*Immunology* 1983 **49** 37

# Comparison between multilamellar and unilamellar liposomes in enhancing antibody formation*

P. N. SHEK, B. Y. K. YUNG† & N. Z. STANACEV† *Immunology Group, Biosciences Division, Defence and Civil Institute of Environmental Medicine, Department of National Defence, Downsview, Ontario and † Department of Clinical Biochemistry, Banting Institute, Faculty of Medicine, University of Toronto, Ontario, Canada*

*Accepted for publication 14 October 1982*

**Summary.** The effectiveness of two liposomal preparations, multilamellar vesicles (MLV) and unilamellar vesicles (ULV), in enhancing specific antibody formation was compared in this study. The MLV and ULV, prepared in the presence of bovine serum albumin (BSA), were composed of dimyristoyl-lecithin, cholesterol and dicetyl phosphate in a molar ratio of 7:2:1. Excess free BSA was separated from liposome-associated BSA by Blue Sepharose CL-6B column chromatography. The presence of appropriate lamellar structures for each liposome preparation was demonstrated by electron microscopy. The simultaneous injection of control mice with free BSA and 'empty' MLV or ULV failed to elicit a BSA-specific plaque-forming cell (PFC) response. In contrast, animals injected with liposome-associated BSA (MLV-BSA or ULV-BSA) generated a vigorous PFC response; the magnitude of the response induced by BSA entrapped in unilamellar vesicles was significantly higher than that in multilamellar vesicles. These results suggest that the lamellar arrangement of liposomal vesicles may play a role in affecting the magnitude of the potentiated antibody response.

## INTRODUCTION

The immunopotentiating effect of liposomes has been the subject of an increasing number of studies in recent years. The biodegradable, non-toxic, and immunostimulating properties of liposomes render them highly valuable as potential vaccine carriers for human use (Allison & Gregoriadis, 1976). The association of a protein antigen with liposomes internally (entrapment) or externally (surface-association) has been shown to be effective in promoting antibody formation (Shek & Sabiston, 1982b). Nevertheless, the entrapment of an antigen within liposomal vesicles offers the distinct advantage of protecting the immunized host from undesirable hypersensitivity reactions (Gregoriadis & Allison, 1974). Other than the site of antigen-association which is important in

* This is Publication Number 83-P-01 from the Defence and Civil Institute of Environmental Medicine.

Abbreviations: B cell, bone-marrow-derived cell; BSA, bovine serum albumin; DCP, dicetyl phosphate; DML, dimyristoyl-lecithin; MLV, multilamellar vesicles; PBS, phosphate-buffered saline; PFC, plaque-forming cells; T cell, thymus-derived cell; ULV, unilamellar vesicles; SC, spleen cells.

Correspondence: Dr P. N. Shek, Immunology Group, Biosciences Division, Defence and Civil Institute of Environmental Medicine, Department of National Defence, 1133 Sheppard Ave. W., Downsview, Ontario, Canada M3M 3B9.

0019-2805/83/0500-0037$02.00

liposome-mediated immunoenhancement, additional factors, like lipid compositions of the liposomal membrane (Heath, Edwards & Ryman, 1976), and surface charge of the vesicles (Allison & Gregoriadis, 1974; Tyrrell, Heath, Colley & Ryman, 1976), may also influence the immunopotentiating capacity. Although a number of studies have been reported regarding the *in vivo* clearance and tissue distribution of unilamellar and multilamellar liposomes (Juliano & Stamp, 1975; Ryman & Tyrrell, 1980), most, if not all, immunological studies on liposomes were conducted using multilamellar vesicles (MLV). This preference in the practically exclusive application of MLV may well be due to their ease of preparation, and apparently adequate effectiveness in potentiating the immune response being examined. Thus, few or no studies have been undertaken to exploit the possible usefulness of unilamellar vesicles (ULV) as an immunopotentiating carrier. The fact that ULV are composed of only a single lipid bilayer renders them more appropriate to be used in future studies in the analysis of the relationship between molecular lipid–protein interactions and the enhancement of immune responsiveness. In this report, experiments were conducted to compare the efficacy of ULV and MLV in promoting the plaque-forming cell (PFC) response against their associated bovine serum albumin (BSA) antigen. A preliminary account of this study has been reported elsewhere (Yung, Stanacev & Shek, 1982).

## MATERIALS AND METHODS

*Animals*

Female A/J mice, 6 weeks old, were purchased from the Jackson Laboratories, Bar Harbour, Maine. They were kept in plastic cages and given standard mouse diet and water *ad libitum*.

*Chemicals and biologicals*

Bovine serum albumin (essentially free of fatty acids) and dicetyl phosphate were purchased from Sigma Chemical Co., St. Louis, MO; cholesterol was from P-L Biochemicals, Milwaukee, WI, and 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine was from Fluka, A.G., Buchs, Switzerland. Octyl-β-D-glucoside was purchased from Calbiochem Co., La Jolla, CA, and Blue Sepharose CL-6B was from Pharmacia, Uppsala, Sweden. [Methyl-$^{14}$C]bovine serum albumin, [4-$^{14}$C]cholesterol and octyl-β-D[UL-$^{14}$C] glucoside were purchased from New England Nuclear Co., Boston, MA. All solvents used were of the highest purity commercially available from Fisher Scientific Company, Toronto, Ontario.

*Preparation of liposomes*

*MLV.* The method described by Bangham, Standish & Watkins (1965) was adapted as follows: lecithin (42·7 μmol), cholesterol (12·2 μmol) and dicetyl phosphate (6·1 μmol) giving a molar ratio of 7:2:1 were dissolved in 25 ml of chloroform. The solution was evaporated to dryness at 37° under reduced pressure by rotary evaporation to form a thin film around the wall of a round-bottom flask. To this film 1 μmol of BSA (previously purified by Blue Sepharose CL-6B column chromatography) in 5 ml of 0·075 M sodium phosphate buffer (pH 7·4) was added and the lecithin was hydrated at 35° (i.e. 12° above the transition temperature). The lipid film was dispersed by vortexing. The suspension was kept at the hydration temperature for 12 hr, filtered through 0·65 micron membrane (Millipore) and applied to a Blue Sepharose CL-6B column for chromatography as described below.

*ULV.* The procedure used for the preparation of unilamellar liposomes was essentially the same as that described by Mimms, Zamphigi, Nozaki, Tanford & Reynolds (1981). A lipid film having the same composition as described above for MLV was obtained and hydrated at 35° with 5 ml of purified BSA (1 μmol) in 0·075 M sodium phosphate buffer (pH 7·4) containing 0·13 M octyl-β-D-glucoside. The molar ratio of glucoside to lecithin was 15:1. The solution was kept at the same temperature against 0·15 M phosphate-buffered saline (PBS), pH 7·4 for 36 hr. In separate experiments using [$^{14}$C]-octyl-β-D-glucoside only 0·04% of octyl-β-D-glucoside was detectable after such a dialysis. A turbid liposome suspension was formed after dialysis and was filtered through 0·65 micron membrane (Millipore) before its application to a Blue Sepharose CL-6B column for chromatography.

For the preparation of liposomes not containing albumin, the lipid film was hydrated using buffer solution only. In order to facilitate the identification of liposomal peaks in column chromatography and the estimation of the protein entrapment by liposomes, 2–4 μg of [$^{14}$C]-cholesterol (specific activity: 0·14 μCi/μg) or 5 μg of [$^{14}$C]-albumin (specific activity: 0·02 μCi/μg) were used in respective experiments.

*Column chromatography of liposomes and BSA.* A

Blue Sepharose CL-6B column (2·6 × 18·5 cm) was pre-equilibrated with either 0·01 M Tris-HCl buffer (pH 7·4) containing 0·17 M NaCl or 0·15 m PBS (pH 7·4). The binding capacity of this column for BSA was 150 mg at 4°. Liposome-associated BSA was separated from free BSA by passing the mixture through the column at a flow rate of approximately 45 ml/hr. Fractions of 4 ml each were collected. BSA retained by the column was eluted with 1·5 M NaCl in Tris or phosphate buffer (pH 7·4). The elution was monitored at 280 nm and aliquots were taken from each fraction for the determination of phosphorus, protein, and radioactivity, if applicable. All operations were performed at room temperature (20–23°) when liposomes were chromatographed. Chromatography of BSA was done at 4°.

*Electron microscopy*

Liposomes were negatively stained with a solution of 2% sodium phosphotungstate (pH 7·4) on carbon-coated copper grids (400 mesh) and examined in a Philips EM-300 electron microscope operated at 60 kV. The size distribution of liposomal particles was determined at a magnification of × 20,000–30,000 and the magnifications used to examine the lamellar structures are specified in the legend of the appropriate figure.

*Radioactive measurements*

Radioactivity was measured with a Packard Tri-Carb Model 3003 scintillation spectrometer. Buhler's scintillation fluid (1962) was used in which the efficiency for $^{14}C$ counting was about 45%.

*Other measurements*

All spectrophotometric measurements were obtained using a Zeiss PMQ-II spectrophotometer equipped with a digital photometer indicator PI-2. The content of liposomal phosphorus was measured by the method of Bartlett (1959) and protein determination was performed in the presence of 1% sodium dodecylsulphate as described by Markwell, Haas, Bieher & Tolbert (1978).

*Preparation of cell suspension and haemolytic plaque assay*

These procedures were conducted as previously described (Shek & Shabiston, 1982a).

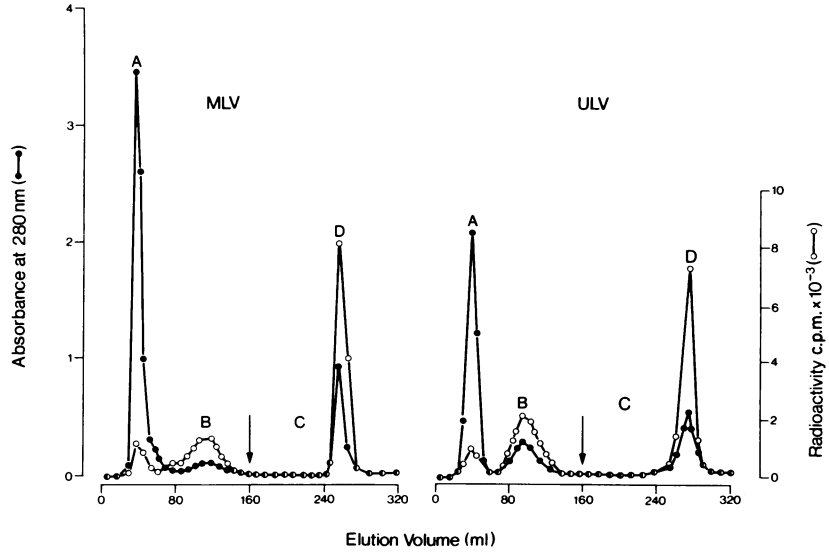


**Figure 1.** Blue Sepharose CL-6B chromatography of liposomal preparations. Liposomes, MLV or ULV, were prepared in the presence of BSA and 0·1 μCi of [$^{14}C$]-methylated BSA was added as a tracer. Each liposome–albumin mixture was passed through a Blue Sepharose CL-6B column (2·6 × 18·5 cm), previously equilibrated with PBS (0·15 M, pH 7·4), at a flow rate of approximately 45 ml/hr. After the elution of the initial two peaks with the starting buffer, the residual materials retained by the column were eluted with a buffer containing 1·5 M NaCl. The start of the high-salt elution is indicated by the inverted arrow. Fractions of 4 ml each were collected. The elution profile was monitored by the absorbance at 280 nm and by the recovered radioactivity of each fraction.

## RESULTS

### Preparation and characterization of BSA-associated MLV and ULV

The methods used for the preparation of MLV and ULV were found appropriate for the entrapment of proteins. The swelling of each lipid preparation in the presence of a BSA solution resulted in a significant encapsulation of the antigen ($107 \pm 10$ $\mu$g BSA/mg lipid for MLV; $105 \pm 10$ $\mu$g BSA/mg lipid for ULV).

*Column Chromatography*. Blue Sepharose CL-6B was used for the removal of excess BSA not entrapped by the liposomes. A clear-cut separation of liposome-associated BSA (peak A) from free BSA (peak D) was achieved (Fig. 1). In all cases, the recovery of lecithin applied to the column was about 70–75% in fractions yielding peak A, and the protein recovered in peak D was about 75% (Fig. 2). Approximately 6% of the original amount of protein was recovered with the liposomes eluted in peak A.

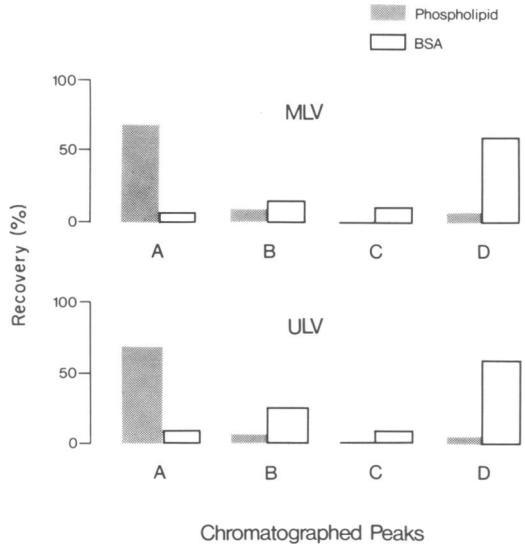


**Figure 2.** Recovery of BSA and phospholipid in fractions obtained from Blue Sepharose CL-6B column chromatography. Quantitative determinations of protein and phospholipid (see 'Materials and Methods') were performed on aliquots taken from fractions of each of the chromatographed peaks (A, B, C, and D) corresponding to those in Fig. 1. Each bar represents the summation of the BSA or phospholipid, expressed as a percentage of the original input to the column, recovered from all the fractions of each peak.

In addition to the two fractions (peaks A and D) discussed above, two minor and rather broad peaks (B and C) were also observed (Fig. 1). The amount of lecithin in these fractions was low (peak B) or negligible (peak C) and the fractions contained mainly protein (Fig. 2). These two protein peaks were not observed upon re-elution of previously chromatographed BSA in control studies. In all likelihood, the appearance of these peaks may be, at least in part, associated with the somewhat labile ester-structure of DML, which can yield lyso-DML reacting with the albumin. This may result in the formation of micellar protein moieties which emerged in peaks B and C. Neither chemical characterization nor immune testings were peformed with these materials.

*Electron Microscopy*. The morphology and the particle size distribution of BSA-associated MLV and ULV were analysed by electron microscopy (Fig. 3). In both liposome preparations, vesicular structures were clearly visible. Multilamellar or unilamellar organization of lipid bilayers was detected in the preparation of liposomes depending on whether glucoside was absent or present as described. The distribution of particle size showed that the average diameter of BSA-associated MLV and ULV was $330 \pm 70$ nm and $240 \pm 50$ nm, respectively. The range of size variations for each liposome preparation is indicated by the particle size distribution histograms (Fig. 3, lower panels).

### Comparison of the PFC response engendered by MLV-BSA and ULV-BSA

A/J mice were given two intraperitoneal injections, 3 weeks apart, of either MLV-BSA or ULV-BSA, each containing 30 $\mu$g of protein. Control animals were injected with a similar dose of chromatographed free BSA together with either empty MLV or ULV. The spleens of the immunized animals were harvested for the BSA-specific PFC assay 3–5 days after antigenic challenge. It can be seen from Fig. 4 that separate but simultaneous injections of free BSA and liposomal vesicles failed to induce a detectable PFC response. In contrast, the BSA antigen entrapped within either MLV or ULV was able to stimulate a vigorous anti-BSA PFC response in the immunized host. The kinetics of the PFC response generated by the two liposomal preparations indicate that ULV is more potent than MLV in promoting antibody formation to a protein antigen.

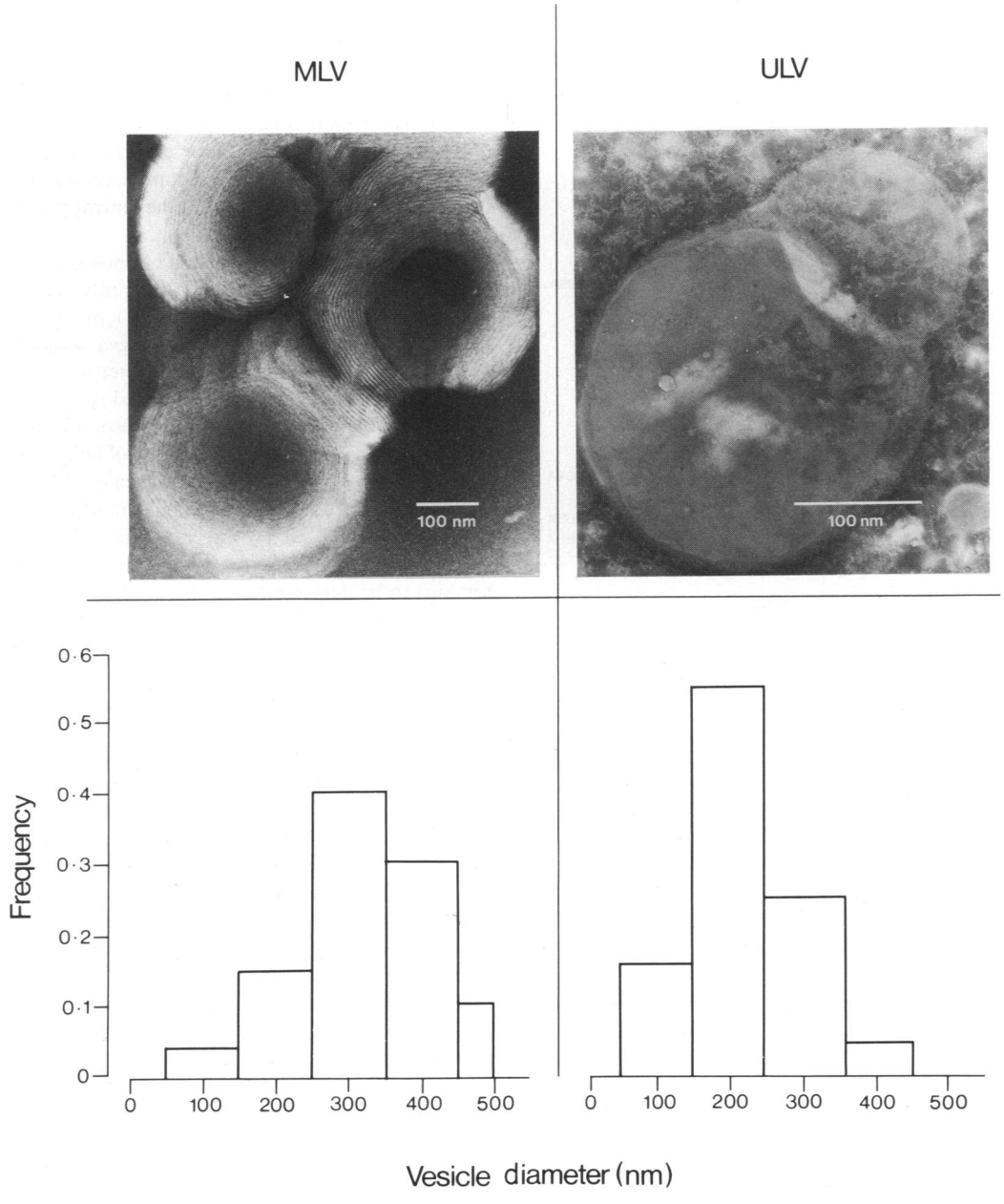


**Figure 3.** Electron micrographs and size distribution histograms of MLV and ULV prepared in the presence of BSA. The liposomal preparations were negatively stained with sodium phosphotungstate and were examined for lamellar structures at a magnification of $\times$ 130,000 and $\times$ 270,000 for MLV and ULV, respectively. Diameters of 150 randomly chosen vesicles were measured for each liposome preparation. Liposomes with diameters greater than 500 nm or smaller than 50 nm were mostly non-vesicular and were excluded from this analysis. The mean vesicle diameter was $330 \pm 70$ nm for MLV and $240 \pm 50$ nm for ULV.

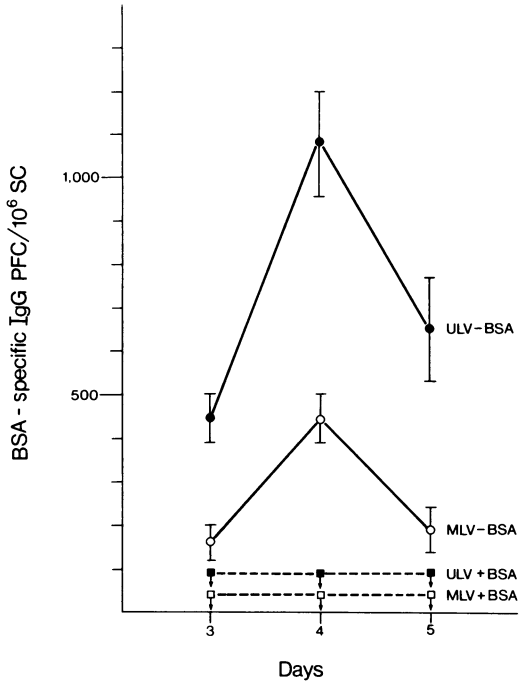


**Figure 4.** Effectiveness of MLV-BSA and ULV-BSA in potentiating the BSA-specific PFC response in A/J mice. Animals were given two intraperitoneal injections, 3 weeks apart, of either MLV-BSA or ULV-BSA each containing 30 $\mu$g of protein and 300 $\mu$g of lipid. Control animals were given separate but simultaneous injections of BSA (30 $\mu$g) and liposomes, MLV or ULV (300 $\mu$g). The splenic BSA-specific IgG PFC response of all immunized animals was evaluated 3–5 days after the second injection of antigen. Each point represents the mean response $\pm$SEM of eight animals.

## DISCUSSION

Among all the potentials of liposomes, it is likely that their application as an effective vaccine carrier may be realized first. Despite repeated reports about the efficacy of liposomal carriers in potentiating the immune response (see review by Alving & Richards, 1983), the exact mechanism of liposome-mediated immunopotentiation remains unknown. Nevertheless, in terms of cellular involvement, it has been established that the promotion of antibody formation by liposome-associated protein antigens required the participation of T cells (Shek & Sabiston, 1982a) and macrophages (Shek & Lukovich, 1982). Although certain physico-chemical characteristics of liposomes can affect their efficiency in immune enhancement (Tyrrell *et al.*, 1976), the possible difference between MLV and ULV of comparable sizes in promoting the antibody response to a protein antigen has not been analysed. The objective of this study was to determine the usefulness of ULV as an immunopotentiating carrier. The results obtained indicate that large ULV ($240 \pm 50$ nm) are much more effective than MLV ($330 \pm 70$ nm) of the same lipid composition in potentiating the PFC response to the entrapped BSA antigen (Fig. 4).

With respect to the preparation of liposomes, DML was used for both MLV and ULV, mainly because of the low transition temperature (23°) of this lipid. Liposomes were also negatively charged with dicetyl phosphate and stabilized with cholesterol, since this combination has been demonstrated to be more efficient in potentiating the antibody response (Allison & Gregoriadis, 1974). The molar ratio of lipids, used in our preparation of liposomes, has been established to be effective in promoting antibody formation (Allison & Gregoriadis, 1976). However, whether this ratio is indeed the optimum for immune enhancement remains to be determined. In this study we have used the classical method of Bangham for the preparation of multilamellar liposomes (Bangham *et al.*, 1965). Among several available methods for the preparation of unilamellar liposomes, we have opted for that described by Mimms *et al.* (1981), mainly because no organic solvents are employed and an almost complete removal of octyl-glucoside, used as a non-ionic detergent in the preparation, can be achieved. Morphological characterization by electron microscopy confirmed the presence of appropriate lamellar structures for the MLV and ULV preparations (Fig. 3).

The affinity chromatography using Blue Sepharose CL-6B was employed in order to separate free BSA from liposome-associated BSA, since it was reported that serum free from albumin could be successfully prepared by this technique (Travis & Pannell, 1973). Our results demonstrate that a clear-cut separation of liposome-BSA from free BSA can indeed be achieved (Fig. 1). A significant amount of BSA was co-eluted with the liposomes in the void volume (peak A, Fig. 1), and no such co-elution was observed when BSA was mixed with preformed liposomes in a control experiment. Thus, the eluted BSA in the void volume must be associated with the liposomes. We are not in a position to exclude the presence of a small amount of liposomes not containing albumin, since the 'empty' liposomes would also be eluted in the same fraction under our experimental conditions. However, since an excess of

BSA was present during the preparation of these liposomes, it is unlikely that the population of 'empty' liposomes could be very significant.

The precise mechanism responsible for the observed difference in immunopotentiating capacity between MLV and ULV remains to be established. Since the two liposomal preparations have identical lipid composition and overlapping particle-size distribution, the major difference between the ULV and MLV appears to be the structural organization of their lipid bilayers (Fig. 3). Although most of the BSA antigens were expected to be entrapped within the aqueous compartments of the liposomes, it is conceivable that some protein molecules might have interacted hydrophobically with the lipid bilayers. On the basis of our previous studies with phospholipid vesicles and cytochrome-c oxidase (Denes & Stanacev, 1978, 1979) and apolipoproteins (Vaughan, Breckenridge & Stanacev, 1980a, b), it is reasonable to expect that some BSA molecules must have been partially embedded in the phospholipid bilayers of the liposomes. The precise distribution of BSA molecules entrapped within the aqueous compartment and incorporated in the phospholipid bilayer is under experimental examination. Such information will enable us to analyse the importance of the nature of association between the protein antigen and the lipid vesicle, at the molecular level, in immunopotentiation.

Our current finding, that large ULV are a preferable immunopotentiating carrier, should pave the way for their more frequent use in the future. Further modifications of the large ULV for antigen encapsulation may be fruitful in our search for an effective immunological carrier with potent adjuvant properties.

## ACKNOWLEDGEMENTS

This work was supported by the Department of National Defence and the Medical Research Council of Canada. The authors wish to thank Ms D. Kerrigan for technical assistance and Ms D. Gardham for the preparation of this manuscript.

## REFERENCES

ALLISON A.C. & GREGORIADIS G. (1974) Liposomes as immunological adjuvants. *Nature (Lond.)*, **252**, 252.

ALLISON A.C. & GREGORIADIS G. (1976) Liposomes as immunological adjuvants. In: *Recent Results in Cancer Research* (eds G. Mathe, I. Florentin and M. C. Simmler), p. 58. Springer-Verlag, Heidelberg.

ALVING C.R. & RICHARDS R.L. (1983) Immunologic aspects of liposomes. In: *The Liposomes* (ed. N. Ostro). M. Dekker Inc., New York. (In press.)

BANGHAM A.D., STANDISH M.M. & WATKINS W. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. mol. Biol.* **13**, 238.

BARTLETT G. (1959) Phosphorus assay in column chromatography. *J. biol. Chem.* **234**, 466.

BUHLER D.R. (1962) A simple scintillation counting technique for assaying $^{14}CO_2$ in a Warburg flask. *Ann. Biochem.* **4**, 413.

DENES A.S. & STANACEV N.Z. (1978) Spin-label study of the relation between enzymatic activity and lipid-protein organization in reconstituted cytochrome c oxidase. *Can. J. Biochem.* **56**, 905.

DENES A.S. & STANACEV N.Z. (1979) Thermally induced changes in reconstituted and membranous cytochrome c oxidase. *Can. J. Biochem.* **57**, 238.

GREGORIADIS G. & ALLISON A.C. (1974) Entrapment of proteins in liposomes prevents allergic reactions in preimmunised mice. *FEBS Lett.* **45**, 71.

HEATH T.D., EDWARDS D.C. & RYMAN B.E. (1976) The adjuvant properties of liposomes *Biochem. Soc. Trans.* **4**, 129.

JULIANO R.L. & STAMP D. (1975) The effect of particle size and charge on the clearance rate of liposomes and liposome encapsulated drug. *Biochem. biophys. Res. Commun.* **63**, 651.

MARKWELL M.A.K., HAAS S.M., BIEHER L.L. & TOLBERT N.E. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Ann. Biochem.* **87**, 206.

MIMMS L.T., ZAMPHIGI G., NOZAKI J., TANFORD C. & REYNOLDS J.A. (1981) Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. *Biochem.* **20**, 833.

RYMAN B.E. & TYRRELL D.A. (1980) Liposomes—Bags of potential. *Essays in Biochem.* **16**, 49.

SHEK P.N. & LUKOVICH S. (1982) The role of macrophages in promoting the antibody response mediated by liposome-associated protein antigens. *Immun. Lett.* **5**, 305.

SHEK P.N. & SABISTON B.H. (1982a) Immune response mediated by liposome-associated protein antigens. I. Potentiation of the plaque-forming cell response. *Immunology*, **45**, 349.

SHEK P.N. & SABISTON B.H. (1982b) Immune response mediated by liposome-associated protein antigens. II. Comparison of the effectiveness of vesicle-entrapped and surface-associated antigen in immunopotentiation. *Immunology*, **47**, 627.

TRAVIS J. & PANNELL R. (1973) Selective normal removal of albumin from plasma by affinity chromatography. *Clinica Chim. Acta*, **49**, 49.

TYRRELL D.A., HEATH T.D., COLLEY C.M. & RYMAN B.E. (1976) New aspects of liposomes. *Biochim. biophys. Acta*, **457**, 259.

VAUGHAN D.J., BRECKENRIDGE W.C. & STANACEV N.Z. (1980a) Reconstitution of lipoproteins. I. Lipid–protein interaction of high density apoproteins, purified apoA-I and apoA-II with dimyristoyl-lecithin and dimyristoyl-

lecithin:cholesterol vesicles studied by isomeric spin-labelled lecithins. *Can. J. Biochem.* **58,** 581.

VAUGHAN D.J., BRECKENRIDGE W.C. & STANACEV N.Z. (1980b) Reconstitution of lipoproteins. II. Lipid-protein interaction between dimyristoyl-lecithin and dimyristoyl-lecithin:cholesterol vesicles and purified apolipoprotein C-I and C-$III_2$ studied by isomeric spin-labelled lecithins. *Can. J. Biochem.* **58,** 592.

YUNG B., STANACEV N.Z. & SHEK P.N. (1982) Immunopotentiation mediated by multilamellar and unilamellar liposomes. *Proc. Can. Fed. Biol. Soc.* **25,** 167. (Abstract No. 657).