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Enhanced lymph node delivery and immunogenicity of hepatitis B surface antigen entrapped in galactosylated liposomes

Chong-Kook Kim *, Eun Ju Jeong[1]

College of Pharmacy, Seoul National University, San 56-1, Shinlim-Dong, Kwanak-Ku, Seoul 151-742, South Korea

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Abstract

The purpose of this work is to increase the lymph node delivery and the immunogenicity of hepatitis B surface antigen (HBsAg) in vivo. HBsAg was entrapped in the dried liposomes with their surfaces modified with galactose. Pharmacokinetics and organ distribution of free HBsAg alone, HBsAg mixed with aluminum phosphate, HBsAg entrapped in ungalactosylated liposomes and galactosylated liposomes (GalL) were studied. For each sample, the anti-HBsAg titres were measured by RIA. Most HBsAg in GalL existed in an antibody-available form. In rats, HBsAg in GalL administered to right thigh muscles, resided in the injection sites longer than did free HBsAg alone or HBsAg mixed with aluminum phosphate. Also, GalL delivered higher amounts of HBsAg to the regional lymph nodes than did other formulations: the area under the concentration-time curve of HBsAg in the regional lymph nodes given in GalL was 16, 2.4, and 2.2-fold higher than that in free form, aluminum phosphate mixture and ungalactosylated liposomes, respectively. The immunogenicity of HBsAg given in GalL showed a good correlation to its enhanced delivery to the lymph nodes. HBsAg in GalL boosted the formation of antibodies 40-fold higher than did free HBsAg, whereas HBsAg mixed with aluminum phosphate and HBsAg in ungalactosylated liposomes increased the titre by 21- and 13-fold, respectively. Taken together, it is concluded that the galactosylated liposomes can target HBsAg to the regional lymph nodes, rich in the antigen-presenting cells and enhance the immunogenicity of HBsAg more efficiently than do the conventional aluminum phosphate or the ungalactosylated liposome formulations. © 1997 Elsevier Science B.V.

Keywords: Galactosylated liposomes; Liposomes; Dried liposomes; Hepatitis B surface antigen; Immunoadjuvant; Lymph node delivery; Pharmacokinetics

1. Introduction

Hepatitis B is a disease of global importance with more than 300 million carriers of the hepati-

* Correspondence author.

[1] Present address: Pharmaceutical Division, Cheil Foods and Chemicals Inc., Ichon-Kun, Kyonggi-Do, 467-810, Korea.

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tis B virus worldwide (Maynard, 1990). Hepatitis B virus also causes up to 80% of primary liver cancer, one of the most significant causes of cancer mortality. To prevent the development of hepatitis B virus infection to more serious stages, it is essential to immunize the body against hepatitis B virus by boosting the level of the antibodies against hepatitis B surface antigen (HBsAg). For the acceleration of the formation of anti-HBsAg antibodies, HBsAg may be needed to be exposed for a prolonged period to the immune system such as the lymph nodes, rich in the antigen-presenting cells (Austyn, 1989).

To increase the retention of HBsAg in the body and stimulate the immune system, the mixture of HBsAg and aluminum salt has been commercially used. The use of aluminum salt as an adjuvant, however, can result in side effects such as inflammation in the injection sites (Gupta et al., 1993). In addition, there is increasing concern about the toxicity of aluminum (Winship, 1993; Abreo and Glass, 1993) since aluminum may be linked with a number of disorders including Alzheimer's disease, Parkinson's dementia and osteomalacia (Exley and Birchall, 1992). Therefore, it is necessary to develop an adjuvant which targets the antigen to the immune cells without a side effect, and increases the exposure of HBsAg to the immune cells by residing in the target sites for longer periods.

Among alternative immunoadjuvants, liposomes have been considered to be promising, given their proven low toxicity, effective adjuvant properties (Audera et al., 1991), and ability to liberate their contents into the cells over a sustained period (Couvreur et al., 1991). Furthermore, liposomes, with an appropriate type of sugar groups on their surfaces, can target specific cells with surface receptors for the sugar (Garcon et al., 1988; Gregoriadis, 1992). Of various sugars, galactose has its receptors on antigen-presenting cells. The galactose receptors are shown to mediate internalization of antigens which have galactose moieties and increase antigenicity (Manca, 1992).

In this study, therefore, HBsAg was entrapped in the dried liposomes with galactose moieties on their surfaces, and investigated whether the galactosylated liposomes carrying HBsAg localize to the lymph node and enhance the immunogenicity of HBsAg in vivo.

2. Materials and methods

2.1. Materials

The pre-S rich hepatitis B surface antigen expressed in recombinant mammalian cell culture (HBsAg) were kindly provided by Cheil Foods and Chemicals (Ichon, Korea). Na ^{125}I (100 mCi/ml) was purchased from Amersham (Buckinghamshire, UK). Cloramine-T derivatized polystyrene beads (Iodo-beads®) was from Pierce (Rockford, IL, USA). 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 Lab (North Chicago, IL, USA). Stearylamine (SA), lactobionic acid hemicalcium salt, sodium taurocholate, trehalose and egg phosphatidylcholine (PC) were obtained from Sigma Chemical (St. Louis, MO, USA). *N*-stearyl lactobionamide (*N*-SLBA) was synthesized by conjugating the amino group of stearylamine to the carboxyl group of lactobionic acid as described previously (Kim et al., 1992, 1996). All other chemicals were of the reagent grade and used without further purification.

2.2. Animals

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

2.3. Radioiodination of HBsAg

Two chloramine-T derivatized polystyrene beads were washed with 1 ml of 50 mM phosphate buffer (pH 7.4) and added to 10 μl of Na ^{125}I solution (100 mCi/ml) diluted with 150 μl of 50 mM phosphate buffer (pH 7.4). The mixture was vortexed at room temperature for 5 min, and

then 60 μl of HBsAg (1 mg/ml in pH 7.4 phosphate buffered saline, PBS) was added to the mixture and incubated for 10 min with occasional vortexing. The radioiodinated HBsAg ([^{125}I]HBsAg) was then purified using Sephadex G-50 presaturated with 1% bovine serum albumin. The radioactivity and presence of HBsAg of each fractions were detected by γ-scintillation counter (Auto-gamma 5000, Packard Instrument, Meriden, CT, USA) and enzyme immunoassay (EIA) as described previously (Kim and Jeong, 1995), respectively. The fractions containing [^{125}I]HBsAg were pooled, kept at 4°C and used within 10 weeks after preparation.

2.4. Preparation of dried liposomes containing HBsAg

The procedure for entrapping HBsAg into dried liposomes has been described previously (Kim and Jeong, 1995). In brief, 120 mg of lipid mixture composed with PC:SA (molar ratio, 9:1) or PC:SA:N-SLBA (molar ratio, 8:1:1) was dissolved in about 1.2 ml of anhydrous chloroform. After the organic solvent was removed on a rotary evaporator, 1.2 ml of PBS (10 mM, pH 7.4) was added to the dried lipid film, and the mixture was vortexed to resuspend the lipid. The suspension was sonicated at 30 μW for 10–20 min in an ice bath. The resultant small unilamellar vesicles (SUVs) suspension was centrifuged at 7500 g in a microcentrifuge (Sarstedt MH 2, Sarstedt, Germany) for 15 min at room temperature to remove large lipid aggregates. The supernatant (1 ml) was mixed with an equal volume of PBS (10 mM, pH 7.4) containing 50–900 μg of HBsAg. In some experiments, [^{125}I]HBsAg 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 over night at a vacuum under 0.2 torr. The lyophilized preparation was rehydrated with 2 ml of PBS (10 mM, pH 7.4) to generate dehydration-rehydration vesicles. The resulting dehydration-rehydration vesicles were sized by extrusion through polycarbonate filters (0.4 μm pore size, Nuclepore, Cambridge, MA, USA). 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/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 was flushed with nitrogen, sealed and stored in a refrigerator. The dried liposome was observed by scanning electron microscopy (JEOL JSM-T200 scanning microscope, JEOL, Tokyo, Japan). The resuspended liposome 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). Before use, the dried liposomes was resuspended with distilled water by vortexing for about 30 s.

Entrapping efficiency of HBsAg was calculated on the basis of ^{125}I radioactivity of the liposomes after the chromatographic separation of resuspended dried liposomes through Sepharose CL-4B column (Kim and Jeong, 1995).

The surface-available liposomal HBsAg and the total liposomal HBsAg were estimated by the enzyme immunoassay using AUSZYME® kit (Kim and Jeong, 1995) before and after lysis of the liposome with sodium taurocholate (final concentration of 27 μg/ml), respectively. Phospholipid concentration of liposomes was determined by the ammonium ferrothiocyanate technique (Stewart, 1980).

2.5. Animal experiment

Free HBsAg (200 μl), the mixture of HBsAg and aluminum phosphate (AP), HBsAg entrapped in ungalactosylated liposomes (PC:SA = 9:1) and galactosylated liposomes (PC:SA:N-SLBA = 8:1:1) were administered intramuscularly to the center of the right thigh muscle (musculus rectus) of rats with the dose of 3 μg HBsAg/rat after an overnight fast.

At each time point (1 min, 2 h, 8 h, 2 day, and 7 day after dosing), three rats of each group were killed and various organs (right thigh muscle, right and left iliac lymph nodes, liver, spleen, kidney, lung, heart, thymus, thyroid gland, stomach and intestine) were removed immediately. The organs were rinsed with saline solution, blotted-

dry with filter paper and weighed. Approximately 0.1–0.2 g of tissue slices was excised and placed in a γ-scintillation counter for the determination of radioactivity.

Separately, 12 rats (3 rats per group) were housed individually in the metabolic cages and their urine was collected every 24 h for 7 days. The volume of urine was measured and an aliquot (2 ml) was transferred to an assay tube. Urinary excretion was determined by measuring the radioactivity of urine.

2.6. Humoral immunity

Three doses (3 μg/rat/dose) of free HBsAg, HBsAg mixed with aluminum phosphate (HBsAg:AP = 1:200), HBsAg entrapped in ungalactosylated liposomes (PC:SA = 9:1; 6.83 ± 0.39 μg HBsAg/mg lipid) and HBsAg in galactosylated liposomes (PC:SA:N-SLBA = 8:1:1; 7.46 ± 0.56 μg HBsAg/mg lipid) were administered intramuscularly into the center of right thigh of rats with a 4 week interval between doses. Serum samples were taken from the tail vein 1 week after each dosing. For each sample, the anti-HBsAg titres were measured by RIA using AUSAB® kit (Kim and Jeong, 1995).

2.7. Pharmacokinetic data analysis

The area under the concentration-time curve from time 0 to 7 days ($AUC_{0\ 7\ days}$) and the area under the amount-time curve from time 0 to 7 days ($AUQ_{0\ 7\ days}$) were estimated by the trapezoidal rule (Kim and Hwang, 1993). The AUC and the AUQ, respectively, refer to the unweighed and the weighed time averaged exposure of each organ to the antigen (Gupta and Hung, 1989).

Two indices for the evaluation of lymphatic uptake of the antigen from the injection site were calculated based on AUC;

$$R/L = AUC_{RLN}/AUC_{LLN}$$

$$R/S = AUC_{RLN}/AUC_{serum}$$

2.8. Statistical analysis

Anti-HBsAg titres among the groups were statistically evaluated with analysis of variance. Unpaired t-test was performed between the ungalactosylated liposome-treated group and the galactosylated liposome-treated group.

3. Results and discussion

3.1. Characterization of liposomes

Ungalactosylated control liposomes (ConL) composed of PC and SA (molar ratio of 9:1) showed an entrapping efficiency of 44.6%. The presence of SA (10 mol %) in the bilayers of liposomes improved the entrapping efficiency of HBsAg by 2.5–3.0-fold compared with liposomes composed of PC alone (44.6 vs. 18.02) (Kim and Jeong, 1995). It might be due to the electrostatic interaction of positively charged SA with negatively charged HBsAg at pH 7.4 (Manesis et al., 1979). Galactosylated liposomes (GalL) composed of PC, SA and N-SLBA (molar ratio of 8:1:1) entrapped 54.0% of initially loaded HBsAg.

To characterize whether HBsAg locates in the outer leaflet of liposomes or in the internal aqueous spaces, the antigenicity of liposomal HBsAg in the absence and presence of a detergent, sodium taurocholate, was compared. Table 1 shows that the concentrations of HBsAg recognized by antibodies were not different significantly between intact and detergent-lysed liposomes.

Table 1
The amounts of antibody-available HBsAg in intact and lysed liposomes

Compositions	Molar ratio	HBsAg in intact liposomes (μg/mg lipid)	HBsAg in lysed liposomes (μg/mg lipid)
PC:SA	9:1	7.12 ± 0.23	6.83 ± 0.39
PC:SA:N-LBA	8:1:1	7.32 ± 0.04	7.46 ± 0.50

Liposomes were lysed with sodium taurocholate

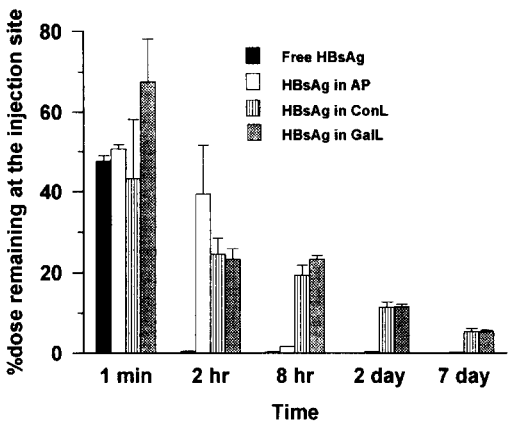

Fig. 1. HBsAg remaining in the injection sites after intramuscular administration. The dose was equivalent to 3 μg of HBsAg per rat, and radioiodinated HBsAg was used as a tracer. The data are the arithmatic mean values $\pm$ S.D.

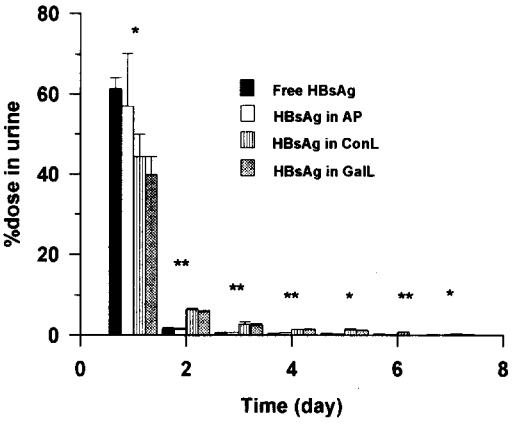

Fig. 3. Urinary excretion of HBsAg after intramuscular injection. The dose was equivalent to 3 μg of HBsAg per rat, and radioiodinated HBsAg was used as a tracer. The data are the arithmatic mean values $\pm$ S.D. (*: $p < 0.05$, **: $p < 0.01$ among the groups).

Such a similarity suggests that HBsAg in liposomes might be entrapped as either partially imbedded in the lipid bilayers and/or electrostatically bound to the outer leaflet of lipid bilayers as an antibody-available form, rather than encapsulated in the internal aqueous spaces. Manesis et al. (1979) also suggested that the HBsAg particles are interdigitated between the phospholipid molecules of the bilayers in multilamellar vesicles through hydrophobic interaction. The high surface-availability of HBsAg was also reported for neutral ungalactosylated liposomes (Kim and Jeong, 1995).

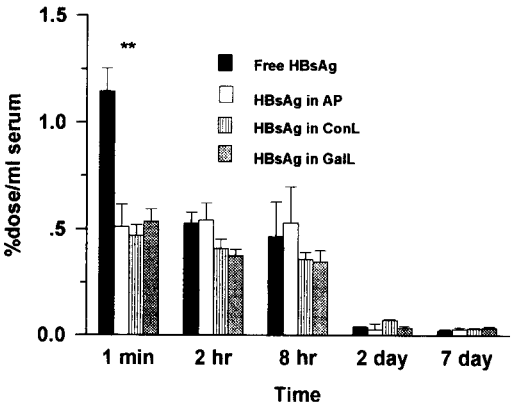

Fig. 2. Serum concentration-time profiles of HBsAg after intramuscular injection. The dose was equivalent to 3 μg of HBsAg per rat, and radioiodinated HBsAg was used as a tracer. The data are the arithmatic mean values $\pm$ S.D. (**: $p < 0.01$ among the groups).

The size distribution of resuspended liposomes was determined by photon correlation spectroscopy (LPA-3100, Otsuka Electronics, Japan). The addition of N-SLBA did not affect significantly the size distribution of PC:SA (9:1) liposomes. The size of liposomes composed of PC:SA (9:1) was 421 $\pm$ 213 nm. Similarly, liposomes composed of PC:SA:N-SLBA (8:1:1) sized 335 $\pm$ 214 nm. The slightly smaller size of the galactosylated liposomes might be resulted from the higher curvature of the liposomal outer layers due to the bulky galactose moiety of N-SLBA.

3.2. Clearance from the injection site

As shown in Fig. 1, more than 99% of free HBsAg was cleared from the injection site within 2 h. HBsAg mixed with AP disappeared from the injection site slower than did free HBsAg alone due to depot action of AP. At 8 h after injection, nevertheless, only about 2% of HBsAg mixed with AP remained at the injection site. HBsAg, entrapped in either ConL or in GalL, resided in the injection sites longer than did HBsAg alone or mixed with AP. At one min after injection, 70% of

Table 2
Area under the HBsAg concentration-time curve ($AUC_{0\text{-}7\ days}$) (%dose·h/ml or g) and area under the HBsAg amount-time curve ($AUQ_{0\text{-}7\ days}$) (%dose·h) of four different formulations in each organs

Organ	Free HBsAg	HBsAg in AP	HBsAg in ConL	HBsAg in GalL
RLN	46.05[a] (0.51)[b]	306.10 (3.53)	328.90 (3.59)	720.99 (8.07)
LLN	40.11 (0.46)	49.09 (0.45)	93.75 (1.00)	81.09 (0.77)
Serum	18.37 (95.91)	18.68 (96.29)	18.00 (88.64)	14.78 (73.55)
Injection site	22.13 (63.01)	117.50 (284.60)	832.93 (1955.00)	754.21 (1833.00)
Spleen	6.22 (2.99)	7.35 (3.70)	6.20 (2.90)	11.18 (4.11)
Lung	8.94 (14.09)	10.16 (12.82)	9.87 (15.75)	7.69 (9.41)
Thymus	7.04 (2.34)	5.35 (2.13)	7.42 (2.06)	8.13 (2.56)
Heart	4.09 (2.95)	4.96 (2.88)	6.50 (3.13)	6.78 (3.11)
Thyroid gland	6.89 (3.00)	8.17 (3.48)	7.73 (2.76)	8.44 (2.84)
Liver	7.21 (57.96)	6.70 (53.64)	8.33 (63.49)	11.34 (88.66)
Kidney	8.69 (16.94)	9.58 (18.36)	12.42 (22.54)	19.42 (38.91)
Stomach	22.96 (31.90)	22.03 (29.71)	17.08 (21.00)	16.37 (20.08)
Small intestine	10.02 (71.51)	6.99 (51.10)	6.73 (42.62)	7.29 (48.64)
Large intestine	4.79 (13.19)	4.95 (14.01)	5.01 (12.18)	5.18 (12.83)

The radioiodinated HBsAg was used as a tracer.
[a] $AUC_{0\text{-}7\ days}$.
[b] $AUQ_{0\text{-}7\ days}$.

HBsAg in ConL, and 42% of HBsAg in GalL remained in the injection site. The higher clearance of HBsAg in GalL compared with clearance in ConL in the initial phase from the injection site might result from the size difference (335 ± 214 vs. 421 ± 213 nm) and increased uptake of GalL by surrounding tissues. From 2 h after injection, HBsAg in both ConL and GalL showed a slower phase of disposition with a terminal half-life of 66.5 and 67.0 h, respectively. The biphasic disposition pattern of liposomal HBsAg given by intramuscular (i.m.) route agrees with the previous report (Ohsawa et al., 1985) where they observed that liposomal inulin given by i.m. injection disposed biphasically from the injection sites.

3.3. Serum pharmacokinetics

The serum concentration-time profiles of HBsAg in AP, ConL, or GalL did not differ significantly from that of free HBsAg, although free HBsAg was absorbed to serum in the higher rate than other formulations at 1 min after intramuscular dosing (Fig. 2). $AUC_{0\text{-}7\ days}$ values of free HBsAg alone, HBsAg mixed with AP, HBsAg entrapped in ConL and GalL were 18.37, 18.68, 18.00 and 14.78% dose·h/ml, respectively. $AUC_{0\text{-}7}$ days of HBsAg in GalL is slightly lower than other formulations. It indicates that higher amounts of HBsAg in GalL disposed in tissues other than serum compared with other formulations.

3.4. Urinary excretion

The total urinary excretion of HBsAg was not significantly different among the groups: free HBsAg, HBsAg in AP, HBsAg in ConL and HBsAg in GalL showed 65 ± 4, 60 ± 16, 57 ± 8 and 52 ± 6% of the injected doses over 7 days, respectively (Fig. 3). However, the daily excretion was significantly different among the groups ($p < 0.05$). Over 99% of the total urinary excretion was excreted during the inital 24 h in the group treated with free HBsAg and HBsAg mixed with AP. In contrast, the radioactivity was continuously excreted during 7 days in the liposome treated groups.

3.5. Tissue distribution

Table 2 lists the organ distribution of HBsAg by four different formulations. HBsAg in GalL showed slightly higher distribution to spleen, liver and kidney rich in fixed macrophages than the

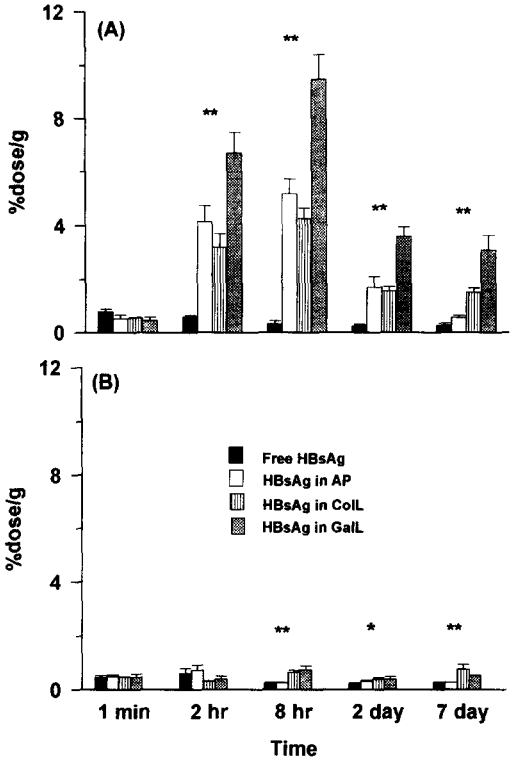

Fig. 4. Distribution of HBsAg to the right (A) and the left (B) lymph nodes. The dose was equivalent to 3 μg of HBsAg per rat, and radioiodinated HBsAg was used as a tracer. The data are the arithmatic mean values $\pm$ S.D. (*: $p < 0.05$, **: $p < 0.01$ between the ungalctosylated and galactosylated liposome-treated groups).

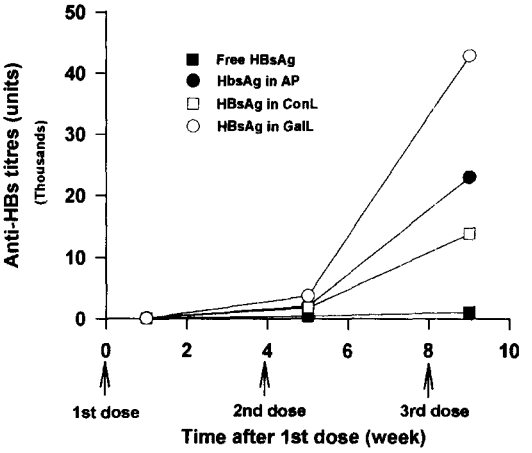

Fig. 5. Anti-HBs titres of four different formulations. Anti-HBsAg antibody titres were measured using RIA at 1 week after each dosing (3 μg/rat/dose). The data are the geometric mean values $\pm$ S.D. of six experiments.

other formulations based on $AUQ_{0\text{-}7\ days}$. But in some organs such as stomach and lung, HBsAg in GalL showed lower $AUC_{0\text{-}7\ days}$ and $AUQ_{0\text{-}7\ days}$. The sum of $AUQ_{0\text{-}7\ days}$ in the group treated with free HBsAg, HBsAg mixed with AP, HBsAg entrapped in ConL and GalL is 376.7, 576.7, 2237 and 2134% dose·h, respectively. The sum of $AUQ_{0\text{-}7\ days}$ in the group treated with free HBsAg and HBsAg mixed with AP was far less than those in liposome treated groups. These coincide with the results: most of the HBsAg in the former groups were excreted during the first day but those in the later groups were continuously excreted during 7 days.

3.6. Uptake and retention of HBsAg in lymph nodes

The lymph node distributions of HBsAg given in various formulations were measured both in the right iliac lymph node (RLN) and in the left iliac lymph node (LLN). After i.m. injection to the right thigh muscle, HBsAg in AP or in liposomes distributed mainly to RLN (Fig. 4A) rather than LLN even in the later time points such as 2

Table 3
The R/L and R/S ratios of four different formulations

Ratio	Free HBsAg	HBsAg in AP	HBsAg in ConL	HBsAg in GalL
R/L	1.1	6.2	3.5	8.9
R/S	2.5	16.0	18.0	48.0

The ratios were calculated based on the $AUC_{0\text{-}7\ days}$ values of each organ.
The radioiodinated HBsAg was used as a tracer.

and 7 days (Fig. 4B). GalL delivered HBsAg to RLN greater than did other formulations: the $AUC_{0\text{-}7\ days}$ of HBsAg in RLN given in GalL was 16- fold higher than that in free form (720.90 vs. 46.05% dose·h/g), and over 2-fold higher than that in AP mixture or in ConL (720.90 vs. 306.10 or 328.90% dose·h/g) (Table 2).

In addition, HBsAg in GalL showed the highest R/L and R/S ratios compared with other formulations (Table 3). It might be explained by that higher amounts of HBsAg in GalL could be rapidly taken up by fixed macrophages in regional lymph node than the other formulations after injection. With enhanced delivery, the increased lymph node residence time of HBsAg given in GalL may prolong the exposure time of HBsAg to antigen-presenting cells, which are plentiful in the lymph nodes.

3.7. Immunogenicity of liposomal HBsAg

The immunogenicity of HBsAg was measured after three doses of each formulation with 4 weeks apart. At 1 week after the second dose, HBsAg in AP, ConL and GalL induced anti-HBs in all animals with RIA units of 2090, 1860, and 3759, respectively (Fig. 5). At 1 week after the third injection, HBsAg in free form induced 1090 units of anti-HBs titres. Compared with free HBsAg, HBsAg given in AP and in ConL increased the titre by 21- (1090 vs. 23073) and 13-fold (1090 vs. 13911), respectively. In contrast, GalL enhanced the formation of antibody by 40-fold (1090 vs. 42918).

The superior immunogenicity of HBsAg in GalL might be related to the increased residence of HBsAg in the lymph nodes. Dal Monte and Szoka (1989) also proposed that the in vivo immunoadjuvant effect of liposomes may be mainly due to the antigen delivered to the antigen-presenting cells and the depot effect of the liposomes not taken by antigen-presenting cells.

In conclusion, our results indicate that GalL can target HBsAg to the regional lymph nodes, rich in the antigen-presenting cells and enhance the immunogenicity of HBsAg more efficiently than do the conventional aluminum phosphate or the ungalactosylated liposome formulations.

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