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Characterization of immunogenic properties of haptenated liposomal model membranes in mice

III. SPECIFICITY OF DELAYED-TYPE HYPERSENSITIVITY AND ANTIBODY FORMATION

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Summary. This paper describes the specificity of delayed-type hypersensitivity (DH) and antibody formation in the mouse to the tripeptide-enlarged hapten, 3-(p-azobenzenearsonate)-N-acetyl-L-tyrosylglycylglycine (A). Hapten A was coupled to phosphatidylethanolamine (PE) and incorporated into liposomal membranes (A–PE-liposomes). DH was measured as footpad swelling and antibody formation by the enumeration of direct plaque-forming cells in the spleen.

A–PE-liposomes mixed with the cationic, surface-active lipid, dimethyl dioctadecyl ammonium bromide (DDA) induce, on intracutaneous injection in mice, hapten-specific DH without a contribution by the carrier. With other haptenated liposomes it was not possible to induce DH to those haptens, including the closely related hapten 3-(p-azobenzenesulphonate)-N-acetyl-L-tyrosylglycylglycine (S).

A–PE- and S–PE-liposomes evoke, after intravenous injection in mice, a humoral response. The antibody formation to A–PE-liposomes was thymus-independent. In this response a considerable cross reaction between haptens A and S was observed.

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INTRODUCTION

Snippe, Johannesen, Inman & Merchant (1978) described the induction of delayed-type hypersensitivity (DH) after intracutaneous (i.c.) immunization of mice with several tripeptide-enlarged hapten–bovine serum albumin (BSA) complexes mixed with the adjuvant, dimethyl dioctadecyl ammonium bromide (DDA). A broad array of cross reactions between these enlarged hapten–carrier complexes showed a relative lack of specificity in the DH responses.

In an accompanying paper (van Houte, Snippe, Peulen & Willers, 1981) we described the coupling of the tripeptide enlarged hapten A (3-(p-azobenzenearsonate)-N-acetyl-L-tyrosylglycylglycine) to phosphatidylethanolamine (PE) and the incorporation of this conjugate into liposomal membranes (haptenated liposomes). Injection of A–PE-liposomes mixed with DDA induced DH to hapten A after i.c. injection in mice. From a previous study (van Houte, Snippe & Willers, 1979) it was known that several other haptenated liposomes were able to evoke a thymus-independent, hapten-specific humoral immune response after intravenous (i.v.) injection in mice.

The purpose of the present study was to investigate if tripeptide-enlarged, hapten-PE conjugates other than A-PE, alone or incorporated into liposomes, can also induce a state of DH. Furthermore, the humoral

response to A-PE-liposomes is investigated. The specificity of both DH and antibody formation was studied in mice immunized with a number of different haptenated liposomes.

MATERIALS AND METHODS

Mice

Inbred female BALB/c mice and inbred female F_1 (hybrid BALB/c ♂ × Swiss ♀) mice were raised and maintained in the Laboratory of Microbiology, State University, Utrecht, The Netherlands. Nude B10LP/Cpb (nu/nu) mice and their heterozygous littermates (nu/+) were purchased from the Central Institute for the breeding of Laboratory Animals (CPB-TNO), Zeist, The Netherlands. The mice were used at an age of about 10 weeks (weight approximately 20 g).

Preparation of derivatives

The haptens employed in this study are listed in Table 1. The tertiary butyloxycarbonyl hydrazides of these haptens (hapten Boc hydrazides) were coupled to soybean L-α-phosphatidylethanolamine (PE, mol.wt 735, Sigma Chemical Company, Saint Louis, Miss., U.S.A.) as described (van Houte *et al.*, 1979). A and S Boc hydrazides were synthesized according to the procedure of Inman, Merchant & Tacey (1973a). J and N Boc hydrazides were obtained commercially from Biosearch, San Rafael, Calif., U.S.A.

Liposomes

In all experiments 5 mol% haptenated liposomes, containing 5 nmol hapten–PE, were used. These haptenated liposomes were prepared as described previously (van Houte *et al.*, 1979).

Hapten–carrier complexes

The A_{13}–BSA and S_{33}–BSA antigens were prepared as described by Inman, Merchant, Claflin & Tacey (1973b).

The Ficoll and ovalbumin (OVA) antigens derivatized with the haptens A (A_{45}–Ficoll, A_7–OVA) and S (S_{61}–Ficoll) were a gift of Dr J. K. Inman (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md, U.S.A.) and were prepared according to the procedure of Inman (1975). The subscripts refer to the number of moles of hapten per mole of carrier. The keyhole limpet haemocyanin antigen (A_6-KLH) was also a gift of Dr J. K. Inman and was prepared as described by Inman *et al.* (1973b).

DH response

DH was induced and assayed in BALB/c mice as described in an accompanying paper (van Houte *et al.*, 1981).

Humoral response

For each experiment, groups of 5 F_1 mice were used. Haptenated liposomes, dispersed in 0·5 ml phosphate buffered saline (PBS, 0·01 M phosphate buffer, pH 7·2, containing 0·14 M NaCl) were injected in the lateral tail vein. 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). Indicator erythrocytes, optimally derivatized with the haptens A, S or J, were prepared according to the procedure of Inman *et al.* (1973b).

Statistical analysis

Results are expressed as the arithmetic mean of n independent observations ± 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

Specificity of DH to hapten A

Intracutaneous immunization of mice with A–PE-liposomes mixed with the adjuvant DDA resulted in DH to hapten A at days 5 and 6 (van Houte *et al.*, 1981). To determine the specificity of this DH, groups of mice were immunized i.c. with A–PE-liposomes mixed with 100 μg DDA. Five days after immunization these mice were elicited in the left hind footpad with haptenated liposomes. The increase in footpad thickness was measured 24 h later. Table 2 shows that the DH to A–PE-liposomes was hapten specific since no footpad swelling could be detected in mice elicited with J–PE- or S–PE-liposomes. However, when BSA was used as a carrier both for immunization and elicitation a strong cross-reactivity between haptens A and S was observed (Table 2 and Snippe *et al.*, 1978). Immunization of mice with A–PE-liposomes mixed with DDA and elicitation with hapten A coupled to BSA or OVA (A_{13}–BSA, A_7–OVA) resulted in a strong

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

TGG series
A = 3-(p-azobenzenearsonate)-N-acetyl-L-tyrosylglycylglycine
S = 3-(p-azobenzenesulphonate)-N-acetyl-L-tyrosylglycylglycine

AGG series
J = N-(2,4-dinitrophenyl)-β-alanylglycylglycine
N = N-(4-hydroxy-3-iodo-5-nitrophenylacetyl)-β-alanylglycylglycine

*Structural formulae of the enlarged haptens are presented in Inman, *et al.* (1973a).

DH of the same magnitude as that induced in the homologous DH. On the other hand, immunization with A_{13}–BSA (10 μg) mixed with DDA and elicitation with A–PE-liposomes resulted in a moderate DH. A_{45}–Ficoll elicited a moderate DH in mice immunized with A–PE-liposomes and DDA.

Induction and specificity of DH to hapten S

Hapten S differs only from hapten A in the arsonate group which in the former hapten is replaced by a sulphonate group (Table 1). Therefore it is very likely that the S–PE conjugate or S–PE-liposomes could also induce DH. Groups of mice were immunized i.c. with 5 nmol of free S-PE conjugate or the same amount of conjugate incorporated into liposomes, mixed with the adjuvants DDA (100 μg) or FCA (0·1 ml) or with PBS. Control groups received PBS or non-haptenated liposomes with DDA or FCA. Five days later all mice were elicited with S–PE-liposomes. Table 3 shows that free S–PE mixed with PBS, FCA or DDA did not induce significant DH. Neither incorporation of S–PE into liposomes nor mixing with PBS or FCA or DDA resulted in induction of DH. However, injecting mice with the hapten–carrier complex S_{33}–BSA mixed with DDA immunized mice for elicitation not only with the homologous antigen but also with A_{13}–BSA. No DH was measured if S–PE-liposomes were used as immunizing and/or eliciting antigen. J–PE- and N–PE-liposomes mixed with DDA were also not able to induce DH in the homologous system 5 days after immunization (Table 3).

Humoral immune response to haptenated liposomes

The results presented in Table 2 and 3 show that only A–PE-liposomes could induce hapten-specific DH. To compare the immunogenicity of haptenated liposomes in the humoral response, mice were injected i.v. with 5 nmol free A–PE conjugate or haptenated liposomes (containing either 5 nmol A–PE or S–PE or J–PE)

Table 2. Specificity of DH to hapten A

Immunization	Adjuvant	Elicitation	Footpad swelling (mm ± SEM)	*P*
A–PE-liposomes	DDA	A–PE-liposomes	1·16±0·15	<0·001
A–PE-liposomes	DDA	J–PE-liposomes	0·22±0·03	0·1
A–PE-liposomes	DDA	S–PE-liposomes	0·24±0·04	0·1
A_{13}–BSA (10 μg)	DDA	A_{13}–BSA (10 μg)	1·60±0·12	<0·001
A_{13}–BSA (10 μg)	DDA	S_{33}–BSA (10 μg)	2·04±0·08	<0·001
A_{13}–BSA (10 μg)	DDA	S–PE-liposomes	0·19±0·03	0·2
A_{13}–BSA (10 μg)	DDA	BSA (10 μg)	0·13±0·01	0·3
A–PE-liposomes	DDA	A_{13}–BSA (10 μg)	1·71±0·11	<0·001
A–PE-liposomes	DDA	S_{33}–BSA (10 μg)	0·24±0·04	0·1
A–PE-liposomes	DDA	A_{45}–Ficoll (10 μg)	0·72±0·12	<0·001
A–PE-liposomes	DDA	A_7–OVA (10 μg)	1·10±0·12	<0·001
A_{13}–BSA (10 μg)	DDA	A–PE-liposomes	0·73±0·09	<0·001
PBS	DDA	A–PE-liposomes	0·15±0·03	–

Groups of mice ($n = 7$) were immunized i.c. and elicited 5 days later with the antigen combinations indicated in the table. *P* values test the significance of the difference between groups of mice immunized with PBS and DDA and groups immunized with the different antigens.

Table 3. Induction and specificity of DH to hapten S

Immunization	Adjuvant	Elicitation	Footpad swelling (mm ± SEM)	*P*
S–PE (5 nmol)	PBS	S–PE-liposomes	0·09 ± 0·04	0·3
S–PE (5 nmol)	FCA	S–PE-liposomes	0·12 ± 0·02	0·4
S–PE (5 nmol)	DDA	S–PE-liposomes	0·07 ± 0·04	0·2
S–PE-liposomes	PBS	S–PE-liposomes	0·07 ± 0·02	0·2
S–PE-liposomes	FCA	S–PE-liposomes	0·11 ± 0·02	0·4
S–PE-liposomes	DDA	S–PE-liposomes	0·16 ± 0·07	0·4
Non-haptenated liposomes	DDA or FCA	S–PE-liposomes	0·15 ± 0·03	0·4
PBS	DDA or FCA	S–PE-liposomes	0·13 ± 0·05	—
S_{33}–BSA (10 μg)	DDA	S_{33}–BSA (10 μg)	1·97 ± 0·10	< 0·001
S_{33}–BSA (10 μg)	DDA	S–PE-liposomes	0·12 ± 0·04	0·4
S–PE-liposomes	DDA	S_{33}–BSA (10 μg)	0·21 ± 0·04	0·1
S_{33}–BSA (10 μg)	DDA	A_{13}–BSA (10 μg)	1·67 ± 0·12	< 0·001
S_{33}–BSA (10 μg)	DDA	A–PE-liposomes	0·08 ± 0·02	0·2
S_{33}–BSA (10 μg)	DDA	BSA (10 μg)	0·24 ± 0·05	0·1
S–PE-liposomes	DDA	A–PE-liposomes	0·07 ± 0·03	0·2
J–PE-liposomes	DDA	J–PE-liposomes	0·09 ± 0·03	0·3
N–PE-liposomes	DDA	N–PE-liposomes	0·16 ± 0·06	0·4

Groups of mice ($n = 7$) were immunized i.c. and elicited 5 days later with the antigen combinations indicated in the table. *P* values test the significance of the difference between groups of mice immunized with PBS and adjuvant and groups immunized with the different antigens.

without DDA, and 4 days later the number of direct, A-, S- or J-specific plaque-forming cells in the spleens of these mice were determined. Table 4 shows that A–PE needs to be incorporated into the liposomal bilayer to induce a significant humoral response 4 days after immunization. A–PE-liposomes and S–PE-liposomes induced large numbers of hapten-specific PFC not only in the homologous but also in the heterologous systems (Table 4). The response on J–PE-liposomes was rather specific for hapten J.

A high degree of cross-reactivity between haptens A and S in the PFC response could also be observed when these haptens were coupled to Ficoll.

In a previous paper (van Houte *et al.*, 1979) we

Table 4. Humoral immune response to haptenated liposomes

Immunization	Direct PFC × 10^{-3}/spleen ± SEM, specific for			
	SRBC	A–SRBC	S–SRBC	J–SRBC
PBS	0·20 ± 0·07	0·18 ± 0·07	0·20 ± 0·06	0·18 ± 0·04
Non-haptenated liposomes	0·20 ± 0·05	0·40 ± 0·03	0·30 ± 0·04	0·20 ± 0·04
A–PE (5 nmol)	0·42 ± 0·32	0·77 ± 0·12	n.t.	0·51 ± 0·06
A–PE-liposomes	0·15 ± 0·08	17·1 ± 1·8	14·9 ± 0·8	0·43 ± 0·05
S–PE-liposomes	1·1 ± 0·2	14·3 ± 1·5	25·5 ± 1·9	0·63 ± 0·14
J–PE-liposomes	0·10 ± 0·07	1·5 ± 0·3	0·44 ± 0·12	26·8 ± 1·9
A_{45}–Ficoll (10 μg)	0·19 ± 0·07	20·8 ± 2·3	12·1 ± 2·0	1·9 ± 0·5
S_{61}–Ficoll (10 μg)	0·12 ± 0·04	8·1 ± 0·8	16·6 ± 1·3	0·75 ± 0·18

Groups of mice ($n = 5$) were immunized i.v. as indicated in the table. Individual spleens were assayed for direct PFC responses 4 days after immunization.
n.t. = not tested.

Table 5. Direct PFC response to A–PE-liposomes in nude (nu/nu) and heterozygous (nu/+) mice

Immunization	Direct A-specific PFC $\times 10^{-3}$/spleen ± SEM		
	Nude (nu/nu)	Heterozygous (nu/+)	*P*
A–PE-liposomes	7·7±0·9	6·0±0·7	0·087
A_{45}–Ficoll (10 μg)	11·1±1·3	8·3±1·0	0·063
A_6–KLH (50 μg)	0·9±0·3	6·2±0·8	<0·001

Groups of mice ($n=5$) were immunized i.v. with A–PE-liposomes or 10 μg A_{45}–Ficoll or i.p. with 50 μg A_6–KLH on bentonite. Individual spleens were assayed for direct A-specific PFC responses 4 days after immunization. *P* values test the significance of the difference between the nude (nu/nu) and heterozygous (nu/+) mice.

showed that J–PE-liposomes behave like T-cell independent antigens. In order to study if A–PE-liposomes could induce a humoral immune response in mice deficient in thymus-derived (T) lymphocytes, nude mice (nu/nu) were immunized with A–PE-liposomes, A_{45}–Ficoll and A_6–KLH (Table 5). Heterozygous littermates (nu/+) were used as controls. The specific PFC response did not differ significantly in nude (nu/nu) and heterozygous (nu/+) mice for either the thymus-independent antigen A_{45}–Ficoll or the A–PE-liposomes (Table 5). When the two different groups of mice were immunized with A_6–KLH, however, the A-specific PFC-response of the nude (nu/nu) group was about seven times lower than that of the heterozygous (nu/+) group.

DISCUSSION

In a recent paper (van Houte *et al.*, 1981) it was described that i.c. injection in mice with A–PE conjugate or A–PE-liposomes mixed with DDA induced DH at days 5 and 6. The use of the cationic, surface-active compound, DDA (Dailey & Hunter, 1974; Snippe, Belder & Willers, 1977) was necessary to induce this DH. We argued that the presentation of hapten A in a liposomal or micellar structure is required to evoke a cellular immune response in mice to that hapten.

In this paper the specificity of DH induced by A–PE-liposomes mixed with DDA was studied. The DH reaction was hapten specific. In the elicitation reaction A–PE-liposomes could be replaced by A_{13}–BSA, A_7–OVA and A_{45}–Ficoll (Table 2). This strong hapten specificity only occurred in mice immunized with A–PE-liposomes and not in A_{13}–BSA immunized mice (Table 2 and Snippe *et al.*, 1978). In the latter instance cross-reactivity with S_{33}–BSA was observed. It is very likely that in this experiment carrier determinants are involved in DH. In order to obtain more information on the specificity of DH to hapten A, DH was studied to hapten S, in which the arsonate group is replaced by a sulphonate (Table 1). Alkan, Williams, Nitecki & Goodman (1972) found that 3-(p-azobenzenesulphonate)-L-tyrosine (ABS-Tyr) and FCA also induced DH in guinea-pigs to ABS-Tyr and observed only a small cross-reactivity between 3-(p-azobenezenearsonate)-L-tyrosine (ABA-Tyr) and ABS-Tyr. Nauciel (1972) did not find cross-reactivity between ABA-Acetyl-Tyr and ABS-Acetyl-Tyr in an *in vitro* stimulation test of guinea-pig lymph node cells. Ray & Ben-Sasson (1979) however, found that T cells from guinea-pigs immunized with ABA-Acetyl-Tyr could be stimulated *in vitro* with ABS–guinea-pig serum albumin. In our hands no DH could be induced with S–PE or S–PE-liposomes neither with DDA nor FCA. However, hapten S is capable of inducing DH when it is coupled to BSA (S_{33}–BSA) and the elicitation is given with the homologous antigen but not with the heterologous S–PE-liposomes (Table 3). In an accompanying paper (van Houte *et al.*, 1981) we suggest that the hydrocarbon chains of DDA become incorporated into the liposomal bilayer upon mixing of A–PE-liposomes with DDA. This results in liposomes with a strongly electropositive charge at their surfaces provided by the

polar head group of DDA. Such positively charged liposomes might induce a better interaction between hapten and T lymphocyte than reached by A–PE-liposomes without DDA. The sulphonate group in S–PE-liposomes is more negatively charged than the arsonate. This would result in a stronger interaction between hapten S and the positive head group of DDA, thus reducing the incorporation of DDA into the S–PE-liposomes. In this complex DDA does not promote the interaction between hapten and T lymphocytes and no DH will be induced. The specificity of DH induced with A–PE-liposomes can be explained by the absence of a contribution to the DH of an immunogenic carrier. It should, however, be borne in mind that this specificity was observed with haptenated liposomes which are not able to induce DH themselves under the conditions used.

In a previous paper (van Houte *et al.*, 1979) we described that J–PE- and N–PE-liposomes evoke a thymus-independent, hapten-specific humoral immune response after i.v. injection in mice. Conjugates of J or N with BSA mixed with DDA induced DH (Snippe *et al.*, 1978). It was not possible to induce DH with these haptens incorporated into liposomes (Table 3). In general it is difficult to induce DH to thymus-independent antigens like pneumococcal polysaccharides (Turk & Parker, 1977), lipopolysaccharides and flagellin (Parish, 1971). This might also explain the incapacity of J–PE- and N–PE-liposomes to induce DH. On the other hand A–PE-liposomes also behave as a thymus-independent antigen in the humoral response: inducing only production of IgM antibodies (data not given) and stimulating antibody formation in nude mice (Table 5). It might be possible that, for induction of DH to other haptenated liposomes, the physicochemical and chemical properties of the hapten or of the complexes have to be changed. It is also still possible that the lack of DH response of BALB/c mice to S–PE-, N–PE- and J–PE-liposomes is genetically determined. However, previous experiments (Snippe *et al.*, 1978) indicate that J_{30}- and N_{43}–BSA induce strong DH reactions in BALB/c mice and no correlation could be observed between H-2 haplotype and the intensity of the DH reaction in J_{31}–BSA immunized mice (Snippe, Johannesen, Lizzio & Merchant, 1980). A possible explanation for the failure to demonstrate a relationship between H-2 haplotype and immune response (Ir) genes for DH to J_{31}–BSA in mice might relate to the complexity of the BSA carrier used. Therefore experiments are now in progress to test the induction of DH with hapten A, S, J or N coupled to *non-immunogenic* liposomes in mice strains of a different H-2 haplotype.

A totally different explanation for the peculiar behaviour of A–PE-liposomes may be found in the toxicity of the arsonate group. This enables hapten A to inhibit its own metabolism which results in a longer persistence.

No serum antibodies to hapten A could be detected in mice after i.c. immunization with A–PE or A–PE-liposomes and DDA (data not given). It is possible, however, to induce a humoral response to hapten A by changing the route of immunization. Intravenous injection of mice with A–PE-liposomes resulted in a humoral response to hapten A (Table 4). In this experiment the A–PE conjugate had to be incorporated into the liposomal membrane but no adjuvant (DDA or FCA) was required. A considerable cross-reaction between haptens A and S in the humoral response was observed when they were coupled to liposomes or Ficoll (Table 4). As the response on these antigens in thymus independent, the cross reactions in the humoral response are very probably on the B lymphocyte level.

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