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Use of Liposomes as an Immunopotentiating Delivery System: in Perspective of Vaccine Development

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Liposomes have been widely used to deliver antigens to the antigen-presenting cells (APCs) and also to modify their immunological behaviour in model animals. We recently demonstrated the potential of yeast lipid liposomes to undergo membrane–membrane fusion with cytoplasmic membrane of the target cells. Interestingly, studies in the present report revealed that antigen encapsulated in yeast lipid liposomes could be successfully delivered simultaneously into the cytosolic as well as endosomal processing pathways of APCs, leading to the generation of both $CD4^+$ T helper and $CD8^+$ cytotoxic T cells. In contrast, encapsulation of same antigen in egg phosphatidyl-choline (PC) liposomes, just like its free form, has inefficient access to the cytosolic pathway of major histocompatibility complex (MHC) I dependent antigen presentation and failed to generate antigen specific $CD8^+$ cytotoxic T-cell response. However, both egg PC as well as yeast lipid liposomes have elicited strong antigen specific antibody responses in immunized animals. These results imply usage of liposome encapsulated antigen as potential candidate vaccine capable of eliciting both cell mediated as well as humoral immune responses.

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INTRODUCTION

The protein antigens delivered to APCs, normally undergo processing within the endosomes to produce the peptides that bind to MHC class-II molecules. The MHC II processing and presentation of the antigen results in the activation of $CD4^+$ T cells [1, 2]. On the other hand, $CD8^+$ T cell responses can be elicited by the endogenous antigens, which are processed within the cytosol and produce peptides, which bind to MHC-I molecules [2]. Nevertheless, exogenous antigen can also enter the class I processing pathway, if it is delivered in a vehicle that can fuse either with the plasma membrane under normal physiological conditions or with the endosomal membrane at low pH [2–4, 16, 17]. Since both cytotoxic as well as T-helper cells are crucial for protecting the host against intracellular pathogens to mount an effective immunity against such infections, it is important to develop vaccines that generate strong $CD4^+$ and $CD8^+$ T-cell responses [5–7].

In our previous study, we isolated the fusogenic lipids from yeast cells and utilized them for the first time to prepare liposomes [8]. We demonstrated that the yeast lipid liposomes very effectively fuse with the membrane of macrophages. In the present report we established that antigen encapsulated in the liposomes could be readily introduced into the cytoplasmic as well as endosomal compartments of the target cells. Moreover, these studies have revealed that antigen entrapped in these liposomes could generate strong OVA-specific, $CD4^+$ T helper as well as $CD8^+$ cytotoxic T cells. The T-helper response was chiefly of Th-2 type, as evidenced by the secretion of interleukin (IL)-4 and immunglobulin (Ig)G1-isotype [9]. On the contrary, the same antigen after encapsulation in egg PC liposomes was able to induce humoral immune response mainly.

MATERIALS AND METHODS

Animals. Inbred female BALB/*c* mice (8–10-weeks-old), of 20 ± 2 g of weight, were obtained from the Institute's Animal House Facility.

Chemicals and reagents. Egg PC was prepared using the standard method [10]. Cholesterol (chol) was purchased from Centron Research Laboratory, Mumbai, India. Ovalbumin (OVA) was from Sigma Chemical Company (St. Louis, MO, USA). Antibodies to IL-2 (HB 8794), IL-2 receptors (CRL 1698 and TIB 222), IA^d (MKD6), Mac 2

(TIB 166), L3T4 (GK1.5), lyt 2.2 (TIB 210) and IL-4 (11B11) were used as culture supernatants of the hybridomas. The P815 cells, an MHC matched (H-2^d) mastocytoma cell line, EL-4 cells, an MHC disparate (H-2^b) lymphoma cell line, HT-2 cell line (CRL-1841) and hybridomas used in the study were procured from ATCC (Rockville, MD, USA). [^{3}H]-thymidine and [^{51}Cr] sodium-chromate were bought from Bhabha Atomic Research Center, Mumbai, India.

Preparation of liposomes. The yeast lipid was isolated from *Saccharomyces cerevisiae* cells as described elsewhere [8]. The liposomes were prepared essentially by following the published procedure [11]. Briefly, egg PC/cholesterol (2 : 1 molar ratio, total 20 mg) or yeast lipids (20 mg) were reduced to thin dry film. The film was hydrated followed by sonication in a bath-type sonicator for 2 h at 4 °C, under N_2 atmosphere. The liposomes thus formed were mixed at this stage with an equal volume of OVA (30 mg/ml). The mixture was flash frozen and thawed (3 cycles), and then lyophilized. The free-flowing, dried powder obtained was rehydrated with distilled water (120 μl) and finally re-constituted with phosphate-buffered saline (PBS). The preparation was centrifuged at 14 000 × ***g*** and the pellet was further washed at least 3 times with PBS to remove the traces of the unentrapped solute. The protein entrapped in the liposomes was estimated as described elsewhere [8]. Briefly, the liposomes (given volume) were lysed with 10% Triton X-100 solution (the final concentration of Triton X-100 was maintained 1%). The mixture of solutions A and B of the Bicichoninic acid (BCA) reagent was added to the lysed liposomes (released protein) and then incubated at 37 °C for 45 min. The absorbance was measured at 570 nm wavelength. The protein concentration was calculated using a standard curve of OVA plotted in the presence of Triton X-100.

Immunization. The immunological studies were performed in inbred female BALB/*c* mice. Each group was consisted of five animals. The animals were immunized with different preparations (free OVA or entrapped in liposomes) of antigen on various days. The following immunizing schedules were adapted for administration of OVA into the animals, for different immunological studies.

Cell preparation. (a) CD8$^+$ T cells. Different groups of BALB/*c* mice were injected separately, through an intravenous route, with a total of three doses (day 0, 7, and 14) of free OVA, OVA encapsulated in egg PC/chol or yeast lipids liposomes and also with free OVA mixed with empty liposomes [100 μg OVA (which correspond to 500 nmoles of lipid)/animal/week] for 3 weeks. On day 21, the animals (five animals each group) were sacrificed and spleens were removed aseptically. The cells were prepared as described elsewhere [12]. The cells obtained from different animals in a given group were pooled, purified and used in cytotoxicity assay. The enriched population stained with anti-CD8 antibodies (Ab), was > 98% pure, as evaluated by FACScan.

(b) CD4+ T cells. Animals were immunized with single dose of free OVA, OVA encapsulated in egg PC/chol or yeast-lipid liposomes (100 μg OVA/animal) through intravenous route. On day 7, the animals (five animals each group) were sacrificed and their spleens were removed aseptically and CD4$^+$ T cells were isolated as described elsewhere [12]. For lymphokine assay experiments, the animals were immunized intravenously using two different immunizing schedules. In one set, the animals (five animals each group) were immunized with single dose of OVA (100 μg OVA/animal), while in another set, the animal were immunized with total three doses of OVA (100 μg OVA per animal, on day 0, 7, and 14). On day 21, the animals were sacrificed and CD4$^+$ T cells were isolated as described above. The enriched population of CD4$^+$ T cells were stained with anti-CD4 and CD3 Abs and the purity was > 98%, as revealed by FACScan.

CD8$^+$ T lymphocyte response. (a) Target cells. BALB/*c* mice were injected with thioglycollate broth. On day 4, the macrophages were isolated from the peritoneal exudate cells (PEC) by adherence on Petri plates. The harvested cells (2×10^7 cells/ml) were washed 3 times with Hank's balanced salt solution (HBSS) and incubated at 37 °C for 3–4 h with either free OVA, OVA entrapped in egg PC/chol or encapsulated in yeast-lipid liposomes. The cells were again washed 3 times to remove free antigen. This was followed by incubation with ^{51}Cr (100 μCi/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.

(b) Cytotoxicity assay. The ^{51}Cr-labelled macrophages/P815 cells (5×10^3/well) were used as target cells. The antigen primed target cells were incubated with CD8$^+$ T cells (effector cells isolated from the spleen of the five mice were pooled, and used for assay) at an effector to target (E/T) ratios of 2.5 : 1–20 : 1. The cells were incubated at 37 °C for 6 h, after completion of incubation period, the cells were pelleted at 3000 × ***g*** (15 min at 5 °C), and the amount of ^{51}Cr released was determined by measuring the radioactivity in the supernatant. The experiments were performed three times and the error bars represent standard deviation of the means from three different experiments. The total ^{51}Cr release was calculated by treating an aliquot of the target cells with Triton X-100 (10% final concentration). The spontaneous release of ^{51}Cr in the supernatant was determined by incubating the labelled macrophages for 6 h. An amount of auto-release was subtracted from the total release to determine the extent of macrophage lysis. In most of the experiments, the autorelease was less than 25%. The percentage specific release was calculated as the (mean sample cpm-mean spontaneous cpm/mean maximum cpm-mean spontaneous cpm) × 100%.

CD4+ T-cell proliferation. The CD4$^+$ T cells (2×10^4/well) obtained from pool of the splenic cells of five mice from different groups, were cultured in triplicate wells. The cells were incubated with macrophages (6×10^4/well) pulsed with different doses (0.001–100 μg/ml) of free OVA or encapsulated in egg PC/chol or yeast lipid liposomes. The macrophages were treated with mitomycin C (50 μg/ml). The cultures were incubated for 72 h at 37 °C/7% CO_2. The cells were pulsed with 1.0 μCi[^{3}H]-thymidine for 16 h before harvesting with an automatic cell harvester (Skatron, Tranby, Norway). The [^{3}H]-thymidine incorporation was measured by a standard liquid scintillation counting method. The results are expressed as mean cpm of triplicate cultures.

Lymphokine assays. The cultures were set up as described for Th cell proliferation. The supernatants were collected after 48 h for estimation of IL-2, IL-4 and interferon (IFN)-γ levels. The IL-2 and IL-4 were assayed using the HT-2 cell line, while IFN-γ was estimated by its ability to inhibit the proliferation of WEHI-279 cells [13]. The lymphokines data were expressed as pg/ml in the culture supernatants as computed by comparison with the standard curve plotted using r IL-2/rIL-4 and rIFN-γ (Genzyme, Cambridge, MA, USA).

Determination of OVA-specific IgG isotypes by ELISA. The production of OVA-specific antibodies was measured in the sera of the different groups of mice. The animals were injected, intravenously, with two doses of free OVA, OVA entrapped in the egg PC/chol or yeast lipid liposomes (100 μg OVA/animal) on day 0 and 7, and bled on day 14 to monitor the presence of antibodies as described earlier [13]. Briefly, 96 well microtitre plate was incubated overnight with 50 μl of OVA (25 μg/ml) in carbonate-bicarbonate buffer (0.05 M, pH 9.6) at 4 °C. After the usual washing and blocking steps the plate was finally incubated with 1 : 500 dilutions of test and control sera at 37 °C for 2 h. After excessive washing of the plate, it was further incubated with 50 μl of biotinylated goat antimouse IgG1 and IgG2a antibodies. The plate was incubated at 37 °C for 1 h. After the usual washing steps, 50 μl of streptavidin-HRP were added to each well and the plate was

incubated at 37 °C for 1 h. The plate was washed again before adding 50 μl ABTS (Sigma) and was finally incubated at 37 °C for 20 min. The reaction was terminated by the addition of 50 μl of 70% H_2SO_4. The absorbance was read at 492 nm with a microtitre plate reader (Eurogenetics, Torino, Italy).

Statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA) following Dunnet's *t*-test method. $P < 0.05$ was considered statistically significant.

RESULTS

Delivery of the antigen entrapped in the fusogenic yeast-lipid liposomes elicits effective immune response

$CD8^+$ T-lymphocyte response. Since our preliminary experiments demonstrated that the yeast-lipids liposomes possess a strong fusogenic character, the protein entrapped in liposomes, in principle, should be delivered into the cytosol of APCs for presentation via the MHC class I pathway. We evaluated the potential of yeast-lipid liposomes entrapped OVA, to undergo MHC-I processing and presentation to generate a $CD8^+$ T-cell response. Initially the animals were immunized with different doses of antigen entrapped in yeast liposomes (10–100 μg OVA/per dose/per animal/total three doses, each at week interval). It was found that a dose of 100 μg per animal induced cytotoxic T lympocyte (CTL) response, which generated 30–40% target lysis at an effector to target ratio of 10 : 1 (data not shown). This dose was selected for subsequent studies performed for ^{51}Cr release assay. Interestingly, immunization with OVA entrapped in the yeast-lipid liposomes, but not other forms of OVA viz. free OVA or OVA entrapped in egg PC/chol liposomes and free OVA mixed with yeast-lipid empty liposomes (sham liposomes), generated cytotoxic T cells. A considerably high degree (30–40%) of macrophage lysis occurred when the OVA was encapsulated in the yeast-lipid liposomes, as compared to less than 1% specific lysis in free OVA or OVA incorporated into the egg PC/chol liposomes or sham yeast-lipid liposomes ($P < 0.001$) (Fig. 1A). We also found concentration optima for antigen (Fig. 1B) for percentage specific lysis of the target cells.

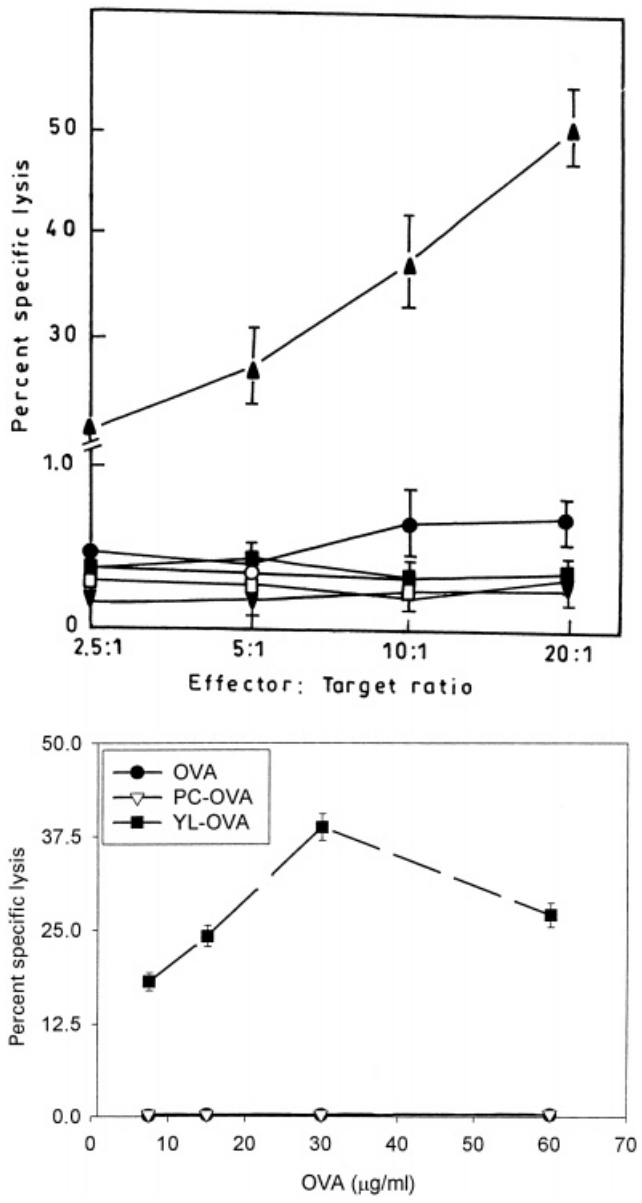
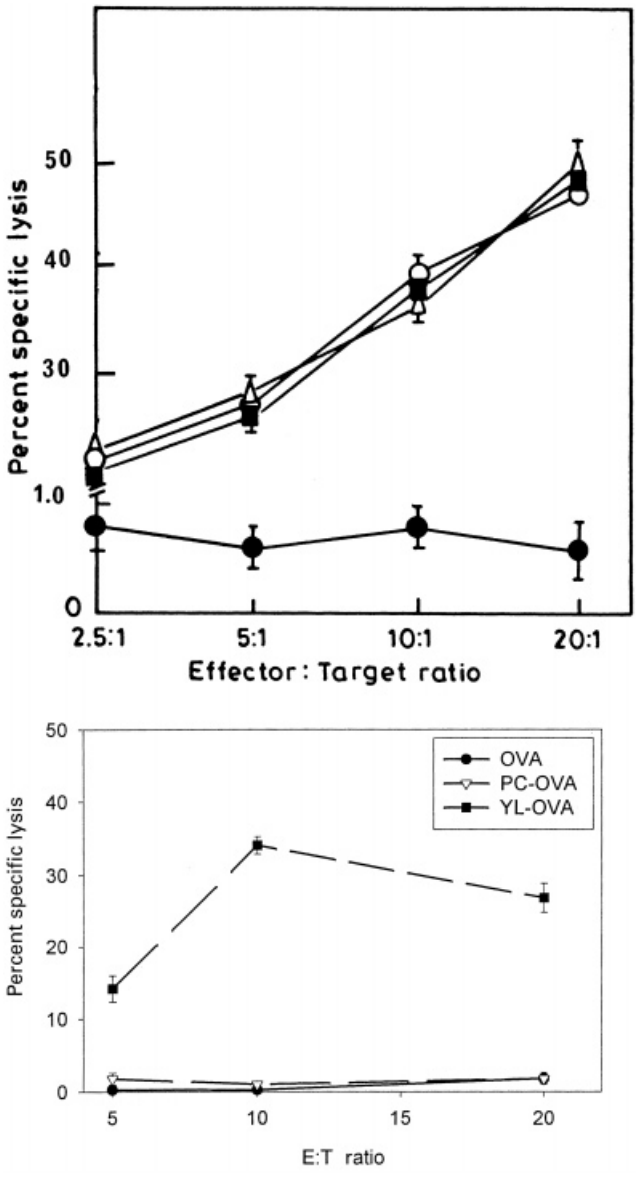


Fig. 1. (A) Induction of antigen specific cytotoxic T lympocyte (CTL) activity by immunization with yeast lipid liposomes containing different forms of ovalbumin (OVA). The BALB/*c* mice were immunized with saline (□); Sham yeast liposomes (Sham YL)(O); Free OVA (▼); yeast liposomes mixed with OVA (YL + OVA) (■); OVA encapsulated in Egg PC liposomes (PC-OVA) (●) and OVA encapsulated in yeast liposomes (YL-OVA) (▲), as described in Materials and Methods. The effector cells were isolated from the spleen of five mice, belonging to the different groups above. The cells were pooled, purified and used for target cell lysis assay. The target cells (macrophages) were pulsed with saline, Sham ES, Free OVA, Yeast liposomes mixed with OVA (YL + OVA), OVA encapsulated in Egg PC liposomes (PC-LIP-OVA) and OVA encapsulated in YL liposomes (YL-LIP-OVA), respectively (30 μg OVA/2 × 10^7 cells), and labelled with ^{51}Cr. The lytic activity was measured by the incubation of suitable effector cells with target cells (primed with matching formulation of OVA). The results are represented as, percent specific lysis (^{51}Cr release) of the target cells. Each value represents the mean of three determinations ± S.D. Data are representative of five independent experiments performed with similar results. (B) Dose response of antigen on the induction of target cell lysis. The macrophages were sensitized with different concentrations of free OVA, OVA loaded in either egg PC/chol liposomes (PC-OVA) or yeast lipids liposomes (YL-OVA) and then incubated with OVA reactive $CD8^+$ T lymphocytes isolated from the animals immunized with OVA, PC-OVA, and YL-OVA, respectively. The E/T ratio was 10 : 1. In the case of control wells, target cells incubated with effector cells (OVA reactive $CD8^+$ T cells) in the absence of antigen or with sham liposomes (no antigen), YL mixed with OVA and unrelated antigen (lysozyme entrapped in yeast lipids liposomes), there was not more than 2% of ^{51}Cr release. Values represent the mean of three determinations ± S.D. The experiment was performed three times with similar results.

Results shown in Fig. 2(A) confirm the phenotype of the effector cells. The pretreatment of the purified effector cells with anti-$CD8^+$ antibodies, followed by treatment with baby rabbit complement, abolished target cell lysis. We further demonstrated that macrophages, primed with free or egg PC liposomes encapsulated OVA, can not act as target cells (Fig. 2B). The effector cells, obtained from the animals immunized with yeast lipid liposomes, were allowed to incubate with the target cells, which were primed with different forms of OVA, i.e. the free OVA, OVA encapsulated either in egg PC liposomes or yeast liposomes. The ^{51}Cr release assay shows that OVA-specific CTL successfully distinguished the target cells, which were incubated with yeast lipid liposomes for antigen presentation, however, egg PC liposomes pulsed target cells were not recognized by the same CTLs ($P < 0.001$).

The results discussed in Table 1 demonstrated that, beside macrophages, yeast lipid induced effector cells can lyse other target cells with equal propensity. However, the recognition of target cells is confined to the MHC compatible cells only, thus, effector cells were able to induce lysis of P815, an MHC matched (H-2^d) mastocytoma cells, while allogenic EL-4 cells (H-2^b) were not recognized at all by the same effector cells.



$CD4^+$ T-cell response

Antigen entrapped in the yeast-lipid liposomes enhances $CD4^+$ T cells proliferation. The T cells of the mice immunized with antigen encapsulated in the yeast-lipid liposomes induced significantly higher proliferation as compared to T cells obtained from animals immunized with egg PC/chol liposomes (Fig. 3). T-cell responsiveness to antigen was observed in a dose-dependent manner. Control cultures containing cells obtained from either OVA-immunized animals or the groups immunized with PBS or fusogenic lipids only (sham liposomes with no OVA), gave background levels of < 2000 cpm of ^3H-thymidine incorporation.

Antigen encapsulated into yeast-lipid liposomes predominantly enhances the production of IL-4. The animals immunized with single dose of antigen encapsulated in the yeast-lipid liposomes showed significantly higher levels of IL-4 (512 pg/ml) as compared to animals inoculated with egg PC liposomes (216 pg/ml) (Fig. 4A). In contrast, only marginal secretions of IL-2 and IFN-γ were observed when yeast-lipid liposomes (26 pg/ml IL-2, 20.27 pg/ml IFN-γ) and egg PC/chol liposomes (12.4 pg/ml IL-2, 15.69 pg/ml IFN-γ) were used for immunization (Fig. 4B,C). No detectable amount of lymphokines was observed in the case of control animals inoculated either with PBS, sham egg PC/chol or yeast-lipid-liposomes alone (no antigen). However, the animals which were

Fig. 2. (A) OVA encapsulated in yeast lipid liposome can induce T lymphocytes with $CD8^+$ phenotype. Effector cells obtained from the OVA (encapsulated in yeast liposomes) primed animals were pretreated with anti-$CD4^+$ or anti-$CD8^+$ monoclonal antibodies (MoAbs) followed by incubation with baby rabbit complement. The effector cells were incubated with peptide pulsed, ^{51}Cr loaded target cells. The lysis of the target cells was measured by ^{51}Cr release assay. Anti-$CD8^+$ antibody pretreatment (●); anti-$CD4^+$ antibody pretreatment (■); pretreatment with complement alone (O); and no pretreatment (Δ). Values are mean of three determinations ± S.D. Data are representative of five different experiments with similar results. (B) Yeast liposomes provide an efficient means of sensitizing target cells to class I restricted CTL recognition. BALB/*c* mice were immunized with OVA encapsulated in yeast liposomes. Cytotoxic T cells from the spleen of five mice were pooled, and used as effector cells for cytotoxic assay. The target cells were primed with various formulations of OVA viz. free OVA (●); OVA encapsulated in egg PC liposomes (∇); and OVA encapsulated in yeast lipid liposomes (■) and incubated with effector cells, isolated from YL-OVA treated animals (at the effector to target ratio of 10 : 1). The lysis of the target cells was measured by ^{51}Cr release assay. Data are mean of three determinations ± S.D.

Table 1. Antigen specificity and MHC restriction of the OVA specific cytotoxic T lymphocytes

Targets	Treatment	Specific lysis (%)
P815	None	0.45 ± 0.12
P815	Free OVA	0.30 ± 0.10
P815	Sham YL*	0.48 ± 0.13
P815	YL + OVA†	0.54 ± 0.09
P815	PC-OVA	0.78 ± 0.20
P815	YL-OVA‡	38.5 ± 2.20
P815	YL-Lysozyme	0.65 ± 0.15
EL-4	Free OVA	0.52 ± 0.10
EL-4	PC-OVA	0.65 ± 0.18
EL-4	YL-OVA	0.42 ± 0.08
EL-4	YL-Lysozyme	0.42 ± 0.10

The BALB/*c* mice were immunized with yeast liposome encapsulated OVA, and effector cells were isolated and purified as described in the text. The MHC restriction as well as antigen specificity of the generated CTLs was demonstrated by ^{51}Cr release assay. The target cells, P815 (H-2^{d}) and EL-4 (H-2^{b}) were pulsed with different forms of OVA and lysozyme (an unrelated antigen) followed by ^{51}Cr labelling, as described in Materials and Methods. The antigen primed target cells were incubated with effector cells at effector to target ratio of 10 : 1. Each value represents mean of three determinations ± SD. The experiment was performed four times with similar results.* Liposome without antigen; †yeast liposome simply mixed with OVA; ‡OVA encapsulated in yeast liposomes.

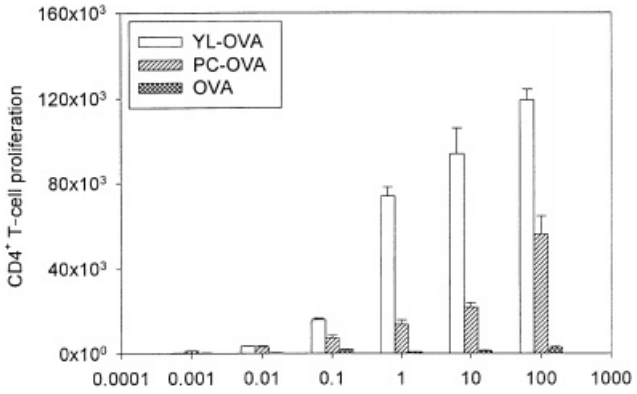


Fig. 3. Antigen incorporated in yeast-lipid-liposomes augmented the proliferation of OVA-specific Th cells. CD4^{+} T cells (2×10^{4}/well) were isolated from the groups of five animals immunized with OVA encapsulated either in yeast-lipid-liposomes (YL-OVA) or egg-PC/chol liposomes (PC-OVA) and cultured with the OVA, YL-OVA and PC-OVA pulsed macrophages (6×10^{4} cells/well). After 72 h, [^{3}H]-thymidine was added, and its incorporation was measured 16 h later by liquid scintillation spectroscopy. The control cultures consisting of Th cells + medium (no OVA), macrophages + medium, Th cells + yeast-lipid sham liposomes (no OVA), medium + Th cells obtained from the animals immunized with placebo (phosphate-buffered saline) showed < 2000 cpm. The data represents mean cpm of three determinations ± S.D. The experiment was repeated four times with similar results.

boosted three times with YL liposomes demonstrated a substantial increase in the IL-2 and IFN-γ level, while the IL-4 level had decreased to some extent (Fig. 4).

Humoral immune response

Enhancement of the secretion of IgG1 isotype by immunizing the animals with the antigen encapsulated in the liposomes of the fusogenic lipids derived from yeast. The significant increase in the level of antibodies was detected in sera of the animals, which were primed with OVA entrapped in the yeast-lipid-liposomes as compared to the animals, inoculated with egg PC/chol liposomes (Fig. 5). However, mainly IgG1 antibodies were generated in both the cases, while the IgG2a type of antibody were induced to lesser extent only ($P < 0.05$). No IgG1 and IgG2a isotypes were detected in the control group of mice injected with either sham yeast-lipid/egg PC/chol liposomes (no antigen) or PBS.

DISCUSSION

Protective immunity against intracellular pathogens (e.g. tuberculosis, leprosy, leishmaniasis, malaria, viruses, etc.) and cancer is considered to be controlled essentially by cell-mediated immunity. The important role of CD4^{+} and CD8^{+} T lymphocytes has been suggested by several studies of murine and human infections [6, 7, 14, 15]. The majority of the vaccines available to date, mainly induce the humoral immune responses, conversely the generation of cytotoxic T cells always remains a major uphill task against most of the infections. Earlier investigators have attempted the use of virosomes as well as pH-sensitive liposomes for delivery of protein antigens into the cytosol [16–19]. Nevertheless, such vehicles proved to be toxic to the host and were additionally not simple to prepare, and were thus restricting their use as a vehicle to deliver the newer generation of vaccines against intracellular infections.

We have shown earlier that the antigen incorporated into liposomes results in the increased production of IL-4 but not IFN-γ [20]. In another report, we recently demonstrated that yeast liposomes can readily deliver their contents to cytosol of the APCs [8]. In the present study, we considered it to be of interest to exploit the inherent membrane–membrane fusion tendency of yeast lipids in the development of liposome-based vaccines. The antigen encapsulated in liposome was allowed to interact with the APCs. The following four major findings have emerged from the study. Firstly, the antigen entrapped in the yeast-lipid vehicles effectively delivers the contents into the class I and class II antigen processing pathways; secondly, the effector CD8^{+} cytotoxic T cells were stimulated; thirdly, CD4^{+} T-helper cells, predominantly of Th2 phenotype, were generated; fourthly, a predominant secretion of IgG1 isotype was observed.

The results of the present study demonstrate that the

immunogenicity of OVA is controlled by the nature of adjuvant/antigen carrier used for immunization (Fig. 1A). While the antigen entrapped in the yeast lipid liposomes elicits antigen specific CTL generation, other forms of antigen, e.g. OVA entrapped in egg PC liposomes fail to do so. Furthermore, the processing and presentation depends on the mode of the antigen delivery, as evident from our results that OVA specific CTLs, obtained from animals immunized with yeast liposomes, can induce lysis of target cells which were primed *in vitro* with yeast liposomes. The same effector cells do not lyse target cells, which were primed *in vitro* with egg PC liposomes or free OVA (Fig. 2B). In other words, these experiments prove that target cells primed with OVA encapsulated in egg PC liposomes can not be recognized by effector cells, i.e. OVA specific CTLs isolated from mice immunized with OVA encapsulated in yeast liposomes.

One can argue that macrophages can not be ideal APCs for determining the lytic activity of the CTLs, especially for conventional egg PC liposomes. In order to rule this possibility out, we repeated the same study using dendritic as well as syngenic P815 cells as APCs for performing the CTL assay. We found that the OVA-YL primed P815 cell, in manner similar to macrophages, can be specifically lysed by the OVA specific effector cells. These results also demonstrate the MHC-resticted lysis of the target cells, as MHC mismatched allogenic targets (EL-4 cells) were not identified by same effector cells. Furthermore, the CTL response was antigen specific, as the target cells which were primed with lysozyme (an unrelated antigen) were not recognized by the OVA specific CTLs (Table 1).

To confirm the phenotype of the effector cells, these were pretreated with anti $CD8^+$ antibodies, before using them for ^{51}Cr release assay. The result shows that such pretreatment abolish CTL response, which confirms the $CD8^+$ phenotype of the generated T lymphocytes (Fig. 2A). Typical of the effector cell mediated target lysis, the ^{51}Cr release assay depends on the concentration of antigen, used for the *in vitro* antigen presentation (Fig. 1B).

Moreover, the results of the present study, in conformity with the earlier reports, show that endocytosis of the antigen and generation of $CD4^+$ T-cell response is the only predominant

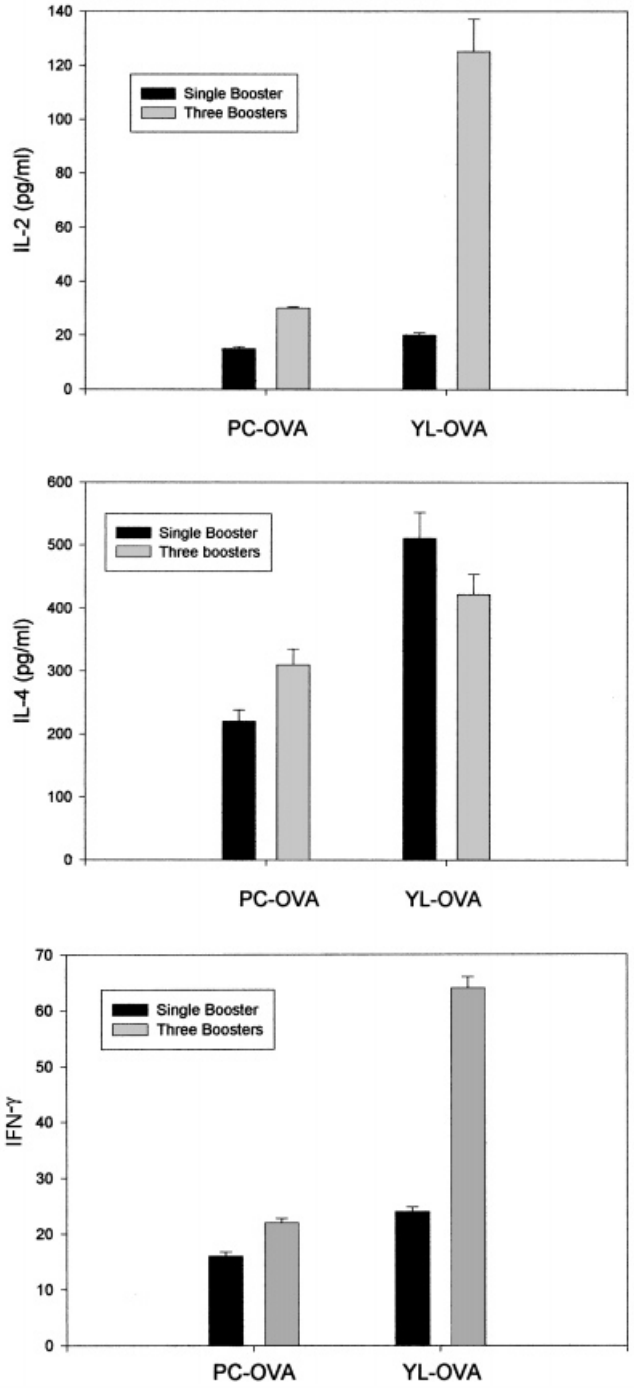


Fig. 4. OVA loaded liposomes prepared from fusogenic lipids of yeast enhances IL-4 level. The cultures were set as mentioned in the legend to Fig. 3. The levels of different lymphokines were monitored in the following manner. (A) The interleukin (IL)-2 content in culture SN was measured by its ability to induce the proliferation of HT-2 cells. The cells were incubated in 96 well plate (1×10^4/well) in the presence of culture SNs (treated with anti IL-4 antibody [11B11, 1 μg/ml] to block IL-4), for 16 h at 37 °C, pulsed with 0.5 μCi[^{3}H]-thymidine and harvested 8 h later. The thymidine incorporation was measured by liquid scintillation spectrometry. The IL-2 content was computed from standard plot of rIL-2, and expressed as pg/ml. (B) The IL-4 content was determined using same method as for that of IL-2, except that here, IL-2 was neutralized by anti-IL-2 MoAb (cocktail of 1 : 12 dilution of culture SN of CRL 1698, HB 8794 and TIB 222). The proliferation of HT-2 was assayed in similar manner and data were computed with the standard plot of rIL-4. All data were calculated from the mean cpm of triplicate observations and expressed as pg/ml ± S.D., after back calculation. (C) The secretion of IFN-γ was measured by its ability to inhibit the proliferation of WEHI-279 cells. The data are computed in manner similar to that of IL-2, and represented as mean of triplicate observations ± S.D. Data are representative of three different experiments performed with similar results.

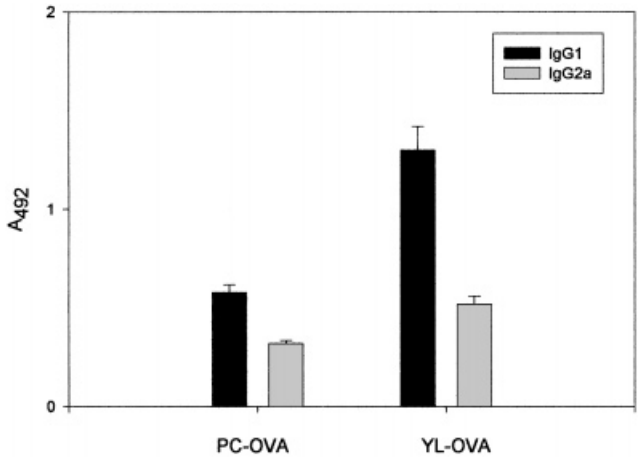


Fig. 5. Yeast lipids liposomes increase the secretion of IgG1 isotype. The BALB/*c* animals were immunized with different preparations of OVA as described in Materials and Methods. Sera (1 : 500 dilution) obtained from the control and experimental animals were analyzed for the presence of OVA-specific IgG isotype by the ELISA method as described in Materials and Methods. The levels of the two major isotypes of IgGs were expressed as absorbance (A_{492}) of the coloured complex developed in the immunosorbent assay. The values expressed are the mean of the absorbance of the sera of five different animals ± S.D. The control animals immunized with phosphate-buffered saline (PBS), free OVA or sham liposomes (no antigen), could not induce any detectable level of IgG isotype.

mode, when egg PC liposomes interacts with the APC [2, 16, 17]. In contrast, the yeast lipid liposomes appear to interact with the target cells by both endocytosis as well as membrane fusion mode. Thus, this helps in the activation of not only $CD4^+$ T-helper cells but also $CD8^+$ cytotoxic T cells. It is worth to mention here that the $CD4^+$ T cell proliferative responses generated using the yeast lipids liposomes were better than those observed with the egg PC liposomes.

Both the $CD4^+$ T cell and $CD8^+$ T-cell responses seem to be required for an effective control of the intracellular parasitic infections. Current evidence suggests that in addition to T-helper cells that activate macrophages, in fact, it is primarily the T-cytotoxic cells, which play crucial role in the protection. Even though, optimum levels of $CD4^+$ T-cell response were generated, yet the disease develops in the absence of CTL cells [5, 6]. Thus, encapsulation of the protective antigens in the yeast lipids liposomes may elicit strong CTL response that may be necessary for protection against intracellular infections.

Furthermore, the use of yeast liposomes leads to the generation of Th2 cells and CTLs. Th2 cells are known to provide help to B cells leading to the production of antibodies, while Th1 cells are crucial for the activation of the CTLs [9]. The Th1/Th2 shift is crucial to the effective immunity and it is likely that many interlocking factors will contribute to that decision [7]. The yeast liposome mediated strong CTL generation, in spite of the observed polarization of the $CD4^+$ T-cell response in favour of the Th2 cells that can be attributed to the physical targeting of the entrapped antigen to the cytosol of the APCs. The yeast liposomes like other particulate substances are avidly endocytosed by APCs and activate $CD4^+$ T cell. However, it can not be excluded that in addition to the endocytosis, a substantial portion of the entrapped antigen is delivered to the cytosol of the target cells, and form the basis of the observed antigen specific CTL response. The peculiar behaviour of the yeast lipid liposome based delivery system, might function in a way that could be a more desirable feature in activation of both cell mediated as well as humoral immune responses. Such a strategy could be appropriate for elimination of virus mediated infections. It is a well known fact that both CTLs and the neutralizing antibodies are essential for imparting protective immunity against the viruses as well as other pathogens [5, 6, 21, 22].

It may be inferred from our study that the lipids derived from *S. cerevisiae* possess a strong fusogenic character. The yeast lipid liposomes can effectively deliver antigen into the class I and class II processing compartments of APCs and consequently generating a strong antigen-specific $CD8^+$ (cytotoxic) and $CD4^+$ (helper) T cells. Such considerations, which could selectively enhance the CTL and the T-helper cells are pertinent in designing liposomes-based vaccines, and will hence subserve in the protection against infectious diseases.

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