

## Characterization of immunogenic properties of haptened liposomal model membranes in mice

### II. INDUCTION OF DELAYED-TYPE HYPERSENSITIVITY

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**Summary.** This paper describes the induction of delayed-type hypersensitivity (DH) in the mouse and guinea-pig to haptened liposomes. The tripeptide-enlarged hapten 3-(p-azobenzearsonate)-N-acetyl-L-tyrosylglycylglycine (A) was coupled to phosphatidylethanolamine (PE) and incorporated into liposomal membranes (A-PE-liposomes). In mice DH was measured as footpad swelling and in guinea pigs by skin testing.

To induce hapten A-specific DH in mice with A-PE-liposomes the application of the cationic, surface-active lipid, dimethyl dioctadecyl ammonium bromide (DDA) was necessary. The use of Freund's complete adjuvant (FCA) did not result in the induction of DH to hapten A. In guinea-pigs, however, FCA and DDA had equally good adjuvant properties in the induction of DH. The time course of the DH and the optimal time interval between immunization and elicitation were determined for the mouse system. Also, the effect of dose and epitope density was studied in that system. Cyclophosphamide treatment, before immunizing mice with A-PE-liposomes and DDA, resulted in greatly impaired DH, probably caused by the short

lifetime of the integrity of liposomes after intracutaneous administration to mice.

The results make it very likely that presentation of hapten A in a liposomal or micellar structure is required to induce a cellular immune response to this hapten in mice.

### INTRODUCTION

In a preceding paper (van Houte, Snippe & Willers, 1979) the humoral immunogenicity of haptened liposomes was studied. A simple coupling method for tripeptide-enlarged haptens to phosphatidylethanolamine (PE) and the incorporation of these conjugates into liposomal membranes (haptened liposomes) was described. These haptened liposomes evoked a thymus-independent, hapten-specific humoral immune response after intravenous injection in mice.

Nicolotti & Kinsky (1975) and Nicolotti, Kochibe & Kinsky (1976) showed that the low molecular weight compound 3-(p-azobenzearsonate)-tyrosine (ABA-Tyr), linked through the carboxyl group to PE (ABA-Tyr-PE), induced not only anti-ABA antibodies but also delayed-type hypersensitivity (DH). Incorporation of ABA-Tyr-PE into a liposomal membrane was not obligatory, but the use of Freund's complete adjuvant (FCA) was required. They further found that

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presentation of the ABA group on bovine serum albumin (BSA) resulted in the production of antibodies without DH (Nicolotti & Kinsky, 1975). On the other hand Mattern & Leskowitz (1977) demonstrated that coupling of hydrocarbon chains to the amino group of the ABA-Tyr molecule resulted in DH which decreases in strength with increasing length of the chain. All results mentioned thus far were obtained with the ABA-Tyr group in guinea pigs.

Snippe, Johannesen, Inman & Merchant (1978) coupled to BSA the enlarged hapten A, (3-(p-azobenzearsonate)-N-acetyl-L-tyrosylglycylglycine), which is very similar in structure to ABA-Tyr. They were able to induce a homologous DH reaction in mice to this hapten if the cationic, surface-active lipid, dimethyl dioctadecyl ammonium bromide (DDA) was used as an adjuvant.

In the present study the immunogenicity of liposomes, haptenated with hapten A, in mice and guinea-pigs is investigated. To study the optimal conditions for the induction of DH to the hapten A, the effect of the adjuvants DDA and FCA, the time course of the DH, and the effect of dose of A-PE-liposome and of epitope density are investigated.

## MATERIALS AND METHODS

### Animals

Inbred female BALB/c mice were raised and maintained in the Laboratory of Microbiology, State University, Utrecht, The Netherlands. The mice were used at an age of about 10 weeks (weight approximately 20 g). Random-bred, white, female guinea-pigs (weight approximately 300 g) were purchased from the Central Institute for the breeding of Laboratory Animals (CPB-TNO), Zeist, The Netherlands.

### Preparation of derivatives

The structural formula of hapten A used in this study is presented in Fig. 1. The tertiary butyloxycarbonyl hydrazide of this hapten (A Boc hydrazide) was coupled to soy bean L- $\alpha$ -phosphatidyl-ethanolamine (PE, mol.wt. 735, Sigma Chemical Company, Saint Louis, Missouri) as described before (van Houte *et al.*, 1979). A Boc hydrazide was synthesized according to the procedure of Inman, Merchant & Tacey (1973).

### Liposomes

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

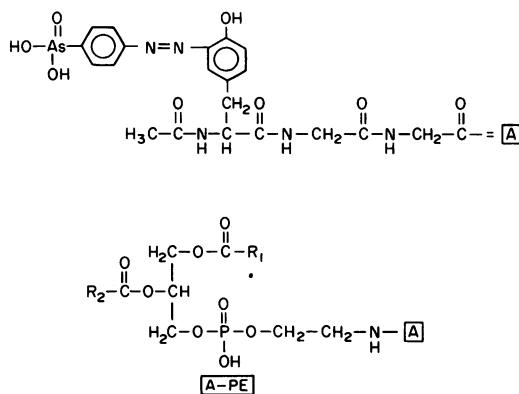


Figure 1. Structural formula of the tripeptide-enlarged hapten A and the A-PE conjugate.

priate amounts of the N-substituted PE derivative (A-PE; Fig. 1) into a basic lipid mixture containing synthetic dipalmitoyl lecithin, cholesterol, octadecylamine (stearylamine) and A-PE conjugate in molar ratios of 14:2:3:1. These haptenated liposomes were prepared as described previously (van Houte *et al.*, 1979).

### Hapten-carrier complexes

The Ficoll and ovalbumin (OVA) antigens, derivatized with hapten A (A<sub>45</sub>-Ficoll and A<sub>7</sub>-OVA), were a gift of Dr J. K. Inman (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland) and were prepared according to the procedures of Inman (1975) and Inman, Merchant, Claflin & Tacey (1973) respectively. The subscripts refer to the number of moles of hapten per mole of carrier.

### Induction, elicitation and measurement of delayed-type hypersensitivity

*In mice.* For each experiment, groups of seven BALB/c mice were used. A-PE-liposomes, dispersed in 0.1 ml phosphate buffered saline (PBS, 0.01 M phosphate buffer, pH 7.2, containing 0.14 M NaCl) and mixed with a suspension of 100  $\mu$ g dimethyl dioctadecyl ammonium bromide (DDA, Eastman Kodak, Rochester, New York) in 0.1 ml PBS, were injected intracutaneously (i.c.) on the abdomen at four separate sites. In some experiments the liposomes were emulsified in 0.1 ml Freund's complete adjuvant (FCA) containing killed *Mycobacterium tuberculosis* H<sub>37</sub>R<sub>a</sub> (Difco Laboratories, Detroit, Michigan) instead of DDA.

DH reactions were determined by measuring the increase in footpad thickness (footpad swelling test) as described by Kerckhaert, van den Berg & Willers (1974) using a semi-electronic paw meter (van Dijk, Versteeg & Hennink, 1976). The eliciting dose of antigen suspended in 0.05 ml PBS was injected into the left hind footpad. A footpad swelling of 0.25 mm is regarded as positive. The results are expressed as the increment of the footpad thickness in 1 mm units.

*In guinea-pigs.* A-PE-liposomes, dispersed in 0.2 ml PBS and mixed with 100 µg DDA in 0.2 ml PBS or 0.2 ml FCA, were injected subcutaneously (s.c.) in the footpads of guinea pigs (0.1 ml/footpad).

DH reactions were determined by skin testing. Three weeks after immunization depilated lateral sites were injected intradermally with the eliciting dose of antigen suspended in 0.1 ml PBS. Skin tests were read after 24 h and two parameters were measured: (1) diameter of erythema in millimetres and (2) increase in double skin thickness in millimetres with a manual caliper (Oditest; H. C. Kröplin, Schlüchteren, West Germany).

#### *Cyclophosphamide treatment*

Cyclophosphamide (CY) was obtained from Koch-Light Laboratories Ltd, (Colnbrook Buckinghamshire). The mice received an intraperitoneal (i.p.) injection of CY (300 mg/kg) in 0.5 ml PBS, 8 h before i.c. immunization with A-PE-liposomes.

#### *Histology*

Twenty-four hours after elicitation, sections of the left hind footpad of mice were stained with haematoxylin and eosin and examined histologically by Dr A. A. van den Broek, Laboratory of Histology, State University of Groningen, The Netherlands.

#### *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 used to analyse the statistical significance of the results. Values of *P* over 0.05 are considered to be not significant.

## RESULTS

### Induction of DH to hapten A

Groups of mice were immunized i.c. with 5 nmol of free A-PE conjugate or the same amount of conjugate incorporated into the bilayers of 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 after immunization mice were elicited in the left hind footpad with 5 mol% A-PE-liposomes containing 5 nmol A-PE. The increase in footpad thickness was measured 24 h later. Table 1 shows that free A-PE mixed with PBS did not induce significant DH 5 days

**Table 1.** Induction of DH to hapten A in mice

Immunization	Adjuvant	Footpad swelling (mm ± SEM)	<i>P</i>
A-PE (5 nmol)	PBS	0.21 ± 0.04	0.1
A-PE (5 nmol)	FCA	0.12 ± 0.06	0.3
A-PE (5 nmol)	DDA	1.15 ± 0.14	<0.001
A-PE-liposomes	PBS	0.43 ± 0.04	<0.001
A-PE-liposomes	FCA	0.22 ± 0.04	0.1
A-PE-liposomes	DDA	1.16 ± 0.15	<0.001
A Boc (5 nmol)	DDA or FCA	0.08 ± 0.03	0.1
Non-haptenated liposomes	DDA or FCA	0.15 ± 0.02	0.5
PBS	DDA or FCA	0.15 ± 0.03	—
A45-Ficoll (10 µg)	DDA or FCA	0.08 ± 0.03	0.1

Groups of mice (*n* = 7) were immunized i.c. as indicated in the table. Haptenated liposomes were 5 mol% and contained 5 nmol A-PE. All mice were elicited at day 5 with 5 mol% haptenated liposomes, containing 5 nmol A-PE. The footpad swelling was measured 24 h later. *P*-values test the significance of the difference between groups of mice immunized with PBS and adjuvant and groups immunized with the different antigens.

**Table 2.** Induction of DH to hapten A in guinea-pigs

Elicitation	Skin reaction* in guinea-pigs immunized with A-PE-liposomes mixed with the adjuvants					
	FCA		DDA		PBS	
	Erythema	Induration	Erythema	Induration	Erythema	Induration
A-PE-liposomes	11.0 ± 1.3	0.92 ± 0.25	9.7 ± 1.2	0.90 ± 0.13	1.9 ± 0.9	0.13 ± 0.07
Non-haptenated liposomes	1.2 ± 0.6	0.02 ± 0.02	1.3 ± 0.6	0.02 ± 0.02	1.0 ± 0.5	0.01 ± 0.01
PBS	0	0.02 ± 0.02	0	0.01 ± 0.01	0	0.01 ± 0.01
A <sub>45</sub> -Ficoll (10 µg)	10.5 ± 1.4	0.83 ± 0.24	10.3 ± 1.2	0.75 ± 0.11	0	0.07 ± 0.03
A <sub>7</sub> -OVA (10 µg)	10.0 ± 1.2	0.67 ± 0.15	9.7 ± 0.6	0.55 ± 0.16	1.7 ± 0.6	0.10 ± 0.05

Groups of guinea-pigs ( $n=3$ ) were immunized s.c. in the footpads with 5 mol% A-PE-liposomes (containing 100 nmol A-PE) mixed with the adjuvants DDA (100 µg) or FCA (0.2 ml) or with PBS. After 3 weeks the animals were elicited as indicated in the table; 5 mol% A-PE-liposomes (containing 5 nmol A-PE) were used. Erythema and induration were measured 24 h later.

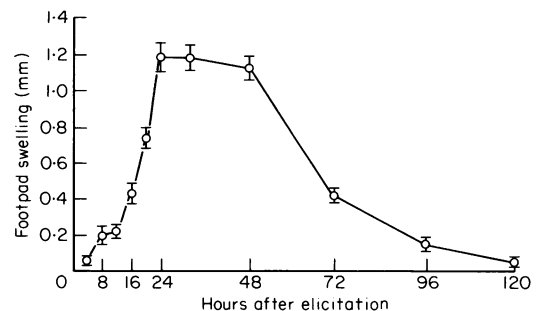
\* The diameter of the erythema is given in mm ± SEM. The induration is measured as the increase in mm of double skin thickness ± SEM.

after immunization. A-PE-liposomes mixed with PBS induced a moderate but significant footpad swelling. However, both A-PE-liposomes mixed with DDA and free A-PE mixed with DDA induced a strong DH of the same magnitude. Mixed with FCA neither A-PE-liposomes nor free A-PE induced a significant DH 5 days after immunization. Hapten A (A Boc hydrazide) mixed with DDA or FCA did not induce DH. Similar immunizations with the thymus-independent antigen A<sub>45</sub>-Ficoll (Inman, 1975) did not result in induction of DH to hapten A (Snippe *et al.*, 1978; Table 1).

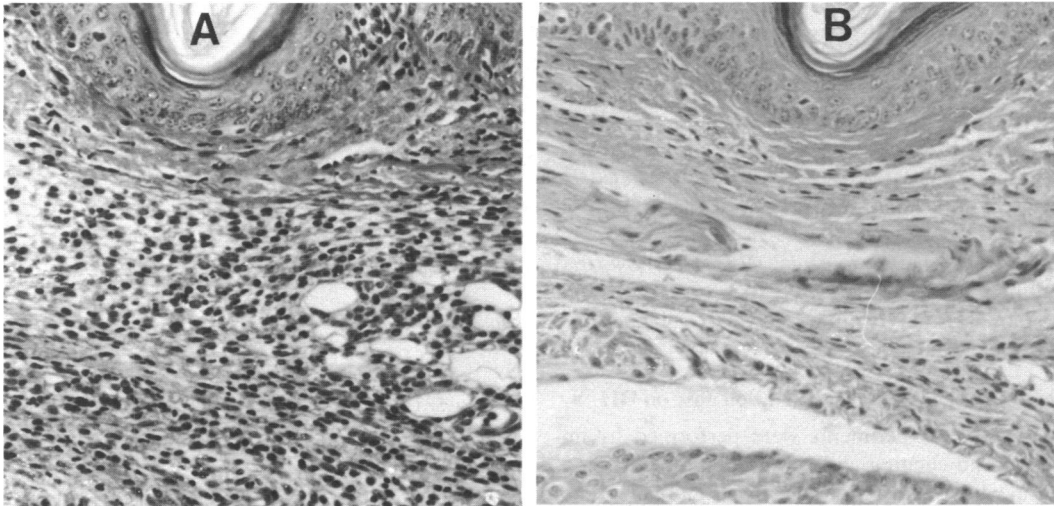
Nicolotti & Kinsky (1975) reported that immunization of guinea-pigs with ABA-Tyr-PE-liposomes and FCA resulted in DH to the ABA-Tyr compound. We also studied in guinea-pigs the induction of DH to hapten A with A-PE-liposomes mixed with the adjuvants FCA and DDA. Groups of guinea pigs were immunized s.c. in the footpads with 5 mol% A-PE-liposomes (containing 100 nmol A-PE) mixed with FCA (0.2 ml) or DDA (100 µg) or with PBS. Three weeks after immunization these animals were skin tested with 5 mol% A-PE-liposomes (containing 5 nmol A-PE), A<sub>45</sub>-Ficoll (10 µg), A<sub>7</sub>-OVA (10 µg) and non-haptenated liposomes. The diameter of erythema and the increase in double skin thickness were measured 24 h later. Table 2 shows that A-PE-liposomes mixed either with FCA or DDA induced DH to hapten A which was of the same order of magnitude. A-PE-liposomes mixed with PBS did not evoke DH.

#### Time course of DH after elicitation

Mice were immunized i.c. with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) mixed with 100 µg DDA. Five days after immunization these mice were injected in the left hind footpad with 5 mol% A-PE-liposomes, containing 5 nmol A-PE. The increase in footpad thickness was measured at different hours after elicitation. Figure 2 shows the typical picture of a delayed-type hypersensitivity reaction: the footpad swelling becomes positive 16 h after injection and increases steadily with time until it reaches a maximum value between 24 and 48 h. Four days after injection



**Figure 2.** Time course of DH after elicitation. Groups of mice ( $n=7$ ) were immunized i.c. with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) mixed with DDA and elicited 5 days later with 5 mol% A-PE-liposomes (containing 5 nmol A-PE). The footpad swelling was measured at different times after elicitation. Vertical bars indicate SEM.



**Figure 3.** Histology of DH in mice to A-PE-liposomes 24 h after elicitation. Mice were immunized i.c. with A-PE-liposomes (containing 5 nmol A-PE) mixed with 100  $\mu$ g DDA. Five days later these mice were elicited with (A) A-PE-liposomes, containing 5 nmol A-PE or with (B) non-haptenated liposomes. Sections were stained with haematoxylin and eosin. Note the marked infiltration of mononuclear cells in (A). Magnification:  $\times 400$ .

the DH reaction was no longer detectable. The footpad swelling between 4 and 12 h was not yet positive which suggests that no antibody mediated hypersensitivity was involved. In further experiments the footpad swelling was measured 24 h after elicitation.

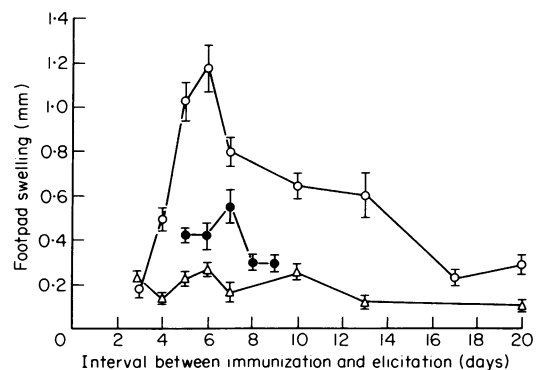
### Histology

Mice were immunized i.c. with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) mixed with 100  $\mu$ g DDA. Five days after immunization these mice were injected with 5 mol% A-PE-liposomes, containing 5 nmol A-PE. Control mice were injected with non-haptenated liposomes. Histological examination of the skin test site 24 h after elicitation shows a dense infiltrate of primarily mononuclear cells (Fig. 3A). Some polymorphonuclear cells are also seen.

### Effect of varying the interval between immunization and elicitation on DH

In order to determine the interval between immunization and elicitation necessary to induce a maximal footpad swelling, mice were immunized i.c. with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) mixed with 100  $\mu$ g DDA or PBS. With different intervals after immunization these mice were elicited in the

left hind footpad with 5 mol% A-PE-liposomes (containing 5 nmol A-PE). Figure 4 shows that no or only a moderate response appeared when the mice were tested 3 or 4 days after immunization, but a maximum response appeared on days 5 and 6. A response was no longer detectable 17 days after immunization. In further experiments the skin test was made 5 days



**Figure 4.** The effect on DH of varying the interval between immunization and elicitation. Groups of mice ( $n=7$ ) were immunized i.c. with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) mixed with DDA ( $\circ$ ), FCA ( $\Delta$ ) or PBS ( $\bullet$ ) and tested at different intervals with 5 mol% A-PE-liposomes (containing 5 nmol A-PE). The footpad swelling was measured 24 h later. Vertical bars indicate SEM.

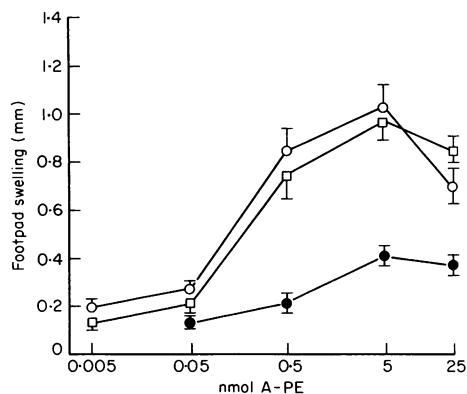
after immunization. When mice were immunized i.c. with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) in 0.1 ml FCA and elicited on day 5 with the same antigen, no DH reaction could be detected (Table 1). Since FCA is known to possess a depot function for an antigen—making it slowly available to the immune system (Borek, 1977)—we also studied the effect of varying the interval between immunization with 5 mol% A-PE-liposomes in 0.1 ml FCA and elicitation with the same antigen. Figure 4 shows that extending the interval from 3 to 20 days did not result in induction of DH to hapten A.

#### Effect of immunization and elicitation dose on DH

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

The effect of the immunizing dose on footpad swelling was studied by i.c. immunization of groups of mice with 5 mol% A-PE-liposomes containing a graded dose of A-PE with or without 100  $\mu$ g DDA and with different amounts of free A-PE conjugate mixed with 100  $\mu$ g DDA. Five days after immunization these mice were injected in the left hind footpad with 5 mol% A-PE-liposomes (containing 5 nmol A-PE). The increase in footpad thickness was measured 24 h after elicitation. Figure 5 shows that a maximum response was induced with liposomes mixed with DDA containing a total amount of 0.5–5 nmol A-PE. Immunization with 0.5–25 nmol of free A-PE mixed with DDA resulted in a maximum response of the same magnitude as induced by A-PE-liposomes and DDA. A-PE-liposomes injected without DDA induced a very moderate footpad swelling with doses of 5 and 25 nmol A-PE (Fig. 5).

The effect of the eliciting dose on footpad swelling was studied by i.c. immunization of groups of mice with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) mixed with 100  $\mu$ g DDA. Five days after immunization these mice were injected in the left hind footpad with 5 mol% A-PE-liposomes containing a graded dose of A-PE. The increase in footpad thickness was measured 24 h after elicitation. A maximum footpad swelling was induced by injecting with liposomes containing a total amount of 5 or 25 nmol A-PE (Fig. 6). The latter is the maximum amount that could be incorporated into 5 mol% A-PE-liposomes which

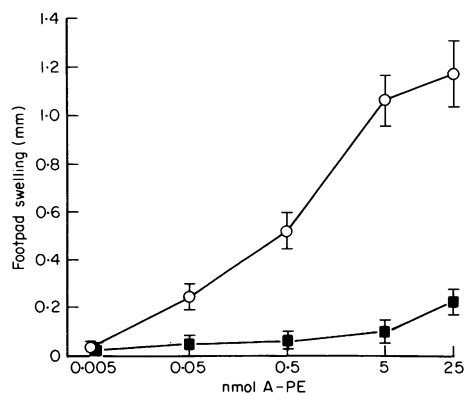


**Figure 5.** The effect of immunization dose on DH. Groups of mice ( $n=7$ ) were immunized i.c. with 5 mol% A-PE-liposomes (containing a graded dose of A-PE as indicated on the abscissa) mixed with DDA ( $\circ$ ) or PBS ( $\bullet$ ) or with free A-PE and DDA ( $\square$ ). Five days later the mice were tested with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) and the footpad swelling was measured 24 h after elicitation. Vertical bars indicate SEM.

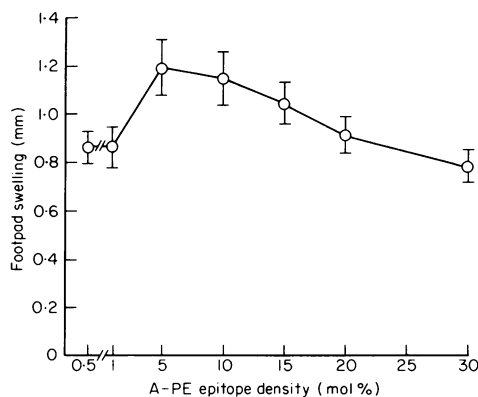
had to be suspended in 0.05 ml PBS (injection volume).

#### Effect of epitope density on DH

The effect of varying epitope density of haptened liposomes used for immunization, while maintaining a fixed dose of A-PE (5 nmol) was studied. The epitope



**Figure 6.** The effect of testing dose on DH. Groups of mice ( $n=7$ ) were immunized i.c. with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) mixed with DDA ( $\circ$ ) or with DDA alone ( $\bullet$ ). Five days later the mice were injected with 5 mol% A-PE-liposomes containing a graded dose of A-PE as indicated on the abscissa. Footpad swelling was measured 24 h after elicitation. Vertical bars indicate SEM.

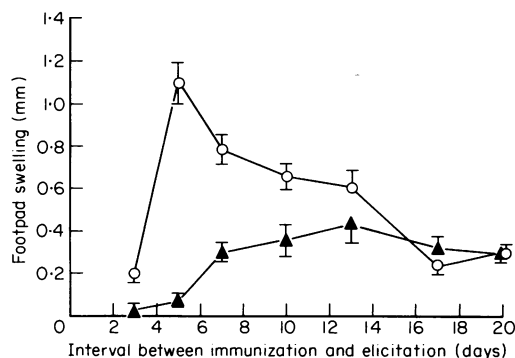


**Figure 7.** The effect of A-PE epitope density on DH. Groups of mice ( $n=7$ ) were immunized i.c. with DDA and A-PE-liposomes containing a fixed dose of A-PE (5 nmol). The epitope density was varied by changing the lecithin/A-PE molar ratio of the liposome. Five days later the mice were tested with 5 mol% A-PE-liposomes (containing 5 nmol A-PE-liposomes) and the footpad swelling was measured 24 h after elicitation. Vertical bars indicate SEM.

density was varied by changing the lecithin/A-PE molar ratio of the liposome. A maximum footpad swelling was obtained with liposomes containing 5–15 mol% A-PE (Fig. 7).

#### Effect of CY treatment on DH

Snippe *et al.* (1978) reported that CY injection preceding the i.c. immunization with hapten-BSA com-



**Figure 8.** The effect of CY treatment on DH. Groups of mice ( $n=7$ ) were immunized with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) mixed with DDA 8 h after i.p. injection of CY (▲) or PBS (○). Elicitation with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) was performed by footpad injection at varying time intervals after immunization. The footpad swelling was measured 24 h later. Vertical bars indicate SEM.

plexes mixed with DDA resulted in a delay in the onset of DH which finally reached values very similar in magnitude to those obtained without CY. We studied the effect of CY treatment on DH to A-PE-liposomes. Groups of mice received an i.p. injection of CY (300 mg/kg) in PBS or PBS alone and 8 h later an i.c. injection of 5 mol% A-PE-liposomes (containing 5 nmol A-PE) mixed with 100  $\mu$ g DDA. Testing with 5 mol% A-PE-liposomes (containing 5 nmol A-PE) was performed at varying intervals after immunization. Figure 8 shows that DH of CY treated animals was greatly impaired during almost the whole period. Control animals showed normal DH with a maximum footpad swelling on day 5.

## DISCUSSION

Leskowitz, Jones & Zak (1966) showed that the low molecular weight substance 3-(p-azobenzene-*ortho*-N-acetyl-L-tyrosine (ABA-Ac-Tyr) could induce pure DH to this molecule without concomitant antibody production in guinea-pigs. For the induction of DH the use of FCA was necessary (Leskowitz *et al.*, 1966; Degrand & Raynaud, 1973). In the present study the tripeptide-enlarged hapten A, which is very similar to ABA-Tyr used by Nicolotti & Kinsky (1975), was coupled to phosphatidylethanolamine (PE), incorporated into liposomes and used in combination with the adjuvants FCA or DDA. The adjuvant properties of the cationic, surface-active lipid, DDA, are well documented by Dailey & Hunter (1974) and Snippe, Belder & Willers (1977). Intracutaneous immunization of mice with A-PE or A-PE-liposomes mixed with DDA resulted in a significant DH 5 and 6 days later (Fig. 4). The use of DDA but not the incorporation of A-PE into liposomes was necessary to induce DH (Fig. 4; Table 1). We were unable to induce significant DH in mice with A-PE or A-PE-liposomes mixed with FCA.

In guinea-pigs, however, FCA and DDA had equally good adjuvant properties in the induction of DH with A-PE-liposomes (Table 2). This confirms and extends the results of Nicolotti & Kinsky (1975) and Nicolotti *et al.* (1976) who found that FCA was required to induce DH to the ABA-Tyr-PE conjugate in guinea-pigs and that incorporation of the conjugate into liposomes did not further enhance the DH. These results suggest that FCA is not a generally applicable adjuvant for the induction of DH in mice. Recently it was found that injection of mice with killed *Listeria monocytogenes* mixed with DDA induced DH and

acquired cellular resistance against a lethal dose of *L. monocytogenes* (van der Meer, Hofhuis & Willers, 1979). No such results were found if FCA was used instead of DDA.

The requirement of DDA for the induction of cellular responses in mice suggests that a certain micellar structure of the antigen is required. Recent studies of Lim & Fendler (1979) and Kano, Romero, Djermouni, Ache & Fendler (1979) have shown that ultrasonic dispersal of dimethyl dioctadecyl ammonium chloride resulted in the formation of liposome-like structures. It is very probably that A-PE and DDA together form micelles or that the hydrocarbon chains of DDA become incorporated into the liposomal bilayer upon mixing of liposomes with DDA. The results in both cases are micelles or liposomes with a strong electropositive charge at their surfaces provided by the polar head group of DDA. Such a positively charged micelle or liposome might induce a better interaction between haptens and a thymus derived lymphocyte (T cell) than would a micelle of A-PE alone (which did not induce DH) or a A-PE-liposome without DDA (which only induced moderate DH). The complex nature of FCA does not permit us to hypothesize on the adjuvant-antigen-cell interactions.

To characterize the cellular response to hapten A in more detail, the time course of DH and the effect of dose and epitope density were studied. The footpad swelling to A-PE-liposomes peaked at 24-48 h after elicitation. On histological examination of the lesion primarily a mononuclear cell infiltrate was seen (Fig. 3A). These results, and the absence of footpad swelling at 6-8 h, suggest a pure DH without an Arthus-type reaction. Further conditions for reaching optimal DH consisted of immunization with 5 nmol A-PE (Fig. 5), free or incorporated into 5 mol% haptened liposomes (Fig. 7) and mixed with 100 µg DDA (Fig. 5), followed 5 or 6 days later (Fig. 4) by an eliciting injection of 5-25 nmol A-PE incorporated into liposomes (Fig. 6). These conditions correspond with those which we described earlier for the induction of a humoral response on intravenous injection of mice with liposomes; 5 mol% haptened liposomes containing 5 nmol hapten-PE induced a maximal humoral response (van Houte *et al.*, 1979). These findings suggest that the route of immunization and processing of antigen determine the type of immune reaction induced (Miller, Wetzig & Claman, 1979). The dose of antigen is in this instance of minor importance.

Treatment of mice with CY before immunization

with sheep red blood cells, or hapten-carrier complexes in FCA, results in a delay in the onset but finally in an enhanced DH caused by a T cell education in the absence of bone-marrow derived lymphocytes (B cells) (Kerckhaert *et al.*, 1974; Snippe, Willems, Graven & Kamp, 1975). CY treatment preceding immunization with hapten-carrier complexes mixed with DDA results only in a delayed DH (Snippe *et al.*, 1977, 1978). Using A-PE-liposomes as antigen, no recovery of the DH could be observed in CY-treated mice (Fig. 8). Little is known about the lifetime of the integrity of liposomal membranes after i.c. administration to mice. Mauk & Gamble (1979) recently showed that in *in vivo* experiments in mice, unilamellar phosphatidylcholine/cholesterol liposomes remain intact for nearly 10 h after i.c. injection and are thereafter rapidly degraded. Our result with A-PE-liposomes in CY-treated mice can be explained by the fact that the liposomal structure is no longer intact at the time the T cells recover from the CY treatment (after about 2 days; Aisenberg, 1967). As a consequence, no induction of DH to hapten A can take place. This result is in line with our assumption that a micellar or liposomal structure of the antigen is required to induce DH to hapten A in mice.

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