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Development of dried liposome as effective immuno-adjuvant for hepatitis B surface antigen

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Abstract

Dried liposomes containing recombinant hepatitis B surface antigen (HBsAg) were developed to enhance the immunogenicity of HBsAg and to retain the stability of liposomal HBsAg during storage. Vesicles containing HBsAg were prepared by extruding the dehydration-rehydration vesicles with entrapped HBsAg through 400 nm polycarbonate filter. The sized liposomes were lyophilized in the presence of trehalose (4 g/g lipid) as a cryopreservative. Dried liposomes were rehydrated in distilled water with brief vortexing prior to use. When rehydrating the dried liposomes after 1 year of storage at 4°C, the vesicles showed a size distribution similar to that obtained before lyophilization, and retained about 70% of HBsAg immunogenicity. Dried liposomes containing HBsAg showed significantly earlier sero-conversion ($p < 0.05$) and higher titre compared to the free HBsAg and the mixture of HBsAg and aluminum phosphate. Taken together, these results suggest that the dried liposomes, with high stability and enhanced immunogenicity, can be further developed as potential immuno-adjuvants for HBsAg.

Keywords: Dried liposome; Immunoadjuvant; Hepatitis B surface antigen; Stability

1. Introduction

Liposomes are attractive as promising new-generation immuno-adjuvants (Allison and Gregoriadis, 1990; Gregoriadis, 1992). Since the immuno-potentiating effect of liposomes on tetanus toxoid was reported (Allison and Gregoriadis, 1974), the role of liposomes as effective immuno-adjuvant for various antigens has been confirmed (Van Rooijen and Van Nieuwmegen, 1980; Kersten et al., 1988; Alving and Richards, 1990; Pillips and Emili, 1992). Practical use of liposomes as immuno-adjuvant requires the antigen to be entrapped without loss of antigenicity and the final liposomal product should be stable with respect to antigen leakage and aggregation of the liposomes during storage. In addition, the handling of the final preparation must be easy and simple.

Dehydration-rehydration vesicles have been used for peptide drugs (Kirby and Gregoriadis, 1984; Alino et al., 1990) and antigens such as tetanus toxoid (Davis and Gregoriadis, 1987) and

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Leishmania major antigen (Kahl et al., 1989). Dehydration-rehydration vesicles have many advantages such as relatively high entrapping efficiency and little potential damage to the entrapped material by organic solvents or detergents during preparation (Cullis et al., 1987). Dehydration-rehydration vesicles are usually sized by extrusion (New, 1990) or microfluidization (Gregoriadis et al., 1990) because the size distribution of liposomes plays a critical role in the pharmacokinetics of both the liposomes and the entrapped drug in vivo (Hwang, 1987). The sized dehydration-rehydration vesicles are then separated from unentrapped antigen before use. However, the processes for size control and separation need special instruments and skill which are not readily available at the place of vaccination such as a local hospital or public health center.

In this study, we prepared stable dried liposomes (DL) with entrapped antigen which could be easily handled at the place of vaccination, by lyophilizing the sized and separated dehydration-rehydration vesicles in the presence of trehalose, since it has been used as a cryopreservative for cell lines and for liposome-entrapped small molecules such as isocitrate (Crowe et al., 1985, 1986; Madden et al., 1985; Crowe and Crowe, 1988), but not for vaccines. Pre-S rich hepatitis B surface antigen (HBsAg) particles (Youn and Samanta, 1989) were used as a model antigen. Further, in vitro physico-chemical properties of dried liposomes containing HBsAg such as entrapping efficiency and stability were studied. Humoral immunity and delayed-type hypersensitivity of this dried liposomes were also compared with free HBsAg and the mixture of HBsAg and aluminum phosphate.

2. Materials and methods

2.1. Materials

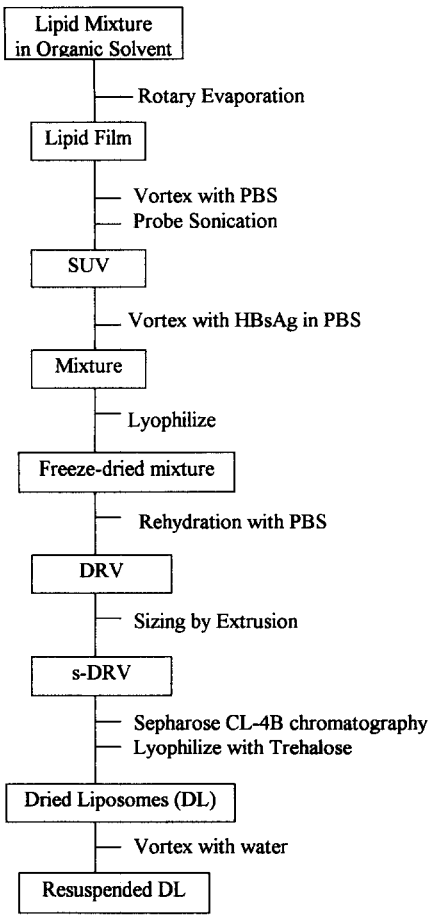
The pre-S rich hepatitis B surface antigen particles expressed in recombinant mammalian cell culture (HBsAg) were kindly donated by Cheil Foods and Chemicals Inc. (Ichon, Korea). Sepharose CL-4B was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Egg phosphatidylcholine, trehalose, sodium taurocholate and bovine serum albumin (BSA, fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Enzyme immunoassay (EIA) kits (AUSZYME® monoclonal) and radio immunoassay (RIA) kits (AUSAB®) for the detection of HBsAg and anti-HBs, respectively, were purchased from Abbott Laboratories (North Chicago, IL, U.S.A.). All other chemicals were of reagent grade and were used without further purification.

2.2. Experimental animals

Male Wistar Albino rats (250 ± 50 g body weight) were obtained from the Laboratory Animal Center, Seoul National University (Seoul, Korea). The rats had access to tap water and food (Cheil Foods and Chemicals Inc., Ichon, Korea) ad libitum.

2.3. Preparation of dried liposome (DL)

The dehydration-rehydration vesicles with entrapped HBsAg were prepared according to the method of Kirby and Gregoriadis (1984) with minor modifications (Scheme 1). In brief, 120 mg of egg phosphatidylcholine (PC) was dissolved in about 1.2 ml of anhydrous chloroform. After the organic solvent was removed on a rotary evaporator, 1.2 ml of phosphate-buffered saline, pH 7.4 (PBS) was added to the dried lipid film, and the mixture was vortexed to resuspend the lipid. The suspension was sonicated at 30 μ for 10–20 min in an ice bath. The resultant small unilamellar vesicle (SUV) suspension was centrifuged at 15000 rpm for 15 min at room temperature to remove large lipid aggregates. The supernatant (1 ml) was mixed with an equal volume of PBS containing 50–900 μg of HBsAg. The radioiodinated HBsAg (^{125}I-HBsAg) from the AUSAB® kit was used as a tracer. The mixture was immediately frozen at -70°C in a deep freezer for 2 h. The preparation was then lyophilized overnight at a vacuum under 0.2 Torr. The lyophilized preparation was rehydrated with 2 ml of PBS to



Scheme 1. Preparation of the Dried Liposomes containing HBsAg.

generate dehydration-rehydration vesicles. The resulting dehydration-rehydration vesicles were sized by extrusion through polycarbonate filters (0.4 μm pore size, Nucrepore, Cambridge, MA, U.S.A.). The sized dehydration-rehydration vesicles were separated from unentrapped material with Sepharose CL-4B column (50 ml) chromatography using PBS as eluent (20 ml/cm^2 per h). The sized and separated dehydration-rehydration vesicles were mixed with trehalose (4 g/g lipid) and lyophilized as described above. The final dried liposome (DL) was flushed with nitrogen, sealed and stored in a refrigerator. Before use, DL was resuspended with distilled water by vortexing for about 30 s.

2.4. Preparation of multilamellar vesicles (MLVs)

MLVs were prepared by mechanical resuspension (New, 1990). The dried lipid film equivalent to 50 mg of phosphatidylcholine was rehydrated by vortexing for 15 min with 0.5 ml of PBS containing 50 μg of HBsAg. The resultant MLVs were sized by the extrusion and separated from unentrapped material as described previously.

2.5. Electron microscopy

The DL was observed by scanning electron microscopy (JEOL JSM-T200 scanning microscope, JEOL, Tokyo, Japan). The resuspended DL was examined by negative stain transmission electron microscopy (JEOL-200 CX, JEOL, Tokyo, Japan) using 2% ammonium molybdate dissolved in 2% ammonium acetate buffer (Kim et al., 1992).

2.6. Calculation of entrapping efficiency

After Sepharose CL-4B chromatography of the liposomes containing ^{125}I-HBsAg, the entrapped HBsAg concentraion was determined by measuring the radioactivity of ^{125}I. The entrapping efficiency of HBsAg within liposomes was calculated from the following equation;

$$\text{Entrapping efficiency (\%)} = [RL/(RL + RA)] \times 100$$

where RL and RA are the radioactivities of the liposomal fraction and the unentrapped antigen fraction, respectively.

2.7. Measurement of surface-available antigen

The surface-available liposomal HBsAg and the total liposomal HBsAg were estimated by the enzyme immunoassay before and after lysis of the liposomes. The liposomes were lysed with sodium taurocholate (final concentration of 27 μg/ml). The concentration of phosphatidylcholine was determined by the ammonium ferrothiocyanate technique (Stewart, 1980).

2.8. Enzyme immunoassay (EIA) of HBsAg

The concentrations of HBsAg were measured using the AUZYME® monoclonal kit. The test samples and standard HBsAg solution were diluted with 1% BSA in PBS to final concentrations of 0.5–5.0 ng/ml. Briefly, 50 μl of conjugate (mouse monoclonal anti-HBs-horseradish peroxidase (HRPO) conjugate, 0.2 g/ml in Tris buffer) and one bead coated with mouse monoclonal anti-HBs was added to 200 μl of positive control (human HBsAg, 9 ± 2 ng/ml in Tris buffer), negative control (recalcified human plasma, nonreactive for HBsAg and anti-HBs) and the diluted test samples. The beads were incubated at 40°C for 3 h, then washed with distilled water and immediately transferred to assay tubes. 300 μl of freshly prepared *o*-phenylenediamine (OPD) substrate solution (OPD HCl 2.56 mg/ml in citrate-phosphate buffer containing 0.02% hydrogen peroxide) was pipetted into each assay tube. The tubes were incubated at room temperature for 30 min. The reaction was terminated by the addition of 1 ml of 1 N sulfuric acid. Absorbances of controls, standard solutions and test samples were determined at 492 nm against substrate blank.

2.9. Humoral immunity

Rats were randomly divided into three groups ($n = 6$). 200 μl of free HBsAg, the antigen mixed with aluminum phosphate (AP) (antigen/AP = 1:200) and the resuspended DL in saline solution were injected intramuscularly into the center of the right thigh muscle (musculus rectus) of the rats with the dose of 3 μg HBsAg/rat at week 0, 4 and 8. Blood was obtained from the tail vein at 1, 5 and 9 weeks after the first injection and the anti-HBs titre was determined by RIA.

2.10. Radio immunoassay (RIA) of anti-HBs

Anti-HBs titre was measured by RIA using the AUSAB® kit. In brief, 200 μl of positive control (recalcified normal human plasma, reactive for anti-HBs, 512 ± 200 RIA units/ml), negative control (recalcified normal human plasma, nonreactive for anti-HBs and HBsAg) and test serum were added to each human HBsAg coated bead. After 18 ± 2 h incubation at room temperature, the beads were washed with distilled water. The beads were then incubated in 200 μl of ^{125}I-HBsAg (0.74 μCi/ml in 0.01 M Tris buffer containing 20% recalcified normal human plasma) at room temperature for 4 h. The beads were washed again and transferred to assay tubes. Radioactivity of each assay tube was measured in a γ-scintillation counter (Auto-gamma 5000 series, Packard Instrument Co., Meriden, CT, U.S.A.). Reactive and nonreactive specimens were determined by relating net cpm of the unknown to 2.1-times the mean net cpm of the negative control. The estimated RIA unit values were determined by calculating the ratio of the unknown specimen to the positive control and selecting the estimated RIA unit value from the table in the AUSAB® kit manual.

2.11. Delayed-type hypersensitivity

Rats were randomly divided into three groups ($n = 6$). 200 μl of free HBsAg in saline solution, the antigen mixed with AP (antigen/AP = 1:200) and the resuspended DL were administered subcutaneously into the right back paw of the rats with a dose of 3 μg HBsAg/rat. 200 μl of saline solution was injected to the left back paw as a control. The increase in each foot thickness was measured using a micrometer (Mitutoyo Corp., Japan) at 24 and 48 h after the injection.

2.12. Statistical analysis

All data are expressed as mean ± standard deviation. The experimental data in vitro and anti-HBs titre were analyzed for statistical significance ($p < 0.05$) by ANOVA. Sero-conversion rate was analyzed by chi-square test.

3. Results and discussion

3.1. Entrapping efficiency of HBsAg in the DL

The DL was more efficient than the MLVs in entrapping HBsAg (18.02 vs 3.73%). Manesis et

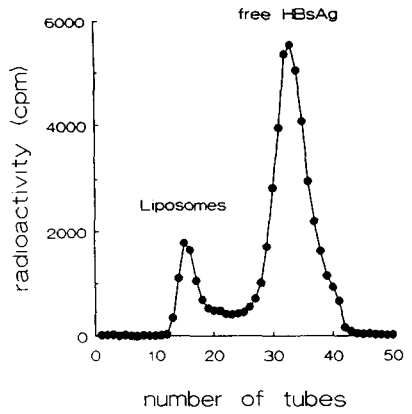


Fig. 1. Elution pattern of the liposomes with entrapped HBsAg (first peak) and free HBsAg (second peak) by a Sepharose CL-4B column (50 ml) eluted with pH 7.4 phosphate-buffered saline solution (20 ml/cm^2 per h). ^{125}I-HBsAg was used as a tracer.

al. (1978, 1979) reported higher efficiencies of MLVs in entrapping HBsAg with a different MLV lipid composition than the MLV in this experiment. However, there is a possibility that Manesis et al. overestimated the entrapping efficiency, since the liposomal peak considerably overlapped the unentrapped HBsAg peak after Sepharose CL-2B column chromatography. In contrast, unentrapped HBsAg was clearly separated from the liposomes using the Sepharose CL-4B column in the present study as shown in Fig. 1; this would partially explain the apparent lower entrapping efficiency of our liposomes than those of Manesis et al.

3.2. Surface availability of HBsAg in the DL

The total liposomal HBsAg measured after the lysis of liposomes by sodium taurocholate was not significantly different from the surface-available HBsAg measured in the absence of sodium taurocholate (4.69 ± 0.009 vs 4.11 ± 0.119 μg HBsAg/mg lipid). This might be attributed to the preparation procedure of the DL and the physico-chemical properties of the HBsAg particles such as the lipid coat and large size of the HBsAg (average diameter of 22 nm) (Youn and Samanta, 1989). The intimate contact between the hydrophobic HBsAg particles and the lipid bilayers of the SUVs in the dried state might facilitate hydrophobic interaction between them. It was observed that the DL is irregularly shrunken in particles from the scanning electron micrograph. The transmission electron micrograph of the resuspended DL shows that the DL is of the typical spherical shape of liposomes, and the HBsAg is entrapped on the inner surface of DL and associated with lipid layers. During the rehydration process, when the lipid bilayers rehydrate, fuse and reseal to form vesicles (New, 1990), most of the HBsAg might be entrapped as either partially imbedded in or adsorbed to the lipid bilayers of the DL. Manesis et al. (1979) also suggested that the HBsAg particles are interdigitated between the phospholipid molecules of the bilayers in MLVs through hydrophobic interaction.

3.3. Effect of trehalose on the retention of HBsAg during resuspension of the DL

The retention of the liposomal HBsAg after resuspension of the DL was determined by measuring the radioactivity of ^{125}I after Sepharose CL-4B chromatography of the resuspended liposomes entrapping ^{125}I-HBsAg as a tracer. The retention of liposomal HBsAg was dramatically increased (68.4 vs 9.74%) by lyophilizing the liposomes in the presence of trehalose (4 g/g lipid). This result indicates that trehalose is an effective cryopreservative for liposomes entrapped HBsAg. There is a possibility that the retention of HBsAg was underestimated, since passing the liposomes through the column may destabilize them (Crowe et al., 1985). However, there was no practical method except gel chromatography to separate leaked HBsAg from liposomal HBsAg, since most of HBsAg was settled down by the minimal centrifugal force needed to separate our liposomes ($450\,000 \times g$ for 2 h).

3.4. Size distribution of the resuspended DL

The size distribution of resuspended DL was determined by photon correlation spectroscopy (LPA-3000 and LPA-3100, Otsuka Electronics, Japan). The diameter of the liposomes resus-

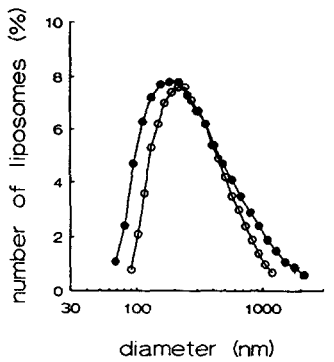


Fig. 2. Size distribution of the dried liposomes containing HBsAg; resuspended immediately after preparation (○), resuspended after 12 months (●).

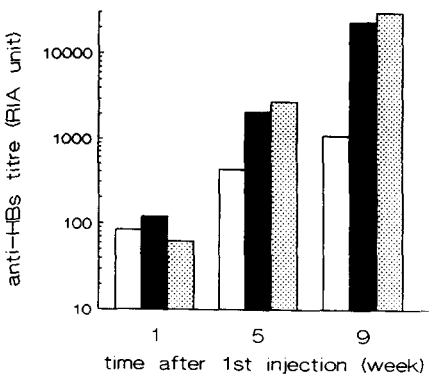


Fig. 3. Anti-HBs geometric mean titre after intramuscular injection to rats: 200 μl of free HBsAg (open bars), the antigen mixed with aluminum phosphate (AP) (antigen/AP = 1:200) (closed bars) and the dried liposomes (stippled bars) in saline solution were injected into the center of the right thigh muscle of the rats ($n = 6$) at week 0, 4 and 8. The dose was equivalent to 3 μg HBsAg per rat.

pended immediately after preparation was 329 ± 216 nm. The size of liposomes varied slightly after storage of the DL for 12 months in the refrigerator (373 ± 353 nm) as illustrated in Fig. 2.

3.5. *Immuno-adjuvanticity of the DL*

The DL showed excellent immuno-adjuvanticity for the entrapped antigen. The group treated with the dried liposomes containing HBsAg showed eariler sero-conversion ($\chi^2 < 0.05$) (Table 1) with higher anti-HBs titre than the groups treated with free HBsAg and HBsAg mixed with AP which has been used as immuno-adjuvant in the commercial hepatitis vaccine as shown in Fig. 3. The delayed-type hypersensitivity was measured to estimate the possible enhancement of cell-mediated immune response by the DL. There were no significant differences in the delayed-type hypersensitivity responses among the groups (Table 2).

In summary, we prepared DL which can be easily reconstituted to the liposomal suspension with the desired size distribution and retention of most of the antigens within the liposomes. The resuspension process of our DL was simple, easy, and not time-consuming. The DL not only preserved but also potentiated the antigenicity of the entrapped antigen. The DL showed enhanced immuno-adjuvant activity to the entrapped antigen compared with the aluminum salt which is the only adjuvant authorized for use in human vaccines (Allison and Gregoriadis, 1990). The DL might be used as new-generation immuno-ad-

Table 1
Anti-HBs sero-conversion rate (SCR) after intramuscular injection to rats

Treatment [a]	SCR at week		
	1	5	9
Free HBsAg	3/6	5/6	6/6
HBsAg with aluminum phosphate	5/6	6/6	6/6
Dried liposomes	6/6 [b]	6/6	6/6

[a] 200 μl of free HBsAg, HBsAg mixed with aluminum phosphate (antigen/AP = 1:200) and the dried liposomes in saline solution were injected into the center of the right thigh muscle with a dose of 3 μg HBsAg/rat at week 0, 4 and 8.
[b] $\chi^2 < 0.05$.

Table 2
Delayed-type hypersensitivity after subcutaneous injection to rats

Treatment [a]	Positive response	
	After 24 h	After 48 h
Free HBsAg	3/6	1/6
HBsAg with aluminum phosphate	4/6	1/6
Dried liposomes	2/6	1/6

[a] Abbreviations as in Table 1.

juvant showing enhanced adjuvanticity and improving the instability of the conventional liposomes.

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