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# Effects of chronic injection of sphingomyelin-containing liposomes on lymphoid and non-lymphoid cells in the spleen. Transient suppression of marginal zone macrophages

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**Summary.** Mice were injected with sphingomyelin/cholesterol or phosphatidylcholine/cholesterol (PC/C) liposomes, from twice up to 10 times, on alternate days. Administration of sphingomyelin/cholesterol (SM/C) liposomes gave rise to hepato and spleno-megaly, micro-granulomatous infections and changes in macrophage numbers and activity in spleen and liver. Enzyme and immuno-cytochemical methods were used, to demonstrate the effect of liposomes on the lymphoid and non-lymphoid cell populations, on cryostat sections of the spleen. Routine histological staining, of sphingomyelin/cholesterol treated animals, showed no drastic changes in morphology or compartmentalization of the spleen, apart from a small enlargement (with some microgranulomas) of the red pulp. No significant differences were found in the presence or localization of T-helper, T-cytotoxic/suppressor, T-total-lymphocytes, B-total-lymphocytes, red pulp macrophages, marginal metallophils, or non-lymphoid dendritic cells. However, a transient suppression of cells expressing marginal zone macrophage surface marker ERTR-9, was observed between the second and eighth (intravenous) administration of sphingomyelin/cholesterol liposomes. Immunization of these animals with trinitrophenyl (TNP)-ficoll, a thymus-independent type-2 antigen which is specifically processed by marginal zone macrophages (MZM), showed that these cells were not suppressed with regard to their immunological function. We conclude that chronic administration of sphingomyelin liposomes influences macrophages, probably through a general phagocytic-system overload, but no permanent or damaging changes in splenic cell populations or immunological functions occur.

Keywords: immunocytochemistry, liposomes, marginal zone macrophages, mice, sphingomyelin, spleen

Liposomes are artificial microscopic (20 nm to microns) spheres built up of concentric phospholipid bilayers separated by aqueous compartments. Liposomes have been proposed as drug carriers (Gregoriadis *et al.* 1975; Gregoriadis 1985) and as immunoadjuvant in vaccines (cf. Van Rooijen & Van Nieuwmegen, 1982). Liposomes used for

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these in-vivo applications must be stable, have a high trapping efficiency for solutes, exhibit a slow rate of clearance from the blood, and possess no or only minimal toxicity. Little evidence is available concerning the possible in-vivo toxicity of liposomes, although, *in vitro*, they may influence proliferation of lymphocytes (Chen & Keenan 1977; Ng *et al.* 1978; Rivnay *et al.* 1978; Grover *et al.* 1986) and cause chromosome abberations (Nuzzo *et al.* 1985).

Inclusion of sphingomyelin (SM) in the liposomal membranes (together with cholesterol) greatly enhances liposomal stability, while at the same time it prolongs the presence of liposomes in the circulation (Hwang *et al.* 1980; Ellens *et al.* 1981; Alving 1982; Beaumier & Hwang 1983). A longer serum half-life for liposomes is desirable in targetting studies (cf. Juliano 1982; Claassen *et al.* 1987), but not in studies involving the reticulo-endothelial system (cf. Alving 1982). We are interested in the first phenomenon, targetting. Chronic administration of SM-containing liposomes to mice has been demonstrated to cause (micro)granulomatous inflammation, hepato and splenomegaly, decrease of phagocytic index and, contrary to this, an increase in the number of phagocytic cells in both spleen and liver (Weereratne *et al.* 1983; Allen *et al.* 1984; Allen & Smuckler 1985). In all cases, these phenomena disappeared within 2 weeks after termination of SM/C liposome administration and phagocytic indices in this recovery period were 50% higher than in control (phosphatidylcholine) animals. Furthermore, Allen and Smuckler (1985) also observed this recovery as early as the eighth injection, and relates these phenomena to phospholipid metabolism. Weereratne *et al.* (1983) suggested that chronic administration of SM/C liposomes could be used as a model for the study of tissue granulomatous inflammation. For this purpose, the spleen is an ideal test system since it has an open circulation (blood) from which the liposomes have free and easy access to the great diversity of splenic cell populations (Van Rooijen & Claassen 1988). The recent development of several monoclonal antibodies to identify these cells greatly facilitates and improves histological studies; in addition, we have developed a method for the analysis *in situ* of the immune response to various experimental antigens (Claassen *et al.* 1986a–c; Van Rooijen *et al.* 1986). In the present study the effect of chronic administration of similar SM/C liposomes (compared to non-toxic PC/C liposomes and untreated animals) on the lymphoid and non-lymphoid cell populations of the spleen is reported, after both intravenous and intraperitoneal administration in mice.

## Materials and Methods

### *Experimental design*

BALB/c or C3H × DBA/2 mice were injected, either intravenously (i.v.) or intraperitoneally (i.p.), either with phosphate-buffered saline, or with PC/C liposomes, or with SM/C liposomes. Liposomes were administered on alternate days, for 2, 4, 6, 8 or 10 times, and groups were killed respectively 4, 8, 12, 16 or 20 days after the first injection. Spleens and livers were removed and snap-frozen in liquid nitrogen. Cryostat (−20°C) sections, of 8 μm thickness were picked up on glass slides, for enzyme and immuno-cytochemistry. Venous tail-blood was collected from liposome-treated or control groups immunized with TNP-ficoll after 0, 4, 6, 11 and 35 days, and sera were analysed for specific serum antibody titres. Histochemical results were quantified on an Artek counter (model 880, Farmingdale, NY, USA) coupled to a video-microscope, and the total stained surface measured by image analysis. Experiments were performed twice with a minimum of four mice (total eight) per group; results were scored on coded samples (blind) with automated data-gathering.

### *Animals and chemicals*

Female mice (C3H×;DBA/2) $F_1$ were obtained from Bomholtgård, Ry, Denmark;

BALB/c mice were bred at TNO, Rijswijk, The Netherlands. Alkaline phosphatase-conjugated goat anti-mouse IgM (or IgM+IgG) was obtained from TAGO, Burlingame, CA 94010, USA. Peroxidase-conjugated rabbit anti-rat-Ig was from Dako, Copenhagen, Denmark. 3,3'-diaminobenzidine tetrahydrochloride, cholesterol (99% pure), naphthyl-AS-BI-phosphate, α-naphthyl acetate, *p*-nitrophenylphosphate, pararosaniline, phosphatidylcholine (VIIE, 99%), sphingomyelin from bovine brain (99%) were obtained from Sigma, St Louis MO, USA. ERTR-9 antibody was the kind gift of Dr W van Ewijk, Rotterdam, The Netherlands; all other antibodies were kindly provided by Dr G. Kraal, Amsterdam, The Netherlands.

*Preparation of liposomes*

Liposomes were prepared as described previously (Claassen *et al.* 1986c), essentially in the same way as used by other authors for SM/C liposomes (Ellens *et al.* 1981; Beaumier & Hwang 1983; Weereratne *et al.* 1983; Allen & Smuckler 1985). Phospholipids (PC:C or SM:C in 1:1 mole-ratio) were dissolved in chloroform/methanol (1:1), and recrystallized twice in choloroform, in a round-bottomed flask. The thin film that formed on the interior of the flask after low-vacuum rotary evaporation at 37°C was dispersed by gentle rotation for 15 min with PBS (0.15 M NaCl/ 10 mM phosphate buffer, pH 7.4). The milky white suspension was kept for 2 h at room temperature and sonicated for 3 min in a Sonicor waterbath sonicator (50Hz, Farmingdale NY). After an additional 2 h at room temperature, the liposome suspension was centrifuged (100 000*g*, 30 min) to remove non-liposomal material. Liposomes were resuspended in PBS and checked for formation and size homogeneity. Animals were injected with 200 μl and 1 mg total lipid per administration.

*Enzyme cytochemical stainings*

Endogenous acid-phosphatase and non-specific esterase activity of macrophages was revealed as described by Eikelenboom *et al.* (1985) with minor modifications. Briefly, sections were air-dried for 1 h, fixed in acetone (p.a.) for 10 min and incubated vertically in substrate solution in coplin jars, rinsed in distilled water, counterstained for 1 min in haematoxylin, dehydrated with alcohol–xylol, and mounted in malinol.

*Substrate solution for acid phosphatase.* Twenty milligrams of naphthyl-AS-BI-phosphate (Pearse 1968) was dissolved in 2 ml *N,N*-dimethylformamide, and added to 34 ml 0.05 M sodium barbital buffer (pH 10). 1.6 Millilitres of hexazotized pararosaniline (64 mg in 2M HCl, heated and filtered (David & Ornstein 1959) was mixed with 1.6 ml 4% sodiumnitrite, added to the substrate/buffer solution, and brought to pH 5.0 with 1M HCl or NaOH. This was incubated for 30 min at 37°C.

*Substrate solution for non-specific esterase.* Ten milligrams of α-naphthyl acetate dissolved in 0.4 ml acetone was added to 40 ml 0.067 M phosphate buffer (pH 5.0). 1.2 Millilitres of hexazotized pararosaniline (see above) was mixed with 1.2 ml of 4% sodium nitrite, added to the substrate/buffer solution and brought to pH 5.8 with 2M NaOH. This was incubated for 30–60 min at room temperature.

*Immunocytochemical stainings*

Sections were air dried and fixed for 10 min in acetone (analytical grade) containing 0.02% hydrogen peroxide (freshly prepared, to inhibit endogenous peroxidases). Sections were incubated, for 1 h, with ascites or culture supernatants of hybridomas producing antibodies against tissue determinants, as listed under Results, Effects of liposomes on histology of the spleen. Sections were washed with PBS (3 × 1 min) and covered with a 1:300 dilution of peroxidase-conjugated rabbit anti-rat/Ig in PBS with 0.1% bovine serum albumin and 1% normal

mouse serum. After 1 h, sections were washed in PBS (3 × 1 min) and peroxidase activity was revealed with 3,3-diaminobenzidine-4(HCl) (20 mg in 40 ml 0.05 M Tris-HCl buffer, pH 7.6; just before use, add 50 μl 3% hydrogen peroxide) within 7–15 min. Staining was enhanced by incubation for 5 min in 0.5% $CuSO_4$/ 0.9% NaCl.

*Detection of serum anti-TNP antibody levels*

Serum antibodies to trinitrophenyl (TNP) were determined using a direct enzyme-linked immunosorbent assay (ELISA; cf. Engvall 1980) as described previously (Claassen *et al.* 1987) but with some modifications. Microtitre plates (Flow Labs, PVC flat-bottomed, 96 wells, # 77-172-05 high activated) were coated overnight at 4°C with 200 μl/well of 5 μg/ml TNP-ovalbumin in 0.1 M sodium carbonate buffer, pH 9.6. Free binding sites were blocked by coating with 0.1% bovine serum albumin in 0.1 M sodium phosphate buffer + 0.9% NaCl, pH (PBSN) for 30 min at room temperature. Plates were washed three times with PBSN+0.1% Tween-20 (PBSNT). Serial doubling dilutions (starting with 1:32) were made from 96 μl serum samples in PBSNT, and incubated for 1 h at 37°C. Plates were washed five times with PBSNT, and a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgM (or IgG) antiserum was added and incubated for 1 hr at 37°C. Plates were again washed three times with PBSNT, once with 0.1 M diethyleneglycol pH 9.6, and 100 μl of substrate solution (1 g/l *p*-nitrophenylphosphate in 10 mM diethyleneglycol, pH 9.6) was added to each well. The absorbance at 405 nm was read after 1 h, on an automated micro-ELISA reader (Organon Teknika, Oss, The Netherlands). Titres were determined, after subtraction of individual backgrounds for normal mouse serum, at 50% of the slope and expressed as $^{-2}$log values. Samples were analysed by the two-sample Student's *t*-test for comparison of two emperical means in a normally distributed population (cf. Sachs 1984).

## Results

*Effects of liposomes on histology of the spleen*

After more than one injection of SM/C liposomes (either i.v. or i.p.) the spleen was markedly enlarged (30–60%); this could be ascribed to the enlargement of the red pulp. More red pulp macrophages (detected with acid-phosphatase activity (Eikelenboom *et al.* 1985)) and megakaryocytes (detected with haematoxylin/morphology (Romeis 1968)) could be found in SM/C treated animals than in PBS or PC/C treated animals. Several microgranulomas were observed in the red pulp; there was no clustering of T cells (detected with Thy-1 Van Ewijk & Nieuwenhuis 1985) and only macrophages and reticulocytes were found in these microgranulomas.

No differences in the number and/or localization patterns of the following cell types were observed after SM/C treatment as compared to the normal (untreated) or PC/C treated mice: marginal metallophils (MOMA-1, Kraal & Janse 1986), T-helper lymphocytes (MT-4, Pierres *et al.* 1984), T-cytotoxic/suppressor lymphocytes (Lyt-2, Ledbetter & Herzenberg 1979), B lymphocytes (anti-mouse immunoglobulins, Braun & Unanue 1980) and non-lymphoid dendritic cells (NLDC-145, Kraal *et al.* 1986; Steinman & Nussenzweig 1980).

However, ERTR-9+ve staining of marginal zone macrophages (MZM Van Vliet *et al.* 1985; Dijkstra *et al.* 1985) was drastically suppressed after i.v. administration of SM/C liposomes (Fig. 1); i.p. administered SM/C or i.v. administered PC/C liposomes showed no such effect. In Fig. 2 we have presented the quantitative results of this phenomenon; these values show that initial loss of ERTR-9 expression is gradually restored to reach normal values after 10 SM/C administrations. No such decrease was observed for MOMA-1 marker of marginal metallophil macrophages in SM/C i.v.-treated (Fig. 2, hatched bars always 100%) or other liposome-treated animals (data not shown).

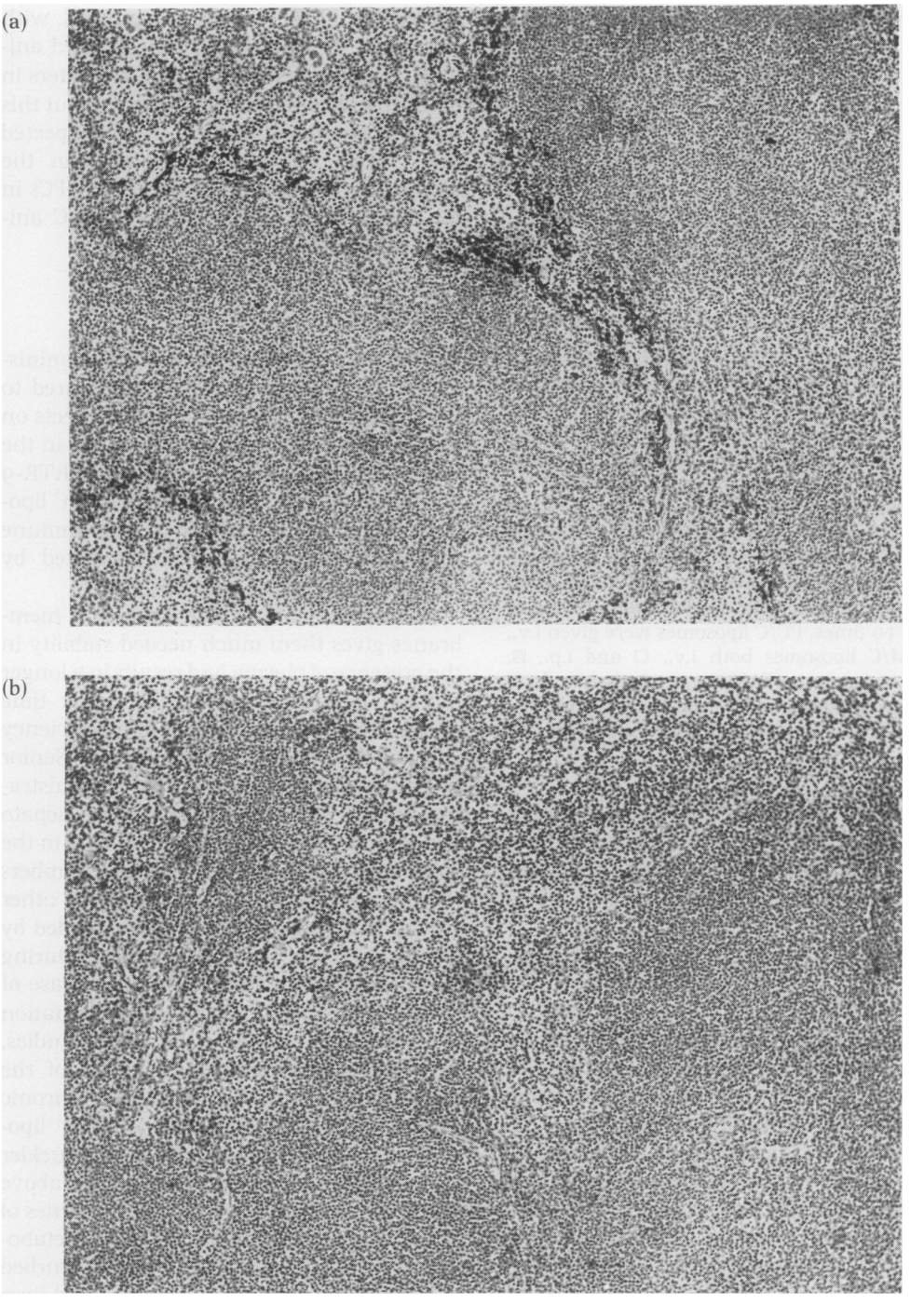


**Fig. 1.** Spleen sections of a representative control PC/C (a) and a SM/C (i.v.) treated (two injections) animal (b), stained for ERTR-9 surface marker of marginal zone macrophages. Note disappearance of ERTR-9 staining in SM/C treated animals (1b), × 80.

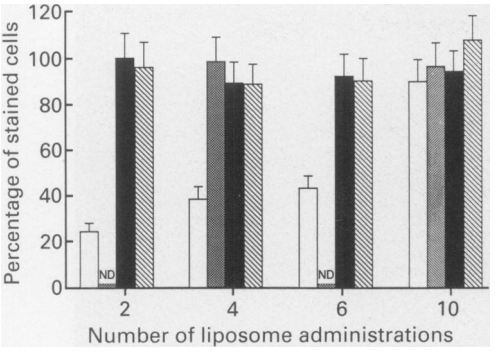


**Fig. 2.** Image analysis of cryostat sections of murine spleen for ERTR-9 positive macrophages (see also Fig. 1), in control (PC/C- liposomes or untreated animals) or SM/C treated mice. Staining is expressed as total area of positive staining, irrespective of staining intensity; mean of 20 values was determined in eight sections from two different animals. Total area of ERTR-9 staining (4.5% of total section surface) in six untreated control animals was used as 100% value. Liposomes were administered on alternate days, twice, 4, 6 or 10 times. PC/C liposomes were given i.v., and SM/C liposomes both i.v., □ and i.p., ▩. MOMA-1 staining of SM/C i.v. treated animals, ■ was also measured as internal immunocytochemical control. Bars represent standard deviation.

Comparison with acid-phosphatase staining showed that the MZM were still present and positive for acid-phosphatase in SM/C treated animals, at all times.

*Effects of liposome treatment on anti-TNP-antibody titres and -AFCs*

In earlier studies we showed that splenic marginal zone cells could be eliminated after i.v. administration of dichloromethylene diphosphonate containing liposomes (cf. Van Rooijen & Claassen 1988). Upon immunization with either thymus-dependent or thymus-independent type 1 or 2, we found that only the immune response against thymus-independent type-2 antigens (soluble or liposomal) was MZ dependent (Claassen *et al.* 1986; 1987). As Fig. 3 shows, no such effects were observed in SM/C (i.v.) animals with only 24% of their MZM still expressing ERTR-9, when compared with untreated, or SM/C (i.p.), PC/C treated animals. A slight elevation of anti-TNP titers in SM/C treated animals was observed, but this was not significant at $P<0.05$. As expected from these results, no differences in the amount or localization of anti-TNP AFCs in the spleen were found between SM/C animals and controls.

## Discussion

In this study it is demonstrated that administration of SM/C liposomes, as compared to PC/C liposomes, has only minimal effects on the lymphoid and non-lymphoid cells in the murine spleen. Even though the ERTR-9 positive MZM were influenced by SM/C liposomes, no negative effects on the immune response against TNP-ficoll (mediated by MZM, B and T-cells) were observed.

Inclusion of SM in the liposomal membranes gives them much needed stability in the presence of plasma and results in a longer half-life *in vivo*, while at the same time preserving a high trapping efficiency (Hwang *et al.* 1980; Ellens *et al.* 1981; Senior & Gregoriadis 1982). Chronic administration of SM/C liposomes to mice led to hepato and spleno-megaly, microgranulomas in the spleen and changes in macrophage numbers (Weereratne *et al.* 1982; 1983). In other studies this was confirmed and extended by noting a decrease in phagocytic index during SM/C treatment, followed by an increase of 50% above control values upon termination of injections. However, in these studies, persisting granulomatous infection of the liver was also found 2 weeks after chronic injection (10 doses) of PC/C 'control' liposomes (Allen *et al.* 1984; Allen & Smuckler 1985). These authors correlated the above findings with specific metabolic properties of the various phospholipids or their metabolites. The latter results and the undisturbed biochemical composition of spleen and liver as described by Weereratne *et al.* (1983) did not indicate a toxic effect but, in our opinon, only a temporary disturbance. Since lipo-

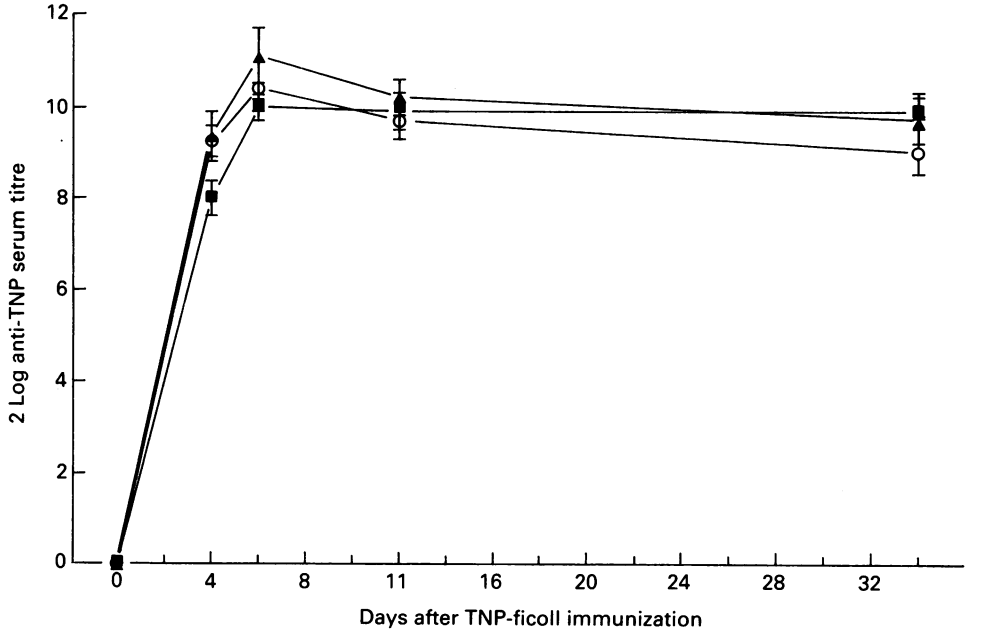


**Fig. 3.** Anti-TNP serum titers (IgM+IgG) after i.v. immunization of SM/C liposome treated mice (SM/C liposomes administered i.v. on day −3 and −1, see Figs 1 and 2). Values are expressed as $^{-2}$log of the serum titers as detected in an anti-TNP ELISA (see Materials and Methods). Values represent mean of four animals; the bar shows standard deviation. ■, Control; ○, phosphatidylcholine; ▲, sphingomyelin.

somes are still very promising as drug carriers or vaccines, precise study of the effects of SM/C and PC/C liposomes on the spleen was desirable.

Changes in one of the splenic compartments alone is not a suitable indicator for drug effects in the spleen. For example, we have shown in previous studies that lipopolysaccharide-treated mice show markedly enlarged white pulp areas. Changes (not detectable with routine stainings) in these animals, however, were found in the inner-PALS where T-cells were substituted by B-cells (Groeneveld & Van Rooijen 1984).

The spleen contains numerous different cell types (recognized by different monoclonal antibodies) as described in the results section. Not only do they all have a specific function but their relative position in the spleen is of paramount importance for normal antigen-processing (Humphrey *et al.* 1984) and immune responses (Claassen *et al.* 1986b,c, 1987; Van Rooijen & Claassen 1988). Routine histological staining, as employed by Weereratne *et al.* (1982, 1983), Allen *et al.* (1984), and Allen and Smuckler (1985), can not reveal all changes in tissue sections of liposome (or any other toxic/harmless substance) -treated mice.

By using specific staining of different cell types in combination with a precise analysis of the splenic architecture in treated and untreated mice, we found almost no changes in the white pulp of SM/C treated mice. It is noteworthy that liposomes were used which were prepared in essentially the same way as described by other authors for SM/C liposomes (Ellens *et al.* 1981; Beaumier & Hwang 1983; Weereratne *et al.* 1983; Allen & Smuckler 1985). However, changes were found in the expression of surface marker ERTR-9 (unique for MZM). The MZM, that lost expression of this marker, are indeed the most likely candidates to do so because they are the first macrophages to clear the blood of antigenic particles. Furthermore these mac-

rophages and the closely associated MZ-B cells (Van Vliet *et al.* 1985) are indispensable for processing the thymus independent type-2 antigens such as neutral polysaccharides (e.g. TNP-ficoll) or haptenated liposomes (Claassen *et al.* 1986b; 1987). Immunization with TNP-ficoll gives a very complete picture of the immunological capacities of the spleen. MZM (Humphrey 1985; Claassen *et al.* 1986b; Sinha *et al.* 1987); T-cells (Mond *et al.* 1980) and B-cells (Kumararatne *et al.* 1981; McLennan *et al.* 1982; Gray *et al.* 1985) are necessary for the formation of specific antibody-forming cells, against TNP-ficoll, in the spleen. Responses against thymus-dependent antigens (most proteins) or thymus-independent type-1 antigens (e.g. lipopolysaccharide) are usually not influenced by changes in (or removal of) the spleen (Amlot *et al.* 1985; Gray *et al.* 1985; Claassen *et al.* 1986b; 1987) and are therefore not suitable to study subtle alterations. In this study we find that the response against TNP-ficoll in SM/C treated animals is not changed when compared to PBS or PC/C control animals, contrary to earlier studies where this immune response could be suppressed to 10% of its normal values (Claassen *et al.* 1986b). In the present study the MZM were not actually eliminated, as was the case in former experiments, but only suppressed with regard to their surface marker ERTR-9. Nevertheless, it is difficult to believe that these macrophages, which are clearly overloaded by the constant supply of SM/C liposomes, could nevertheless still internalize TNP-ficoll and present it to the MZ-B cells. Either the remaining 25% of the MZM which still express (albeit less) ERTR-9 are not involved in liposome processing and can still present TNP-ficoll, or the MZM are not involved at all in presentation of TNP-ficoll but only in its phagocytosis and degradation. The latter explanation seems the more likely, although this would mean that our earlier conclusions obtained by complete elimination of MZM and partial removal of MZ-B cells, need reevaluation (Claassen *et al.* 1986b; 1987).

The suggestion of Weereratne *et al.* (1983) that chronic SM/C liposome administration could be used as a model system for tissue granulomatous infection seems unjustified in the light of the present results. No clustering or even presence of the T-helper or T-cytotoxic/suppressor cells in or around the granulomas was observed as would have been expected in a bona fide inflammation. The increase in red pulp macrophages is only small and can also be observed after overload administration of non-toxic particles like indian ink or latex beads; furthermore it is likely (from their localization and content) that these macrophages perform only non-specific phagocytosis (Claasen & Van Rooijen 1984; e.g. of erythrocytes).

A surprising finding in this study was the fact that the effects on ERTR-9 (MZM marker) were only found after i.v. and not after i.p. administration of SM/C liposomes. These results could be in accordance with the findings of Ellens *et al.* (1981), that the rate of vesicle uptake by spleen and liver of i.p. injected SM/C liposomes was reduced by a factor of 2 to 3, when compared to the i.v. route. Our results indicate that i.v. administered liposomes are mainly cleared by the MZM (as expected), and that SM can temporarily block/suppress these macrophages. These effect are not observed after i.p. administration either because these liposomes do not reach the MZM in sufficient numbers, or because they have already lost or exchanged their lipids with e.g. lymphocytes (Plesser *et al.* 1979) or peritoneal macrophages, resulting in changes in fusion behaviour with other cells (Rivnay *et al.* 1978), in this case with the MZM.

In summary, we have observed no temporary or permanently damaging effects of PC/C or SM/C liposomes if given by the i.p. route, on the immunologically active cells in the murine spleen. A thymus-independent type-2 immune response, which is spleen dependent, showed no changes after chronic liposome treatment by either i.v. or i.p. route. The role of MZM in the specific immune response against this type of antigen needs to

be reconsidered. SM/C liposomes are not suitable as a model for the study of granulomatous inflammation in the spleen.

Although SM/C liposomes do possess some drawbacks, care should be taken not to eliminate them as carriers for drugs and vaccines; they also have some unique and valuable properties for use *in vivo*.

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