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A Study of the Efficacy of Liposomes in Comparison to New and Established Adjuvants in Potentiating the Antibody Response against Hepatitis B Virus Surface Antigen

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SUMMARY **The dehydration-rehydration vesicle (DRV) method was used to encapsulate hepatitis B surface antigen (HBsAg) in phosphatidylcholine (PC) and distearoyl phosphatidylcholine (DSPC) liposomes giving entrapment values of 31.7% and 33.1% respectively. A comparison of antibody levels, as determined by ELISA, in the primary and secondary immune responses in mice immunized twice with 1 μg HBsAg free, or in formulations of PC DRV, DSPC DRV, Syntex Adjuvant Formulation (SAF), alum and Freund's Complete Adjuvant (FCA) showed that by far, FCA was the best adjuvant in both the primary and secondary IgG_1, IgG_{2a} and IgG_{2b} responses. In the secondary response, apart from FCA, DSPC DRV and SAF were equally efficacious and better adjuvants than alum and PC DRV for the IgG_{2a} and IgG_{2b} subclasses. SAF was a better adjuvant for HBsAg than alum, DSPC DRV and PC DRV (in descending order of efficacy) in the secondary IgG_1 response.**

The pharmaceutical industry has long realised the need for safe and efficacious adjuvants for use in a new generation of vaccines. [1] Presently established ones like Freund's complete adjuvant (FCA), although possessing high efficacy, suffer from unacceptable adverse effects like granuloma formation which precludes their use in humans. The only adjuvants currently licenced for use in most countries are the aluminium salts (alum) but their drawbacks are the inadequate stimulation of cell-mediated immunity and sub-optimal stimulation of the antibody response. [2] Active research is thus taking place to develop adjuvants that have none of the deficiencies of their predecessors. With regard to the immunogens, there is a trend towards use of subunit vaccines extracted from viruses or produced by recombinant DNA technology and synthetic peptides. Many of these antigens are relatively weak immunogens so the requirement for an effective adjuvant formulation is even more critical.

Some 200 million people in the Western Pacific region are carriers of the hepatitis B virus (HBV). Cirrhosis and hepatocellular carcinoma are strongly associated with persistent HBV infections. [3] Vaccines presently in use consist of hepatitis B virus surface antigen (HBsAg) adsorbed to alum. [4] The HBsAg is purified from plasma of hepatitis virus carriers or produced by recombinant DNA technology. Three doses of the alum-adjuvanted vaccines elicit protective antibody responses in 90-95% of normal adults in North America and Europe. [5] The sero-conversion rate is much lower in some groups of people especially susceptible to HBV infection, such as intravenous drug users and promiscuous homosexuals. For all HBV immunizations, it would be convenient to reduce the amount of antigen required, and to obtain protective immunity with two rather than three doses of vaccine.

Since the discovery that liposomes function as adjuvants to entrapped diphtheria toxoid, [6] hope arose that liposomes could eventually be clinically used as versatile, non-toxic carriers in vaccines. The appeal of liposomes lies in the eminent amenability to alteration of their bilayer characteristics, enabling parameters such as hydrophobicity, fluidity, charge, size, surface-associated molecules and encapsulated

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material to be varied at will. Other adjuvants such as muramyl dipeptide (MDP) or interleukin-2 may be co-entrapped with the antigen in an effort to further boost adjuvanticity. Syntex adjuvant formulation (SAF) which consists of an emulsion of squalane and a Pluronic polymer in saline has been shown to be efficacious with several antigens in various animal species.[7] In this study, a comparison of the efficacy of the various experimental and established adjuvants in stimulating the immune response to HBsAg is made.

MATERIALS AND METHODS

Entrapment of HBsAg in dehydration-rehydration vesicles (DRV liposomes)

Equimolar egg PC or DSPC (Lipoid), and cholesterol (32 μmoles) (British Drug Houses, UK) were mixed in a 50 ml round-bottomed flask (Quickfit). The lipids were dried to a thin film by evaporation of the solvent at a low speed in a rotary evaporator (Buchi) connected to a running tap water pump. Two ml of phosphate-bufferred saline (PBS, pH 7.4) was added to the dried lipid film. The flask was lowered into a bath sonicator (Kerry, UK) and subjected to bursts of sonication while being manually rotated to form MLV. To convert MLV into small unilamellar vesicles (SUV), sonication of the MLV samples using a probe sonicator (MSE) was performed at room temperature.[8] One ml of the SUV formed were mixed with 1 ml of ^{125}I-labelled recombinant hepatitis B virus surface antigen (HBsAg) (gift from Dr. G M Dusheiko, Academic Medicine, Royal Free Hospital, London, UK) at a concentration of 200 μg/ml water to generate DRV liposomes by overnight lyophilization followed by two-step rehydration with 0.1 ml distilled water and 0.9 ml PBS (pH 7.4).[9] The amount of HBsAg encapsulated in DRV was determined by measuring ^{125}I radioactivity in the pellets after washing three times in 8 ml PBS by centrifugation at 10,000 × g for 30 min.

Preparation of HBsAg-SAF vaccine

A 2-times concentration of SAF emulsion (Syntex Research, Palo Alto, California, USA) was prepared by emulsifying 10% (v/v) squalane and 5% (v/v) Pluronic L121 in pH 7.2 PBS containing 0.4% Tween 80. The vehicle components were completely emulsified so that no residual Pluronic L121 remained unincorporated. A solution containing a 2-times concentration of HBsAg and threonyl-MDP was prepared in PBS. The 2-times HBs-Ag-threonyl-MDP solution was then added to an equal volume of the 2-times SAF emulsion and mixed gently. The resulting vaccine had the appearance and consistency of milk and was injected through a 25 gauge needle.

Animal immunization experiments

BALB/c mice in groups of five were immunized intramuscularly twice (with a 28-day interval between injections) with 0.1 ml containing 1 ug of HBsAg either free or in the following adjuvant formulations: 1) thoroughly mixed with an equal volume of FCA, 2) adsorbed onto alum (Engerix B, from Smith Kline and French) 3) PC DRV and 4) DSPC DR. Blood samples were obtained from the tail veins 1 day before and every 14 days after administration of the vaccine formulations. Serum samples were kept at -20°C until they were assayed for anti-HBsAg antibodies by the enzyme-linked immunosorbent assay (ELISA),[10] using an antigen concentration of 10 μg of HBsAg/ml carbonate-bicarbonate buffer (pH 9.6) to coat the wells of the microtitre plates.

RESULTS

To encapsulate HBsAg in multilamellar liposomes composed of equimolar phospholipid and cholesterol, the DRV method was used. Table 1 shows the percentage entrapment values of HBsAg in PC and DSPC DRV.

Fig. 1 compares the efficacies of the various adjuvant formulations in boosting the primary and secondary immune responses against HBsAg while Table 2 shows the mean serum IgG_1, IgG_{2a} and IgG_{2b} titers obtained in the secondary

Table 1. Entrapment of HBsAg in DRV liposomes.
HBsAg was entrapped in DRV liposomes generated from SUV liposomes composed of equimolar phospholipid and cholesterol. Entrapment values shown are the means of two separate preparations.

DRV composition	Total HBsAg added (μg)	% entrapped	μg HBsAg/μmol phospholipid
PC:Chol	200	31.7	3.96
DSPC:Chol	200	33.1	4.14

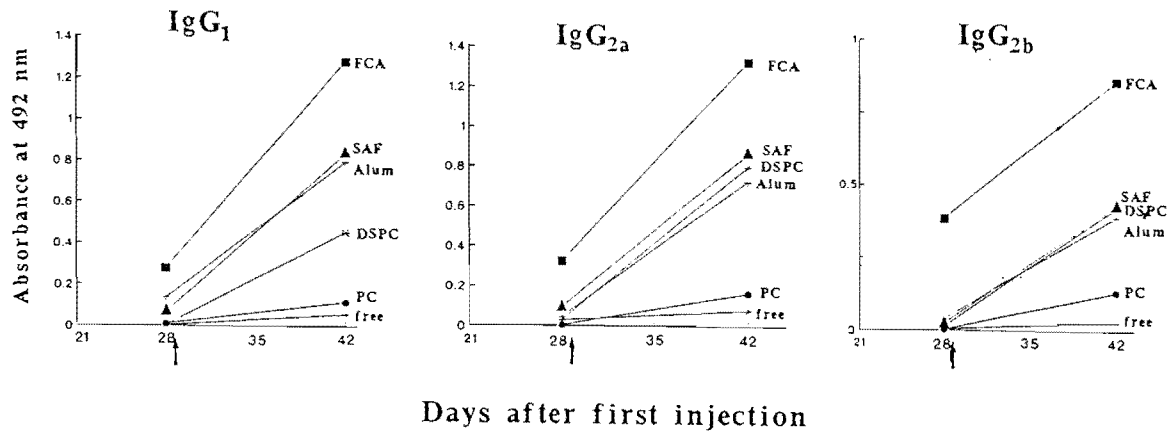

Fig. 1 Primary and secondary antibody responses to HBsAg in various adjuvant formulations. BALB/c mice in groups of five were injected intramuscularly twice (with a 28-day interval between injections) with 1 μg HBsAg free (×) or in formulations of PC DRV (•), DSPC DRV (*), alum (+), SAF (▲) and FCA (■). Animals were bled just before and 14 days after each injection. Anti-HBsAg IgG_1, IgG_{2a} and IgG_{2b} responses were measured in sera by ELISA. The arrow indicates the day on which the booster injection was given.

response for the best liposomal formulation i.e. DSPC DRV. These are compared to alum, the only adjuvant in clinical use, and to free HBsAg.

Table 2. Titration of the secondary antibody response to HBsAg formulated with various adjuvants

Sera obtained from mice immunized with HBsAg free, or formulated with alum or in DSPC DRV were titrated by doubling dilutions. Results are expressed as the means in each group of the highest titer of serum that yielded an optical density of 0.200 as determined by ELISA

Antibody isotype	Mean serum titre		
	Alum	DSPC DRV	Free
IgG_1	4800	667	67
IgG_{2a}	1633	2283	283
IgG_{2b}	110	150	15

DISCUSSION

Encapsulation of HBsAg in PC and DSPC DRV

The original method for the formulation of multilamellar vesicles devised by Bangham [11] suffered from the disadvantage of low encapsulation efficiencies. Later work which uncovered new liposomal morphologies such as the large unilamellar vesicle (LUV) overcame the initial problem of poor entrapment efficiency but introduced new conditions, which may be detrimental to the conformation of critical epitopes, such as subjection of the antigen to contact with organic solvents, [12] high temperatures or sonication.

A relatively recently devised method [9] circumvents all the aforementioned problems, with the added advantage of being easily amenable to industrial scale-up. The DRV method is a gentle one where SUV, which may be preformulated and stored for prolonged periods at 4°C, are mixed with the antigen to be entrapped as and when required and then freeze-dried. Subsequent two-step rehydration results in the encapsulation of large amounts of the antigen by the phospholipid bilayers which were flattened and put into intimate contact with the antigen in the lyopholization process.

Using this method, 31.7% and 33.1% of the total amount of HBsAg added to the SUV preparation were encapsulated in PC and DSPC DRV respectively. These values are similar

to the percentage entrapment values of many predominantly hydrophilic solutes such as tetanus toxoid[9,10] for the concentration of phospholipid used in the formation of SUV. Preliminary data (unpublished) obtained while trying to entrap tumour necrosis factor-beta in DRV indicates that one can raise encapsulation efficiencies to close to 100% by raising the amount of phospholipid used in the initial formulation of MLV to the maximum concentration permissible for these structures to form. There is no doubt that this strategy would also encapsulate much greater amounts of HBsAg but others have shown that the antigen to lipid ratio employed in liposomal vaccines is instrumental in determining the magnitude of the antibody response and that no great advantage, apart from reducing antigen loss, would be obtained from injecting a higher antigen to lipid ratio in terms of eventual antibody formation.

Primary and secondary antibody responses to various adjuvant formulations containing HBsAg

A comparison of the magnitudes of the primary and secondary IgG_1, IgG_{2a} and IgG_{2b} responses to HBsAg either free or formulated with various adjuvants was made by ELISA on serum samples taken from immunized BALB/c mice. What is most evident in the comparison is the fact that FCA was by far the best adjuvant for HBsAg in both the primary and secondary responses for all the antibody subclasses studied. This is not surprising as FCA has been the adjuvant of choice in animal immunization protocols for many decades, being able to stimulate both humoral and cellular immunity well. However, because of its tendency to form persistent granulomas which may ulcerate, its clinical use is out of the question.

As all immunization protocols require at least two injections to produce long-lasting immunity, the secondary response is much more important and will be dealt with here. It was found that SAF and DSPC DRV performed equally well, with the DSPC DRV formulation yielding titers about ten times greater than those obtained for the control free antigen in the IgG_{2a} and IgG_{2b} subclasses (Table 2), proving themselves to be as effective as alum and much better than PC DRV for the dose of HBsAg employed. The efficacy of DSPC and PC liposomes in relation to each other may initially seem to be at odds with data obtained with tetanus toxoid as the model antigen,[10] but our recent study using influenza virus A/Sichuan surface antigen entrapped in PC and DSPC DRV in a range of doses revealed that in the secondary response, DSPC DRV were more effective adjuvants for low doses of the antigen whilst PC DRV surpass the efficacy of DSPC DRV for high concentrations of encapsulated antigen (unpublished data). For IgG_1, SAF was seen to be better than alum and both forms of DRV. It must be born in mind, though, that SAF is a composite formulation,[7] consisting of squalane, Pluronic L121 and threonyl-MDP each of which have strong adjuvant activity on their own whereas liposomes are merely composed of phospholipids and cholesterol which have no adjuvanticity before formulation into three-dimensional structures that entrap the antigen. Thus, liposomes have immense potential in the sense that other adjuvants may be co-entrapped in their aqueous compartments, charged lipids may be incorporated into their bilayers or ligands may be grafted onto their surfaces for targeting to antigen-presenting cells such as macrophages. These approaches have already been shown to be successful for interleukin-2,[13] a novel positively-charged lipid BisHOP[14] and mannose,[15] respectively. If combined in the same formulation to synergise the adjuvant effect of liposomes on HBsAg, these composite DRV may well prove to be superior to SAF.

In a recent study[16] using bovine serum albumin as the model antigen, the immunoadjuvant action of PC DRV was compared with that of a variety of other adjuvants. Antibody levels in both the primary and secondary responses were similar for DRV (with or without co-entrapped threonyl-MDP) and alum. Moreover, significantly higher responses were obtained with DRV without threonyl-MDP (primary) and DRV with threonyl-MDP (primary and secondary) compared to those seen with FCA and threonyl-MD alone. These results lend further evidence to the comparable efficacy of liposomes as compared to well-established adjuvants.

In view of the active ongoing research in adjuvant and vaccine development, more studies to compare the efficacies of the various new adjuvant formulations with established industry standards are required. This would complement data on each new adjuvant formulation studied on various disease entities in isolation. This paper has preliminarily evaluated two new adjuvants, namely DRV liposomes and SAF in comparison to alum and FCA with respect to their efficacy in boosting the antibody response against HBsAg. Results show that the new adjuvants perform well in comparison to the most widely used clinical adjuvant, alum. Thus, they hold great promise in fulfilling the need for a new generation of safe and effective adjuvants.

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