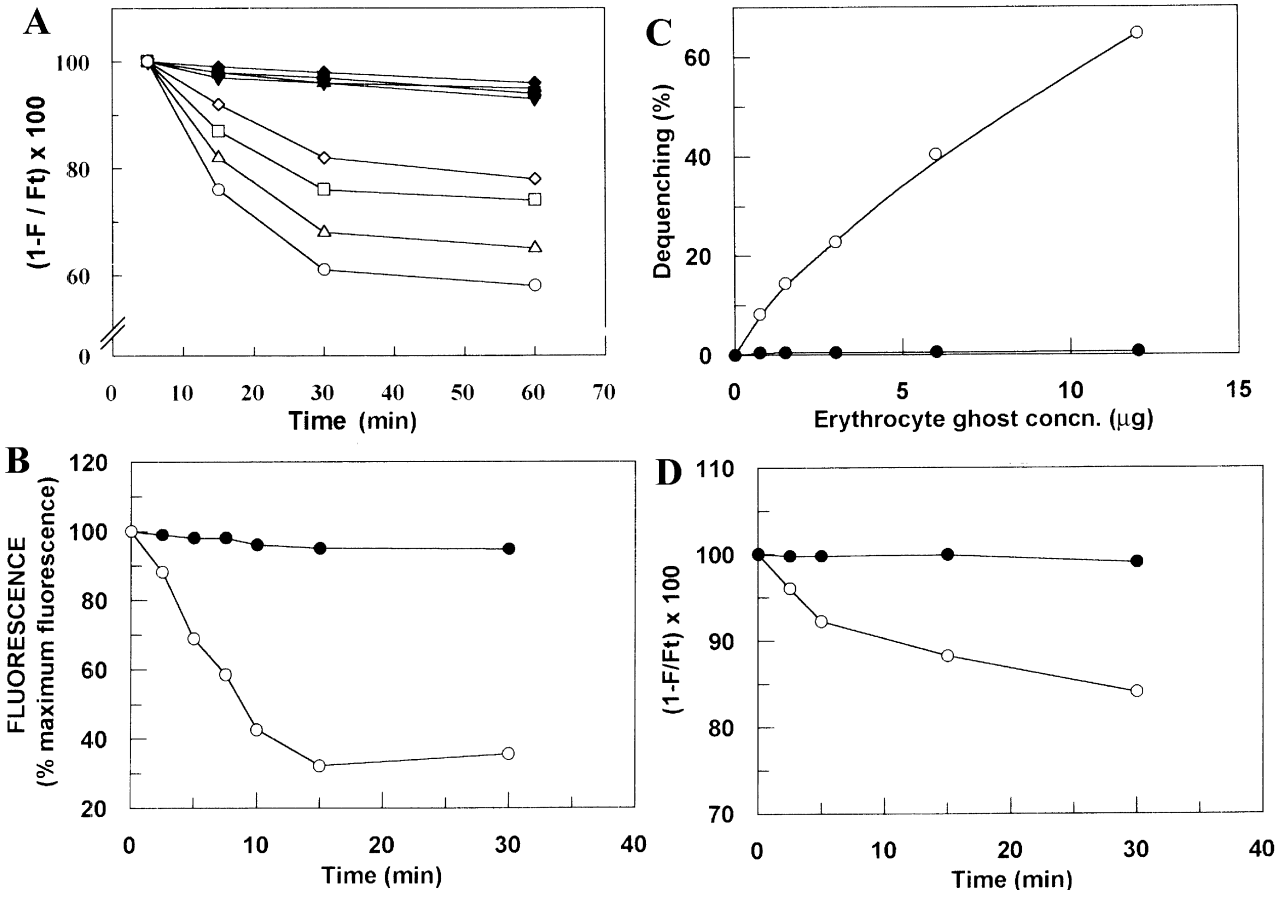

Fig. 1. Efficiency over time of RET between NBD and rhodamine incorporated onto the surface of LUVs upon mixing labeled LUVs with increasing amounts of unlabeled LUVs (A) **and time-dependent quenching of ANTS fluorescence after mixing ANTS-containing LUVs with DPX-containing LUVs in a ratio of 1 : 10 (B).** (A) Filled symbols, egg PtdCho; open symbols, yeast lipids; diamonds, unlabeled : labeled LUV ratio = 1; rectangles, unlabeled : labeled LUV ratio = 2; triangles, unlabeled : labeled LUV ratio = 4; circles, unlabeled : labeled LUV ratio = 10. Experiments were also carried out by diluting the NBD/rhodamine labeled egg PtdCho LUVs (600 nmol lipid P) with the unlabeled yeast lipid LUVs (6000 nmol lipid P) or the NBD/rhodamine labeled yeast lipids LUVs (600 nmol lipid P) with the unlabeled egg PtdCho LUVs (6000 nmol lipid P). The incubations were carried out for 60 min at 37 °C. The RET efficiency in the experiments decreased to 50.73 ± 3.67% and 79.99 ± 2.2%, respectively. Values are the mean of three measurements. (B) Egg PtdCho LUVs, ●; yeast lipid LUVs, ○. Values are the mean of three measurements. (C) Interactions of the NBD-labeled yeast lipid LUVs with the erythrocyte membrane vesicles. Yeast lipids (or egg PtdCho) LUVs (750 nmol lipid P per mL) containing 5 mol percentage NBD-PE in their bilayers were interacted with varying concentrations of right-side-out erythrocyte membrane vesicles at 37 °C for one hour. The percentage of NBD dequenching was calculated as given in Fig. 2B. Egg PtdCho LUVs, ●; yeast lipids LUVs, ○. Values are the mean of three measurements. (D) Efficiency over time of RET between NBD and rhodamine incorporated onto the surface of LUVs upon mixing labeled LUVs (600 nmol lipid P) with the erythrocyte membrane vesicle (3.5 μg protein). Egg PtdCho LUVs, ●; yeast lipids LUVs, ○. Values are the mean of three measurements.

aqueous contents. The lipid mixing was followed by measuring RET between NBD (photon donor) and rhodamine (photon acceptor). These probes were grafted on the LUVs surface by including NBD-PE and Rh-PE in egg PtdCho or yeast lipids. The LUVs containing these two fluorophores (labeled vesicles) were diluted with increasing amounts of the LUVs free of these fluorophores (unlabeled vesicles). The efficiency of RET was calculated by measuring the NBD fluorescence, as described in Materials and methods. As the efficiency of RET between these fluorophores has been shown to depend on their concentration in the vesicle bilayer [26], it was expected that any fusion between the labeled and unlabeled LUVs would lead to dilution of the probes and consequently to a reduction in RET efficiency. Unlike the egg PtdCho LUVs, the RET in the case of the yeast lipid LUVs decreased with an increase in the incubation time as well as in the concentration of the unlabeled LUVs; the efficiency decreased to about 60% when the unlabeled : labeled yeast lipid LUVs ratio was 10 (Fig. 1A). However, this efficiency decreased to only 80% when the labeled yeast lipid LUVs were diluted with a 10-fold excess of the egg PtdCho LUVs. The 'probe dilution' method is known to be insensitive to vesicle aggregation [30] and the fluorescent probes employed (NBD and rhodamine) have been shown not to undergo any exchange between the phospholipid vesicles [31]. We infer that the observed decrease in the RET efficiency is certainly due to vesicle–vesicle fusion. To further examine the validity of this conclusion, we monitored the mixing of the aqueous contents during fusion by measuring quenching of the ANTS fluorescence by the water soluble quencher DPX.

Incubation of the ANTS containing yeast lipid LUVs with a 10-fold excess of DPX-containing LUVs resulted in about 70% quenching of the ANTS fluorescence in 15 min (Fig. 1B). Unlike this finding, no such quenching of the ANTS fluorescence was observed when ANTS-containing egg PtdCho LUVs were incubated with 10-fold excess of the DPX-containing egg PtdCho-LUVs in identical conditions. These results clearly indicate that the yeast lipid LUVs have a strong tendency to undergo fusion with each other as well as with egg PtdCho LUVs. While the extent of membrane fusion seems to vary from 40–70% depending on the assay used, only about 20% fusion of yeast lipid vesicles was observed with egg PtdCho LUVs.

LUV–erythrocyte membrane fusion. Fusion of the yeast lipid LUVs with the erythrocyte membrane vesicles was studied by including a self-quenching concentration (5 mol percentage) of NBD-PE [32] in the yeast lipids or egg PtdCho LUVs bilayer. The NBD fluorescence dequenching was measured by the fluorescence light microscopy and fluorometry, subsequent to the fusion of these LUVs with the erythrocyte membrane vesicles. These results indicate that unlike the egg PtdCho LUVs, yeast lipid LUVs readily fuse with erythrocyte membrane vesicles, leading to about 60% dequenching of the NBD fluorescence in a concentration-dependent (Fig. 1C) as well as a time-dependent (Fig. 2B) manner. To further establish our findings, we measured RET between NBD and rhodamine after incubating the yeast lipid (or egg PtdCho) LUVs labeled with these two fluorophores with a limited amount of the erythrocyte membrane vesicles. Figure 1(D) shows that even under these conditions, the yeast lipid LUVs, unlike the egg PtdCho LUVs, undergo membrane fusion with the erythrocyte membrane to an extent of about 20%. The transfer of fluorescent probe to the erythrocyte ghost was further confirmed by fluorescence micrographs shown in Fig. 2A.

Fusion of yeast lipid DRVs with cells

Transfer of water-soluble and membrane fluorescent markers. Fusion of the yeast lipid DRVs with the macrophage cell line J774 A1 was followed by monitoring the transfer of both the

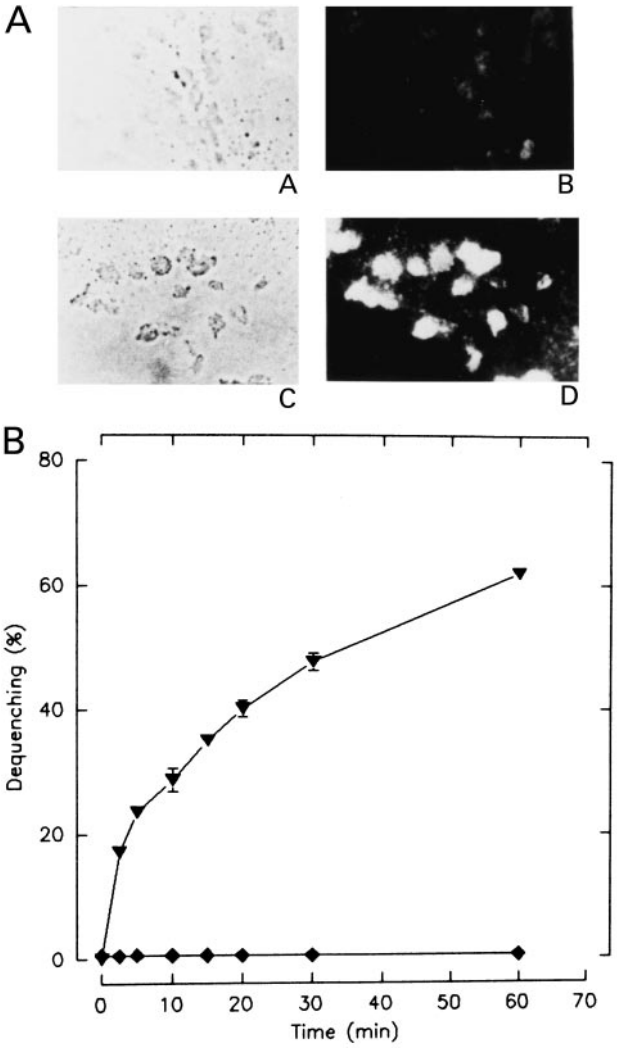

Fig. 2. Interactions of NBD-labeled yeast lipids LUVs with the erythrocyte membrane vesicles. Yeast lipids (or egg PtdCho) LUVs (750 nmol lipid P per mL) containing 5 mol percentage NBD-PE in their bilayers were interacted with right-side-out erythrocyte membrane vesicles (1×10^8 vesicles per mL) at 37 °C for varying periods of time. The NBD fluorescence was monitored up to 60 min incubation, using the excitation and emission wavelengths of 470 nm and 520 nm, respectively. The percentage of NBD dequenching was calculated as follows: % dequenching = $100 \times (F - Fo) / (Ft - Fo)$, where F, Fo and Ft are the fluorescence intensities at time 't', 0 min, and after adding Triton X-100 (1% final concentration), respectively. After completing 60 min of incubation, the erythrocyte membrane vesicles were pelleted by centrifugation at 1200 *g*, and washed several times with NaCl/P_i. The fluorescence associated with these vesicles was observed using a fluorescence light microscope. (A) A and D, phase contrast and fluorescence light micrographs, respectively, of the erythrocyte membrane vesicles interacted with egg PtdCho LUVs for 60 min; C and D, phase contrast and fluorescence light micrographs, respectively, of the erythrocyte membrane vesicles interacted with yeast lipids LUVs for 60 min. (B) Time-dependent NBD-fluorescence dequenching upon interaction of NBD-labeled LUVs with erythrocyte membrane vesicles. Egg PtdCho LUVs, ◆ yeast lipids LUVs, ▼.

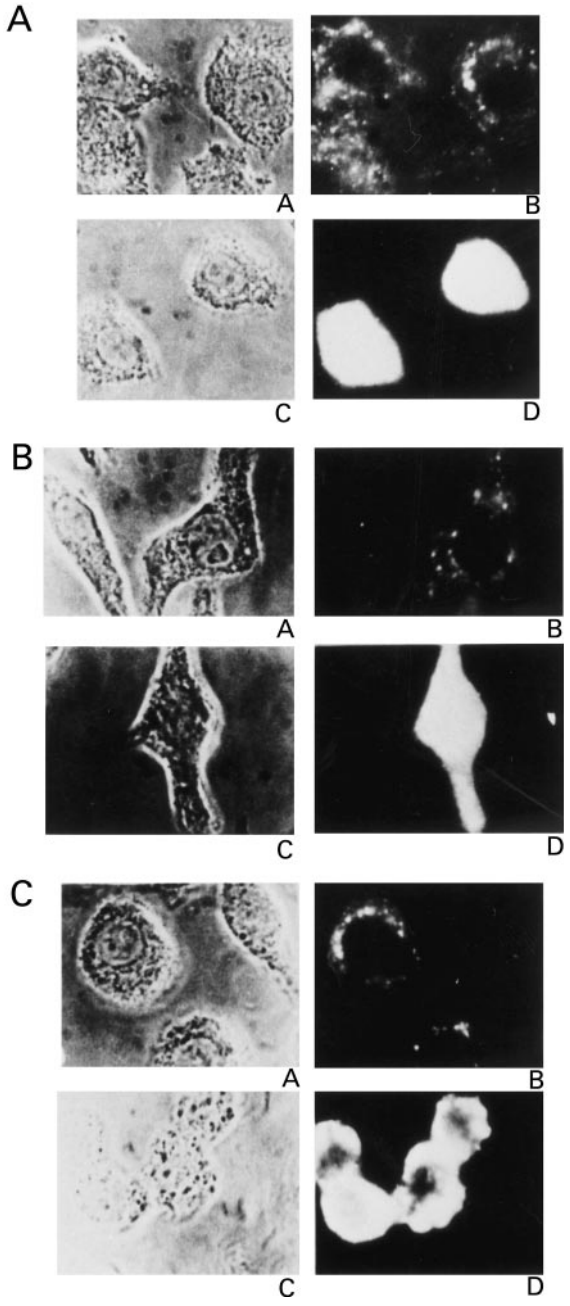

Fig. 3. Interactions of J774 A.1 cells with DRVs loaded with fluorescent solutes. (A) A and B, phase contrast and fluorescence light micrographs, respectively, of the macrophages interacted for 60 min at 37 °C with egg PtdCho/chol DRVs loaded with calcein. C and D, phase contrast and fluorescence light micrographs, respectively, of the macrophages interacted for 60 min at 37 °C with yeast lipids DRVs loaded calcein. Almost identical light micrographs were observed when the macrophages were interacted with calcein loaded DRVs in the presence of 100 μM chloroquine or at 4 °C. (B) A and B phase contrast and fluorescence light micrographs, respectively, of the macrophages interacted for 60 min at 37 °C with egg PtdCho/chol DRVs loaded with FITC-dextran. C and D, phase contrast and fluorescence light micrographs, respectively, of the macrophages interacted for 60 min at 37 °C with yeast lipid DRVs loaded with FITC-dextran. (C) A and B, phase contrast and fluorescence light micrographs, respectively, of the macrophages interacted for 60 min at 37 °C with egg PtdCho/chol DRVs loaded with FITC-PHA. C and D, phase contrast and fluorescence light micrographs, respectively, of the macrophages interacted for 60 min at 37 °C with yeast lipids DRVs loaded with FITC-PHA.

water soluble fluorescent solutes and fluorescent membrane markers from DRVs to the cells. For this purpose, the yeast lipid (or egg PtdCho) DRVs loaded with calcein, FITC-dextran or FITC-PHA were incubated with J774 A1 cells. After removing DRVs that did not interact, the cells were observed using a fluorescence light microscope. In each case, a significant amount of these solutes was transferred by the yeast lipid DRVs but not by the egg PtdCho/chol DRVs to the cytoplasmic compartment of the cells by membrane fusion (Fig. 3). To further corroborate our results, the yeast lipids (or egg PtdCho/chol) DRVs containing 5 mol percentage of NBD-PE in the bilayers were allowed to interact with J774 A1 cells. The transfer of the NBD fluorescence from DRVs to the macrophage membrane was analyzed by the fluorescence light microscopy (results not shown) and flow cytometry. A substantial amount of the NBD fluorescence was transferred to the plasma membranes of the cultured macrophages after their incubation with the NBD-labeled yeast lipid DRVs. The FACScan data revealed that the macrophage-associated NBD fluorescence, after incubating the cells with the yeast lipid DRVs, was at least 20 times higher than that observed with the egg PtdCho/chol DRVs (Fig. 4). Further, our findings also suggest that the incubation of the cells with the yeast lipid vesicles in the presence of 100 μM chloroquine or at 0 °C did not appreciably affect the NBD-PE transfer (data not shown).

Intracellular distribution of ^{125}I-labeled lysozyme. To analyze the interactions between the yeast lipid vesicles and J774 A1 cells, we incubated the cells with yeast lipid DRVs containing ^{125}I-labeled lysozyme. The intracellular distribution of lysozyme was measured as described in the Materials and methods. About 15.0% of the total cell associated ^{125}I-labeled lysozyme was localized in the cytosolic portion of the macrophages that were incubated with the yeast lipid DRVs loaded with ^{125}I-labeled lysozyme (Table 1). In contrast, only 1.8% of the cell-associated radioactivity could be detected in the cytoplasmic compartment of the cells that were treated with the ^{125}I-labeled lysozyme loaded egg PtdCho/chol DRVs. These results clearly suggest that the yeast lipid DRVs possess a strong fusogenic character that enables them to deliver the entrapped solutes into the cytosolic compartment of the antigen presenting cells.

Inhibition of the macrophage protein synthesis by ricin A. To further examine whether the membrane–membrane fusion constitutes one of the major modes of interaction between the yeast lipid DRVs and the J774 A1 cells, we also studied the effect of ricin A on macrophage protein synthesis. The cells were incubated with DRVs loaded with the toxin. As without the B-chain, ricin A is incapable of entering the cytosolic compartment of the cells [33]. To exert any biochemical effect on protein synthesis, ricin A needs a delivery system which can undergo fusion with the plasma membrane of macrophages and thereby deliver ricin A into the cytosyl. Our results indicate that, unlike with free or egg PtdCho/chol DRVs, ricin A when entrapped in yeast lipid DRVs did effectively inhibit macrophage protein synthesis in a dose dependent manner. (Fig. 5).

The delivery of the antigen entrapped in the fusogenic yeast lipid DRVs elicits an effective immune response

For an exogenous antigen to elicit the CD8$^+$ T lymphocyte response, the antigen must be delivered into the cytosol of the antigen presenting cells [10,11]. The strong fusogenic character

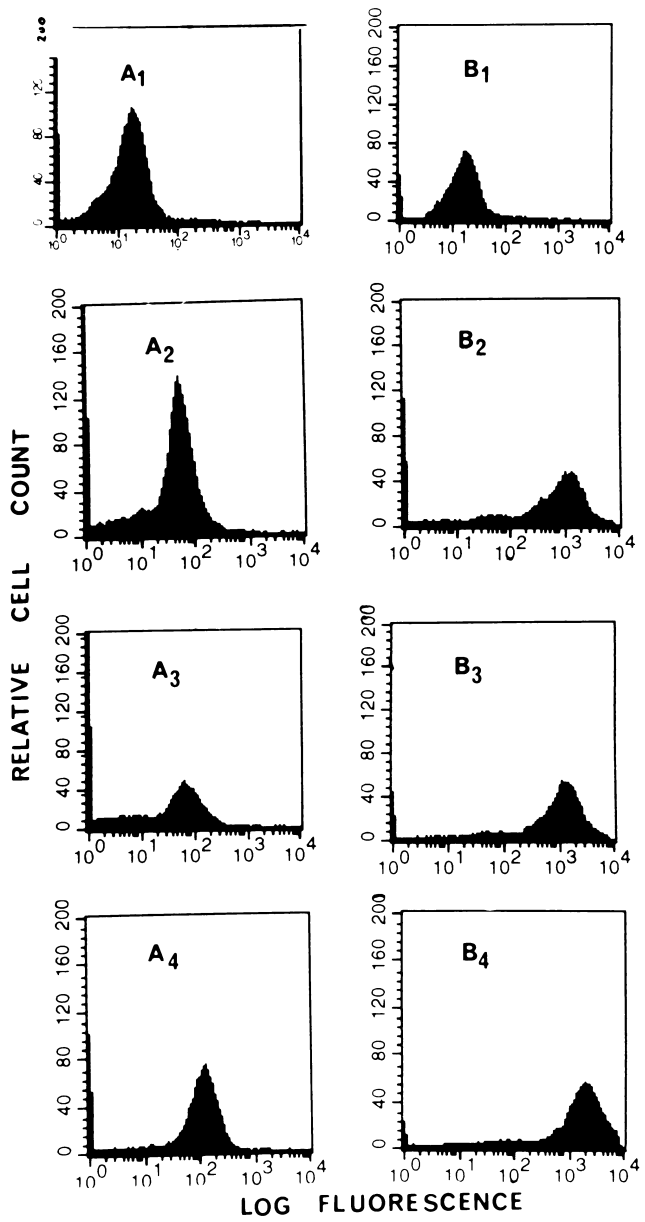

Fig. 4. FACS analysis of J774 A.1 cells after their interaction with DRVs labeled with NBD-PE. A_1, A_2, A_3 and A_4 show cells interacted with NBD-labeled egg PtdCho/chol DRVs for 0 min, 15 min, 30 min and 120 min, respectively. B_1, B_2, B_3 and B_4 show cells interacted with NBD-labeled yeast lipid DRVs for 0 min, 15 min, 30 min and 120 min, respectively.

of the great lipid DRVs is likely to facilitate cytosolic delivery of the entrapped protein, and in principle, should generate a strong $CD8^+$ T cell response. To examine the validity of this prediction, we evaluated the ability of ovalbumin entrapped in the yeast lipid DRVs to generate a $CD8^+$ T cell response. The antigen-specific $CD8^+$ cytotoxic T cell response was measured by ^{51}Cr release assay. Interestingly, immunization with the ovalbumin entrapped in the yeast lipid DRVs, but not other forms of antigen (free ovalbumin or sham liposomes, or free ovalbumin mixed with sham liposomes or ovalbumin entrapped in egg PtdCho/chol DRVs) led to the generation of cytotoxic T cells, as documented by the lysis of the target cells. A considerably higher degree (30–40%) of macrophage lysis occurred when the ovalbumin was encapsulated in the yeast lipid DRVs than when in free form or in egg PtdCho/chol DRVs (Table 2). The percent specific lysis of the target cells depends on the target : effector ratio as depicted in Fig. 6. Furthermore, these results also confirm the antigen specificity of CTLs generated. Unrelated antigen (lysozyme) when incubated with antigen presenting cells were not recognized by CTLs obtained from ovalbumin primed animals (Fig. 6).

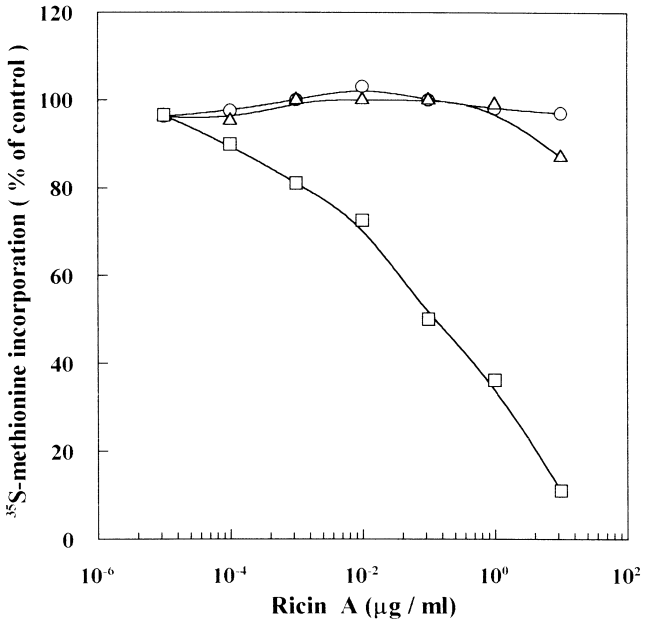

Fig. 5. Inhibition of the cellular protein synthesis by ricin A after J774 A.1 cells were interacted with ricin A-loaded yeast lipid DRVs. Free ricin A, ○; ricin A loaded in egg PtdCho/chol DRVs, △ ricin A loaded in yeast lipids DRVs, □. Values are the mean of three experiments.

Table 1. Subcellular distribution of ^{125}I-lysozyme introduced in J774 A.I cells using yeast lipid DRVs as carriers.

	Percent distribution of radioactivity[a]			
Lipid vesicles	Nucleus/plasma membrane	Cytosol	Endosome/lysosome	Other organelles
Egg PtdCho/chol	12.5 ± 1.40	1.80 ± 0.50	71.23 ± 3.20	13.60 ± 1.70
Yeast lipids	61.11 ± 3.40	15.41 ± 1.20	20.41 ± 2.90	2.63 ± 0.50

[a] Values shown are means of three experiments ± SD.

Table 2. Ovalbumin-specific $CD8^+$ T lymphocyte response generated by ovalbumin loaded yeast lipid DRVs in Balb/c mice. The ovalbumin-specific $CD8^+$ T lymphocyte response was ascertained by measuring the lysis of the antigen sensitized macrophages by the antigen-specific $CD8^+$ T cells isolated from ovalbumin immunized mice, which in turn was determined by measuring the ^{51}Cr release. Results shown are of four independent experiments. Each value is a mean of three determinations ± SD.

Antigen	Percent ^{51}Cr release
Saline	0.14 ± 0.07
	0.35 ± 0.05
	0.27 ± 0.16
Free ovalbumin	0.02 ± 0.04
	0.00 ± 0.00
	0.50 ± 0.28
	0.25 ± 0.03
Plain yeast lipid (sham) DRVs	0.08 ± 0.04
	0.76 ± 0.15
Ovalbumin mixed with sham DRVs	0.45 ± 0.20
	0.84 ± 0.16
Ovalbumin encapsulated in egg PtdCho/chol DRVs	0.78 ± 0.29
	0.80 ± 0.16
	0.00 ± 0.00
	0.00 ± 0.00
Ovalbumin encapsulated in yeast lipid DRVs	37.45 ± 1.40
	38.85 ± 3.45
	30.00 ± 3.45
	33.10 ± 1.80

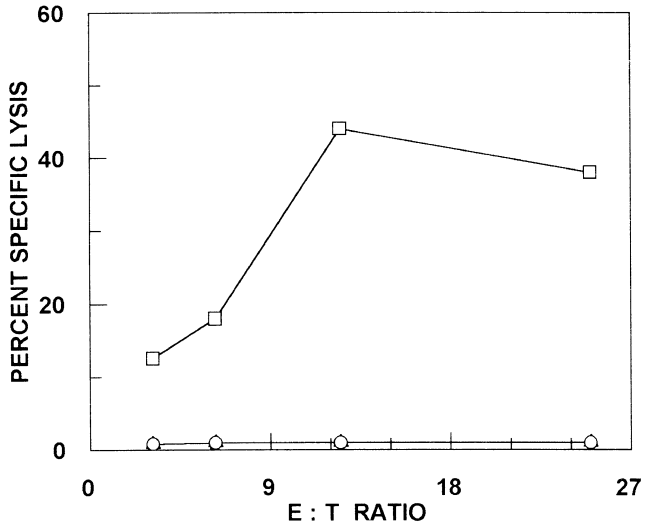

Fig. 6. Effect of the $CD8^+$ T lymphocyte/macrophage ratio on lysis of the ovalbumin-sensitized macrophages by $CD8^+$ T lymphocytes derived from mice immunized with different forms of ovalbumin. Free ovalbumin, △ ovalbumin loaded in egg PtdCho/chol DRVs, ○; ovalbumin loaded in yeast lipids DRVs, □. In the case of control wells, target cells incubated with effector cells (ovalbumin reactive $CD8^+$ T cells) in the absence of antigen or with sham liposomes (no antigen) and unrelated antigen (lysozyme entrapped in yeast lipids liposomes) did not show more than 2% of ^{51}Cr release.

DISCUSSION

A number of pathogens adapt intracellular parasitism as a survival strategy that protects them against the action of host humoral defense mechanisms. Protective immunity against these intracellular pathogens (for example, tuberculosis, leprosy, leishmaniasis, malaria, etc.), which thrive inside the hostile environment of the cells of an immunocompetent host, is considered to be essentially through cell-mediated immunity. Important roles for $CD4^+$ and $CD8^+$ T lymphocytes have been suggested by several studies of murine and human infections [34–37]. The generation of CTLs occurs when the antigen is delivered into the cytosol of the antigen presenting cells. In contrast, T-helper cells recognize antigens that are delivered exogenously and are processed in the class II antigen-processing pathway. Exogenous antigens do not ordinarily enter the class I processing pathway and thus do not activate $CD8^+$ CTLs. However, they can enter the class I processing pathway if they are delivered in a vehicle that has the property of undergoing fusion either with the plasma membrane, under normal physiological conditions or with the endosomal membrane at low pH.

We have shown earlier that antigen incorporated into conventional liposomes results in the enhancement of interleukin-4 (IL-4) and IgG-1 secretion [38]. In the present work, we have attempted to deliver exogenous antigen into the cytosol of target cells using yeast lipid liposomes. The following major findings have emerged from this study; yeast lipid vesicles undergo efficient fusion with the cell membrane; the macromolecules entrapped in the liposomes can be delivered into the cytosol of the target cells; the antigen entrapped in the yeast lipid vehicles effectively delivers the content into the class I antigen processing pathway; and effector $CD8^+$ cytotoxic T cells are stimulated.

The present study shows that vesicles formed of a yeast lipid extract have an inherent tendency to undergo spontaneous membrane fusion. These vesicles fuse not only with each other but also with egg PtdCho/chol vesicles, albeit to a lesser extent. The extent of fusion with egg PtdCho liposomes was about 20%, as judged by measuring the RET between NBD-PE and Rh-PE embedded in the yeast lipid vesicle bilayer. The RET assay is known to be insensitive to vesicle aggregation [30] and ensurees that the probes do not undergo exchange between the membranes [31]. In the present study, the observed decrease in RET efficiency upon incubation of yeast lipid vesicles containing NBD-PE/Rh-PE with unlabeled yeast lipid (or egg PtdCho/chol) vesicles or the erythrocyte membrane vesicles, is directly used to calculate the extent of membrane fusion. Similarly, the ANTS/DPX mixing used for assaying the mixing of the aqueous content of the interacting vesicles appears to be quite reliable, as quenching of the ANTS fluorescence by DPX has been shown to be highly dependent on DPX concentration, and it does not occur upon leakage of DPX into the medium [30]. Further, the NBD-fluorescence dequenching assay used to monitor the interactions of NBD-labeled yeast lipid vesicles with the erythrocyte membrane vesicles (or macrophages) would only monitor the membrane fusion, as NBD-PE has been shown to be a nonexchangeable fluorescent lipid probe by earlier investigators [31].

The fusion of the yeast lipid vesicles is not only restricted to the model membrane systems but it could also occur with live cells, such as macrophages. This is based on our observations that transfer of both the water soluble fluorescent solutes and the fluorescent membrane markers from the yeast lipid vesicles to J774 A1 cells was considerably higher (at least 20-fold) than

with egg PtdCho/chol vesicles. This transfer remained virtually unaffected by lowering the incubation temperature from 37 °C to 4 °C or by incorporation of 100 μM of chloroquine in the incubation mixture. Furthermore, nearly 15% of cell-associated ^{125}I-lysozyme could be localized in the cytosolic fraction after J774 A1 cells were interacted with ^{125}I-lysozyme-loaded yeast lipid DRVs as opposed to about 2% using egg PtdCho/chol DRVs. Unusually high incorporation of radioactivity (61%) was seen in the plasma membrane/nuclear fraction of the interacted cells (Table 1). This may be considered as the result of the multilamellar nature of the yeast lipid DRVs, which have a tendency to settle upon centrifugation even at lower ***g***-values. This is also evident from the fact that repetition of the same study but substituting DRVs with LUVs resulted in a remarkable increase in cytosolic delivery (up to 30%), and only 13% of the total cell-associated radioactivity was localized in plasma membrane/nuclear fraction (data not shown).

The results of the present investigation suggest that the yeast lipid DRVs could undergo spontaneous membrane fusion with J774 A1 cells, resulting in the delivery of the entrapped material into their cytoplasmic compartment. This is further established by our present finding that ricin A encapsulated in yeast lipid DRVs, but not in free form or in egg PtdCho/chol DRVs, strongly inhibited cellular protein synthesis upon its interactions with J774 A1 cells. Our observation is further substantiated by the fact that ovalbumin loaded in the yeast lipid DRVs, but not in free form or in egg PtdCho/chol DRVs, generated a strong ovalbumin-specific $CD8^+$ T lymphocyte response [10,11].

It appears that antigen is only endocytosed when egg PtdCho/chol vesicles interact with the APC. In contrast, the yeast lipid vesicles appear to interact with the cells by both endocytosis and membrane fusion, thus helping in the activation of both T-helper cells as well as CTLs. It has long been appreciated that both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells are required for immunity to virulent *M. tuberculosis*. The requirement of $CD4^+$ recognition of infected macrophages is very much illustrated by the synergy between HIV and mycobacteria: HIV infection desolates the body's $CD4^+$ T-helper cell population and allows the Mycobacteria to grow unhindered. Besides, MHC-II or CD4 deficient mice are susceptible to infection even with the avirulent Bacillus Calmette-Guerin (BCG) strain of *Mycobacterium bovis* [39]. With this in mind, we extended our study to determine the role of yeast lipid vesicles in the elicitation of $CD4^+$ T-helper cells. The secretion of IL-2, IL-4 and interferon-γ (IFN-γ) was evaluated in the supernatant of cells obtained from immunized animals. We observed that antigen encapsulated in yeast lipid DRVs generated strong $CD4^+$ T lymphocyte responses as well (data not shown). Even the $CD4^+$ T cell response generated by using the yeast lipid vesicles was better than that observed with the egg PtdCho/chol DRVs. As both $CD4^+$ and $CD8^+$ T lymphocyte responses seem to be required for effective control of the mycobacterial infections, especially tuberculosis [40], it may be inferred that encapsulation of protective mycobacterial antigens [41,42] in yeast lipid vesicles may further improve their effectiveness against these infections.

Lipid vesicles have been widely employed as vehicles to deliver drugs and other biologically active substances to cells both *in vitro* and *in vivo* [43–46]. Also, their use as vaccine and nucleic acid carriers has been well documented [44,46–48]. In addition, antisense nucleotide sequences entraped in lipid vesicles have been successfully delivered to their target cells [46]. As yeast lipid vesicles are easy to prepare and should presumably be nontoxic to humans, their use as vehicles for introducing biologically active substances, especially nucleic acids and proteins, into the cytoplasmic compartment of cells could prove advantageous over the use of other lipid vesicles.

It is not yet clear what factor gives rise to the strong fusogenic character of the yeast lipid extract. However, earlier studies with polyethylene glycol-resistant cell mutants have shown that some of these mutants can readily undergo spontaneous membrane fusion, and that the increased fusogenic tendency of these cells is correlated with an increased saturation of the fatty acyl chains as well as an increased neutral ether-linked lipid content [49]. Furthermore, a glycero-diPtdCho derivative linked to a polyol and an amino acid has been shown to be the main factor responsible for fusion of *Mycoplasma* to T lymphocytes [50,51]. But no such fusogenic lipid factors have so far been identified in *S. cerevisiae*. However, the lipid content of this yeast does include significant quantities of anionic phospholipids, lysolipids, monoglycerides and phosphatidylethanolamine [52], all of which are known to be fusion inducers. Alternatively, membrane fusion can be induced by fusogenic proteins present in cellular membranes [53]. Our preliminary studies have, however, indicated that the fusogenic character of yeast lipids is not influenced by passing the lipid mixture through a silica gel column, using chloroform/methanol (1 : 1, v/v) as the eluant, or by the prolonged digestion of the lipid extract with pronase (data not shown). Although this argues against the possible role of some fusogenic proteins, which could be present as contaminants in the yeast lipid extract, imparting a fusogenic character on the lipid mixture, further studies are clearly required to reach a definite conclusion.

In summary, this study demonstrates that lipids derived from *S. cerevisiae* possess a strong fusogenic character. The vesicles formed from these lipids readily fuse with target cells, delivering a variety of water-soluble solutes, including macromolecules, to the cytoplasmic compartment. The quantities of the delivered solutes are sufficient enough to exhibit an intracellular biochemical effect as evidenced by inhibition of the cellular protein synthesis by ricin A loaded in the yeast lipid vesicles. Also, ovalbumin encapsulated in these vesicles generated a strong ovalbumin-specific $CD8^+$ T lymphocyte response, as well as generating the $CD4^+$ T cell response.

Finally we can conclude that the yeast lipid liposomes should certainly provide a safe and promising adjuvant for inducing cell-mediated immunity when attenuated live vaccines are unavailable or impractical.

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