View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1669 (2005) 170–181

BIOCHIMICA ET BIOPHYSICA ACTA BBA

http://www.elsevier.com/locate/bba

Phospholipid diversity: Correlation with membrane–membrane fusion events

F. Deeba[a], H. Nasti Tahseen[a], K. Sharma Sharad[a], N. Ahmad[b], S. Akhtar[a], M. Saleemuddin[a], O. Mohammad[a,*]

[a]*Inter-disciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, 202002 India*
[b]*Faculty of Pharmacy, Jamia Hamdard, New Delhi-62, India*

Received 3 July 2004; received in revised form 7 February 2005; accepted 7 February 2005
Available online 11 March 2005

Abstract

The transport of various metabolically important substances along the endocytic and secretory pathways involves budding as well as fusion of vesicles with various intracellular compartments and plasma membrane. The membrane–membrane fusion events between various sub-compartments of the cell are believed to be mainly mediated by so-called "fusion proteins". This study shows that beside the proteins, lipid components of membrane may play an equally important role in fusion and budding processes. Inside out (ISO) as well as right side out (RSO) erythrocyte vesicles were evaluated for their fusogenic potential using conventional membrane fusion assay methods. Both fluorescence dequenching as well as content mixing assays revealed fusogenic potential of the erythrocyte vesicles. Among two types of vesicles, ISO were found to be more fusogenic as compared to the RSO vesicles. Interestingly, ISO retained nearly half of their fusogenic properties after removal of the proteins, suggesting the remarkable role of lipids in the fusion process. In another set of experiments, fusogenic properties of the liposomes (subtilosome), prepared from phospholipids isolated from *Bacillus subtilis* (a lower microbe) were compared with those of erythrocyte vesicles. We have also demonstrated that various types of vesicles upon interaction with macrophages deliver encapsulated materials to the cytosol of the cells. Membrane–membrane fusion was also followed by the study, in which a protein synthesis inhibitor ricin A (that does not cross plasma membrane), when encapsulated in the erythrocyte vesicles or subtilosomes was demonstrated to gain access to the cytosol.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Subtilosomes; Inside out vesicles; Fusion; Membranes

1. Introduction

Besides limiting the boundary of the cell from rest of the universe, plasma membrane plays several other important biological functions [1]. Several lines of evidences suggest that the composition of membrane is crucial for various physiological activities that rely on membrane–membrane fusion viz., fertilization, phagocytosis, exocytosis, and cell division, etc. [1,2]. In fact, the membranous organelles present in the cytoplasm are part of a dynamic, integrated network in which materials are shuttled back and forth from one part of the cell to another. Most of these shuttling pathways involve membrane–membrane fusion. These include secretory or

Abbreviations: ISO; Inside out vesicles; RSO; Right side out vesicles; LUV; Large unilamellar vesicles; EL; Erythrocyte lipids; OVA; Ovalbumin; PC; Phosphatidylcholine; PS; Phosphatidylserine; PE; Phosphatidylethanolamine; R18; Octadecylrhodamine B-chloride; NBD–PE; L-(Phosphatidylethanolamine–*N*-(4-nitrobenzo-2-oxa-1,3-diazole); Rh–PE; *N*-(Lissamine rhodamine B sulfonyl)phosphatidylethanolamine; ANTS; L-Aminonaphthalene-3,6,8-trisulfonic acid; DPX; *N*,*N*'-*p*-Xylylenebis (pyridinium bromide); DMEM; Dulbecco's modified Eagle medium; HBSS; Hanks balanced salt solution; FCS; Fetal calf serum; CTL; Cytotoxic T lymphocyte

* Corresponding author. Tel.: +91 571 2720388; fax: +91 571 2721776.
E-mail address: owais_lakhnawi@yahoomail.com (O. Mohammad).

0005-2736/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2005.02.009

exocytic pathways in which materials are synthesized in endoplasmic reticulum or Golgi complex, and transported to various destinations like plasma membrane, lysosomes or vacuoles, etc. The endocytic pathways operate in opposite direction where materials move from exterior of the cell to subcellular compartments such as endosomes or lysosomes [3,4].

Many investigations regarding the mechanism and regulation of vesicular transport have been undertaken in cell free systems to emulate inter-compartmental transport [5]. These studies suggest that biological molecules such as proteins and nucleotides take active part in the distinct interaction steps that are crucial for various transport events [6–8]. As per the SNARE hypothesis molecules such as heterotrimeric G-proteins, SNAP, NSF, and SNARE etc. play significant role in intracellular trafficking [9]. In order to decipher molecular mechanisms involved in the membrane–membrane fusion events, much of the focus has been made on the role of proteins; in contrast lipid counterparts received little attention. However, the marked variation in lipid composition in between the primitive and more evolved organisms on one hand, and highly efficient energy driven intricate mechanisms operative in the maintenance of membrane lipid asymmetry in higher eukaryotes on the other hand, clearly suggests the important role of lipids in various membrane related processes (cf. membrane–membrane fusion). For example, the asymmetric distribution of lipids between two leaflets of the plasma membrane of the erythrocytes is quite apparent, where outer leaflet contains the bulk of sphingomyelin and PC (both bilayer forming neutral lipids) while the inner leaflet has preponderance of PS, PI and PE [10–12].

Earlier we demonstrated the fusion potential of *Escherichia coli* (escheriosomes) and yeast lipid liposomes with the target cells [12,13]. In the present study, we have tried to evaluate the fusion efficiency of erythrocytic vesicles (inside out and right side out) and liposomes derived from the lipids of *Bacillus subtilis* in perspective of their lipid composition. The fusogenic potential of various forms of vesicles was established using conventional fusion assay methods. To further establish that the membrane–membrane fusion constitutes a major mode of interaction between various vesicles and target cells, we studied vesicles mediated cytosolic delivery of ricin A to the interacting macrophages. In absence of chain B, ricin A is incapable of entering the cytosolic compartment of the cell to inhibit protein synthesis.

In an analogy with fusion events taking place in professional antigen presenting cell, the various vesicles were used as an efficient tool to deliver model antigen ovalbumin (OVA) into cytosol as well. Interestingly, vesicle mediated fusion with plasma membrane of antigen presenting cells was found to by pass endocytic mode of macromolecules delivery that ensues elicitation of strong cell mediated immune responses.

2. Materials and methods

2.1. Materials

Human blood was obtained from the Blood Bank of J.N. Medical College, Aligarh, India in heparinized tubes. Egg phosphatidylcholine was prepared using the standard procedure [14]. Cholesterol was purchased from Centron Research Laboratory, Bombay, India, and crystallized three times with methanol prior to its use. Nutrient Broth was obtained from Hi Media Laboratories, Bombay, while Dextrose was the product of S.D. Fine Chemicals, Boisar, India. DMEM, HBSS and FCS were obtained from Life Technologies (Grand Island, NY). Deglycosylated ricin A, ovalbumin, human serum albumin (HSA), Percoll, Sephadex G-75, G-50 and Sepharose 6B were procured from Sigma Chemical (St. Louis, MO). ANTS and DPX were bought from Molecular Probes. The fluorescent probe R18, Rh–PE and NBD–PE (Avanti polar lipids) were kind gift from Dr. Anu Puri (NIH, Frederick, MD). [^{35}S] L-methionine was bought from Bhabha Atomic Research Center, Trombay, India. J 774 A.1, a macrophage cell line was procured from American Type Culture Collection (Rockville, MD) and was grown in DMEM (pH 7.2) containing L-glutamine (4 mM), sodium pyruvate (110 mg/l), penicillin (100 U/ml), streptomycin sulfate (100 μg/ml) and sodium bicarbonate (3.7 g/l) in 75 ml plastic bottles (Costar, MA, USA) at 37 °C under 7.5% CO_2.

2.2. Methods

2.2.1. Preparation of erythrocyte membrane vesicles

Erythrocytes were isolated from human blood by removing plasma and buffy coat after centrifugation at 800 ×*g* (15 min, 4 °C). Subsequently, for preparation of erythrocyte vesicles, two different protocols were used. For preparation of right side out vesicles, the cells were lysed by treating with lysis buffer (sodium phosphate buffer 5 mM, pH 8.0) and then resealed after washing three times in isotonic PBS (10 mM phosphate, 150 mM saline, pH 7.4) [10]. The erythrocyte membrane vesicles thus prepared were pelleted at 12,000 ×*g* (15 min., 4 °C). After washing several times, the preparation was resuspended in PBS for further use. Inside out vesicles were prepared as described earlier [15]. The isolated erythrocytes were hypotonically lysed with 25 volumes of chilled 10 mM Tris–HCl (pH 7.0) and then centrifuged at 22,000 ×*g* for 10 min at 4 °C. The supernatant was aspirated carefully and the tube was tipped and rotated on its axis so that loosely packed ghosts slide away from the hard button rich in contaminating proteases. The membranes were washed and rinsed with the same buffer till white ghosts were obtained. The sealed vesicles were prepared by suspending the ghosts in 40 volumes of 0.5 M Tris–HCl (pH 8.0) for 2 h, washed twice and centrifuged at 22,000 ×*g* for 30 min at 4 °C. The membranes were vesiculated by passing through 27 gauge

needle 15–20 times. The vesicles thus formed contained right side as well as inside out vesicles. The suspension was diluted in 0.5 M phosphate buffer (pH 7.2) and layered upon an equal volume of Dextran Barrier solution. Centrifugation for 2 h at 40,000 rpm resolved the mixture into a pellet and a band floating on the top of the barrier. The top band was collected, washed and diluted in the same buffer and pelleted at 28,000 $\times g$. The supernatant contained the inside out vesicles. The sidedness of ghosts was verified by membrane markers acetylcholinesterase and glyceraldehydes 3-phosphate dehydrogenase for inside out and right side out vesicles respectively following protocols described elsewhere [15]. The membrane lipids of erythrocytes were isolated following published protocol as modified in our lab [11].

2.2.2. Protease treatment of erythrocyte ghosts

The erythrocyte ghosts were digested with 5 μl of 100 μg/ml pronase stock for 2 h at 37 °C in PBS, and washed with cold PBS three times. The efficiency of the proteolytic treatment was visualized on SDS-PAGE of the treated ghosts using Coomassie blue staining.

2.2.3. B. subtilis lipids

B. subtilis was cultured in nutrient broth (1% peptone, 0.3% beef extract, 0.3% yeast extract and 1% sodium chloride; pH 7.4). The cells were harvested from mid-log phase (18–20 h). Phospholipids were isolated by the method of Bligh Dyer, as modified by Kumar and Gupta [11]. The liposomes were prepared as described elsewhere [12].

2.2.4. Lipid vesicles

LUVs were prepared essentially by the freeze–thaw method [16]. The outer diameter of these vesicles was found to be 160 ± 50 nm as measured by electron microscopy. Reconstituted liposomes (EL-DPG) were prepared by using mixture of erythrocyte lipid and DPG in ratio of 9:1 respectively, following published protocol as modified in our lab [17].

2.2.5. Dequenching assay

Erythrocytic vesicles were labeled with R18 (octadecylrhodamine B-chloride) as described elsewhere [5]. Briefly, an injection of 10 μl of an ethanolic solution of R18 (15 mM) was dispensed into vesicles suspended in 1 ml of phosphate buffer saline (1 mg protein/ml, 1.3 mM phospholipid) with vigorous mixing. The mixture was incubated in ice for 1 h in the dark. Labeled vesicles were separated from unlabeled population by size exclusion chromatography using a G 75 column (20×1 ml). The R18 containing vesicles were diluted with unprobed vesicles in a molar ratio of 10:1. Subsequent to vesicle–vesicle fusion, the fluorescence dequenching was measured by fluorometry using thermostated SLM Aminco Bowman Series 2 in time dependant manner. Fluorescence was measured with 1 s time resolution at 560 and 590 nm excitation and emission wavelengths respectively. Similarly, self quenching concentration (5 mol%) of Rh–PE was incorporated in the subtilosomes and other types of liposomes following published protocol as modified in our lab [12]. Briefly, NBD–PE or Rh–PE were mixed with various types of lipid in 5 mol% ratio and subsequently dissolved in chloroform methanol. Subsequently, the mixture was reduced to thin dry film with the help of slow jet of nitrogen gas. The film was hydrated with saline buffer followed by sonication in bath type sonicator to get labeled liposome. The fluorescence dequenching was measured by mixing unprobed liposomes with probed liposomes in a molar ratio of 10:1. The fluorescence associated with various types of the labeled vesicles was monitored up to 20 min time period using excitation and emission wavelengths of 536 and 585 nm for Rh–PE and 450 and 520 nm for NBD–PE, respectively. The excitation wavelength was chosen to be 20 nm below the absorption maxima of Rh so as to allow a better resolution between the scattered light peak and the Rh emission peak, and also to minimize the direct excitation of rhodamine. The percent dequenching was calculated as follows:

$$\% \text{ dequenching} = 100 \times (F - F_0)/(F_t - F_0)$$

where F, F_0, F_t are the fluorescence intensities at time 't', 0 min, and after disrupting the vesicles with Triton X-100 (1% final concentration), respectively. The Rh–PE was replaced with NBD–PE to perform various fusion assays used in the present study, both probes yielded same fusion efficiency pattern, however for sake of simplicity only Rh–PE results were reported.

2.2.6. Aqueous contents mixing assay

For the aqueous contents mixing experiments both ISO and treated ISO vesicles incubated in 1.5 ml of 10 mM phosphate buffer (pH 7.5), containing ANTS (25 mM) and NaCl (40 mM) or DPX (90 mM) respectively were allowed to undergo 20 cycles of freezing and thawing followed by brief sonication in bath type sonicator at 4 °C. The entrapped ANTS (or DPX) from free ANTS (or DPX) was separated by size exclusion chromatography using Sephadex G 75 column. The loading of probe in RSO vesicles was performed in identical conditions except that unloaded probe was separated by centrifugation at 10,000 $\times g$ at 4 °C. In another experiment, LUVs were prepared by hydrating *B. subtilis* lipids or egg PC (15 μmol lipid P) with 1.5 ml of 10 mM Tris–HCl (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 Sephadex G-50 column.

Quenching of the ANTS fluorescence by DPX was monitored to follow the mixing of the aqueous contents of various types of the vesicles (RSO, ISO, treated ISO, subtilosomes, reconstituted erythrocyte lipid and egg PC liposomes) undergoing fusion [18]. Given specie of ANTS-containing vesicles was mixed with an excess (10-fold) of the DPX-containing vesicles (of same lipid composition) 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 min 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%. The excitation and emission wavelengths used were 380 and 540 nm, respectively.

2.2.7. Macrophage–vesicle interaction

The interaction of the macrophages with the subtilosome and ISO vesicles was monitored by observing the transfer of fluorescently labeled membrane markers from the vesicles to the macrophages. Membranes of vesicles/liposomes were fluorescently labeled by incorporating R18/Rh–PE or NBD–PE (5 mol%). The J774 A.1 (1×10^6 cells) were cultured overnight on a sterile cover slip in DMEM containing 10% FCS. The cells were washed with DMEM and incubated at 4 °C for 2 h. After washing once with FCS-free DMEM, the cells were pulsed with labeled liposomes (600 μmol lipid) for 60 min in FCS-free DMEM at 37 °C. The fixed macrophages were observed under Leitz fluorescence microscope at ×100, using I 3 filter.

2.2.8. Inhibition of protein synthesis in macrophages by ricin A

The J774 A.1 cells (1×10^6 cells/well) were cultured overnight in a 24-well plate at 37 °C. Next day, the cells were incubated separately with five different forms of toxin viz., free ricin A, ricin A encapsulated in: *B. subtilis* lipid liposomes, RSO, ISO, treated ISO and egg PC vesicles for 1 h. The cells were washed, and then pulsed with [^{35}S] L-methionine (1 μCi / well) in a total volume of 200 μl/well for next 2 h at 37 °C. The cells were washed twice with DMEM, and treated with 7 M guanidine (50 μl/well). The final volume was made up to 200 μl/well with PBS. The suspension was vortexed, and small aliquot of the cell lysate (20 μl), was withdrawn in Eppendorff tubes. The lysate was subsequently treated with TCA (25%, 100 μl) and BSA (1%, 50 μl), followed by centrifugation at 4 °C. The precipitate was washed with 10% TCA and counted for β emission in a Rack Beta Scintillation Counter, after suspending in 10 ml of scintillation fluid.

2.2.9. Immunization

The immunological studies were performed in inbred female Balb/C mice ($n = 5$ each group). The animals were immunised with various forms of OVA viz. i) free OVA, ii) OVA encapsulated in egg Ptd\Cho liposomes iii) OVA encapsulated in subtilosomes iv) OVA encapsulated in RSO v) OVA encapsulated in ISO vi) OVA encapsulated in treated ISO vii) sham ISO vesicles and viii) sham subtilosomes.

2.2.10. $CD8^+$ T lymphocyte response

2.2.10.1. Cell preparation. Different groups of Balb/C mice were injected separately, as described above with a total three doses (day 0, 7, and 14) of OVA encapsulated in various types of vesicles or its free form [100 μg OVA] for 3 weeks. On day 21, the animals (five animals each group) were sacrificed and spleens were taken out aseptically. The effector cells were prepared as described elsewhere [19]. The cells obtained from different animals in a given group were pooled, purified and used in cytotoxicity assay. The enriched population stained with anti-$CD8^+$Ab, was >98% pure, as evaluated by FACScan.

2.2.10.2. Target cells. Balb/c mice were injected with thioglycollate broth. On day 4, the macrophages were isolated from the peritoneal exudate by adherence on petri plates. The harvested cells (2×10^7cells/ml) were washed 3 times with HBSS and incubated at 37 °C for 3–4 h with either free OVA, OVA entrapped in various types of vesicles. The cells were again washed 3× 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 in CO_2 incubator. The cells were finally washed with RPMI solution and were used as target cells.

2.2.10.3. Cytotoxicity assay. The^{51}Cr-labelled macrophages (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, 5 °C) and the amount of ^{51}Cr released was determined by measuring the radioactivity in the supernatant. 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 labeled macrophages for 6 h. Amount of auto-release was subtracted from the total release to determine the extent of macrophage lysis. In most of the experiments, the auto-release was less than 25%. The percent specific release was calculated as the (mean sample cpm—mean spontaneous cpm/mean maximum cpm−mean spontaneous cpm) × 100%. The experiments were performed three times with same results.

2.2.11. Immunoblot assay

In order to confirm the cytosolic delivery of the intact protein molecules, the J 774 A.1 cells were allowed to interact with vesicle encapsulated form of HSA for 20 min. After stipulated time period, the un-interacted vesicles were removed by thorough washings. Subsequently, the cells were lysed and the extract was separated on a 10% SDS-PAGE as described earlier [20]. The protein was electrophoretically blotted onto nitrocellulose paper, and blocked

with PBS (1 M Phosphate buffer saline, pH 7.4) containing 3% skimmed milk. The blot was incubated with 1:200 dilution of mouse sera (sera containing Ab against HSA) in 0.05% Tween-PBS at 37 °C for 90 min, washed three times with PBS-T (1 M PBS, 0.05% Tween-20), and incubated with a 1:1000 dilution of HRP-conjugated goat anti-mouse immunoglobulin (Sigma Immunochemicals, USA) for 90 min at 37 °C. The strips were washed three times, incubated with substrate [0.3% DAB (Sigma) in PBS with 0.4% H_2O_2] till development of color, and finally washed extensively with triple distilled water.

2.2.12. Statistical analysis

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

3. Results

3.1. Effect of enzyme treatment

The efficiency of the proteolytic treatment among various vesicles (RSO, ISO and Subtilosomes) was observed on a 10% SDS-PAGE gel. As shown in Fig. 1, following protease treatment ISO as well as RSO vesicles showed distinct protein profiles (compare lanes 1 and 2, 3 and 4) while subtilosomes being made exclusively of lipids were free from any protein content (lane 5).

3.2. Vesicle–vesicle fusion

3.2.1. Dequenching assay

Vesicle–vesicle fusion was studied by including a self-quenching concentration (5 mol%) of R18 or Rh–PE in various types of vesicles. Fusion of the labeled vesicles with overwhelming numbers of unprobed vesicles resulted in a significant dequenching of the fluorescence in all types of vesicles except in case of egg-phosphatidyl-choline. Both ISO as well as RSO vesicles were also found to have fusion tendency that was more prominent for the former (~35%) than later (~12%) vesicles. The pronase treated ISO vesicles (with extensive loss of membrane proteins) also showed a substantial dequenching (~18–20%), suggesting the retention of fusion capacity. Among various types of vesicles used in the present study, the subtilosomes were found to possess maximum fusion efficiency (~60%), while reconstituted liposomes made of erythrocyte lipid and DPG (EL-DPG) were found to have around ~45–50% fusion efficiency (Fig. 2).

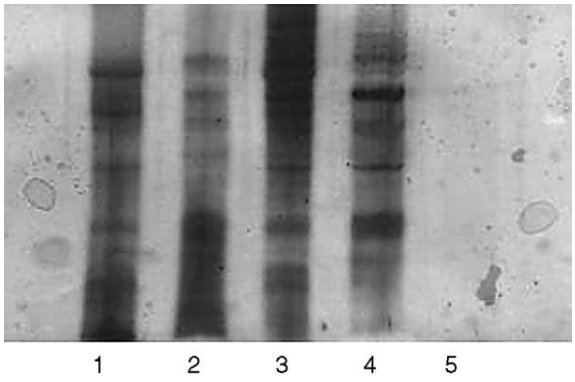

Fig. 1. SDS-PAGE profile of proteins within membranes of various vesicles. The effect of protease treatment among various vesicles (RSO, ISO and Subtilosomes) was observed on a 10% SDS-PAGE gel. Lane 1 and 3 represents RSO and ISO vesicles; lane 2 and 4 shows protease treated RSO and ISO, respectively while lane 5 represents subtilosomes.

3.2.2. Content mixing assay

Fusion potential of erythrocyte ghosts (ISO and RSO) and subtilosome were validated by monitoring the mixing of aqueous contents during fusion by measuring quenching of the ANTS fluorescence by DPX. Incubation of ANTS containing vesicles with 10-fold excess of DPX containing vesicles resulted in approximately ~58% quenching in the case of subtilosomes, while there was only 30–35% quenching in case of ISO vesicles within 5–10 min. The reconstituted vesicle made of erythrocyte lipid and DPG have around ~46% fusion efficiency that matches well with dequenching assay data. However, no such quenching of the ANTS fluorescence was observed when ANTS containing egg PC-LUVs were incubated with 10-fold excess of the DPX-containing egg PC-LUVs in identical conditions (Fig. 3).

3.3. Fusion of vesicles with target cells

3.3.1. Fusogenic vesicles mediated transfer of membrane fluorescent markers to the macrophages

Fusion of the erythrocyte vesicles and bacterial lipid vesicles with the macrophage cell line J774 A.1 was followed by monitoring the transfer R18 and NBD–PE markers respectively from these vesicles to the target cells. Vesicles loaded with these markers were allowed to interact with J774 A.1 cells. The transfer of the probes to the macrophages was analyzed by the fluorescence light microscopy. The results demonstrated that interaction with egg PC liposomes resulted in punctate type of fluorescence, while NBD/R18 fluorescence was mainly associated with membrane of the target cells when transferred from fusogenic vesicles (subtilosomes or inside out vesicles) to the macrophages (Fig. 4). Further, our findings also suggest that the incubation of the cells with the fusogenic subtilosomes or ISO vesicles in the presence of 100 μM chloroquine or at 0 °C did not appreciably affect the NBD/R18 transfer (data not shown).

3.4. Inhibition of the macrophage protein synthesis by ricin A

For further analysis of the membrane–membrane fusion as a major mode of interaction of fusogenic vesicles with the

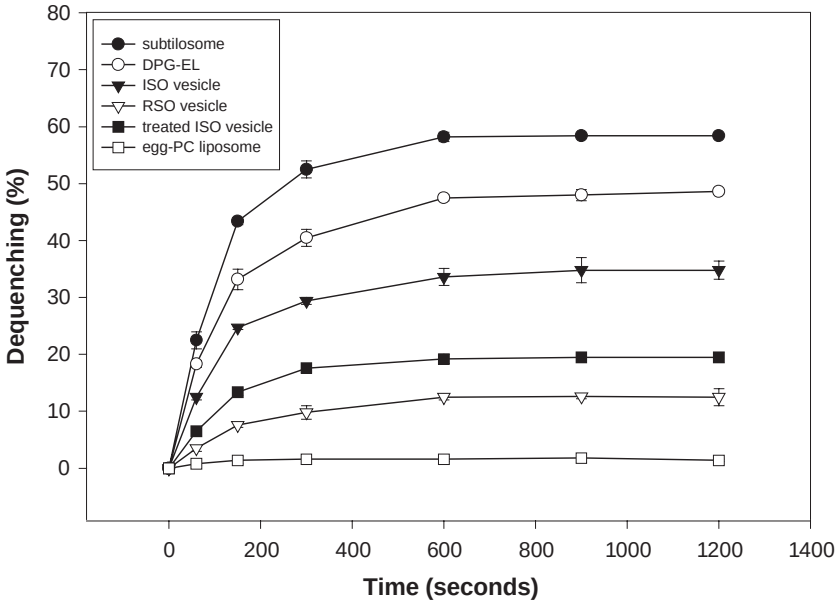

Fig. 2. Interaction of R18 labelled erythrocytic vesicles and Rh–PE labeled *B. subtilis* liposomes with their unlabeled counterparts. Subtilosomes or egg PC LUVs (750 nmol lipid ml^{-1}) containing 5 mol% Rh–PE and erythrocytic vesicles containing R18 (corresponding to 750 nmol lipid ml^{-1}) were allowed to interact with unlabeled form of the same types of the vesicles in a ratio of 1:10. The fluorescence associated with various types of the labeled vesicles was monitored up to 20 min time period using excitation and emission wavelengths of 536 and 585 nm, respectively. Percent dequenching of Rhodamine was calculated as follows: Percent Dequenching $= 100 \times (F - F_0)/(F_t - F_0)$, where F, F_0, F_t are the fluorescence intensities at time T, 0 min and after adding Triton X-100 (1% final concentration), respectively. Reconstituted erythrocyte lipid liposome (EL) were found to behave like treated ISO vesicles (~18%). Values are means of three independent experiments ± S.D.

J774 A.1 cells, the effect of ricin A on the macrophage protein synthesis was studied by incubating macrophages with various types of the vesicles loaded with toxin protein. Ricin A, a plant toxin consists of two polypeptide chains viz. chain A and chain B. A chain without B is not capable of entering the cytosolic compartment of the cells [21]. Any inhibitory effect of ricin A loaded vesicles on macrophage synthesis confirms the membrane–membrane fusion as possible mode of the vesicle–macrophage interaction. It was shown that effect of ricin A is more remarkable in case

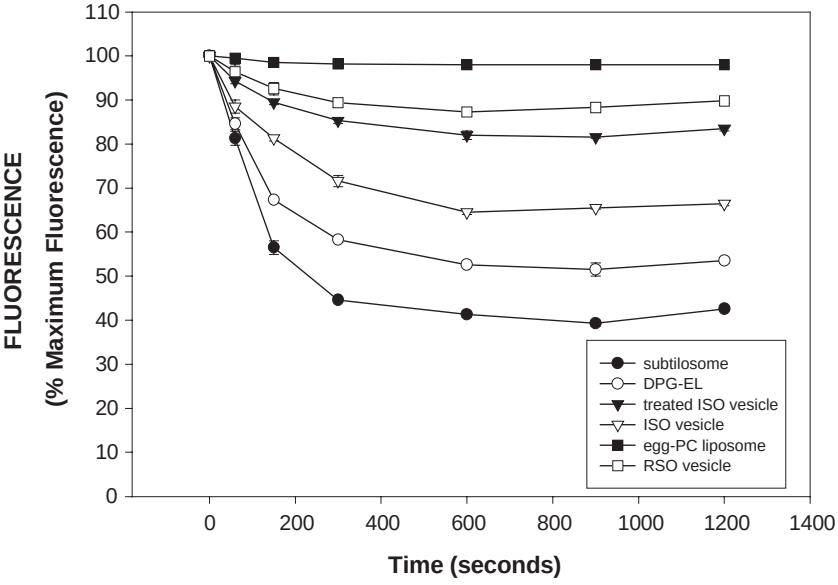

Fig. 3. Time dependent effect on the efficiency of fusion as evidenced by content mixing assay. Various forms of vesicles were loaded with ANTS and subsequently allowed to interact with excess (10 times) of the same species of the vesicles that was loaded with DPX (quencher of flouorophore). Time course for vesicle–vesicle fusion was monitored by measuring ANTS fluorescence as described in Materials and methods section. The excitation and emission wavelengths used were 380 and 540 nm, respectively. The fusion efficiency of reconstituted erythrocyte liposomes was found to be of the order of treated ISO vesicles. Values are the means of three independent experiments ± S.D.

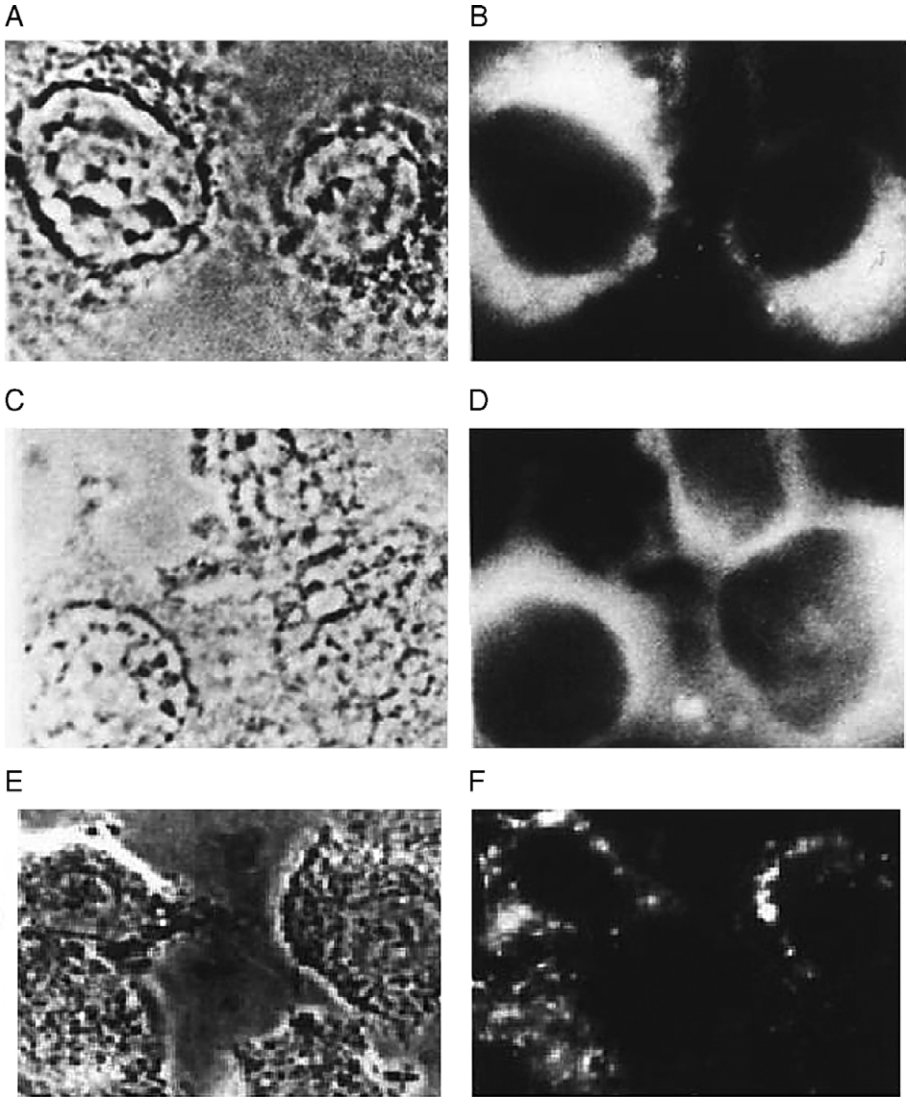

Fig. 4. Fluorescence light micrographs of the macrophages J774 A.1 after their interaction with lipid vesicles labeled with fluorescent probes. Phase contrast and florescence light micrographs, (A, B) respectively, of the macrophages interacted for 60 min at 37 °C with Rhodamine labeled subtilosomes. Phase contrast and fluorescence light micrographs, (C, D) respectively, of the macrophages interacted with R 18 labeled inside out erythrocytes vesicles. Non fusogenic egg PC liposomes interacted with target cells through endocytic mode only, resulted in punctate type fluorescence (E, F). Almost identical light micrographs were observed when the macrophages were interacted with Rhodamine labeled subtilosomes or in side out vesicles in the presence of 100 μM chloroquine or at low temperature (4 °C).

of subtilosomes followed by ISO, treated ISO and RSO erythrocyte vesicles respectively as shown in Fig. 5. Free ricin and ricin encapsulated in egg-PC liposomes showed no significant inhibitory effect.

3.5. $CD8^+$ T lymphocyte response

Keeping into consideration the fact that if the vesicles used in the present investigation possess a strong fusogenic character, in principle they should deliver the entrapped protein into the cytosol of the APCs for presentation via MHC class I pathway. We evaluated the potential of the vesicle entrapped OVA, to undergo MHC-I processing and presentation to generate a $CD8^+$T cell response. Initially, animals were immunized with varying doses of antigen entrapped in various types of vesicles (10–100 μg OVA/dose/animal, total three doses each at week interval). It was found that a dose of 100 μg/animal induced 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 these vesicles, but not other forms of OVA viz. OVA-IFA or OVA entrapped in egg PC/Chol liposomes, successfully generated cytotoxic T cells. A considerably high degree (~40%) of target cell lysis occurred when the OVA was encapsulated in the subtilosomes, followed by inside out vesicles (~30%), the treated inside out (~24%) and the right side out vesicles (~18%) as compared to less than 1% specific lysis in

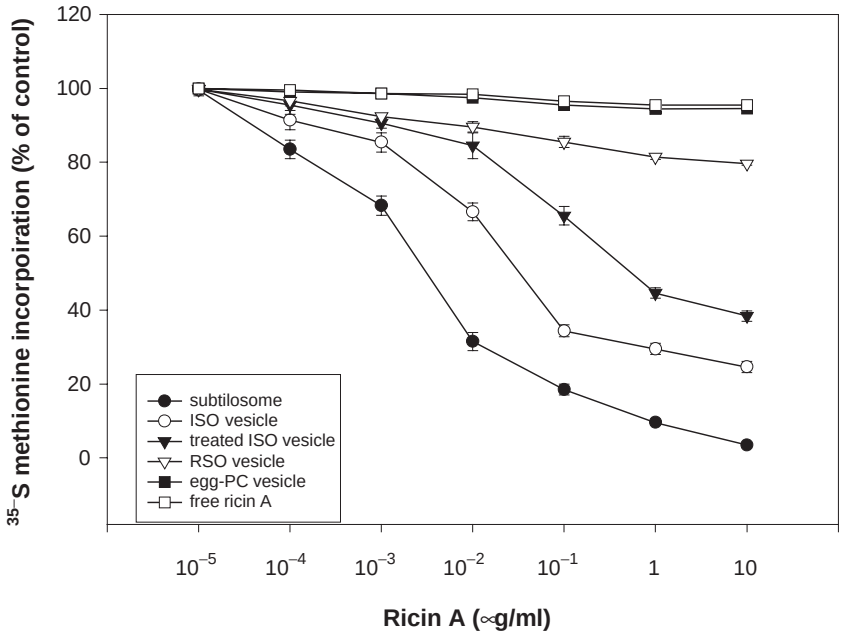

Fig. 5. Fusogenic vesicles mediated cytosolic delivery of ricin A. Inhibition of cellular protein synthesis in macrophages that were allowed to interact with ricin A loaded vesicles. Values are mean of three independent experiments ± S.D.

OVA-IFA or OVA incorporated into the egg PC/Chol liposomes ($P<0.001$) (Fig. 6). The result of the present study clearly demonstrated that target cells primed with OVA encapsulated in fusogenic vesicles were able to process them via MHC I pathway ensuing recognition by effector cells, while other forms of OVA (free or egg PC encapsulated) failed to do so.

3.6. Immunoblot assay

To further confirm the delivery of vesicle entrapped macromolecules into cytosol of the target cells by the vesicles used, Western blotting was performed using cytosolic fraction as described in Materials and methods. As shown in Fig. 7, it was observed that various types of

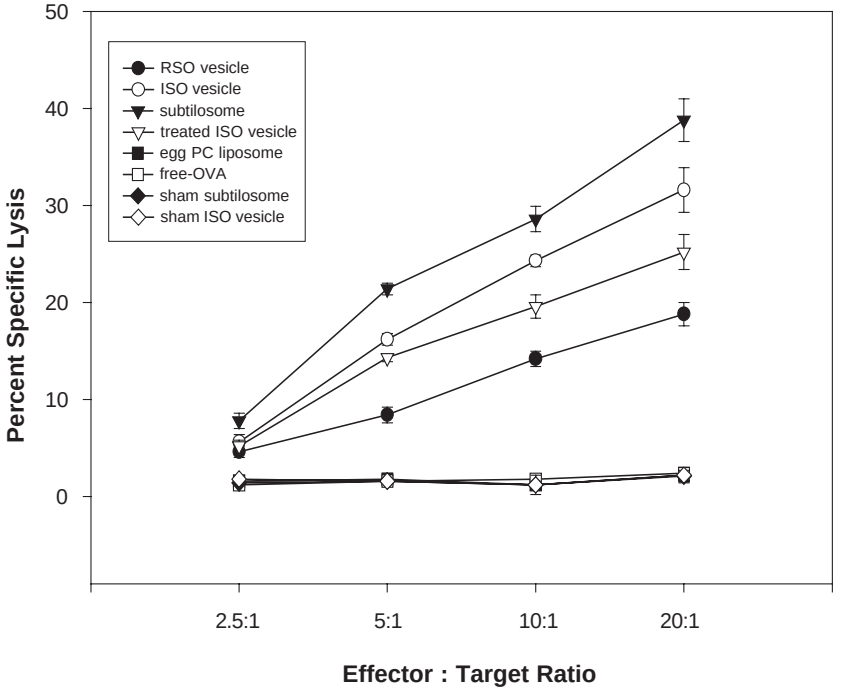

Fig. 6. Induction of antigen specific CTL activity by immunisation with various types of vesicles containing ovalbumin. The results are represented as, percent specific lysis (^{51}Cr release) of the target cells. Each value represents the mean of three determinations ± S.D. Data are representative of three independent experiments performed with similar results. The target cells incubated with the unrelated antigen lysozyme did not recognize OVA specific CTLs.

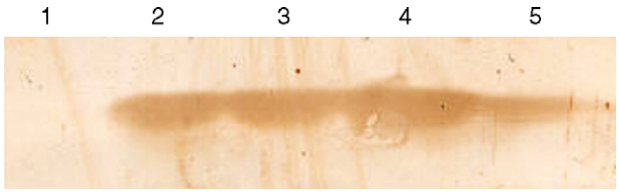

Fig. 7. Immunoblot analysis of cytosolic extract of macrophages after interaction with the vesicles containing HSA. The macrophages were incubated with the vesicles containing model protein (HSA) for 20 min. After removing un-interacting vesicles by extensive washing, the cells were lysed by nitrogen cavitation method. The extracts obtained were assayed on immunoblots (10 μg of protein/lane) separated by SDS-PAGE on 10% gradient gel. (lane 2) subtilosome, (lane 3) ISO, (lane 4) treated ISO and (lane 5) RSO vesicles containing HSA. Macrophage extract that was not incubated with HSA was used as a negative control (lane 1).

fusogenic vesicles were excellent in the intact delivery of HSA into the target cells.

4. Discussion

An interesting correlation between the plasma membrane lipid compositions of the living organisms and their generation time can be made. For example, bacteria such as *E. coli*,*Bacillus megaterium* and *B. subtilis* have preponderance of anionic lipids viz. PG and DPG (in combination of PE) in their plasma membranes and have very short generation time of the order of 20–25 min [22,23]. On the other hand, membranes of relatively more evolved *Saccharomyces cerevisiae*or *Candida albicans* have greater variety of phospholipids with lower percentage of anionic lipids (e.g. PG, PI, PS, DPG) and the organisms have a generation time of approximately 2 h. Since, both the classes of organisms multiply by binary fission, it can be presumed that the presence of anionic lipids facilitates the fusion of the membranes essential for high duplication rates [13]. Unlike the lower organisms, the more evolved eukaryotes have neutral phospholipids as major membrane components and have generation times of the order of days (cf. ~22 h). While eukaryotes also contain some anionic lipids along with PE in their plasma membranes, these are confined to the specific leaflet of the membrane resulting in their asymmetric distribution. The asymmetry is initially established in the endoplasmic reticulum and is maintained throughout the life of cell as the membrane passes from one organelle to the other [24]. Wide diversity also exists in the distribution of lipids in the membranes of various subcellular organelles [25]. The architectural differences in the lipid composition as well as evolutionary trend followed in distribution of membrane lipids clearly signifies nature's strategy to carry out certain biologically important events.

In the present investigation, we compared the fusion efficiencies of various types of membrane vesicles (prepared from lipids of prokaryotic and eukaryotic origin) using epifluorescence as well as other membrane–membrane fusion assay methods. In the content mixing assay, marked decrease in fluorescence results when ANTS containing fusogenic vesicles fuse with the excess of DPX containing vesicles of same lipid composition. Among various types of vesicles used in the present study, quenching of fluorescence was highest in subtilosome indicating their strong fusogenic potential (Fig. 3). The content mixing assays are quite reliable because the quenching of the ANTS fluorescence by DPX has been shown to be highly dependent on DPX concentration and it does not occur upon the leakage of DPX into the medium [26]. The strongly fusogenic nature of PE, PG and DPG has been earlier reported using dequenching studies [22,23]. Among the erythrocyte vesicles, ISO vesicles were more fusogenic than the RSO vesicles. While, treatment of erythrocyte vesicles with pronase affected the fusogenic properties of erythrocyte vesicles, nevertheless, around 40–50% fusion efficiency was found to be still retained by ISO vesicles. It may be attributed to the presence of fusogenic lipids in their membrane (Fig. 2). It seems the nature of lipids present in the outer surface of ISO may be crucial since pronase treatment completely abolished the fusogenic activity of RSO vesicles, which are containing predominantly choline phospholipids such as phosphatidyl choline and sphingomyelin in their outer membrane (data not shown). In another set of experiments, reconstituted erythrocyte lipid (EL) liposomes showed fusogenic properties comparable with those of pronase treated ISO vesicles (~20%). Since asymmetric distribution of lipids is not retained in liposomes, it seems PS and PE were positioned on their outer leaflet, leading to H_{II} phase formation that in turn facilitates membrane–membrane fusion. The EL turned strongly fusogenic (~48%) upon incorporation of DPG further showing the significance of anionic lipids in membrane–membrane fusion events. Interestingly, this is the natural composition of membrane lipids of most of the prokaryotes as well [25].

The fusion of subtilosome as well as erythrocyte vesicles was also followed by monitoring the transfer of fluorescent membrane markers to the target cells (J774 A.1). For this purpose, the *B. subtilis* lipid (or egg PC) LUVs containing 5 mol% of NBD–PE (or Rh–PE) in the bilayers, and erythrocyte vesicles labeled with R18, were allowed to interact with J774 A.1 cells and observed under fluorescence light microscopy. While the egg-PC liposomes were found to be taken up by endocytosis ensuing punctate type of fluorescence pattern, in contrast subtilosome or inside out vesicle mediated transfer of NBD–PE/R18 fluorescence was mainly associated with membrane of the target cells (Fig. 4). Further, our findings also suggest that the incubation of the cells with liposome/vesicles in the presence of 100 μM chloroquine or at 0 °C did not appreciably affect the transfer of probe (Fig. 4). These results strongly indicate that the subtilosomes and inside out erythrocyte vesicles could undergo spontaneous membrane fusion with the macrophages resulting in the delivery of their entrapped material into the cytoplasmic compartments of target cells. This was further supported by the finding that ricin A encapsulated in

fusogenic vesicles strongly inhibited cellular protein synthesis upon its interaction with J774 A.1 cells, while its free or egg PC lipid vesicles encapsulated form failed to do so (Fig. 5). Similarly, immunoblot assay also supported fusion-mediated delivery of encapsulated probe HSA where subtilosomes, RSO, ISO and treated ISO vesicles were able to deliver this probe to the target cells (Fig. 7). The apparent higher intensity (although statistically insignificant) of the blot in ISO treated cells as compared to the cells incubated with other types of vesicles could be explained on the premise that beside membrane–membrane fusion and typical endocytosis mediated delivery, the presence of phosphatidyl serine on the surface of ISO vesicle might be leading to overwhelming intake of the probe by the macrophages [24]. Apparently, the limited incubation time does not allow complete digestion of HSA, thereby leading to its accumulation in the treated cell that was subsequently visualized in immunoblotting. In contrast, egg PC liposomes are presumably taken up exclusively by simple endocytic mode leading to the complete degradation of entrapped antigen.

Immunological studies were also used to substantiate the fusogenic properties of various vesicles. It is now well established that professional antigen presenting cells viz. macrophages as well as dendritic cells are tailored to eliminate pathogens or foreign substances from the systemic circulation [27]. In fact, such cells have an active endocytic mode for engulfing alien substances leading to their effective elimination from the body of the host. The proteinaceous substances are chopped off to small peptides that are presented along with the MHC-II molecules on the surface of antigen presenting cells. The whole assembly is recognised by helper class of T lymphocytes that eventually activate either humoral or cell mediated immunity depending upon various other intrinsic as well as external factors. The other class of T lymphocytes that are meant for killing of pathogen harboring cells (rather direct killing of pathogens) recognize MHC- I peptide complex present on the surface of APCs [28]. In general these peptides are derived from endogenous intracellular proteins, which are digested into small fragments with the help of proteasome machinery. Nevertheless exogenous antigens can also enter class I degradation pathway provided they breach plasma membrane barrier to gain access to the proteosomal assembly present in the cytosol of the nucleated cells [28–31]. In order to demonstrate the fusogenic potential of various vesicles using immunological techniques, we entrapped OVA inside the vesicles and used them for immunization of animals. Our study demonstrates that vesicle entrapped antigens can successfully elicit class I mediated immunological responses (CTL generation). This is possible in situations where the antigen is delivered to the cytosol of the antigen presenting cells. This indirectly suggests that a soluble antigen like OVA that normally does not activate CTLs (because of endocytic uptake) can be made accessible to proteasome via its delivery through fusogenic vesicles. Keeping into consideration the ability of various vesicles used in the study to deliver antigenic macromolecules to immune cells leading to the preferential activation of cell mediated immunity, they seem to offer a novel strategy for protection against intracellular pathogens as well [32].

The observations discussed in the preceding lines substantiate our hypothesis that vesicles made of lipid obtained from lower organisms or those with unique composition simulating natural distribution pattern of the lipids in endocytic vesicles have strong fusogenic properties. Moreover, pronase treatment of RSO and ISO vesicles led to digestion of proteins that were present on the external surface of specific population of vesicles. The set of proteins present on the surface of ISO vesicles facilitate membrane fusion as revealed in Fig. 1. It seems these proteins along with anionic lipids play important role in membrane–membrane fusion of vesicles during exocytosis. Further, the results of the present investigation indicate that both ISO as well as RSO vesicles were able to successfully fuse with the target cells. However, presence of fusogenic lipids in the outer leaflet, as in case of inside out vesicles, renders them strong fusogenic properties. Earlier Vidal and Hoekstra [5] have studied fusogenic potential of vesicles obtained from reticulocytes but failed to observe significant fusion of these vesicles with PS/PC liposomes. In contrast, the present study clearly demonstrates high fusion efficiency of PS rich inside out vesicles. The apparently conflicting results could be explained on the premise that Vidal's group used PS/PC liposomes for the fusion assay, while we used two different populations (labeled and unlabeled) of inside out vesicles with natural composition. Presumably, the presence of strongly fusogenic PE in combination with PS might be imparting higher fusion efficiency to ISO vesicles observed in this study. In fact, in concordance with the reports from other groups, the fusion data involving vesicle formed of mixture of DPG and reconstituted erythrocyte lipid highlight the role of anionic lipids in membrane–membrane fusion events [18,24]. This is further supported from our earlier studies where presence of PE along with anionic DPG/PG was found to impart strong fusogenic efficiency to escheriosomes [12].

Many of the attributes regarding fusion of naturally occurring vesicles have been focused on proteins part mainly. However, pattern of lipid diversity among various species and their characteristic distribution among the two leaflets of various membranes implies that nature has adopted it as a strategy to carry out various functions using common infrastructure. The features such as maintenance of membrane asymmetry, the commonness of luminal components of the exocytic vesicles (as well as other organelles such as endoplasmic reticulum) to that of outer leaflet of plasma membrane carries significance of biological importance to accomplish certain coveted goal. Similarly, occurrence of specific phospholipids that form efficient combination to impart fusogenicity in lower organisms may

also be of biological relevance. Recently the role of dynamin in fission events taking place in Golgi apparatus has been demonstrated [33]. However, even the fusogenic proteins still need assistance from phospholipids that may readily undergo fusion under physiological condition. It therefore seems not unlikely that involvement of proteins in fusion is a late evolutionary event and presumably this was an adaptation for efficient execution of many biological functions.

In fact, membrane–membrane fusion that is crucial for many biological events in living system, has been conceptually viewed as local point event that involves a small area of interacting membranes of the dimensions less than 20 nm in diameter. This certainly implies that fusion requires a local restructuring of the interacting lipid bilayers, that actually include relatively few lipid molecules (hundred to few thousands). Beside lipids, the protein counterpart can also invoke bio-membrane destabilization, and thereby inducing membrane–membrane fusion. In order to overcome repulsive hydration forces arising from water, tightly bound to the lipid head groups, the attractive hydrophobic forces between the hydrocarbon interiors of the membrane has to rely on certain factors that include extreme membrane curvature as well as local changes in lipid composition. It seems among various phospholipids, PE binds with surrounding water molecules less tightly because of its shape and charge and hence requires less energy to bypass repulsive hydration forces that ensues fusion of the approaching bilayers [34,35]. Beside, PE (H_{II} phase lipid) also facilitates formation of membrane defects in the bilayer thereby allowing closer proximity of the bilayer of two approaching vesicles [34,35].

In the present study, we have compared fusogenic properties of subtilosome to that of escheriosome, and found that both have comparable fusogenic potential. Interestingly, combination of erythrocyte lipids and DPG (DPG-EL) was found to induce strong fusogenic properties at par with that of escheriosomes. Our data provide direct evidence that proteins are not an absolute prerequisite for membrane fusion; as artificial lipid vesicles (subtilosomes and DPG-erythrocyte lipid vesicles) can be induced to fuse in the absence of added proteins and that too with far more higher efficiency. While fusion behavior of ISO vesicles can be explained on the basis of PS/PE induced charge neutralization, cross linking of membranes, local dehydration and finally the induction of local defects in lipid packing. On the other hand H_{II} phase forming PE (*E. coli* or *B. subtilis* etc.) may rely on factors such as diacylglycerol (DG), unsaturated fatty acid and lyso phospholipids to facilitate membrane fusion events. It appears that higher fusion efficiency of escheriosome or subtilosome is mainly because of low head group hydration of PE that facilitate membrane adhesion by reducing the hydration repulsion, while its negative curvature preference facilitate the formation of highly curved concave semifusion intermediates and stimulate membrane fusion. However, to make the system more efficient, the PE mediated fusion is further assisted by DPG or PG as well.

Finally, the data of the present study support the notion that lipid bilayers can fuse in the complete absence of proteins. In fact net negative monolayer curvature facilitated by H_{II} phase lipids remains the key factor in formation of lipidic fusion intermediates. While lower prokaryotes may owe their membrane fusion events to the lipid components, the eukaryotes seek help of special protein molecules that acts as fusogen or produce some product that are fusogenic in nature and help in reducing the energy barrier to membrane–membrane fusion.

Acknowledgements

We extend our gratitude to Dr. C. M. Gupta (Director, CDRI, Lko) and Anu didi for their valuable suggestions, and indebtedness to Dr. Krishnakumar for critically reviewing the manuscript. Thanks are also due to Dr. J. N. Agrewala, (IMTECH, Chd.) and Dr. G. C. Mishra (NCCS, Pune) for allowing us to avail the Tissue Culture facilities and supply of immunochemicals. We are also thankful to CST (Govt. of India) for the financial support.

References

[1] D. Voet, J.D. Voet, C.W. Pratt, in: M. Cliff (Ed.), In the Fundamentals of Biochemistry, John Wiley and Sons, Inc, New York White, 1999, pp. 239–278.
[2] J.M. White, Membrane fusion, Science 258 (1992) 917–924.
[3] D.W. Andrews, A.E. Johnson, The translocon: more than a hole in the membrane, Trends Biochem. Sci. 21 (1996) 365–369.
[4] S.D. Emr, V. Malhotra, Membranes and sorting, Curr. Opin. Cell Biol. 9 (1997) 475–476.
[5] M. Vidal, D. Hoekstra, In vitro fusion of reticulocyte endocytic vesicles with liposome, J. Biol. Chem. 270 (1995) 17823–17829.
[6] E.S. Sztul, P. Melancon, K.E. Howell, Targeting and fusion in vesicular transport, Trends Cell Biol. 2 (1992) 381–386.
[7] P.A. Takizawa, V. Malhotra, Coatmers and SNAREs in promoting membrane traffic, Cell 75 (1993) 593–596.
[8] U. Pley, P. Parham, Clathrin: its role in receptor-mediated vesicular transport and specialized functions in neurons, Crit. Rev. Biochem. Mol. Biol. 28 (1993) 431–464.
[9] T. Sollner, S.W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, J.E. Rothman, SNAP receptors implicated in vesicle targeting and fusion, Nature 362 (1993) 318–324.
[10] P. Williamson, L. Algarin, J. Bateman, H.R. Choe, R.A. Schlegel, Phospholipid asymmetry in human erythrocyte ghosts, J. Cell. Physiol. 123 (1985) 209–214.
[11] A. Kumar, C.M. Gupta, Red cell membrane abnormalities in chronic myeloid leukaemia, Nature 303 (1983) 632–633.
[12] N. Ahmad, A.K. Masood, M. Owais, Fusogenic potential of prokaryotic membrane lipids: implication in vaccine development, Eur. J. Biochem. 268 (2001) 5667–5675.
[13] M. Owais, C.M. Gupta, Liposome mediated cytosolic delivery of macromolecules and its possible use in vaccine development, Eur. J. Biochem. 267 (2000) 3946–3956.
[14] W.S. Singleton, M.S. Gray, M.L. Brown, A method for adsorbent fractionation of cottonseed oil for experimental intravenous fat emulsions, J. Am. Oil Chem. Soc. 42 (1965) 53–56.

[15] T.L. Steck, R.S. Weinstein, J.H. Strous, D.F. Wallach, Inside out red cell membrane vesicles: preparation and purification, Science 168 (1970) 255–257.
[16] J.A. Hayward, D.M. Levine, L. Neufeld, S.R. Simon, D.S. Johnston, D. Chapman, Polymerised liposomes as stable oxygen carriers, FEBS Lett. 187 (1985) 261–266.
[17] N. Ahmad, M.A. Khan, M. Owais, Liposome mediated antigen delivery leads to induction of $CD8^{+}$T lymphocyte and antibody responses against V3 loop region of HIV gp 120, Cell. Immunol. 210 (2001) 49–55.
[18] H. Ellens, J. Bentz, F.C. Szoka, $H^{+}$and Ca^{++} induced fusion and destabilization of liposomes, Biochemistry 24 (1985) 3099–3106.
[19] B.B. Mishell, S.M. Shiigi, Selected Methods in Cellular Immunology, W.H. Freeman and Company, New York, 1980, pp. 23–24.
[20] U.K. Laemmli, Cleavage of structural proteins during assembly of the head of bacteriophage T_4, Nature 227 (1970) 680–685.
[21] Y. Endo, K. Mitsui, M. Motizuki, K. Tsurugi, The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and characteristics of the modification in 28S ribosomal RNA caused by the toxins, J. Biol. Chem. 262 (1987) 5908–5912.
[22] J.B.M. Rattray, in: C. Ratledge, S.G Wilkinson (Eds.), Microb. Lipids, vol. 1, Academic Press, London, 1988, pp. 555–597.
[23] M.K. Jain, Components of biological membranes, Introduction to Biological Membranes, 2nd ed., John Wiley, New York, 1988, pp. 1–15.
[24] F.A.Z. Robert, A.J. Schroit, Pathophysiologic implications of membrane phospholipid asymmetry in blood cells, Blood 89 (1997) 1121–1132.
[25] Gerald Karp, Cell and Molecular Biology: Concepts and Experiments, 2nd ed., John Wiley and sons, Inc, New York White, 1999, pp. 122–172.
[26] N. Duzgunes, T.M. Allen, J. Fedor, D. Papahadjopoulos, Lipid mixing during membrane aggregation and fusion: why fusion assays disagree, Biochemistry 26 (1987) 8435–8442.
[27] E.R. Unanue, P.M. Allen, The basis for the immunoregulatory role of macrophages and other accessory cells, Science 236 (1987) 551–557.
[28] T.J. Braciale, L.A. Morrison, M.T. Sweetser, J. Sambrook, M.J. Gething, V.L. Braciale, Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes, Immunol. Rev. 98 (1987) 95–114.
[29] A. Hayashi, T. Nakanishi, J. Kunisawa, A novel vaccine delivery system using immunopotentiating fusogenic liposomes, Biochem. Biophys. Res. Commun. 261 (1999) 824–828.
[30] M. Fukasawa, Y. Shimizu, K. Shikata, Liposome oligomannose-coated with neoglycolipid, a new candidate for a safe adjuvant for induction of $CD8^{+}$ cytotoxic T lymphocytes, FEBS lett. 441 (1998) 353–356.
[31] C.V. Harding, D.S. Collins, O. Kanangawa, O. Kanagawa, E.R. Unanue, Liposome-encapsulated antigens engender lysosomal processing for class II MHC presentation and cytosolic processing for class I presentation, J. Immunol. 147 (1991) 2860–2863.
[32] F.M. Syed, M.A. Khan, T.H. Nasti, N. Ahmad, M. Owais, Antigen entrapped in the escheriosomes leads to the generation of $CD4^{+}$ helper and $CD8^{+}$ cytotoxic T cell response, Vaccine 21 (2003) 2383–2393.
[33] K.N.J. Burger, R.A. Demel, S.L. Schmid, B. de Kruijff, Dynamin is membrane-active: lipid insertion is induced by phosphoinositides and phosphatidic acid, Biochemistry 39 (2000) 12485–12493.
[34] P.R. Cullis, B. de Kruijff, The polymorphic phase behaviour of phosphatidylethanolamines of natural and synthetic origin: a 31P NMR study, Biochim. Biophys. Acta 513 (1978) 31–42.
[35] D.P. Siegel, Inverted micellar intermediates and the transitions between lamellar, cubic and inverted hexagonal lipid phases: I. Mechanism of the L alpha-H_{II} phase transition, Biophys. J. 49 (1986) 1155–1170.