HEPATITIS B SURFACE ANTIGEN-CONTAINING LIPOSOMES ENHANCE HUMORAL AND CELL-MEDIATED IMMUNITY TO THE ANTIGEN

Emanuel K. MANESIS, Colin H. CAMERON* and Gregory GREGORIADIS

Division of Clinical Sciences, Clinical Research Centre, Harrow, Middx. HA1 3UJ and **Virology Laboratory, Middlesex Hospital Medical School, London W1P 7PN, England*

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1. Introduction

Immunological response to hepatitis B surface antigen (HB_sAg) and to other viral antigens varies considerably not only among species, but also among individuals of the same species [1]. High antibody titres and a relatively uniform response can often be achieved by the use of adjuvants. However, complete Freund's adjuvant, lipopolysaccharide endotoxins and polyanions can induce local and systemic toxicity which prevents their application in man. On the other hand, adjuvants such as aluminium precipitates or water-in-oil emulsions already used in human vaccines lack in efficiency and duration of effect or have unacceptable local reactions [2]. In this report, in view of the importance of developing a hepatitis B vaccine [1], we have (a) successfully incorporated HB_sAg into liposomes [3] and (b) tested the immunogenicity of liposomal HB_sAg in guinea pigs. We found that liposomes act as powerful immunological adjuvants to the antigen.

2. Materials and methods

HB_sAg was purified from pooled high titre positive donor sera by gel filtration using Sepharose 4B (Pharmacia) followed by two successive $CsCl_2$ gradient ultracentrifugations in a Beckman SW 50L swinging rotor at 124 000 × *g* for 72 h. Following dialysis against 0.01 M sodium phosphate buffer (pH 7.2) containing 0.9% NaCl (PBS), the resulting preparation consisted of the 22 nm particles. ^{125}I-labelled HB_sAg (0.74 μCi/ml) was from the AUSAB kit (Abbott Diagnostics GmbH, Langen) and chromatographed as above before use. Dimyristoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine and sphingomyelin were purchased from Sigma Chemical Co. (London) and phosphatidylserine from Lipid Products (Surrey). The source and grade of all other lipids used are given in [4]. Multilamellar vesicles (MLV) composed of various lipids (see table 1) were made as in [4,5] using 2–3 ml PBS and 10–150 μ HB_sAg with or without radioiodinated HB_sAg to suspend the dry lipids (22–44 μmol phospholipid). The suspensions were sonicated in a bath-type sonicator for 1.5 h at 4°C. Liposome-associated HB_sAg was separated from the free antigen (a) by centrifugation at 100 000 × *g* for 30 min at 4°C. The liposomal pellet containing the antigen was resuspended in PBS and recentrifuged as above (b) through a CL-2B Sepharose (Pharmacia) column (1 × 46 cm) equilibrated with PBS. Liposomal HB_sAg was eluted from the columns with the void volume and there was very little overlapping with the subsequently eluted unentrapped antigen. In some experiments HB_sAg was entrapped in large unilamellar vesicles (LUV) prepared from phosphatidylserine [6]. Free or liposome-associated HB_sAg was measured by solid phase radioimmunoassay (AUSRIA II, Abbott Diagn. GmbH) using a standard curve made up with known concentrations of HB_sAg provided by the kit. To assay total liposomal HB_sAg it was necessary to disrupt liposomes with sodium taurocholate (Calbiochem, England) which at the concentration used (3.7 mM final) was found superior to Triton X-100, sodium dodecylsulphate and Nonidet LE in terms of liposome solubilization and non-interference (% inhibition) with the radioimmunoassay. A standard curve was

made with known concentrations of HB_sAg provided by the kit in the presence of sodium taurocholate

Outbred, female guinea pigs (Hartley strain, ~300 g) were divided into groups of 6 and injected with free or liposome-associated HB_sAg with or without *Bordetella pertussis* and saponin (British Drug House) as shown in the legends to table 2 and fig 1 Anti-HB_s titres in the sera were determined by solid phase radioimmunoassay (AUSAB, Abbott Diag GmbH). For the measurement [7] of specific delayed type hypersensitivity (DTH) response, 0 5 μg and 2 0 μg HB_sAg in PBS was injected intradermally in two areas of the interscapular region of each animal which were then compared at 48 h with a third area in the same region injected with PBS

3 Results and discussion

Judging from radioactivity measurements, a considerable proportion of the HB_sAg used was associated with MLV (15 4–47 7%) and LUV (20 2%) liposomes and radioimmunoassay measurements gave similar results (table 1) There was no consistent evidence that the nature of the phospholipid used, the molar ratios of the liposomal lipid components or the

Table 1
Incorporation of the hepatitis B surface antigen (HB_sAg) into liposomes

Composition of liposomes	Molar ratio	HB_sAg incorporated into liposomes	
		Assay of ^{125}I (%)	Radioimmuno-assay (%)
(MLV)			
PC, CHOL, DCP[a]	7 2 1	39 1 ± 10 2 (7)	43 1 ± 19 8 (7)
PC, CHOL, DCP	7 2 1	26 5 ± 11 1 (4)	
DPPC, CHOL, DCP	7 2 1	20 9 ± 15 2 (3)	
	7 3 5 1	24 5 ± 9 0 (3)	
	7 7 1	15 8	
DMPC, CHOL, DCP	7 2 1	15 8	
	7 3 5 1	15 4	
	7 7 1	42 4	
DPPE, CHOL, DCP	7 0 1	30 7	
	7 2 1	46 1	
SM, CHOL, DCP	7 2 1	34 6	
	7 3 5 1	47 7	
SM, DPPC	7 2	37 8	
SM, CHOL, DPPC	7 2 1	42 4	
	7 3 5 1	38 2	
(LUV)			
PS		20 2 ± 6 6 (5)	13 0, 10 4

[a] Liposomal HB_sAg was separated from the free antigen by molecular sieve chromatography With all other MLV preparations ultracentrifugation was used Entrapment, estimated by radioactivity measurements or radioimmunoassay, is expressed as % ± SD of the total antigen used Numbers in parentheses denote preparations made In control experiments, after equilibration of preformed MLV with 10 μg HB_sAg (mixed with a tracer) 1 3 ± 1 2% (7 expt) of the antigen was associated with chromatographically purified liposomes Latency in these preparations (% liposomal antigen not available to its antibodies) estimated by radioimmunoassay as the difference between liposomal antigen measured in the presence and absence of detergent was 63 4 ± 14 4 (7 experiments) Latency for 2 LUV preparations was nil

Abbreviations PC, egg phosphatidylcholine, DPPC, dipalmitoylphosphatidylcholine, DMPC, dimyristoylphosphatidylcholine, DPPE, dipalmitoylphosphatidylethanolamine, SM, sphingomyelin, DCP, dicetylphosphate, CHOL, cholesterol, PS, phosphatidyl serine

method of preparing liposomes influenced the extent of antigen entrapment. HB_sAg was truly incorporated (and not simply adsorbed) in MLV obtained by molecular sieve chromatography: (a) in mixing experiments in which buffer-containing preformed liposomes were equilibrated with the antigen and subsequently chromatographed, only 1.2% of the antigen was associated with liposomes (legend to table 1). In contrast, adsorption of HB_sAg onto a variety of MLV prepared by centrifugation was much greater (12–23%, not shown), (b) of the HB_sAg incorporated into chromatographed MLV, 63.4% was latent (unavailable to its antibodies) (table 1). As HB_sAg carries a lipid coat [8], it may be that the latter is interdigitated between the phospholipid molecules of the many bilayers of multilamellar liposomes through hydrophobic bonding with some or all of the antigenic sites of the HB_sAg in the outer bilayer only being available to the antibodies. This could explain the absence of latency in the HB_sAg incorporated in large unilamellar liposomes (table 1). Hydrophobic interaction of the HB_sAg with the bilayers may in fact account for the extensive association of the antigen with liposomes in spite of its large size (16–25 nm) [9].

Body weights of all immunized guinea pigs remained comparable throughout the 80 day observation period and by day 44 (after 3 injections) most animals had developed measurable anti-HB_s titres (table 2). However, judging from the humoral conversion rates, some groups responded much earlier than others. Animals treated with liposomal HB_sAg with and without the adjuvants *B. pertussis* [2] or saponin [10] (pooled groups IV–VI) exhibited an earlier conversion rate than respective animals treated with the free antigen (pooled groups I–III). The difference, which was highly significant ($X_4^2 = 22.1, P < 0.005$), could be attributed to liposomal entrapment per se: when conversion rates in groups of guinea pigs injected with *B. pertussis* (pooled groups II and VI), saponin (pooled groups II and V) or the antigen alone (pooled groups I and IV) were intercompared there was no significant difference ($X_8^2 = 7.01, P > 0.5$).

Analysis (Wilcoxon's test) [11] of the data from the groups immunized with liposomal HB_sAg (fig.1)

Table 2
Humoral conversion rate after immunization with HB_sAg

Group	Day 18 conversion[a]	Day 34 conversion	Day 44 conversion	Day 59 conversion
I	0/5	1/5	1/5	2/4
II	0/6	1/5	3/5	4/5
III	0/6	0/6	4/6	5/6
IV	3/6	5/5	5/5	5/5
V	1/6	5/6	6/6	6/6
VI	4/6	6/6	6/6	6/6

[a] Responders from total in the group

Animals were treated on day 0, 18, 34 and 44 with free HB_sAg alone (I) or mixed with saponin (II) or *B. pertussis* (III) and with liposomal HB_sAg alone (IV) or mixed with saponin (V) or *B. pertussis* (VI). They were bled by cardiac puncture on day 18, 34, 44 and 59. Four animals died during sampling

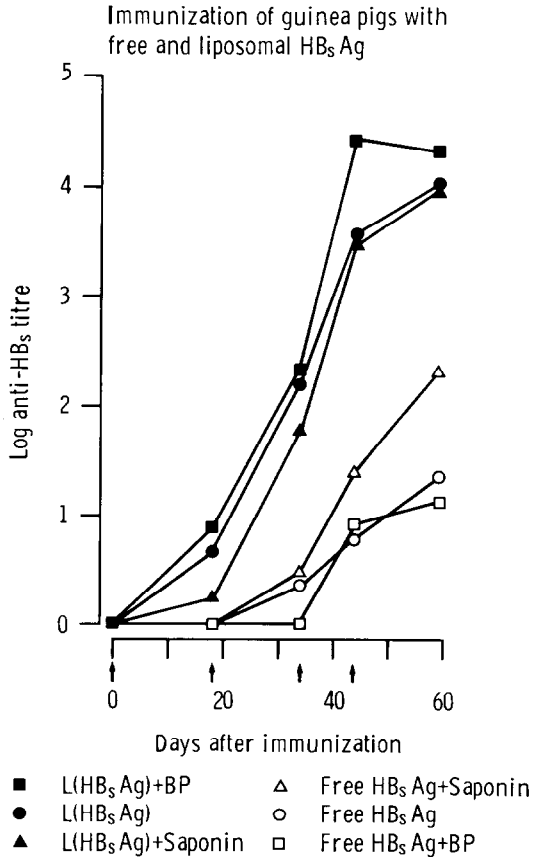

Fig.1. Rise of anti-HB_s titres in guinea pigs immunized with HB_sAg. Animals were treated (arrows) as described in table 2. Each dose (0.6 ml) given subcutaneously in both hind limbs contained 1.4 μg free or liposomal (0.8 mg lipid) HB_sAg and, when appropriate, 1.2×10^{10} bacteria or 50 μg saponin. (○) group I; (△) group II; (□) group III; (●) group IV; (▲) group V; (■) group VI.

revealed that concurrent administration of saponin did not improve titres obtained with liposomes at any of the time intervals examined and a similar conclusion could be made for *B pertussis* except for some effect on day 44 ($P < 0.025$) In addition, at no time interval was there additional effect of *B pertussis* or saponin on the titres (fig 1) obtained with the free HB_sAg Therefore, assessment of immunization was carried out by comparing all animals treated with liposomal HB_sAg (pooled groups IV–VI) with all animals treated with the free antigen (pooled groups I–III) It was found that multilamellar liposomes are a potent adjuvant to the incorporated HB_sAg producing a much steeper anti-HB_s rise with mean anti-HB_s titres being at all times significantly (Wilcoxon's test, $P < 0.001$) higher than those obtained in animals injected with the free antigen (fig 1) For instance, following the second challenge, the geometric mean titre in the groups treated with liposomal HB_sAg was 100-times, and after the third challenge 750-times higher than in the groups injected with the free antigen The ratio, however, decreased to 150-times after the fourth immunization probably because response in the groups injected with liposomes had already reached its maximum, while it was still rising (fig 1) in the groups treated with the free antigen We have recently repeated this experiment in an attempt to compare the effect of liposomes to that of other adjuvants Our results confirm the adjuvanticity of liposomes in the present report in terms of both early conversion rates and levels of antibody titres

There is strong evidence that cell-mediated immunity plays a major role in the protection against viral infections [12] Our data (table 3) show that significantly more (8 out of 16) animals injected with liposomal HB_sAg (groups IV–VI) developed DTH reactions than animals (1 out of 15) injected with free antigen (groups I–III) ($P < 0.005$) Furthermore, in contrast to the effects of other adjuvants, no local or systemic reactions, such as local inflammation, granuloma formation, haemolysis or hypersensitivity reactions occurred at any time during the 80 day period of the experiment and for several days thereafter

High titres of antibodies to HB_sAg acquired by active or passive immunization have been shown to confer immunity to natural infection with hepatitis B virus [13] A vaccine against hepatitis B will be closer to its realization when questions of safety and efficiency have been answered successfully [14] It is almost certain that because of the relatively low and aberrant immunogenicity of the HB_sAg [1,13] or its polypeptides [15], there will be a need for an adjuvant acceptable in man In this context the immunological adjuvant property of liposomes [16] which, alone or in combination with killed *B pertussis*, were shown to promote response to HB_sAg may have practical application Furthermore, the simplicity of HB_sAg incorporation into liposomes composed chiefly of cholesterol and the apparently non-toxic lecithin [17] could render such vaccine suitable for mass trials

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Table 3
Delayed-type hypersensitivity (DTH) to HB_sAg

Group treated with	Positive reaction[a]	Group treated with	Positive reaction
Free HB_sAg	0/4	L(HB_sAg)	3/5
Free HB_sAg + saponin	1/5	L(HB_sAg) + saponin	0/6
Free HB_s + *B pertussis*	0/6	L(HB_sAg) + *B pertussis*	5/5
Total animals	1/15	Total animals	8/16

[a] Animals showing DTH from total in the group

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