Immunology 1979 **37** 505

Characterization of immunogenic properties of haptenated liposomal model membranes in mice

I. THYMUS INDEPENDENCE OF THE ANTIGEN

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Received 23 October 1978; accepted for publication 20 November 1978

Summary. This paper describes a rather simple coupling method for tripeptide enlarged haptens to phosphatidylethanolamine (PE) and the incorporation of these conjugates into liposomal model membranes (haptenated liposomes). These haptenated liposomes evoke a hapten-specific humoral immune response in mice. The magnitude of the response as measured by the appearance of direct plaque forming cells in the spleen is dependent on the route of immunization and the dose and epitope density of the hapten-PE derivatives. It was not possible to evoke an IgG response after either primary or secondary immunization with haptenated liposomes (as measured by the production of indirect plaques or mercaptoethanol-resistant antibody). These data, in addition to the observations that mice depleted of, or deficient in thymus-derived (T) lymphocytes respond to haptenated liposomes, indicate that these haptenated liposomes are T-cell independent antigens.

INTRODUCTION

Complexes of haptens covalently linked to conventional high molecular weight, water-soluble carriers evoke a humoral immune response predominantly specific for the hapten (Mitchison, Rajewsky & Taylor, 1970). The structure of the carrier is decisive for the thymus dependence of the response (Klaus, Janossy & Humphrey, 1975). Uemura, Nicolotti, Six & Kinsky (1974) introduced the use of immunogenic liposomal model membranes in animal systems. These immunogens differ significantly from conventional immunogens as the haptens in liposomes are contributed by an amphipathic compound that is inserted non-covalently into the lipid bilayers which serve a carrier role. This compound is for example a synthetic amino(N)-substituted derivative of phosphatidylethanolamine (PE). Model membranes containing dinitrophenyl - ε - aminocaproyl - phosphatidylethanolamine (DNP-Cap-PE) were used to study the immunogenicity of liposomes in the humoral response of guinea-pigs and mice (Uemera, Claflin, Davie & Kinsky, 1975; Yasuda, Dancey & Kinsky, 1977). The immune response was characterized by the formation of IgM and IgG anti-DNP antibodies of a much narrower range of avidity than antibodies obtained after immunization with DNP-albumin. Moreover the latter authors showed DNP-Cap-PE containing liposomes to be a thymus-independent immunogen.

In order to investigate if a thymus independent humoral response is probably a more general feature

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0019-2805/79/0600-0505$02.00
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of homogeneous liposomes we studied the murine humoral response of several different haptens linked to PE with or without insertion of a tripeptide. These tripeptide enlarged haptens covalently linked to conventional carriers evoke a very specific humoral immune response to the 'immunodominant' distal terminus (Inman, Merchant & Tacey, 1973a). The conjugation of enlarged haptens to PE and the humoral response to their presentation in the lipid bilayers of liposomes is described.

MATERIALS AND METHODS

Mice and immunization

Inbred female F_1 (hybrid BALB/c ♂ × Swiss ♀) mice were raised and maintained in the Laboratory of Microbiology, State University, Utrecht, The Netherlands. Nude BALB/c mice (nu/nu) and their heterozygous littermates (nu/+) were obtained from the Central Animal Laboratory of the University Hospital, Utrecht, The Netherlands. The mice were used at an age of about 10 weeks and immunized as indicated in the experiments. In some experiments the liposomes were emulsified in 0·1 ml Freund's complete adjuvant (FCA) containing killed *Mycobacterium* $H_{37}R_a$ (Difco Laboratories, Detroit, Michigan, USA).

Preparation of derivatives

Soy bean PE (mol. wt 735) was a gift of Dr J. de Gier, Laboratory of Biochemistry, State University Utrecht. The enlarged haptens employed in this study are listed in Table 1. They were kindly provided as tertiary butyloxycarbonyl hydrazides (Boc hydrazides) by Dr J. K. Inman, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland, USA. The hapten Boc hydrazides (6 μmol each) were activated to hapten acyl azides as described by Inman, Merchant, Claflin & Tacey (1973b). The resulting solutions of hapten azides in N,N-dimethylformamide (DMF, Spectrograde, UCB, Brussel, Belgium) were placed in an ice bath (0°) and the pH was adjusted to 8·5–9·5 by adding dropwise a solution of triethylamine (20% v/v in DMF, Merck, Darmstadt, West Germany). Six micromoles (4·4 mg) soy bean PE, dissolved in 4 ml DMF, were added, and the mixture was stirred slowly at 0° for 24 h. The reaction was terminated by pouring the mixture into 30 ml of a cold solution of 20× concentrated phosphate buffered saline (PBS-20×, 0·2 M phosphate buffer, pH 7·2, containing 2·8 M NaCl). After addition of 5 ml of chloroform the mixture was centrifugated 10 min at 1200 *g* at 4° and the chloroform layer was washed once with 30 ml cold PBS (0·01 M phosphate buffer, pH 7·2, containing 0·14 M NaCl) and five times with 30 ml cold distilled water until no free hapten or DMF was detectable spectrophotometrically in the water phase. The final reaction product (tripeptide enlarged hapten-PE) which occurred both in the chloroform phase and as a precipitate in the interphase, was completely dissolved by the addition of 5 ml chloroform and 20 ml methanol following removal of the aqueous layer. The conjugates, dissolved in methanol–chloroform 2:1, were stored under nitrogen at −20° at a concentration of approximately 0·08–0·12 μmol/ml.

The yields of PE-conjugated haptens (J, K and N) were spectrophotometrically determined by measuring the extinction of solutions of these haptens in methanol–chloroform 2:1 at respectively 350, 338 and 425 nm. A calibration curve was made with the aid of the corresponding Boc hydrazides dissolved in methanol-chloroform 2:1. The molar yields of the chromatographically pure conjugates were 40–60% of the starting materials.

TNP-PE was prepared by coupling 2,4,6-trinitrobenzene sulphonic acid (TNBS, BDH, Poole, England) to soy bean PE: 10 mg (26 μmol) of TNBS was dissolved in 15 ml phosphate buffer (0·01 M, pH 8·5), and the resulting solution was mixed with a solution of 10 mg (13 μmol) PE in 30 ml methanol plus 15 ml chloroform. This mixture was stirred vigorously at room temperature for 2 h and 15 ml chloroform and 15 ml phosphate buffer (0·01 M, pH 8·5) were added. Phase separation was performed by centrifugation of the mixture for 10 min at 1200 *g* and the chloroform layer was washed once with 20 ml phosphate buffer (0·01 M, pH 8·5) and two times with 20 ml distilled water. To the resulting chloroform layer 60 ml methanol was added and the TNP-PE, dissolved in methanol–chloroform 2:1, was stored under nitrogen at −20°.

The yield of TNP-PE was spectrophotometrically determined by measuring the extinction of the solution of TNP-PE in methanol–chloroform 2:1 at 338 nm. The concentration of TNP-PE was calculated with the molar extinction coefficient ε of the 2,4,6-trinitrophenyl group ($\varepsilon = 14{,}000\ M^{-1}\ cm^{-1}$; Habeeb, 1966; Means & Feeny, 1971).

Liposomes

Unless otherwise stated, all liposome preparations were actively haptenated by incorporation of appro-

priate amounts of the N-substituted phosphatidylethanolamine derivative into a basic lipid mixture containing synthetic dipalmitoyl lecithin (LEC), cholesterol (CHOL), octadecylamine (stearylamine, ODA) and hapten-PE conjugate in molar ratios of 14:2:3:1. Synthetic 1,2-dipalmitoyl L-3-lecithin was obtained from Koch-Light Laboratories Ltd, Colnbrook, England. Cholesterol was obtained from Brocades-ACF, Maarssen, The Netherlands and octadecylamine from Polysciences, Inc. Warrington, Pennsylvania, U.S.A. These chemicals were dissolved in chloroform at a concentration of 1 μmol/ml and stored under nitrogen at $-20°$.

Liposomes were prepared as described by Bangham, Standish & Watkins (1965) with slight modifications. In brief, appropriate amounts of LEC, CHOL, ODA and hapten-PE, dissolved in chloroform, were added to a 50 ml round-bottom flask. The thin film formed on the walls of the flask after rotary evaporation at 37° was dispersed in PBS by gently shaking for 10 min at 61° (which is 20° above the phase-transition temperature of dipalmitoyl lecithin; Shimshick & McConnel, 1973; Brûlet & McConnel, 1976). The suspension was kept at room temperature for 1 h and then sonicated at 4° in a MSE sonicator (amplitude 7μm) three times for 30 s with intervening periods of 15 s. The sonicated suspension was kept at room temperature for another hour before it was injected into the animals.

Antigens

The Ficoll antigen derivatized with the large hapten J (J_{59}-Ficoll) was a gift of Dr J. K. Inman and was prepared according to the procedure of Inman (1975). The subscript refers to the average number of haptenic groups coupled to 400,000 daltons of original Ficoll. The keyhole limpet haemocyanin antigen (J_{22}-KLH) was also a gift of Dr J. K. Inman and was prepared as described by Inman *et al.* (1973b).

Haemolytic plaque procedures

The number of hapten-specific plaque-forming cells (PFC) in immune spleen-cell suspension was determined by a modification of the Jerne haemolytic plaque technique as described by Merchant & Inman (1977). The technique of Dresser & Wortis (1965) was used to determine the number of indirect (IgG) PFC. Rabbit anti-mouse IgG was prepared according to Zaalberg, van der Meul & van Twisk (1968). While enhancing or facilitating the detection of IgG-secreting PFC, this antiserum (diluted 1:100) simultaneously inhibited 70% of the plaques from IgM-secreting cells. Thus, the indirect, hapten-specific PFC activities have been corrected both for background activity against SRBC and, on an individual basis, for roughly 30% of the corresponding direct PFC activity which remained uninhibited by the facilitating antiserum. Indicator erythrocytes optimally derivatized with the large haptens J, K or N were prepared according to the procedure of Inman *et al.* (1973b). Trinitrophenylated sheep red blood cells (TNP-SRBC) were prepared according to Rittenberg & Pratt (1969).

Antibody detection

Blood was withdrawn from the retro-orbital venous plexus. The presence of antibodies was determined with a haemagglutination assay using indicator erythrocytes optimally derivatized with the large hapten J (Inman *et al.*, 1973b). Two-fold dilutions of antiserum in PBS (supplemented with 1% normal rabbit serum) were prepared in microtitre plates (Greiner, Nürtingen, West Germany). Fifty microlitres of a suspension of 0·5% indicator erythrocytes was added to 50 μl of the diluted antiserum. Haemagglutination was determined after 4h. The reciprocal value of the endpoint of haemagglutination was taken as the titre.

T-cell depletion of mice

Mice were thymectomized at the age of 4 weeks. Five months later they received a whole body irradiation of 850 rad. Irradiation was performed on a teletherapy ^{60}Co unit (Picker, C4M/60). One day later they were reconstituted with 5×10^6 syngeneic bone marrow cells treated with rabbit anti-mouse thymocyte serum (ATS) and guinea-pig complement, both extensively absorbed with IgG_{2b} plasma tumour cells (Snippe, Willems, Graven & Kamp, 1975). The bone marrow cells were suspended in 0·5 ml Eagle's Basal Medium and injected in the lateral tail vein (TXBM mice). Three weeks later, these mice were immunized. Control mice only received whole body irradiation and were reconstituted with ATS-treated bone marrow cells (XBM mice).

Mice were deprived of thymus-derived cells by intravenous (i.v.) injection of 0·1 ml rabbit anti-mouse thymocyte serum (ATS) both one and two days before immunization with J-PE haptenated liposomes.

Statistical analysis

Results are expressed as the arithmetic mean of n independent observations $\pm$ standard error of the mean (SEM). In some experiments, Student's t test

was performed to analyse the statistical significance of the results. Values of P over 0·05 are considered to be not significant.

RESULTS

Immunogenicity of free hapten-PE conjugates versus haptenated liposomes

Groups of mice were i.v. injected with either PBS, 5 nmol of free hapten-PE conjugates or the same amount of conjugates incorporated into the bilayers of liposomes. Table 2 shows that neither free K-PE nor free N-PE induced a significant humoral response 4 days after immunization. Both K-PE and N-PE haptenated liposomes induced large numbers of K- or N-specific PFC respectively. On the other hand liposomes haptenated with 5 nmol of the small hapten TNP-PE were very weakly immunogenic. Neither with TNP-SRBC nor with K-SRBC (tripeptide enlarged TNP) were appreciable numbers of TNP-specific PFC detected. Immunization of mice with 5 mol% K-PE-liposomes (containing 5 nmol K-PE) gave rise to large numbers of TNP-specific PFC both on K-SRBC and on TNP-SRBC. The reaction was specific since only few N-specific PFC were observed in mice immunized with K-PE-liposomes.

Table 1. Letter symbols and chemical designation* for haptens employed in this study

Small hapten
TNP = N-2,4,6-trinitrophenyl

Tripeptide enlarged haptens
J = N-(2,4-dinitrophenyl)-β-alanylglycylglycine
K = N-(2,4,6-trinitrophenyl)-β-alanylglycylglycine
N = N-(4-hydroxy-3-iodo-5-nitrophenylacetyl)-β-alanylglycylglycine

* Structural formulae of the large haptens are presented in the reference of Inman, Merchant & Tacey (1973a).

The immunogenicity of K-PE-liposomes increased after sonication. At day 4 the numbers of K-specific PFC increased about three-fold if sonicated liposomes were used instead of untreated ones.

The response to the thymus independent antigen J_{59}-Ficoll was of a same level as found earlier (Merchant, Snippe, Lizzio & Inman, 1978).

Route of immunization

Groups of mice were immunized with 5 mol% K-PE-liposomes (containing 5 nmol K-PE) either intravenously (i.v.) or intraperitoneally (i.p.) or intracutaneously (i.c.) (Fig. 1). Intravenous immunization resulted in better antibody formation than i.c. or i.p. immunization. A maximal immune response was induced four days after i.v. immunization.

Table 2. Immunogenicity of free hapten-PE conjugates and haptenated liposomes

Antigen	Direct PFC × 10^{-3}/spleen ± S.E.M., specific for			
	SRBC	TNP-SRBC	K-SRBC	N-SRBC
PBS	0·08 ± 0·01	0·10 ± 0·01	0·12 ± 0·01	n.t.
5 nmol K-PE	0·08 ± 0·01	n.t.	0·16 ± 0·01	0·11 ± 0·01
5 nmol N-PE	0·08 ± 0·01	n.t.	0·17 ± 0·02	0·12 ± 0·01
Non-haptenated liposomes	0·07 ± 0·01	n.t.	0·11 ± 0·01	n.t.
K-PE-liposomes (5 mol%, 5 nmol)	n.t.	23·0 ± 1·9	20·0 ± 1·5	1·13 ± 0·1
N-PE-liposomes (5 mol%, 5 nmol)	0·08 ± 0·01	n.t.	0·23 ± 0·01	18·6 ± 1·6
TNP-PE-liposomes (5 mol%, 5 nmol)	n.t.	0·52 ± 0·03	0·61 ± 0·04	n.t.
Non-sonicated K-PE-liposomes (5 mol%, 5 nmol)	0·08 ± 0·01	8·8 ± 0·6	6·3 ± 0·8	n.t.
J_{59}-Ficoll (10 μg)	0·07 ± 0·01	19·2 ± 2·8	21·0 ± 1·8	n.t.

Groups of mice ($n = 5$) were immunized i.v. as indicated in the table; 5 mol% haptenated liposomes, containing 5 nmol hapten-PE were used; individual spleens were assayed for direct PFC responses 4 days after immunization.

n.t. = not tested.

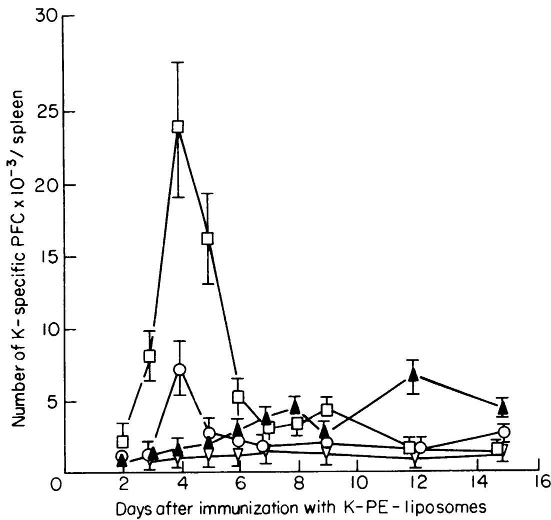

Figure 1. The effect of different routes of immunization. Groups of mice ($n = 5$) were immunized with 5 mol% K-PE haptenated liposomes (containing 5 nmol K-PE) either i.v. (□) or i.p. (○) or i.c. with FCA (▲) or i.c. without FCA (▽). Individual spleens were assayed for direct K-specific PFC response at various times after immunization.

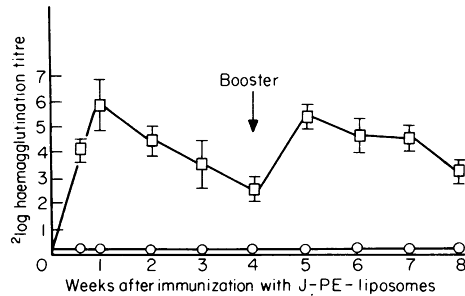

Figure 2. Serum antibody titre after immunization with J-PE-liposomes. Groups of mice ($n = 5$) were immunized i.v. with 5 mol% J-PE haptenated liposomes (containing 5 nmol K-PE) and boosted i.v. 4 weeks later with the same antigen. The mice were bled weekly, and the serum anti-J antibody titre was determined in the absence (□) and presence (○) of 2-mercaptoethanol.

Secondary response

In order to study the secondary response, mice were immunized with 5 mol% haptenated liposomes (containing 5 nmol hapten-PE) at day 0 and boosted 4 weeks later with the same antigen. These mice were bled weekly, and the sera were tested for haemagglutination with J-SRBC.

Figure 2 shows that (1) a maximum antibody titre is attained 1 week after immunization, (2) no enhanced secondary response occurred and (3) no detectable amounts of 2-mercaptoethanol-resistant (IgG) antibody was produced.

To study the secondary response in more detail, groups of mice were used unprimed or primed with J-PE-liposomes or with the highly thymus-dependent antigen J_{22}-KLH at day 0. At day 60 all mice were injected with either J-PE-liposomes or J_{22}-KLH, and the numbers of J-specific PFC (direct and indirect) of these mice were determined. J-PE-liposomes did not sensitize mice for a secondary response to either J-PE-liposomes or J_{22}-KLH (Fig. 3). Boosting with J_{22}-KLH resulted only in indirect (IgG) J-specific PFC if the mice had been primed with J_{22}-KLH.

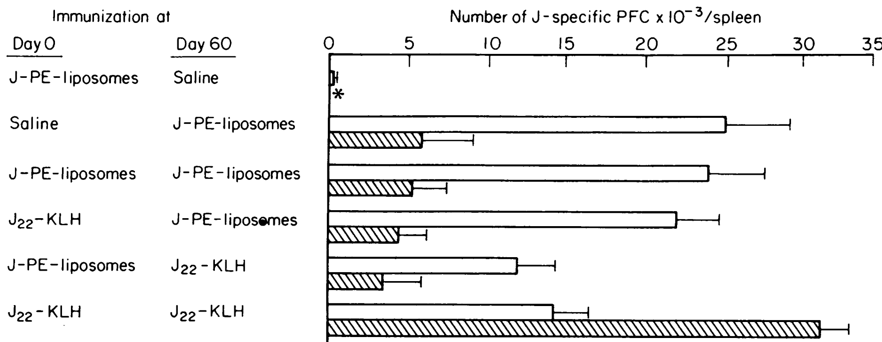

Figure 3. Primary and secondary response to J-PE-liposomes. Groups of mice ($n = 5$) were immunized on day 0 and day 60 with 5 mol% J-PE haptenated liposomes (containing 5 nmol J-PE; i.v.) or with 50 μg J_{22}-KLH on bentonite (i.p.). Individual spleens were assayed for direct (open columns) and indirect (hatched columns) J-specific PFC response 4 days after the second immunization. * No indirect PFC were observed.

Dose dependence

Dose–response experiments were performed by immunization with varying amounts of liposomes in which the epitope density (i.e., molar ratio of hapten-derivative to liposomal lecithin, or mole percentage of haptenation) was kept constant.

Groups of mice were intravenously immunized with 5 mol% K-PE or 5 mol% N-PE haptenated liposomes containing a graded dose of K-PE or N-PE. The response to K and N was studied at day 4. With both antigens a maximum response was induced with liposomes containing a total amount of 3–10 nmol hapten-PE (Fig. 4).

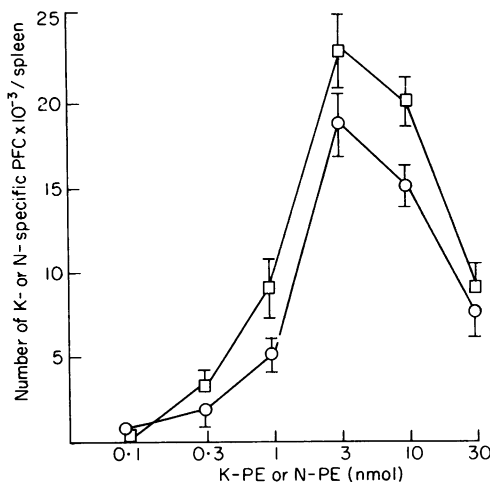

Figure 4. Dose dependence of the response to K-PE and N-PE-liposomes. Groups of mice ($n=5$) were immunized i.v. with 5 mol% K-PE or 5 mol% N-PE haptenated liposomes, containing a graded dose of the hapten-PE as indicated on the abscissa. Individual spleens were assayed for direct, K-specific (□) or N-specific (○) PFC responses 4 days after immunization.

Epitope density

Subsequently, the effect of varying epitope density, while maintaining a fixed dose of K-PE (5 nmol) was studied. The epitope density was varied by changing the lecithin/K-PE molar ratio of the liposome. A maximal PFC response was obtained with liposomes containing 5 mol% K-PE (Fig. 5). This level of haptenation was therefore employed in further experiments. A high dose of low epitope density liposomes did not induce the humoral response.

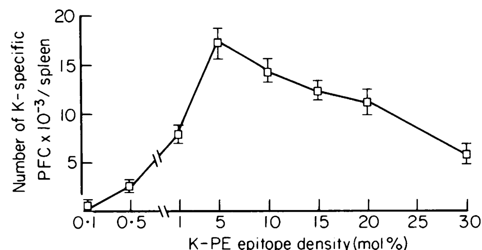

Figure 5. Effect of K-PE epitope density on the response. Groups of mice ($n=5$) were immunized i.v. with K-PE-liposomes containing a fixed dose of K-PE (5 nmol). The epitope density was varied by changing the lecithin/K-PE molar ratio of the liposome. Individual spleens were assayed for direct K-specific PFC response 4 days after immunization.

Thymus independence

The characteristics of the primary and secondary response to J-PE-liposomes correspond with those of thymus-independent antigens. In order to investigate further the thymus dependence of haptenated liposomes, three different experiments with mice deficient in thymus-derived cells were performed. Groups of mice pretreated with ATS (ATS-treated mice) and groups of mice pretreated with normal rabbit serum (NRS, NRS-treated mice) were immunized i.v. with J-PE-liposomes. The effect of the antiserum treatment was tested by injections of the thymus-independent antigen, J_{59}-Ficoll, and the thymus dependent antigens, J_{22}-KLH and 1% SRBC. Table 3 shows that the J-specific PFC response of ATS- and NRS-treated mice immunized with J-PE-liposomes did not differ significantly. On the other hand ATS-treated mice upon immunization with either J_{22}-KLH or 1% SRBC gave a PFC response which is 3·5–6·5 times lower than the response of NRS-treated mice. It should be noted that the J-specific PFC response of ATS-treated mice which were immunized with J_{59}-Ficoll was even higher than that of control NRS-treated mice. This increase might be caused by a removal of T-suppressor cells following ATS-treatment (Baker, Stashak, Amsbaugh & Prescott, 1974).

In a next experiment we used nude BALB/c mice (nu/nu) and the heterozygous littermates (nu/+) as controls. These mice were immunized with non-haptenated and J-PE-liposomes, J_{59}-Ficoll and J_{22}-KLH (Table 4).

The specific PFC response did not differ significantly in nude (nu/nu) and heterozygous (nu/+) BALB/c mice for either the thymus-independent antigen J_{59}-Ficoll or the J-PE-liposomes (Table 4).

Table 3. Direct PFC response to haptenated liposomes in ATS-treated mice

Antigen	Antiserum treatment	Direct PFC × 10^{-3}/spleen ± S.E.M.		
		J-specific	*P*	SRBC-specific
J-PE liposomes (5 mol%, 5 nmol)	ATS	33·5 ± 2·6	0·25	n.t.
J-PE-liposomes (5 mol%, 5 nmol)	NRS	31·3 ± 1·9		n.t.
10 μg J_{59}-Ficoll	ATS	52·7 ± 3·7	0·003	n.t.
10 μg J_{59}-Ficoll	NRS	34·6 ± 3·3		n.t.
50 μg J_{22}-KLH	ATS	6·1 ± 0·6	0·001	n.t.
50μg J_{22}-KLH	NRS	21·1 ± 1·9		n.t.
0·2 ml 1% SRBC	ATS	n.t.	<0·001	1·2 ± 0·1
0·2 ml 1% SRBC	NRS	n.t.		76·3 ± 5·3

Groups of mice ($n = 5$) were deprived of thymus-derived cells by i.v. injection of 0·1 ml ATS both one and two days before immunization. Control groups ($n = 5$) received NRS. The mice were immunized with 5 mol% J-PE haptenated liposomes (containing 5 nmol J-PE; i.v.), 10 μg J_{59}-Ficoll (i.v.), 50 μg J_{22}-KLH on bentonite (i.p.) or 0·2 ml 1% SRBC (i.p.). Individual spleens were assayed for direct PFC responses 4 days after immunization. *P* values test the significance of the difference between the ATS- and NRS-treated mice for each corresponding antigen.

n.t. = not tested.

Table 4. Direct PFC response to haptenated liposomes in nude (nu/nu) and heterozygous (nu/+) mice

Antigen	Direct J-specific PFC × 10^{-3}/spleen ± S.E.M.		
	nude (nu/nu)	heterozygous (nu/+)	*P*
Liposomes	0·10 ± 0·01	0·10 ± 0·01	
J-PE-liposomes (5 mol%, 5 nmol)	21·6 ± 1·9	24·8 ± 2·0	0·14
10μg J_{59}-Ficoll	29·8 ± 2·1	31·9 ± 2·2	0·26
50 μg J_{22}-KLH	1·6 ± 0·2	14·9 ± 1·5	<0·001

Groups of mice ($n = 5$) were immunized i.v. with non-haptenated liposomes or 5 mol% J-PE haptenated liposomes (containing 5 nmol J-PE) or 10 μg J_{59}-Ficoll or i.p. with 50 μg J_{22}-KLH on bentonite. Individual spleens were assayed for direct J-specific PFC responses 4 days after immunization. *P*-values test the significance of the difference between the nude (nu/nu) and heterozygous (nu/+) mice.

Table 5. Direct PFC response to haptenated liposomes in TXBM- and XBM-mice

Antigen	Direct J-specific PFC × 10^{-3}/spleen ± S.E.M.		
	TXBM ($n = 6$)	XBM ($n = 8$)	untreated ($n = 5$)
J-PE-liposomes (5 mol%, 5 nmol)	4·6 ± 1·0	5·5 ± 0·4*	18·7 ± 1·7
10 μg J_{59}-Ficoll	n.t.	n.t.	22·9 ± 1·8

TXBM-mice were thymectomized at the age of four weeks. Five months later they received a whole body irradiation of 850 rad. One day later they were reconstituted with 5×10^6 ATS-treated syngeneic bone marrow cells. XBM-mice only received whole body irradiation and were reconstituted with ATS-treated bone marrow cells.

These mice were immunized i.v. with 5 mol% J-PE haptenated liposomes (containing 5 nmol J-PE) or 10 μg J_{59}-Ficoll.

Individual spleens were assayed for direct J-specific PFC responses 4 days after immunization.

* Does not differ significantly from TXBM; $P = 0·19$.

When the two different groups of mice were immunized with J_{22}-KLH, however, the J-specific PFC-response of the nude (nu/nu) group was about nine times lower than that of the heterozygous (nu/+) group.

In the last experiment TXBM- and XBM-mice were immunized with J-PE-liposomes. Although the J-specific response between these two groups of mice did not differ significantly, the response of the treated animals was only 25–30% of those of untreated mice which were immunized with J-PE-liposomes (Table 5). This decrease in J-specific PFC response is in all likelihood caused by the drastic treatment that the TXBM and XBM mice endured.

DISCUSSION

Liposomal model membranes haptenated by incorporation of DNP-Cap-PE induce a thymus-independent hapten-specific humoral response when administered in saline to mice of several different strains (Yasuda *et al.*, 1977). In the present study we demonstrated that liposomes haptenated with tripeptide enlarged haptens satisfy the principal criteria of T-cell independent immunogens in mice; namely, a predominant production of direct PFC and mercaptoethanol-sensitive (IgM) antibody (Fig. 2); apparent absence of immunological memory (Fig. 3); immunogenicity in mice lacking (Table 4) or deprived of (Tables 3 and 5) thymus-derived cells. Thymus independence of conventional hapten-carrier complexes is thought to be based on the structure of these molecules which are characterized by long chains made up of repeating antigenic determinants. This may be a necessary but insufficient requirement for thymus independence, since several multichain polypeptide immunogens, all possessing repeating antigenic determinants, require both thymus- and marrow-derived cells for eliciting efficient humoral immune response in mice (Mozes & Shearer, 1971). It was postulated by Sela, Mozes & Shearer (1972) that for the thymus independence of the antibody response to thymus-independent immunogens, slow metabolism may be a requirement for a steady multipoint binding to antigenic determinants of the lymphocyte. Furthermore, it has been demonstrated that the anti-hapten responses are carrier-independent i.e. not only thymus-independent immunogens are carriers in this type of response but also non-immunogenic polymers such as hyaluronic acid and poly-γ-D-glutamic acid (Klaus, Janossy & Humphrey, 1975). In haptenated liposomal membranes the carrier is also non-immunogenic.

Several investigations have been made on the interactions of liposomes with mammalian cells (reviewed by Pagano & Weinstein, 1978), but little is known about the metabolism of haptenated liposomes. The results obtained with the enlarged hapten-PE complexes and with DNP-Cap-PE (Yasuda *et al.*, 1977) suggest that at least a number of homogeneous liposomes induce a thymus-independent humoral response. Nicolotti & Kinsky (1975) reported that mono(*p*-azobenzenearsonic acid) tyrosyl-PE-liposomes induce both a cellular and humoral response. The thymus (in)dependence of the humoral response was not investigated but the presence of T-cell epitopes on the liposomes makes the involvement of T-helper cells in the antibody formation very probable (Kochibe, Nicolotti, Davie & Kinsky, 1975). In that instance, thymus independence is not a general feature of homogeneous liposomes.

The humoral response on the haptenated liposomes is specific as only cross reactions between TNP-PE-liposomes and the structurally related complexes with enlarged haptens (J and K) were found. Immunization with liposomes containing enlarged haptens resulted in about thirty to forty times higher PFC numbers compared with those obtained with TNP-PE-liposomes. Kochibe *et al.* (1975) found that immunization with hybrid liposomes results in a significantly greater formation of anti-DNP antibodies than does immunization with homogeneous liposomes (containing only DNP-Cap-PE). It has to be investigated if this also holds for the already high responses on the enlarged haptens. Normal preparations consist mainly of multilamellar liposomes which are heterogeneous vesicles. Huang (1969) has demonstrated that sonication of multilamellar liposomes yields a far more homogeneous and much smaller population (25–40 nm in diameter) of which approximately one half are bounded by a single bilayer. In the present experiments the PFC response decreased three-fold if the liposomes were not sonicated before immunization. Sonicated enlarged hapten-PE-liposomes induced responses of a same level as the highly immunogenic thymus independent J_{59}-Ficoll. These results are at variance with those of Dancey, Yasuda & Kinsky (1978) who found either a reduction or no effect upon sonication of the liposomes. Our results can easily be explained as a dose effect of the number of effective liposomes. From Fig. 4 it can be seen that a sharp peak is obtained with 3–10 nmol of sonicated liposomes.

Lower and higher doses induce far lower responses. This might explain the results of Dancey *et al.* (1978) who did the sonication experiments at the dose which was optimal for non-sonicated liposomes (Yasuda *et al.*, 1977). With a fixed dose of sonicated liposomes, varying epitope densities yielded a peak response at 5 mol% K-PE (Fig. 5). With higher epitope densities the response decreased. This corresponds with the results of Yasuda *et al.* (1977) who found also a maximal response at an epitope density of 5 mol% DNP-Cap-PE.

From our results it is clear that the enlarged haptens inserted within liposomal model membranes provide very suitable immunogens for studying the conditions for controlling the immune response, without interfering contributions of unknown carrier determinants as in the conventional hapten-carrier model.

ACKOWLEDGMENTS

The authors gratefully acknowledge Dr J. K. Inman for constructive criticism of the manuscript, Miss G. Peulen and Mr A. Antonissen for their expert technical assistance.

This study was supported by the Foundation for Medical Research FUNGO which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO), Grant 13-27-34.

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