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The subclass IgG responses of mice to influenza surface proteins formulated into liposomes

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Summary

Unprimed mice and mice primed by prior infection with an H1N1 subtype of influenza virus were used to assess the total and subclass IgG serum antibody responses to influenza virus A/Sichuan/2/87 (H3N2) surface haemagglutinin and neuraminidase proteins incorporated into four different formulations of liposomes. Only one of these liposome preparations, DSPC(B), induced greater total IgG, and subclass IgG1 and IgG2a antibody levels, in sera from both primed and unprimed mice than the aqueous A/Sichuan surface preparations alone administered at equivalent levels of haemagglutinin protein. The same DSPC(B) liposome formulation of A/Sichuan antigens was also the only preparation found to elicit levels of IgG2b and IgG3 subclass antibodies above baseline values in these animals.

Influenza surface proteins; Liposomes; IgG Responses

Introduction

Extensive studies over the last two decades have reported the immune responses to influenza virus vaccines given as whole virus, split virus, or purified, aqueous subunit preparations to experimental animals and man, and

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have determined the value of these vaccines in inducing antibodies and protection from subsequent infection and disease (Virelizier, 1975; Potter, 1982; Johnson et al., 1985). Thus, whole virus vaccines are more reactive than split or subunit vaccine, but are only marginally more immunogenic (Hilleman, 1977; Tyrrell et al., 1981). In times of antigenic drift, the immune responses to the various types of vaccines are approximately equal; however, in all cases immunity to subsequent infection is seen in only 60–90% of volunteers (Meyer et al., 1978), and this level of immunity is disappointingly low. Recent research has aimed to improve the immune response to influenza virus vaccines, and one approach to the induction of higher antibody responses has been to use virus antigens together with adjuvants as carrier systems. The inclusion of alhydrogel has been shown to offer little advantage compared to aqueous subunit vaccines (Potter et al., 1975; Potter et al., 1977; Nicholson et al., 1979); but other, recently developed carrier systems including muramyl dipeptide or derivatives of this material (Allison and Byars, 1986 and 1987), immunostimulating complexes (Morein et al., 1984; Hoglund et al., 1989) and liposomes (Allison and Gregoriadis, 1974; Gregoriadis, 1990) are reported to amplify antibody responses.

The present study reports the immune responses of both primed and unprimed mice to influenza virus haemagglutinin antigen as a subunit preparation encapsulated in four different liposome formulations. Antibodies elicited in mice to these liposome preparations, and as a comparison, to the aqueous influenza virus subunit preparation from which they were derived, were detected by haemagglutination-inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) tests. In addition, the IgG subclass antibody responses of the mice to these preparations was determined, also using an ELISA technique. The study is part of an investigation to compare adjuvant and carrier systems which could be used to enhance the immune response to influenza virus vaccines in man.

Materials and Methods

Virus and virus antigen preparations

Influenza virus A/Sichuan/2/87 (H3N2), kindly supplied by Dr. J.J. Skehel (World Health Influenza Centre, Mill Hill, London), was inoculated into the allantoic sac of 10–11 day embryonated hens' eggs. After 72 h incubation at 33°C, the allantoic fluids were harvested, clarified by centrifugation at 2000 rpm for 15 min, concentrated by ultracentrifugation for 2 h at 15 000 rpm, and the pellet resuspended to approximately 10% of the original volume in phosphate-buffered saline pH 7.2 (PBS). The virus was purified by centrifugation through a sucrose gradient (10–60% sucrose in PBS) for 90 min at 15 000 rpm, gradient fractions with peak haemagglutination (HA) titres were pooled, centrifuged at 25 000 rpm for 2 h, and the resulting deposits suspended in PBS to give a pool of purified virus.

Subunit antigen preparation. Purified virus was disrupted by adding Empigen BB to a final concentration of 0.5% (Crawford et al., 1984; Jennings et al., 1987) and incubating with continual agitation for 18 h at room temperature (RT). After this time, the preparation was centrifuged at 20 000 rpm for 1 h and the supernatant dialysed against PBS for 3 days at 4°C.

Liposome formulations. Four different formulations of liposomes were tested: these were composed of egg phosphatidylcholine (PC) and cholesterol (1:1 molar ratio) (PC liposomes), PC, cholesterol and 1,2-bis(hexadecylcycloxy-3-trimethylaminopropane (BisHOP) (1:1:0.25 molar ratio) (PC (B) liposomes), distearoyl phosphatidylcholine (DSPC) and cholesterol (1:1 molar ratio) (DSPC liposomes) and DSPC, cholesterol and BisHOP (1:1:0.25 molar ratio) (DSPC(B) liposomes). Entrapment of influenza A/Sichuan surface proteins in dehydration-rehydration vesicles (DRV liposomes) which are multilamellar vesicles of mixed sizes (mean diameter in microns: 0.30 ± 0.25) was carried out as described elsewhere (Kirby and Gregoriadis, 1984; Gregoriadis et al., 1987). In brief, 32 μmol of PC (Lipid Products, Redhill, Surrey) or DSPC (Sigma, London) and 32 μmol of cholesterol (British Drug Houses, Poole, Dorset) supplemented when appropriate with 8 μmol of BisHOP (kindly supplied by Syntex Research, Palo, Alto, CA) were dissolved in chloroform. The thin film which formed in the walls of the flask after rotary evaporation at 20°C in a water bath was dispersed with 2.0 ml of distilled water at 20°C (PC) or 58°C (DSPC liposomes) and subjected to bath sonication. This was followed by probe sonication at the respective temperatures with a titanium probe to produce small unilamellar vesicles (SUV) (Kirby and Gregoriadis 1984). After centrifugation at 3000 rpm for 5 min to remove titanium fragments and large liposome aggregates, the supernatants containing the SUV were used to generate DRV liposomes: each supernatant was mixed with 4.0 ml of influenza A/Sichuan subunits (60 μg HA protein/ml) prepared as described above and mixtures were freeze-dried over a 36 h period. The dried liposomal influenza subunit preparations were suspended in 0.1 ml distilled water at 20°C (PC) or 58°C (DSPC liposomes) and allowed to stand for 30 min at the same temperature. A further 3.9 ml distilled water was subsequently added. After 2 h, the resulting DRV suspensions were each centrifuged twice at 20 000 rpm for 30 min in a L-55 Beckman preparative ultracentrifuge. The pellets were then suspended into PBS pH 7.4 containing 0.02% sodium azide and assayed for total protein (Lowry et al., 1951). Table 1 shows that approximately equivalent amounts of HA are entrapped in the different liposome preparations, and that each preparation contains roughly similar quantities of HA per μmol of phospholipid.

Animals. Female male Balb/c mice aged 6–10 weeks from a closed colony at the University of Sheffield were primed by inoculation with $10^{3.0}$ infective doses (ID_{50}) of A/FM/1/47 (H1N1) virus 3 weeks prior to immunisation; random serum samples from these animals all showed HI antibody to the priming virus.

TABLE 1

Entrapment of influenza A/Sichuan proteins into DRV liposomes

DRV Composition	% Entrapped HA (% of the amount used)	μgHA/μmol Phospholipid
PC	46.8 + 1.9 (2)	0.85
PC(BisHOP)	42.1 + 0.4 (2)	0.77
DSPC	42.5 + 3.5 (2)	0.77
DSPC(BisHOP)	37.2 + 1.3 (2)	0.68

DRV liposomes composed of 32 μmol PC or DSPC and equimolar cholesterol, supplemented where appropriate with BisHOP (molar ratio 1:1:0.25) were prepared in the presence of influenza A/Sichuan antigens (60 μg HA/ml).

Primary immunisation was by intramuscular inoculation of 5 or 0.5 μg of viral subunit antigens as an aqueous preparation or as the various liposome formulations, in 0.1 ml vol. Mice were bled at 19 days, and 2 days later all received a booster dose of 0.5 μg virus subunit antigens in an 0.1 ml vol. of the same liposome formulation, or as an aqueous preparation. Animals were bled 35 days following this second immunisation. All sera were stored at −20°C.

Serological tests

(a) Haemaglutination-inhibition (HI) antibody assay. HI tests were performed on microtitre plates (Sterilin, Middlesex), as described previously (Jennings et al., 1981). Briefly, sera were treated with receptor destroying enzyme (RDE) kindly supplied by Dr. J. Wood, National Institute of Biological Standards and Control, South Mimms, Hertfordshire, at 37°C for 18 h, then heated at 56°C for 30 min to remove non-specific inhibitors. Serial 2-fold serum dilutions in 25 μl vol. mixed with an equal volume of 8 HA units of influenza virus, were kept at RT for 30 min; 25 μl of 0.5% of fowl red blood cells (RBC) were added to each well, and the plates left at RT for the cells to settle. HI titres were taken as the reciprocal of the highest serum dilution completely inhibiting haemagglutination.

(b) ELISA test. The immune response induced by each liposome antigen preparation and by the aqueous subunit preparation was assayed using an ELISA (Jennings et al., 1981; Davis and Gregoriadis, 1987). Briefly, microtitre plates (Nunc Live Technology, Paisley, Glasgow) were coated with 200 μl per well of purified influenza virus antigens diluted in carbonate buffer (pH 9.6), and incubated for 18 h at 4°C. The plates were washed 3 times with PBS containing 0.1% Tween 20 (PBS-T), and 100 μl of a single serum dilution (determined by previous titration) diluted in PBS-T containing 1% (v/v) bovine serum albumin, added in triplicate wells and incubated for 1 h at 37°C. After 3 cycles of washing with PBS-T, plates were incubated for 1 h at 37°C with 100 μl of diluted horse radish peroxidase-conjugated goat antibody directed against mouse IgG or specific mouse IgG subclass. The plates were again washed 3 times and developed by adding 100 μl vol. of 0.01 M citrate buffer (pH 5.0)

containing 0.4 mg/ml^{-1} of o-phenylenediamine and 30% H_2O_2 for 20–30 min at RT. The absorbance was read at 492 nm in a Microelisa Autoreader (Anthos Labtec Reader 2001, Denby Instruments Ltd., Billinghurst, Sussex). A number of negative sera were included with each test, and the mean of the results with these sera (+ 3 S.D.) was taken as a cut off value distinguishing between positive and negative results as ELISA absorbance values for each IgG subclass. The statistical significance of the differences between comparable groups was assessed by the one-tailed Mann-Whitney U-test.

Results

Correlation between HI antibody levels and ELISA absorbance values in mouse sera

To determine the correlation between HI antibody levels and the absorbance values by ELISA using gradient-purified whole A/Sichuan/87 influenza virus as antigen, 197 post-immunization or post-infection mouse sera selected at random were tested using both procedures. The results (Fig. 1) indicate a good correlation between the two tests with a correlation co-efficient value (r) of 0.69.

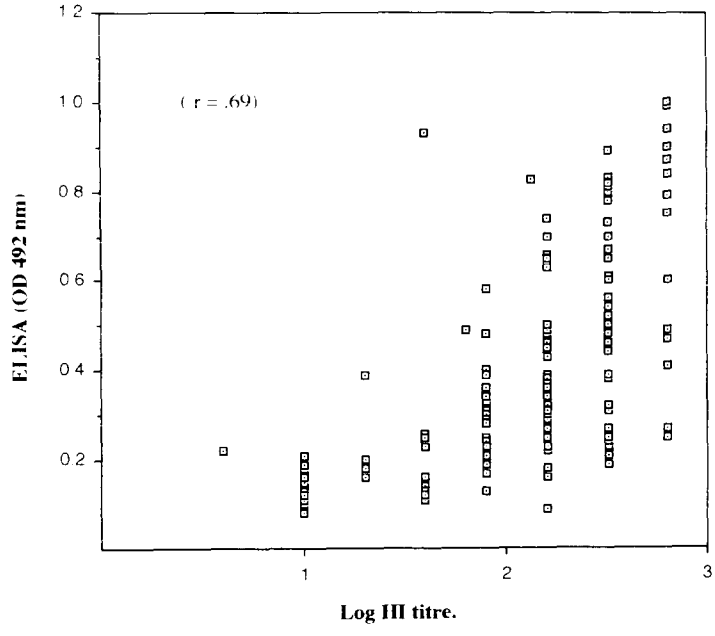

Fig. 1. Correlation between HI titre levels and ELISA absorbance values in sera of mice immunised with influenza virus antigens. (Correlation coefficient, $r = 0.69$).

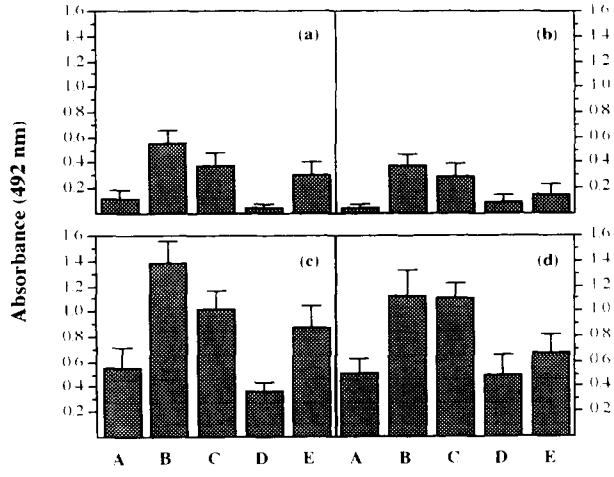

Fig. 2. Serum IgG responses of unprimed mice (8 per group) immunised with influenza subunit antigen preparations in an aqueous form or incorporated into liposomes. (2a and 2c) – 19 days following initial immunisation with 5.0 μg (a), and 35 days following booster immunisation with 0.5 μg (c), of antigen preparation. (2b and 2d) – 19 days following initial immunisation with 0.5 μg (b), and 35 days following booster immunisation with 0.5 μg (d), of antigen preparation. A. DSPC liposomes; B. DSPC(B) liposomes; C. aqueous subunit antigen preparation; D. PC liposomes; E. PC(B) liposomes.

Total serum IgG antibody response to influenza subunit vaccines

The serum IgG antibody response of primed and unprimed mice immunised with aqueous subunit antigen preparation, or subunits incorporated into one of the various forms of liposomes is shown in Figs. 2 and 3. Of the 8 unprimed animals given a single, 0.5 μg, dose of aqueous preparation, 7 developed low levels of IgG antibody detectable by ELISA, with a mean absorbance reading of 0.29 (Fig. 2b); all animals given a single 5.0 μg dose of the same preparation developed higher levels of IgG antibody with a mean absorbance of 0.38 (Fig. 2a). All mice given A/Sichuan subunits formulated into DSPC(B) liposomes, at doses of 5.0 μg or 0.5 μg developed IgG antibody responses, with mean absorbances of 0.55 and 0.38, respectively (Figs. 2a and b). The value of 0.55 was significantly greater ($P<0.01$) than that of 0.38, observed in mice given the aqueous A/Sichuan preparation. The IgG antibody incidence and mean ELISA absorbances in sera from unprimed mice given PC or DSPC influenza antigen liposome formulations were significantly lower ($P<0.01$) than those observed in animals given DSPC(B), PC(B) or aqueous A/Sichuan subunits, while in turn, the IgG antibody levels elicited by the DSPC(B) liposome preparation were significantly greater ($P<0.01$) than those induced by either the PC(B) liposomes or the aqueous preparation (Figs. 2a and b).

Following re-inoculation with 0.5 μg of the same preparation as that given initially, an IgG antibody response measurable by ELISA, was observed in all unprimed mice (Figs. 2c and d). A second immunisation with 0.5 μg of A/Sichuan DSPC(B) liposomes in animals previously given 5.0 μg of this

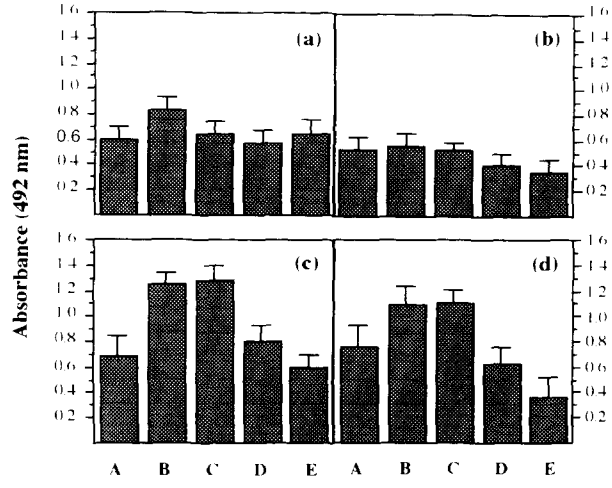

Fig. 3. Serum IgG responses of primed mice (8 per group) immunised with influenza subunit antigen preparations in aqueous form or incorporated into liposomes. (3a and 3c) – 19 days after initial immunisation with 5.0 μg (a), and 35 days following booster immunisation with 0.5 μg (c), of antigen preparation. (3b and 3d) – 19 days following initial immunisation with 0.5 μg (b), and 35 days following booster immunisation with 0.5 μg (d), of antigen preparation. A. DSPC liposomes; B. DSPC(B) liposomes; C. aqueous subunit antigen preparation; D. PC liposomes; E. PC(B) liposomes.

preparation resulted in a mean absorbance value of 1.38 (Fig. 2c); booster immunisation of animals given 5.0 μg of the aqueous A/Sichuan subunits with 0.5 μg of the same preparation elicited a final mean ELISA absorbance of 1.05 (Fig. 2c), significantly lower ($P<0.01$) than that elicited by 2 similar doses of A/Sichuan DSPC(B) liposomes. The mean values elicited by both these preparations were significantly greater ($P<0.01$) than those observed in sera from animals given two doses of PC, PC(B) or DSPC liposome preparations. The results in animals receiving two 0.5 μg doses of the various A/Sichuan antigen preparations (Fig. 2d) showed a similar pattern to that described above; however, the mean ELISA absorbances in mice given either the A/Sichuan DSPC(B) liposomes or the aqueous A/Sichuan antigen preparations were not significantly different, although both were significantly greater ($P<0.01$) than those elicited by all other preparations.

Essentially similar results were obtained in mice previously primed by inoculation with live A/FM1 influenza virus, although all animals produced antibody detectable by ELISA after a single immunisation. Thus, the greatest antibody responses were elicited following initial immunisation with 5.0 μg of the DSPC(B) liposome formulation, and by both DSPC(B) liposomes and the aqueous subunit preparation following booster immunisation (Figs. 3a–d). These responses were significantly ($P<0.01$) greater than the responses elicited by all other liposome formulations tested. However, there were no significant differences between the ELISA antibody responses induced by A/Sichuan DSPC(B) liposomes and the aqueous A/Sichuan antigen preparation.

TABLE 2

Serum IgG subclass antibody responses to influenza subunit antigen preparations in unprimed mice

Immunising material	No. of mice	Dose (μg protein)	19 days following 1st immunisation. Mean IgG subclass ($\pm$ S.D.)				Dose (μg protein)	35 days following 2nd immunisation. Mean IgG subclass ($\pm$ S.D.)			
			IgG1	IgG2a	IgG2b	IgG3		IgG1	IgG2a	IgG2b	IgG3
DSPC liposomes	8	5	0.11*(0.02)	0.1	0.1	0.1	0.5	0.28(0.1)	0.21(0.09)	0.16(0.06)	0.15(0.08)
DSPC(B) liposomes	8	5	0.39(0.08)	0.16(0.02)	0.12(0.02)	0.1	0.5	0.53(0.16)	0.80(0.16)	0.18(0.01)	0.18(0.09)
Aqueous subunit antigens	8	5	0.26(0.03)	0.16(0.02)	0.1	0.1	0.5	0.38(0.12)	0.48(0.12)	0.1	0.1
PC liposomes	8	5	0.09(0.01)	0.11(0.01)	0.1	0.1	0.5	0.16(0.06)	0.24(0.05)	0.1	0.1
PC(B) liposomes	8	5	0.19(0.04)	0.13(0.3)	0.1	0.1	0.5	0.59(0.1)	0.48(0.09)	0.13(0.06)	0.1
DSPC liposomes	8	0.5	0.1	0.1	0.1	0.1	0.5	0.3(0.12)	0.24(0.1)	0.18(0.04)	0.16(0.7)
DSPC(B) liposomes	8	0.5	0.31(0.05)	0.23(0.03)	0.1	0.1	0.5	0.45(0.1)	0.65(0.09)	0.20(0.08)	0.26(0.7)
Aqueous subunit antigens	8	0.5	0.22(0.03)	0.17(0.01)	0.1	0.1	0.5	0.5(0.1)	0.58(0.2)	0.1	0.1
PC liposomes	8	0.5	0.1	0.1	0.1	0.1	0.5	0.16(0.04)	0.31(0.02)	0.1	0.1
PC(B) liposomes	8	0.5	0.13(0.05)	0.1	0.1	0.1	0.5	0.52(0.02)	0.34(0.04)	0.12(0.02)	0.12(0.01)

*ELISA absorbance values.

Serum IgG subclass antibody response to aqueous and liposomal influenza subunit antigen preparations

All sera from both primed and unprimed mice were tested for IgG subclass antibody by ELISA. The results, expressed as ELISA absorbance values, are shown in Table 2 for unprimed mice and Table 3 for primed mice.

With respect to the subclass antibody responses of unprimed mice (Table 2), the greatest responses in both IgG1 and IgG2a subclasses were observed in animals immunised with the A/Sichuan DSPC(B) liposome formulation, and this reflects the results observed for total IgG. As expected, the IgG1 and IgG2a subclass responses of unprimed mice following two doses of the various preparations, were increased compared to those observed in mice with a single dose. In addition, following two doses of DSPC(B) liposomes the ELISA IgG2a antibody level was significantly greater ($P<0.0l$) than those induced by all other preparations. Following two doses, the IgG1 levels induced in mice immunised with either DSPC(B) or PC(B) liposomes, were significantly greater ($P<0.01$) then the IgG1 levels induced by DSPC or PC liposomes. The aqueous preparation also induced significantly greater ($P<0.01$) antibody responses in the IgG1 and IgG2a subclasses than the DSPC or PC liposome formulations. Mean positive ELISA absorbances, detecting antibody in the IgG2b and IgG3 subclasses, were generally only found in sera after booster inoculation, and the highest titre in sera from mice immunised with antigens in the DSPC(B) liposome formulation (Table 2). No responses in these subclasses could be detected following immunisation of unprimed mice with aqueous antigen preparation.

The serum IgG subclass antibody responses of primed mice to the various influenza antigen preparations (Table 3) indicate that these animals responded with generally greater IgG1 and IgG2a subclass levels, than those found in unprimed mice following a single immunisation. There was also less discrimination in antibody levels induced, between the various influenza liposome antigen formulations and the aqueous subunit preparation, after primary immunisation. Nevertheless, the DSPC(B) and DSPC liposome formulations and the aqueous subunits induced significantly ($P<0.01$) greater levels of IgG1 and IgG2a subclass antibodies than the PC(B) or PC influenza antigen liposome formulations following a single 0.5 μg dose. In addition, the DSPC(B) liposome formulation promoted a significantly greater ($P<0.02$) level of IgG2a antibody than aqueous A/Sichuan subunit antigens in primed mice given 5.0 μg doses of these preparations. As in unprimed mice, there were only few and low serum antibody responses in the IgG2b and IgG3 subclasses after the initial immunisation of primed mice (Table 3).

Following booster immunisation of primed mice with 0.5 or 5.0 μg of the various preparations, the responses in both IgG1 and IgG2 subclasses were considerably increased, and the mean ELISA absorbance values as a measure of antibody level elicited by the DSPC(B) influenza antigen liposome formulation or the aqueous antigen preparation in both these subclasses, was significantly greater ($P<0.01$) than those induced by all other preparations

TABLE 3

Serum IgG subclass antibody responses to influenza subunit antigen preparations in primed mice

Immunising material	No. of mice	Dose (μg protein)	19 days following 1st immunisation. Mean IgG subclass ($\pm$ S.D.)				Dose (μg protein)	35 days following 2nd immunisation. Mean IgG subclass ($\pm$ S.D.)			
			IgG1	IgG2a	IgG2b	IgG3		IgG1	IgG2a	IgG2b	IgG3
DSPC liposomes	8	5	0.20*(0.03)	0.24(0.03)	0.1	0.1	0.5	0.39(0.12)	0.31(0.2)	0.12(0.2)	0.16(0.09)
DSPC (B) liposomes	8	5	0.32(0.04)	0.38(0.06)	0.15(0.03)	0.1	0.5	0.70(0.12)	0.81(0.14)	0.27(0.3)	0.27(0.02)
Aqueous subunit antigens	8	5	0.21(0.05)	0.24(0.06)	0.1	0.1	0.5	0.59(0.13)	0.69(0.15)	0.16(0.03)	0.1
PC liposomes	8	5	0.20(0.02)	0.28(0.03)	0.1	0.1	0.5	0.49(0.02)	0.58(0.03)	0.15(0.13)	0.12(0.03)
PC(B) liposomes	8	5	0.24(0.06)	0.22(0.06)	0.1	0.1	0.5	0.24(0.1)	0.22(0.12)	0.13(0.02)	0.14(0.03)
DSPC liposomes	8	0.5	0.30(0.04)	0.24(0.05)	0.1	0.1	0.5	0.34(0.01)	0.26(0.03)	0.1	0.13(0.03)
DSPC(B) liposomes	8	0.5	0.32(0.04)	0.24(0.05)	0.1	0.1	0.5	0.47(0.03)	0.48(0.13)	0.21(0.03)	0.18(0.03)
Aqueous subunit antigens	8	0.5	0.31(0.05)	0.28(0.04)	0.12(0.03)	0.1	0.5	0.54(0.03)	0.59(0.19)	0.12(0.03)	0.1
PC liposomes	8	0.5	0.18(0.01)	0.15(0.02)	0.1	0.1	0.5	0.40(0.03)	0.54(0.24)	0.13(0.01)	0.1
PC(B) liposomes	8	0.5	0.17(0.03)	0.17(0.03)	0.12(0.02)	0.1	0.5	0.21(0.05)	0.21(0.1)	0.12(0.06)	0.1

*ELISA absorbance values.

with respect to mice given the 5.0, followed by 5.0 μg immunisation schedule. With respect to the IgG1 responses of these animals, the DSPC(B) liposomes again induced a significantly ($P<0.05$) greater antibody response than the aqueous preparation. The results in Table 3 show that the subunit aqueous influenza virus antigen preparation, and both the DSPC(B) and PC influenza antigen liposome formulations, boosted IgG1 and IgG2a subclasses and final antibody levels considerably more effectively ($P<0.01$) than the other preparations. As in unprimed mice, none of the preparations used elicited significant responses in primed animals in the IgG2b and IgG3 subclasses following initial immunisation, while the DSPC(B) influenza antigen liposome formulation induced the greatest serum antibody responses in these subclasses following booster immunisation of the primed mice.

Discussion

The ability of four separate preparations of liposomes, differing in lipid composition and formulation, to enhance the immune response of mice to an influenza virus subunit antigen preparation was the subject of the current study; the results were compared to the immune responses obtained using the aqueous subunit preparation alone. Three of the liposome preparations, PC, PC(B) and DSPC, elicited inferior total or subclass IgG serum antibody responses to those induced by the aqueous influenza virus subunit antigen preparation, following either one or two doses in primed mice. Thus, these liposome formulations under the present conditions of experimentation, hold no advantage over the aqueous preparation alone. In contrast one formulation of influenza virus antigens, DSPC(B), incorporating the positively charged lipid BisHOP into liposomes composed of the high melting DSPC, induced significantly greater IgG2a responses in primed mice following a single dose and in unprimed mice following 2 doses, than all other preparations tested, including the aqueous influenza subunit preparation. In addition, following 2 doses of DSPC(B) liposomes formulated with influenza subunits in primed or unprimed mice, it was found possible to detect serum antibody in both IgG2b and IgG3 subclasses; such subclass antibody was not elicited in mice by any other preparation used in the current study. Thus, in almost all the comparative measurements for antibody in the present studies, the DSPC(B) liposome formulation induced the highest levels.

Since one suggested reason for the failure of influenza virus vaccines to induce an immunity in mice comparable to that seen following live virus infection (Clements et al., 1984; Johnson et al., 1986) is that the former do not induce relatively high levels of IgG2a subclass antibody (Balkovic et al., 1987; Tao et al., 1987; Hocart et al., 1988), it is particularly interesting that this antibody subclass is markedly elevated in mice given the DSPC(B) influenza subunit liposome formulation. Studies from this laboratory (Ben Ahmeida et al., 1991), confirm that high levels of IgG2a antibody are produced in mice

following live, intranasal, influenza virus infection, and that these levels are superior to those induced by immunisation of mice via the intramuscular route with an influenza virus subunit preparation administered together with Freunds Complete Adjuvant or aluminium hydroxide gel. Furthermore, those animals receiving live virus infection were more effectively protected against homologous virus challenge than those given subunit preparations with adjuvants (Ben Ahmeida et al., 1992).

The ability of an influenza viral vaccine formulated into DSPC(B) liposomes to produce a high IgG2a response in the mouse could represent an important contribution to protection as this subclass of antibody may act, at least in part, in conjunction with other defence mechanisms (Wechsler et al., 1986; Ezekowitz, 1985; Winkelhake, 1978) against influenza viruses or influenza virus-infected cells, resulting in enhanced protection. Other adjuvants, such as SAF-1 (Byars et al., 1990) and ISCOMs (Lovgren, 1988; Ben Ahmeida et al., 1992) also induce high antibody responses in the IgG2a subclass in mice. The variability of the different liposome formulations used in the present study to elicit circulating IgG antibody, suggests that, although nominally all contain similar amounts of influenza viral glycoproteins, the presentation, distribution or availability of these glycoproteins to the immune system of the host may differ in the different liposome preparations (Gregoriadis, 1992), and that the formulation of the viral proteins into liposomes is critical. Other workers have reported that the induction of antibodies to liposome-incorporated antigens is dependent on the nature of the lipids used, on lipid: antigen ratios, the localisation of the antigen within liposomes as well as the route of immunisation and the animal species employed (Van Houte et al., 1981; Bakouche et al., 1987; Davis & Gregoriadis, 1987; Gregoriadis, 1990). In particular, the DSPC(B) liposomes are composed of phospholipids of high gel–liquid crystalline transition temperature (TC), allowing relatively high accessibility and mobility of incorporated antigens and also relatively high adjuvanticity, due to the presence of BisHOP. In contrast, PC(B), DSPC and PC liposomes have either no BisHOP or lack phospholipids of high TC (Gregoriadis, 1990).

Earlier studies using influenza antigen liposomes in hamsters indicated that antibody responses to DSPC or PC liposomes containing A/Sichuan antigens were markedly greater than those obtained with the aqueous antigens alone (Gregoriadis et al., 1992). Such findings were not apparent in mice in the current study, probably reflecting animal species differences. Alternatively, differences in the assessments used to determine antibody levels may be involved, as although a degree of correlation between the HI and ELISA tests was observed in the present studies, this correlation was not complete.

Thus, although several groups of workers have recently investigated the potential of liposomes used together with other immunomodifiers such as interleukin-2 (Tan and Gregoriadis, 1989), as vehicles for enhancing the immune responses to influenza virus antigens (El Guink et al., 1989; Mbawuike et al., 1990; Nerome et al., 1990), further studies are required to delineate more

clearly both the influenza antigen-liposome-BisHOP formulation and structure, and the capability of such preparations to induce appropriate antibody and cellular immune responses.

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