Immunology 1989 **66** 466–470

The role of macrophages in the immunoadjuvant action of liposomes: effects of elimination of splenic macrophages on the immune response against intravenously injected liposome-associated albumin antigen

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Accepted for publication 11 November 1988

SUMMARY

The primary antibody response to intravenously administered and liposome-associated human serum albumin (HSA) was studied in mice under conditions where no response could be detected against the non-liposome-associated form of the antigen. The positive response against the antigen, entrapped in and/or exposed on the surfaces of liposomes, thus resulted from the adjuvant action of the liposomes. In mice intravenously injected with dichloromethylene diphosphonate (Cl2MDP) also entrapped in liposomes, all red pulp macrophages, marginal metallophilic macrophages and marginal zone macrophages had disappeared from the spleen 2 days after administration. Twenty-two days after such a treatment red pulp macrophages and marginal metallophilic macrophages had reappeared, but marginal zone macrophages were still absent. In mice injected with liposome-associated HSA at 2 days after treatment with the Cl2MDP liposomes, anti-HSA responses were severely depressed, but administration of the liposome-associated antigen 22 days after Cl2MDP liposomes elicited a normal response. These results point to a role of splenic macrophages in the processing of liposome-associated antigens, but marginal zone macrophages, which are located close to the open ends of the white pulp capillaries and thus are the first macrophages to meet the antigens arriving in the marginal zone are not required.

INTRODUCTION

Liposomes may act as carriers for haptens in thymus-independent immune reactions (Yasuda, Dancey & Kinsky, 1977; Van Houte, Snippe & Willers, 1979) and as immunoadjuvant for protein antigens in thymus-dependent (CTD) immune reactions (reviewed by Van Rooijen, 1988). Contrary to haptenated liposomes, liposome-associated (protein) antigens elicit an IgG response in addition to the preceding IgM response (Van Rooijen & Van Nieuwmegen, 1983a; Latif & Bachawat, 1987; Davis, Davis & Gregoriadis, 1987), start the generation of immunological memory (Van Rooijen *et al.*, 1981) and are dependent on the presence of T cells (Shek & Sabiston, 1982a). Recent studies have shown that they also stimulate the antigen-specific interleukin-2 (IL-2) production (Mansour *et al.*, 1988). Apart from lymphoid cells, macrophages have been suggested to play a role in the immune responses against thymus-dependent protein antigens. Evidence has been given that macrophages are involved both in intracellular processing and in MHC-restricted antigen presentation (Unanue, 1984). Evidence for a role of macrophages in mediating the adjuvant effect of liposomes has also been given (Van Rooijen & Van Nieuwmegen, 1979; Shek & Lukovich, 1982).

With respect to liposomes, it is important to distinguish between two forms of association of antigen and liposomes. The antigen may be masked after encapsulation in the aqueous compartments of the liposomes or exposed on their outer surfaces (Van Rooijen & Van Nieuwmegen, 1980, 1982, 1983b; Shek & Sabiston, 1982b; Snyder & Vannier, 1984; Davis & Gregoriadis, 1987; Gregoriadis, Davis & Davies, 1987; Vannier & Snyder, 1988). From this it might be expected that macrophages are required for the processing of liposome-entrapped antigens, since only these cells are able to release the antigen after digestion of the liposomal bilayers by phospholipases in their lysosomal compartments. Intravenously injected antigens enter the spleen by the circulation, and may have their first contact with cells of the immune system in the marginal zone, surrounding the white pulp (Van Rooijen, Claassen & Eikelenboom, 1986).

It was the aim of the present experiments to study the effect of elimination of macrophages in the spleen on the immune responses against liposome-entrapped and liposome-exposed albumin antigens, intravenously injected in mice. Since macrophage subpopulations in the spleen, after initial depletion, show different kinetics of repopulation (Van Rooijen, Kors & Kraal,

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Table 1. The effect of depletion of macrophages in the spleen on the primary antibody response in mice against HSA after intravenous injection with HSA-associated liposomes (mean ± SD $\log_2$ ELISA titres; ND, not detectable; NS, not significant)

Intravenous injection at Day −2	Intravenous HSA-liposomes at Day 0 (per mouse)	Location of liposome-associated HSA	IgM-anti-HSA antibodies at		IgG anti-HSA antibodies at	
			Day 5	Day 7	Day 5	Day 7
Empty liposomes	50 μg HSA, 1·2 mg phospholipid	Entrapped (masked)	5·33 ± 0·52 (6)	7·75 ± 0·42 (6)	9·58 ± 0·38 (16)	11·08 ± 0·74 (6)
C12MDP-liposomes	50 μg HSA, 1·2 mg phospholipid	Entrapped (masked)	4·30 ± 0·82 (10)	7·00 ± 0·75 (10)	7·40 ± 1·88 (10)	8·72 ± 1·56 (10)
			$P<0·05$	$P<0·05$	$P<0·05$	$P<0·01$
Empty liposomes	16 μg HSA, 2·4 mg phospholipid	Surface (exposed)	8·60 ± 0·62 (5)	8·38 ± 0·70 (5)	4·40 ± 1·18 (5)	9·02 ± 1·00 (5)
C12MDP-liposomes	16 μg HSA, 2·4 mg phospholipid	Surface (exposed)	6·43 ± 0·82 (7)	7·11 ± 0·94 (7)	ND (7)	4·63 ± 1·92 (7)
			$P<0·01$	$P<0·05$	$P<0·01$	$P<0·01$
PBS	50 μg HSA, 2·4 mg phospholipid	Both entrapped in and on surface of liposomes	5·8 ± 0·84 (5)	8·8 ± 0·45 (5)	4·0 (1/5) ND (4/5)	12·3 ± 0·45 (5)
C12MDP liposomes	50 μg HSA, 2·4 mg phospholipid	Both entrapped in and on surface of liposomes	5·2 ± 1·30 (5)	6·1 ± 0·89 (5)	3·0 (2/5) ND (3/5)	8·0 ± 0·94 (5)
			NS	$P<0·01$		$P<0·01$
PBS	10 μg HSA, 0·48 mg phospholipid	Both entrapped in and on surface of liposomes	6·0 ± 0·71 (5)	7·3 ± 0·76 (5)	4·0 (3/5) ND (2/5)	10·2 ± 1·68 (5)
C12MDP liposomes	10 μg HSA, 0·48 mg phospholipid	Both entrapped in and on surface of liposomes	3·8 ± 0·45 (5)	5·3 ± 0·45 (5)	ND (5)	7·0 ± 0·35 (5)
			$P<0·001$	$P<0·001$		$P<0·01$
PBS	2 μg HSA 96 μg phospholipid	Both entrapped in and on surface of liposomes	3·8 ± 1·64 (5)	6·4 ± 0·65 (5)	1·0 (1/5) ND (4/5)	8·2 ± 1·79 (5)
C12MDP liposomes	2 μg HSA 96 μg phospholipid	Both entrapped in and on surface of liposomes	3·0 (2/6) ND (4/6)	4·8 ± 0·40 (6)	ND (6)	3·9 ± 1·39 (6)
				$P=0·001$		$P<0·01$

1989) we were also able to study the effect of selective depletion of the subpopulation of 'so-called' marginal zone macrophages on the immune response against the liposome-associated antigens.

MATERIALS AND METHODS

Experimental design

Groups of B6D2 mice aged 6–10 weeks (Bomholtgard, Ry, Denmark) were intravenously injected with empty or dichloromethylene-diphosphonate (C12MDP)-containing liposomes composed of phosphatidylcholine (PC) and cholesterol (CHOL) (Sigma, St Louis, MO) in a molar ratio of 6:1. Two days or 3 weeks later, respectively, complete macrophage depletion or selective depletion of marginal zone macrophages was observed in the spleen of mice injected with C12MDP liposomes (Van Rooijen *et al.*, 1989). Control mice received empty liposomes or phosphate-buffered saline (PBS). Mice were intravenously injected with human serum albumin (HSA; Miles

Table 2. The effect of selective depletion of marginal zone macrophages in the spleen on the primary antibody response in mice against HSA after intravenous injection with HSA-associated liposomes (mean ± SD $\log_2$ ELISA titres)

Intravenous injection at Day −22	Intravenous injection of HSA liposomes at Day 0 (per mouse)	Anti-HSA antibodies at Day 7	
		IgM	IgG
C12MDP liposomes	40 μg HSA 1·2 mg phospholipid	4·5 ± 0·97 (10)	6·70 ± 2·32 (10)
PBS	40 μg HSA 1·2 mg phospholipid	3·8 ± 0·75 (10)	7·35 ± 1·86 (10)
		NS	NS

Labs Inc, Bridgend, U.K.), entrapped in liposomes (Table 1), exposed on liposomal surfaces (Table 1) or both entrapped in and surface exposed on liposomes (Tables 1 and 2) in order to evaluate anti-HSA responses in macrophage-depleted mice. The presence or absence of macrophage populations was checked at the end of each experiment by studying spleen sections of all mice for (i) the total macrophage population and (ii) marginal zone macrophages. IgM and IgG responses at Day 5 and Day 7 after priming were analysed by ELISA and expressed in Tables 1 and 2 as $\log_2$ antibody titre (mean $\pm$ SD). Numbers in parentheses denote the mice used. The *P* value (probability of significance) was derived from Student's *t*-test; ND stands for not detectable (the antibody titre was less than 3 $\log_2$ value).

Preparation of liposomes with entrapped C12MDP

Multilamellar liposomes were prepared as described elsewhere (Van Rooijen & Claassen, 1988). In brief, 75 mg PC and 11 mg CHOL (Sigma Chemical Co., St Louis, MO) were dissolved in chloroform in a round-bottomed flask. The thin film that formed on the interior of the flask after low vacuum rotary evaporation at 37° was dispersed by gentle rotation for 10 min in 10 ml PBS (0·15 M NaCl: 10 mM phosphate buffer, pH 7·4). For C12MDP-containing liposomes, 1·89 g C12MDP were added to 10 ml PBS; more C12MDP could not be dissolved in the buffer. C12MDP was a kind gift of Procter and Gamble, Cincinnati, OH. In order to wash the liposomes, these were centrifuged twice in PBS at 100,000 *g* for 30 min to remove free non-entrapped C12MDP. After washing of the liposomes, these were resuspended in 4 ml PBS and 0·2 ml (containing about 2 mg of liposome entrapped C12MDP) was intravenously injected into the mice.

Preparation of liposome-associated human serum albumin (HSA)

Liposomes with entrapped HSA (Table 1) were prepared as follows. Dehydration-rehydration vesicles (DRV; Kirby & Gregoriadias, 1984), containing both entrapped HSA and HSA on their surface, were prepared by the freeze-drying method, starting with multilamellar vesicles (MLV). To produce liposomes with entrapped antigen, they were consecutively treated with trypsin to remove most of the surface HSA (Shek & Sabiston, 1982b). Liposomes with surface-exposed HSA (Table 1) were prepared by incubating empty MLV with HSA. Liposomes were composed of PC and CHOL in a molar ratio of 7:2. HSA-associated liposomes (Tables 1 and 2) were prepared by freeze-thawing a mixture of empty multilamellar vesicles and HSA three-times (−196°–37°) modified according to Pick (1981).

Characterization of macrophages in the spleen

To check the presence or absence of macrophages in the spleen, these were characterized as described elsewhere (Van Rooijen *et al.*, 1989). Blocks of fresh splenic tissue were frozen in liquid nitrogen and stored at −20°. Cryostat sections of 8–10 μm thickness were fixed in acetone for 10 min and air-dried for at least 30 min. After washing in 0·01 M PBS (pH 7·4) the sections were incubated with culture supernatants of the rat anti-mouse antibody ERTR9 (a kind gift from Dr G. Kraal, Free University, Amsterdam) directed to marginal zone macrophages (Dijkstra *et al.*, 1985), at saturating concentrations in PBS containing 0·1% bovine serum albumin (BSA; Poviet, Tilburg, The Netherlands) for 30 min. After washing thoroughly in PBS, the slides were incubated with a 1:100 dilution of peroxidase-conjugated rabbit anti-rat IgG (Dako, Glostrup, Denmark) in PBS/BSA containing 1% normal mouse serum for 30 min. After washing in PBS again, the peroxidase activity was visualized with 3,3′-diaminobenzidine-tetrahydrochloride (DAB, Sigma) in 0·5 mg/ml Tris-HCl buffer (pH 7·6) containing 0·01% H_2O_2. The sections were stained for 10–15 min at room temperature. Acid phosphatase activity in the sections of spleen tissue, present in lysosomes of all macrophages, was demonstrated as described by Eikelenboom (1978).

RESULTS

Effects of elimination of splenic macrophages on the immune response against HSA entrapped in and/or exposed on liposomes

The elimination of red pulp macrophages, marginal metallophilic macrophages and marginal zone macrophages in the spleen by treatment with liposome-encapsulated C12MDP is generally completed within 1 day. At the end of each experiment the macrophage population as a whole and the subpopulation of marginal zone macrophages in the spleen of all individual mice were studied with respect to their presence or absence. The kinetics of disappearance and reappearance of macrophages in the spleen after treatment with C12MDP liposomes has been described elsewhere in great detail (Van Rooijen *et al.*, 1989), and these results served as a reference to know which macrophage subpopulations were eliminated at the time of antigen administration. In the sera of mice injected with free (non-liposome-associated) HSA in doses from 1 μg to 25 mg, no anti-HSA antibodies were detected within 2 weeks after antigen administration. The positive responses against comparable amounts of HSA entrapped in and/or exposed on liposomes were due to the adjuvant activity of liposomes (Tables 1 and 2). Table 1 shows the effect of pretreatment with liposome encapsulated C12MDP at Day −2; (i) the immune response against liposome entrapped HSA; and (ii) the immune response against HSA exposed on the surfaces of liposomes. Results indicate that antigens coated on liposomes primarily enhance the IgM response, whereas liposome-entrapped antigens primarily enhance the IgG response. Considering the IgM response, HSA exposed on the outer surfaces of liposomes elicited an eight times higher serum antibody concentration at Day 5 than HSA entrapped in liposomes. On the contrary, at the same time interval after antigen administration, liposome-entrapped HSA produced a 32 times higher IgG anti-HSA antibody concentration than did surface-exposed HSA. Considering the normal kinetics of IgM and IgG production during the primary immune response against TD antigens, it is not surprising that these differences were less pronounced at Day 7 after antigen injection. Elimination of macrophages in the spleen by treatment with liposome-encapsulated C12MDP 2 days before antigen injection did result in markedly reduced IgM and IgG anti-HSA antibody titres both at Day 5 and Day 7. This reduction was more impressive for antigen exposed on the outer surfaces of liposomes than for the entrapped antigen (Table 1). Macrophage elimination appeared to influence the IgG response more than the IgM response. Compared to mice pretreated with empty liposomes, mice pretreated with C12MDP liposomes and immunized with HSA exposed on the surfaces of liposomes 2 days later showed an IgG anti-HSA serum

concentration that was 16 times lower at 1 week after immunization. Table 1 also shows the effect of pretreatment with liposome-encapsulated C12MDP at Day −2 on the immune response against different doses of liposome-associated HSA (both entrapped in the liposomes and exposed on their outer surfaces). Although serum antibody levels reached lower values, after immunization with lower doses of liposome-associated HSA, reduction of these values by pretreatment with C12MDP liposomes was similar to that seen after immunization with the higher doses of liposome-associated HSA.

Effects of selective elimination of marginal zone macrophages in the spleen on the immune response against liposome-associated HSA

Table 2 shows the effect of pretreatment with liposome-encapsulated C12MDP at Day −22 on the immune response against liposome-associated HSA (both entrapped in the liposomes and exposed on their outer surfaces). Anti-HSA titres in the mice injected with the batch of HSA-associated liposomes used for the experiment shown in Table 2 were lower than those in the mice injected with the HSA liposomes used for the experiments shown in Table 1. Twenty-two days after intravenous injection of liposome-entrapped C12MDP, red pulp macrophages and marginal metallophilic macrophages in the spleen have repopulated their respective compartments in the spleen, but marginal zone macrophages are still absent (see also Van Rooijen *et al.*, 1989 for details). In some of the mice a small number of marginal zone macrophages had reappeared at the end of the experiments. Their presence or absence was checked in all mice at Day 7, i.e. 29 days after treatment with liposome entrapped C12MDP. Treatment of mice with liposome encapsulated C12MDP at 22 days before immunization with liposome-associated HSA did not result in significantly depressed anti-HSA antibody titres at Day 7 after immunization, as was the case when mice were immunized at 2 days after treatment with C12MDP liposomes.

DISCUSSION

In the sera of control mice injected with free (non-liposome-associated) HSA in doses between 1 μg and 25 mg, no anti-HSA antibodies were detected within 2 weeks of antigen administration. It may thus be concluded that the positive responses against the liposome-associated HSA are due to the adjuvant activity of liposomes. The role of macrophages in mediating this adjuvant activity of liposomes was studied with the aid of an '*in vivo*' macrophage elimination technique. Elimination of all red pulp macrophages, marginal metallophilic macrophages and marginal zone macrophages in the spleen and Kupffer cells in the liver was obtained by intravenous injection of liposome-encapsulated C12MDP 2 days before antigen administration (Van Rooijen & Claassen, 1988). Selective elimination of marginal zone macrophages in the spleen was obtained by intravenous injection of liposome-encapsulated C12MDP 22 days before antigen (Van Rooijen *et al.*, 1989). Elimination of all red pulp macrophages, marginal metallophilic macrophages and marginal zone macrophages in the spleen resulted in markedly reduced IgM and IgG anti-HSA antibody titres. On an average the IgG-anti-HSA concentration in the blood serum of the mice at 7 days after antigen administration was reduced to 5–10% of the normal values. Selective elimination of marginal zone macrophages in the spleen did not result in significantly reduced anti-HSA antibody concentrations at 7 days after antigen administration. It is concluded that macrophages in the spleen or, for example, in the liver (Van Rooijen & Claassen, 1988) are involved in the immune response against liposome-associated HSA, but marginal zone macrophages which are located close to the open ends of the white pulp capillaries (Van Rooijen *et al.*, 1986, 1989) are not required. A surprising finding was that the reduction in antibody production caused by elimination of macrophages was more impressive for antigen exposed on the outer surfaces of liposomes than for antigen entrapped in the aqueous compartments of the liposomes. It can be concluded that the immunogenicity of the entrapped antigen is not completely dependent on the unmasking capability of the local macrophages (disruption of the phospholipid bilayers) as we expected. Since free antigen was not able to elicit an antibody response within 2 weeks, antigen molecules released from liposomes by leakage, influence of serum components, etc. may not be responsible. Obviously, macrophages somewhere in the body, reached by liposome-encapsulated C12MDP in too small amounts for their elimination were consecutively involved in the processing of liposome-entrapped antigens, also in small amounts, but sufficient to evoke a detectable immune response. It can not be excluded, however, that other cells, e.g. particular subsets of B lymphocytes (Unanue, 1984), are able to unmask and process part of the entrapped antigens (probably in the aqueous compartments between the peripheral phospholipid bilayers). Therefore, on the one hand it is clear that our results confirm those of earlier studies (Van Rooijen & Van Nieuwmegen, 1979; Shek & Lukovich, 1982; Garcon *et al.*, 1988) that macrophages are involved in the '*in vivo*' processing of liposome-associated antigens. On the other hand local macrophages seem not to be obligatory for the induction of an immune response against such antigens. However, compared to free protein TD antigens, for which we found no reduction in the antibody responses in similarly macrophage-depleted animals (Claassen, Kors & Van Rooijen, 1986), local macrophages may be considered the most important candidates for unmasking and processing of liposome-associated antigens. It should be realized, however, that processing of antigens may be performed by various cells in different compartments of the body (Unanue, 1984) requiring the presence of T cells close to the processing cell. The T cells may then migrate to other lymphoid organs or compartments in order to stimulate particular subsets of B cells to become antibody-forming cells.

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