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The effect of liposomal charge on the neutralizing antibody response against inactivated encephalomyocarditis and Semliki Forest viruses

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SUMMARY

The primary neutralizing antibody response to encephalomyocarditis (EMC) virus and Semliki Forest virus (SFV) is enhanced by addition of either negatively or positively charged liposomes. The purified u.v. light inactivated viruses were merely mixed with liposomes and injected intraperitoneally (i.p.) into mice. In contrast, neutral liposomes were unable to enhance the primary response to these viruses. Furthermore primary immunization with inactivated SFV mixed with either neutral, positively or negatively charged liposomes was associated with an enhancement of the secondary humoral response after i.p. booster injection of mice with inactivated virus alone. But neutral liposomes seemed to be less effective in this respect than either positively or negatively charged liposomes.

Keywords encephalomyocarditis virus Semliki Forest virus liposomes adjuvant humoral response

INTRODUCTION

Liposomes have adjuvant activity for the humoral immune response against numerous protein antigens including those of viruses (Van Rooijen & Van Nieuwmegen, 1982). The antibody responses in mice against glycoproteins derived from either Epstein–Barr or hepatitis B viruses were considerable enhanced by incorporation of these antigens into liposomes (North *et al.*, 1982; Manesis, Cameron & Gregoriadis, 1979). Furthermore, the immunogenicity of the non-glycosylated capsid proteins of adenovirus carried in liposomes was comparable to equivalent doses administered in Freund's complete adjuvant (Kramp *et al.*, 1979). Most studies on the adjuvanticity of liposomes are performed with viral subunits and generally do not include a comparison of the adjuvant effect of neutral, negatively and positively charged liposomes (Van Rooijen & Van Nieuwmegen, 1982). We did our study with u.v. light inactivated encephalomyocarditis (EMC) virus (picornavirus: naked and non-glycosylated) and Semliki Forest virus (SFV) (alphavirus: enveloped and glycosylated) purified by CsCl gradient ultracentrifugation and column chromatography (Kraaijeveld, Harmsen & Khader Boutahar-Trouw, 1979; Kraaijeveld *et al.*, 1983), which methods favour the selection of whole particles in the virus suspensions used for immunization. The effect of neutral, negatively and positively charged liposomes on the neutralizing antibody response against EMC virus and SFV was determined after intraperitoneal (i.p.) immunization of mice. To demonstrate a clear effect on antibody formation critically low doses of virus antigens were used, which were already known from our earlier studies (Kraaijeveld *et al.*, 1979; Kraaijeveld *et al.*,

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1983). Before i.p. injection diluted inactivated virus was merely mixed with liposomes, which method was shown to be effective in the enhancement of the humoral immune response against human serum albumin and bovine gamma globulin (Van Rooijen & Van Nieuwmegen, 1980).

MATERIALS AND METHODS

Viral antigens. A virulent strain of EMC virus (Bogaerts & Durville-Van der Oord, 1973) was received from Dr W.J.C. Bogaerts (Medical Biological Laboratory TNO, Rijswijk, The Netherlands). A large plaque variant of this strain was used for the preparation of inactivated EMC virus and plaque reduction tests. The avirulent SFV strain MRS MP 192/7 (Henderson *et al.*, 1970) was obtained from Dr K.G. Oei (Royal Tropical Institute of Amsterdam, The Netherlands) and was also used for the production of inactivated viral antigen and plaque reduction tests. General virological methods and the preparation of batches purified inactivated EMC virus and SFV have been described previously (Kraaijeveld *et al.*, 1979, 1983). Doses of virus are expressed in haemagglutinating units (HAU).

Chemicals. Synthetic dipalmitoyl L-α-phosphatidylcholine (DPPC) was obtained from Koch-Light Laboratories, Ltd, Colnbrook, UK. Cholesterol (CHOL) was obtained from Brocades-ACF, The Netherlands; octadecylamine (ODA) from Polysciences Inc. Warrington, Pennsylvania, USA and phosphatidic acid (PA) from egg yolk L-α-phosphatidylcholine was obtained from Sigma Chemical Company, St Louis, Missouri, USA. These chemicals were dissolved in chloroform at a concentration of 1 μmol/ml and stored under nitrogen at −20°C.

Liposomes. Liposomes were prepared, with slight modifications, as described by Bangham, Standish & Watkins (1965). In brief: appropiate amounts of chemicals, dissolved in chloroform, were added with 2·5 ml pure methanol to a 50 ml round bottom flask. The thin film, formed on the walls of the flask after rotary evaporation was dispersed in phosphate-buffered saline (PBS) of pH 7·2 by shaking (Vortex). The suspension was kept at room temperature for 1 h. It was mixed with virus and injected into mice. In this study neutral, positively and negatively charged liposomes were used (Van Houte *et al.*, 1981). Neutral liposomes consisted of CHOL and DPPC; molar ratio, 10:90. Positive liposomes consisted of CHOL, ODA and DPPC, molar ratio 10:15:75. Negative liposomes consisted of CHOL, PA and DPPC with various molar ratio's, respectively; 10:15:75; 10:45:45 and 10:90, without DPPC. Of each liposomal preparation 100 nmol, mixed with virus, was injected into individual mice.

Animals and immunization. Inbred BALB/c mice were bred and maintained in our own animal house; male mice were used at an age of 12 weeks. Blood was obtained by retro-orbital puncture. Mice were injected i.p. with inactivated viruses, with or without added liposomes, in a volume of 0·5 ml PBS.

Determination of neutralizing antibodies. Neutralizing antibodies against SFV and EMC virus were determined with the plaque reduction test. Plaque titration and plaque reduction tests were done in plastic palettes (24 well tissue culture cluster dish; catalogue No. 3524; Costar, Cambridge, Massachusettes, USA). Dilutions were made in a 96 well palette (catalogue No. 3596; Costar). Virus containing fluids were titrated in four-fold series by adding 0·05 ml quantities to four monolayers. After 40 min of adsorption at room temperature 0·3 ml of overlay medium was added. After 24 h (SFV) or 48 h (EMC virus) incubation at 37°C plaques were developed with 0·05 ml of 0·02% neutral red. For plaque reduction tests, 0·025 ml serially diluted and decomplemented (30 min, 56°C) serum was mixed with 0·025 ml of virus and incubated at 37°C for 1 h. A 0·2 ml portion of PBS–peptone was added and 4 × 0·050 ml of each dilution was transferred to monolayers. Controls contained about 100 plaques per 16 mm well. If a serum causes a plaque reduction of 50% at a dilution of 1:10 it is described as an antibody concentration of 10 plaque neutralizing doses (PND_{50}). If no antibodies were detectable, the titre is given as 0·1, which is the lowest level of detection.

Statistical analysis. Results are expressed as the arithmetic mean of n independent observations with the standard error of the mean (s.e.). Statistical significance was assessed by Student's t-test. Values of P over 0·05 are considered to be not significant.

Table 1. Effect of liposomal charge on the development of neutralizing antibodies in mice after primary and secondary intraperitoneal immunization with inactivated SFV

Liposomes*		Mean PND_{50} (s.e.; *n*) for neutralizing antibodies after							
		Primary i.p. immunization with 30 HAU of SFV						Secondary immunization‡ Day 130	
		Day 7		Day 14		Day 106			
Dose (nmol)	Charge	Mean (s.e.; *n*)	*P* value†	Mean (s.e.; *n*)	*P* value	Mean (s.e.; *n*)	*P* value	Mean (s.e.; *n*)	*P* value
none		0·2 (0·1; 8)		1·5 (0·7; 8)		0·2 (0·1; 8)		16 (8; 7)	
100	neutral	1·0 (0·1; 8)		0·9 (0·3; 7)	n.s.	0·2 (0·1; 8)	n.s.	98 (34; 8)	<0·025
100	negative	32 (17 ; 7)	<0·05	31 (14 ; 8)	<0·05	0·4 (0·3; 8)	n.s.	338 (77; 6)	<0·0005
100	positive	34 (12 ; 8)	<0·01	25 (9 ; 8)	<0·025	0·2 (0·1; 8)	n.s.	287 (51; 8)	<0·0005

* Liposomal composition with molar ratio in parentheses were as follows. Neutral: CHOL and DPPC (10:90). Negative: CHOL, PA and DPPC (10:15:75). Positive: CHOL, ODA and DPPC (10:15:75).

† Student's *t*-test; n.s. = statistically unsignificant ($P > 0{\cdot}05$).

‡ i.p. booster at day 123 with 1,500 HAU of SFV.

Table 2. Effect of liposomal charge on the development of neutralizing antibodies in mice after primary i.p. immunization with 1,500 HAU inactivated EMC virus

Liposomes*		Mean PND_{50} (s.e.; *n*) for neutralizing antibodies											
		Day 5			Day 7			Day 10			Day 103		
Dose (nmol)	Charge	Mean (s.e.; *n*)	*P* value†		Mean (s.e.; *n*)	*P* value		Mean (s.e.; *n*)	*P* value		Mean (s.e.; *n*)	*P* value	
none		1·6 (0·7; 5)			11 (4·2; 5)			4·5 (2·7; 5)			3·7 (1·3; 5)		
100	neutral	2·9 (1·6; 5)	n.s.		6·5 (2·8; 5)	n.s.		4·9 (4·3; 5)	n.s.		5·2 (4·8; 5)	n.s.	
100	negative (A)	2·2 (0·5; 5)	n.s.		31 (13; 5)	n.s.		25 (2·8; 5)	<0·001		18 (4·8; 5)	<0·025	
100	negative (B)	2·8 (0·2; 5)	n.s.		62 (43; 5)	n.s.		58 (38; 5)	n.s.		106 (92; 3)	n.s.	
100	positive	17 (4·7; 5)	<0·01		150 (35; 5)	<0·005		44 (5·4; 5)	<0·001		93 (37; 4)	<0·025	
300	positive	9·3 (2·9; 5)	<0·025		98 (32; 5)	<0·025		31 (15; 5)	n.s.		41 (24; 4)	n.s.	

* Liposomal composition with molar ratio in parentheses. Neutral: CHOL and DPPC (10:90). Negative: (A) CHOL and PA (10:90); (B) CHOL, PA and DPPC (10:45:45). Positive: CHOL, ODA and DPPC (10:15:75).

† Student's *t*-test; n.s. = statistically unsignificant ($P > 0·05$).

RESULTS

Groups of mice were immunized i.p. with 30 HAU of purified inactivated SFV alone or mixed with 100 nmol of liposomes of different charge. These mice were serially bled 7, 14 and 106 days later. At day 123 all mice were injected i.p. with 1,500 HAU of SFV as a booster for antibody formation. Seven days later, at day 130, blood was taken for the last time. The mean neutralizing antibody titres of the different groups of mice at the indicated time points are given in Table 1. As expected the specific antibody formation was poor after primary immunization with antigen only. No effect on antibody formation was found with neutral liposomes. By contrast mixing of antigen with either negatively or positively charged liposomes resulted clearly in enhancement of the primary humoral response at day 7 and day 14. Later, at day 106, in almost all mice the neutralizing antibodies had receded to an undetectable level. Mice previously immunized with antigen mixed with either positively or negatively charged liposomes reacted much strongly to a booster immunization compared to control mice. Mice, which previously received neutral liposomes, showed a less but still significantly enhanced humoral response on boosting.

A similar experiment was performed with purified inactivated EMC virus as an antigen. Groups of mice were injected i.p. with 1,500 HAU of EMC virus alone or mixed with liposomes. These mice were serially bled on days 5, 7, 10 and 103 after primary immunization for the determination of neutralizing antibodies in sera of individual mice. As shown in Table 2 neutral liposomes had no enhancing effect on the humoral response against EMC virus; the mean antibody titres at day 5, 7, 10 and 103 were comparable to mean antibody titres observed in control mice. Mixing of EMC virus with positively charged liposomes (100–300 nmol) led to a considerable enhancement of antibody formation. Negatively charged liposomes seemed less effective as adjuvant compared to positively charged liposomes.

DISCUSSION

In this study we demonstrated clearly that mere mixing of low doses of either inactivated SFV or EMC virus with negatively or positively charged liposomes results in enhancement of antibody formation against these viruses after intraperitoneal injection of mice. In this respect neutral liposomes seemed to be not effective. Before inactivation with ultraviolet light the viruses were concentrated and purified by CsCl gradient ultracentrifugation and column chromatography (Kraaijeveld *et al.*, 1979, 1983). The latter methods favor the selection of whole virus particles, which were used as antigens. Both picornaviruses (EMC) and alphaviruses (SFV) have negatively charged surfaces as indicated by the results obtained with gel isoelectric focusing (Putnak & Phillips, 1981; Kennedy, 1974). So, it may be expected that positively charged liposomes have a stronger affinity for these viruses than negatively charged liposomes. However, the different viral proteins composing the virions may have negatively and positively charged parts, which are both important for the integrity of the virion by providing electrostatic bonds between the structural proteins (Hordern, Leonard & Scraba, 1979; Putnak & Phillips, 1981). Therefore there may be positions on these viruses to bind both negatively and positively charged liposomes but not (or fewer) for neutral liposomes. Attachment of liposomes to viruses may be of advantage in the antigen uptake and/or handling by macrophages in the peritoneal cavity. In agreement with this hypothesis is the evidence presented by Shek & Lukovich (1982) that macrophages have an obligatory role in inducing a humoral response to liposome associated protein antigens.

In conclusion, negatively and positively charged liposomes are of advantage in enhancement of the humoral response against low doses of EMC virus and SFV.

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