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Published in:
Biochimica et Biophysica Acta-Biomembranes

DOI:
10.1016/s0005-2736(96)00164-2

Publication date:
1996

ELSEVIER

Biochimica et Biophysica Acta 1285 (1996) 219–228

BBA

Liposomal phosphatidylserine inhibits tumor cytotoxicity of liver macrophages induced by muramyl dipeptide and lipopolysaccharide

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Received 24 May 1996; revised 5 August 1996; accepted 6 August 1996

Abstract

Liposomes can very efficiently deliver immunomodulators to macrophages so as to induce tumor cytotoxicity. Liposomes most widely used for that purpose contain negatively charged lipids, in particular phosphatidylserine (PS), to enhance liposome uptake by the macrophages. We investigated the effect of three negatively charged liposomal lipids on the in vitro activation of liver macrophages to tumor cytotoxicity by muramyl dipeptide (MDP) and lipopolysaccharide (LPS). Both MDP- and LPS-induced tumor cytotoxicity towards murine colon adenocarcinoma cells were strongly inhibited by PS-containing liposomes. Under comparable conditions phosphatidylglycerol (DPPG)-containing or dicetyl phosphate (DCP)-containing liposomes did not inhibit or only marginally inhibited the induction of tumor cytotoxicity. We did not observe PS-mediated inhibition of tumor cell toxicity when the exposure of the macrophages to PS-liposomes was limited to the 4-h activation period prior to addition of the tumor target cells, suggesting that the inhibitory effect is accomplished at the level of the later stages of the activation process. Previously, we showed that macrophages which are activated to tumor cytotoxicity during a 24-h incubation with MDP become refractory to a second activation with MDP. Now we observed that simultaneous incubation with PS-containing liposomes partially prevents this refractoriness, which is also compatible with an interfering action of PS at a relatively late stage in the activation process. We conclude that PS, despite its reported stimulatory effect on liposome uptake by macrophages, can seriously antagonize the effectiveness of immunomodulating agents acting on macrophages. This bears relevance to the use of PS-containing liposomes as a vehicle for such agents. The results are discussed in perspective of earlier reported pharmacological effects of PS and its metabolites.

Keywords: Phosphatidylserine; Tumor cytotoxicity; Cytotoxicity; Muramyl dipeptide; Lipopolysaccharide; Liposome; Macrophage; (Liver)

1. Introduction

Macrophages can be activated to kill tumor cells. Activation can be achieved by exposing macrophages, including Kupffer cells, to a variety of immunomodulators such as muramyl peptides, lipopolysaccharide (LPS), lymphokines and γ-interferon [1–3]. In vivo, however, muramyl dipeptide (MDP) cannot activate

Abbreviations: MDP, muramyl dipeptide; LPS, lipopolysaccharide; PS, phosphatidylserine; DPPG, phosphatidylglycerol; DCP, dicetyl phosphate; FCS, fetal calf serum; PC, phosphatidylcholine; [^{3}H]CE, [^{3}H]cholesteryl oleyl ether; [^{3}H]Thd, [*methyl*-^{3}H]thymidine; POPC, palmitoyloleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine.

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PII S0005-2736(96)00164-2

macrophages to tumor cytotoxicity, presumably due to its rapid renal excretion [4], but upon encapsulation of the MDP in liposomes, the drug is able to cause a significant reduction of hepatic or pulmonary metastatic tumor growth in mice [5–7]. The liposomes presumably act by efficiently delivering the MDP to tissue macrophages, which are the primary target of these vesicles upon i.v. or i.p. injection. A widely used and commercially available liposomal MDP preparation consists of phosphatidylcholine and phosphatidylserine in a 7:3 molar ratio. In our attempts to optimize the level of liver macrophage activation by treatment with liposome-encapsulated immunomodulators, we investigated the effects of different liposomal lipids, in casu the negatively charged constituents and cholesterol, and observed some remarkable differences.

2. Materials and methods

2.1. Animals

Specific-pathogen-free female Wistar or male Wag/Rij rats (Harlan CPB, Zeist, The Netherlands), weighing 160–180 g, 7–9 weeks of age, were used in all experiments.

2.2. Materials

All cultures were grown in RPMI-1640 from Gibco and supplemented with 2 mM L-glutamine (Flow Labs.), penicillin G (100 units/ml), streptomycin (100 μg/ml) (both from Gist-Brocades), and heat-inactivated fetal calf serum (FCS; Gibco). LPS from *Escherichia coli* 0127:B8 was purchased from Difco Lab. MDP and placebo liposomes (palmitoyloleoylphosphatidylcholine (POPC) and dioleoylphosphatidylserine (DOPS) in a 7:3 molar ratio) were generous gifts from Ciba Geigy, Basel. MDP was stored desiccated at 4°C. DNase (grade II) was purchased from Boehringer, Mannheim. Egg phosphatidylcholine (PC, L-α-lecithin from egg yolk), dipalmitoylphosphatidylglycerol (DPPG) and PS from bovine brain were from Avanti Polar Lipids; cholesterol (chol) and dicetyl phosphate (DCP) were purchased from Sigma Chemicals Co., Ltd. [^{3}H]cholesteryl oleyl ether ([^{3}H]CE, spec. act. 1.71 TBq/mmol) and [*methyl*-^{3}H]thymidine ([^{3}H]Thd, spec. act. 185 GBq/mmol) were from Amersham.

2.3. Tumor cell culture

C26 colon adenocarcinoma cells, syngeneic with BALB/c mice were grown as a monolayer in culture medium containing 10% FCS at 37°C in a humidified atmosphere with 5% CO_2 in air.

2.4. Preparation of liposomes

Multilamellar vesicles were prepared as follows. The lipid solutions in chloroform/methanol 9:1 were stored under nitrogen at −20°C. Lipids with or without [^{3}H]CE (18.5 KBq [^{3}H]CE/μmol of lipid) were mixed, dried under reduced nitrogen pressure, dissolved in cyclohexane, and lyophilized. The lipids were then hydrated in pyrogen-free 135 mM NaCl-10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) and mechanically agitated for 10 min at room temperature.

For MDP-liposomes the lipids were hydrated in pyrogen-free 135 mM NaCl-10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) containing 1.7 μg/ml MDP. The latter vesicles were sized by extrusion through two polycarbonate membranes (Nuclepore) of 0.4 μm pore diameter. Vesicles containing MDP were freed from nonencapsulated material on a Sephadex G-100 column (Pharmacia). The encapsulated amount of MDP was determined with a galactosamine determination assay [8] with a minor modification, i.e., addition of 1% of Triton X-100 to the reaction mixture to destroy the liposomes. Liposome preparations were stored under nitrogen at 4°C and used within 1 day after preparation. The lyophilized placebo liposomes from Ciba Geigy were hydrated with phosphate-buffered saline and vortexed for 30 s. These liposomes were stored at 4°C and used within 1 month.

2.5. Isolation of rat liver macrophages

Liver macrophages were isolated by pronase digestion of the liver and purified by centrifugal elutriation as described before [1]. This procedure results in a 90% pure macrophage population, based on Giemsa- and non-specific esterase staining. Contaminating

cells are mostly liver endothelial cells. Liver macrophages ($25 \cdot 10^4$ per well) in 200 μl of culture medium containing 10% FCS were seeded in 96-well microtiter plates (Costar). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air.

2.6. Target cell radiolabeling

Target cells in exponential growth phase were radiolabeled by a 20-h incubation in medium containing [^{3}H]dThd as described before [1].

2.7. In vitro cytolytic assay

Macrophage-mediated cytolysis was assessed by a [^{3}H]dThd-release assay. The macrophages were incubated with the immunomodulators and the liposomes as described in the results. Subsequently 10^4 [^{3}H]dThd-labeled target tumor cells were added to the macrophages in monolayer culture. Radiolabeled target cells were also plated alone, as an additional control. 44 h after the addition of tumor cells, the supernatants were collected and radioactivity was measured in a liquid-scintillation counter. Specific cytolysis was calculated as follows:

$$\% \text{ specific cytolysis} = 100 \times \frac{a-b}{c-b}\%$$

in which a is dpm in the supernatant of target cells cocultured with test macrophages, b is dpm in the supernatant of target cells cocultured with control macrophages, and c is dpm in the total amount of target cells added per well. The amount of radioactivity released from target cells cultured with or without control macrophages was always less than 10% of the amount of radioactivity added to each well.

2.8. Phagocytosis of liposomes

One day after cell isolation, macrophage monolayers were incubated with liposomes containing [^{3}H]CE, as a metabolically inert marker, in the presence of 10% FCS. After 24 h the medium was removed, the wells were washed 4 times with cold phosphate-buffered saline, and the cells were lysed with 0.5 M NaOH. Aliquots were taken for the determination of protein content and radioactivity. Radioactivity data were converted to nmol of liposomal lipid, based on the specific activity of the liposome preparations used.

2.9. Statistical analysis

The two-tailed Student's t-test was used to determine statistically significant differences between groups

3. Results

3.1. Effect of liposomal lipids on MDP and LPS-induced tumor cytotoxic activity of liver macrophages

Addition of liposomes (500 nmol lipid/ml) containing 20–40 mol% of PS significantly affected the

Table 1
Effect of liposomes on MDP-induced macrophage tumor cytotoxicity

Liposome composition [a]	Lipid ratio	dpm in supernatant (% cytolysis) [b,c]	
		Expt. 1 [d]	Expt. 2 [e]
MDP, no liposomes		382 ± 5 (33)	676 ± 60 (43)
eggPC/PS	9:1	381 ± 14 (33)	522 ± 67 (31) *
eggPC/PS	8:2	259 ± 35 (19) *	342 ± 40 (16) *
eggPC/PS	7:3	N.D.	333 ± 23 (15) * *
eggPC/PS	6:4	212 ± 15 (14) * *	214 ± 48 (5) * *
POPC/DOPS	7:3	319 ± 6 (26) * *	421 ± 9 (22) *
eggPC/chol/PS	4:5:1	381 ± 18 (33)	459 ± 24 (25) *
eggPC/chol/PS	3:4:3	251 ± 12 (18) * *	363 ± 29 (17) *
eggPC/chol/DCP	4:5:1	461 ± 45 (41) *	662 ± 47 (42)
eggPC/DPPG	7:3	406 ± 19 (36)	576 ± 38 (35)

[a] Per well $25 \cdot 10^4$ liver macrophages were incubated with MDP (100 μg/ml) in combination with empty liposomes composed of different lipids (500 nmol of lipid/ml). 10^4 [^{3}H]dThd-labeled C26 cells were added per well.

[b] After 48 h ^{3}H-release into the medium was determined in triplicate, between parenthesis the % of cytolysis is given.

[c] dpm presented as mean ± S.D.

[d] Expt. 1, medium release: 88 ± 20 dpm; 10^4 C26 cells added per well containing: 989 ± 85 dpm.

[e] Expt. 2, medium release: 150 ± 28 dpm; 10^4 C26 cells added per well containing: 1369 ± 42 dpm.

* Significantly different ($P < 0.05$) from cytolysis induced by MDP alone.

* * Significantly different ($P < 0.001$) from cytolysis induced by MDP alone.

Table 2
Effect of liposomes on LPS-induced macrophage tumor cytotoxicity

Liposome composition [a]	Lipid ratio	dpm in supernatant (% cytolysis) [b,c]	
		Expt. 1 [d]	Expt. 2 [e]
LPS, no liposomes		805 ± 40 (80)	1196 ± 21 (86)
eggPC/PS	9:1	806 ± 59 (80)	950 ± 20 (66) **
eggPC/PS	8:2	641 ± 28 (61) *	604 ± 34 (37) **
eggPC/PS	7:3	N.D.	577 ± 28 (35) **
eggPC/PS	6:4	401 ± 26 (35) **	443 ± 20 (24) **
POPC/DOPS	7:3	544 ± 14 (51) **	601 ± 79 (37) **
eggPC/chol/PS	4:5:1	722 ± 76 (70)	702 ± 30 (45) **
eggPC/chol/PS	3:4:3	487 ± 31 (44) **	468 ± 48 (26) **
eggPC/chol/DCP	4:5:1	894 ± 55 (89)	1199 ± 27 (86)
eggPC/DPPG	7:3	774 ± 29 (76)	1082 ± 27 (77) *

[a] Per well $25 \cdot 10^4$ liver macrophages were incubated with LPS (100 ng/ml) in combination with liposomes composed of different lipids (500 nmol of lipid/ml). 10^4 [^{3}H]dThd-labeled C26 cells were added per well.
[b] After 48 h ^{3}H-release into the medium was determined in triplicate, between parenthesis the % of cytolysis is given.
[c] dpm presented as mean ± S.D.
[d] Expt. 1, medium release: 88 ± 20 dpm; 10^4 C26 cells added per well containing: 989 ± 85 dpm.
[e] Expt. 2, medium release: 150 ± 28 dpm; 10^4 C26 cells added per well containing: 1369 ± 42 dpm.
* Significantly different ($P < 0.05$) from cytolysis induced by LPS alone.
** Significantly different ($P < 0.001$) from cytolysis induced by LPS alone.

extent of tumor cell lysis induced by MDP and LPS (Tables 1 and 2, respectively). The effect of liposome-incorporated cholesterol, often applied to improve liposome stability, was not consistent. While no significant effect of cholesterol was observed on the MDP-induced cytotoxicity, it showed a tendency to enhance the inhibitory effect of PS on LPS-induced cytotoxicity.

Liposomes in which PS was replaced by DPPG or DCP, both also negatively charged, did not influence, or only slightly, tumor cell lysis. In Experiment 2 (Tables 1 and 2) liposomes containing 30 mol% of DPPG inhibited tumor cell lysis induced by LPS, but not by MDP. However, the extent of inhibition was much lower than that induced by liposomes containing 30 mol% of PS. In contrast, in Experiment 1, DCP-containing liposomes slightly enhanced MDP and LPS induced cell lysis. For DCP the maximal amount incorporated in the liposomes had to be limited to 10%, due to its detergent properties which prevent the formation of liposomes at higher concentrations.

3.2. Effect of PS concentration

Macrophages were incubated with 100 μg/ml of MDP or 100 ng/ml of LPS in combination with liposomes containing 10 to 40 mol% of PS (Figs. 1 and 2). Each liposome formulation was added in two amounts: 0.25 μmol total lipid per ml (open symbols) or 0.5 μmol total lipid per ml (filled symbols). As shown in Figs. 1 and 2, the extent of MDP- and LPS-induced tumor cell lysis was determined not only by the absolute amount of PS but also by the mol fraction of PS in the liposomes. It should be noted that these results refer to the amounts of PS *in the medium* and not to amounts of PS taken up by the macrophages (see also the following two sections, Section 3.3, 3.4).

The levels of cytotoxicity observed vary between experiments (Table 1 vs. Fig. 2). This is inherent to

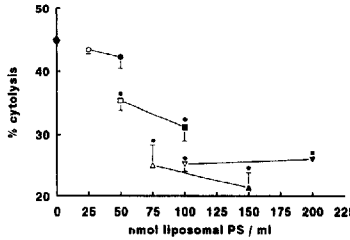

Fig. 1. Inhibition of MDP induced tumor cytotoxicity by PS-liposomes; PS dose response. Liver macrophages were cocultured with ^{3}H-labeled tumor cells in the presence of 100 μg/ml of MDP alone (◆) or in combination with liposomes. Open symbols represent lysis induced in the presence of 0.25 μmol/ml of liposomal lipid and filled symbols lysis induced in the presence of 0.5 μmol/ml of liposomal lipid. Four liposome preparations were used composed of eggPC/PS in ratios of 9:1 (○,●), 8:2 (□,■), 7:3 (△,▲) and 6:4 (▽,▼). After 48 h ^{3}H-release into the supernatant was determined in triplicate. Given is the mean percentage of cytolysis. Bars, S.D. * significantly different ($p < 0.0025$) from cytolysis induced by MDP alone (◆; 45.2%).

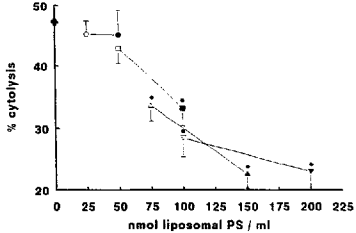

Fig. 2. Inhibition of LPS induced tumor cytotoxicity by PS-liposomes; PS-dose response. Liver macrophages were cocultured with ^{3}H-labeled tumor cells in the presence of 100 ng/ml of LPS alone (◆) or in combination with liposomes. For further details see legend to Fig. 1. * significantly different ($P < 0.0025$) from cytolysis induced by LPS alone (◆; 47.5%).

this type of experiments, due to variations in intrinsic properties of individual macrophage preparations and fluctuations in tumor cell sensitivity. The experiments described were performed over a period of several months.

Activation of liver macrophages was also substantially inhibited by PS when liposome-entrapped rather than free MDP was used to activate the cells (Fig. 3). Four liposome preparations were used with increasing mol fractions of PS. Each preparation revealed a clear tendency towards increasing inhibition with increasing liposome (i.e., PS) concentration. Furthermore, for nearly all points representing the MDP in the 20%-, 30%- and 40%-PS liposomes (squares and triangles), the levels of cytotoxicity were significantly lower than those induced by the MDP in the corresponding lipid doses of the 10%-PS liposomes (circles).

3.3. Effect of lipid composition on the uptake of liposomes by liver macrophages

When comparing the inhibitory effects of different liposome compositions, it should be taken into consideration that liposomes of different composition will be taken up to different extents by the macrophages. In these experiments we determined the uptake of liposomes composed of egg PC with 10 mol% PS, 30 mol% PS or 30 mol% DPPG. Fig. 4 shows the uptake of these liposomes by cultured liver macrophages after incubation for 24 h at 37°C.

The association of PC/PS (7:3) liposomes with macrophages was 2–3-fold higher than that of PC/DPPG (7:3) liposomes and 10-fold higher than that of PC/PS (9:1) liposomes. At 4°C uptake values were only 10–25% of the amount associated at 37°C (not shown), suggesting that the larger part of the radioactivity recovered from the macrophages at 37°C is intracellular.

3.4. Influence of the amount of macrophage-associated liposomes on cytotoxicity

Fig. 5 presents the relation between the extent of MDP-induced tumor cytotoxicity of the amount of macrophage-associated liposomal PS or DPPG. For PC/DPPG liposomes, amounts of up to 300 nmol of DPPG (i.e., as much as 1000 nmol of total lipid) taken up per mg cellular protein in 24 h did not

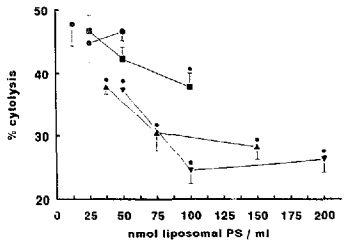

Fig. 3. Inhibition of liposomal-MDP induced tumor cytotoxicity with increasing amounts of liposomal-PS. Liver macrophages were cocultured with ^{3}H-labeled tumor cells in the presence of MDP encapsulated in four differently composed liposomes. The liposomes were composed of eggPC/chol/PS in ratios of 9:10:1 (circles), 8:10:2 (squares), 7:10:3 (triangles) and 6:10:4 (inverted triangles). Open symbols represent lysis induced with 1.25 μg/ml of MDP and 0.25 μmol/ml of total liposomal lipid; half-filled symbols lysis induced with 2.5 μg/ml of MDP and 0.5 μmol/ml of lipid and filled symbols lysis induced with 5 μg/ml of MDP and 1 μmol/ml of lipid. Cytolysis was determined as described in the legend of Fig. 1. Given is the mean percentage of cytolysis. Bars, S.D. * significantly different ($P < 0.0025$) from cytolysis induced by the same amount of total liposomal lipid of eggPC/chol/PS (9:10:1) liposomes.

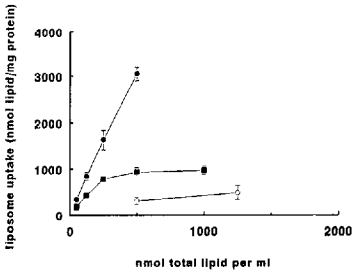

Fig. 4. Uptake of liposomes by rat liver macrophages. Liposomes composed of eggPC/PS (7:3)(●), eggPC/PS (9:1)(○) or eggPC/DPPG (7:3) (■) were labeled with [^{3}H]CE. Increasing amounts of liposomal lipid were added to macrophage monolayers and incubated for 24 h at 37°C in medium containing 10% FCS. Further details in Section 2. Data are expressed as mean uptake in nmol of total lipid per mg of cellular protein of duplicate or triplicate determinations. Bars, S.D.

significantly inhibit tumor cell lysis. By contrast, PS (in PC/PS (7:3) liposomes) taken up by the cells showed an increasing inhibition of tumor cell lysis with increasing amounts of lipid taken up. Inhibition induced by up to 50 nmol cell-associated PS (in 300 nmol PC/PS (9:1) liposomes) was not significant (not shown). The effects of larger amounts of PS uptake by means of these liposomes could not be measured because of toxic effects on the cells due to the amounts of liposomes required to achieve such uptake values, which caused the cells to detach from the culture dishes. The same was observed for PC/PG (7:3) liposomes beyond 300 nmol PG taken up per mg protein (squares).

3.5. Influence of time of addition on the inhibitory effect of PS

In the experiments described above, both the immunomodulators and the liposomes were present all along the activation period of the macrophages and the tumor cell lysis period. To gain more insight in the nature of the inhibitory effect, the macrophages were exposed to PS-liposomes either during the 4-h period before the tumor cells were added, which suffices to induce activation of the cells, or during the 44 h of subsequent cocultivation of macrophages with tumor cells (Figs. 6 and 7). Liposomes (PC/PS (7:3): 0.5 μmol/ml) which are present only during the 4-h incubation with MDP or LPS did not reduce the induced tumor cell lysis significantly, neither for MDP nor for LPS (bars C versus E in Figs. 6 and 7, respectively). Liposomes present during the 44-h coculture period, on the other hand, significantly inhibited the lytic activity of the macrophages. This was true irrespective of the MDP/LPS exposure period: bars D and F, representing the presence of the activator during the initial 4-h period, or bars B with the activator present during the 44-h co-cultivation period. These results clearly indicate that the inhibitory effect of the PS develops during the period after the initial stages of activation. Interestingly, the liposomes do not have to be present simultaneously with the activators to exert their inhibitory action: the development of a full cytotoxic state initiated during a 4-h pre-incubation with the activator can still be effectively blocked by PS liposomes during the sub-

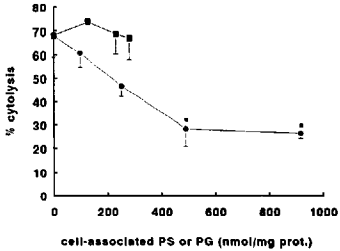

Fig. 5. Inhibition of macrophage-mediated tumor cell lysis in relation to the amount of PS or DPPG associated to the macrophages. Within one experiment liposome uptake and the effect of liposomes on macrophage tumor cytotoxicity was determined. Part of the macrophages were incubated with [^{3}H]CE-containing liposomes to determine uptake, as described in the legend of Fig. 4. The remaining cells were incubated with MDP (100 μg/ml) in the presence of eggPC/PS (7:3) (●) or eggPC/DPPG (7:3) (■) liposomes and cocultured with C26 cells as described in the legend of Fig. 1. Given is the mean percentage of inhibition of MDP-induced tumor cytotoxicity of three individual experiments. Bars, S.D. significantly different ($P < 0.05$) from cytolysis induced by MDP alone (68%).

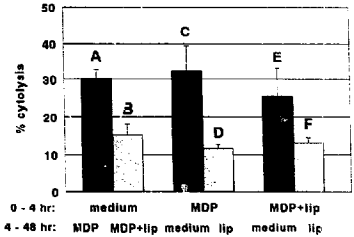

Fig. 6. Effect of PS-liposomes on MDP-induced tumor cytotoxicity; presence of PS-liposomes during the 4-h activation period or the 48-h macrophage/tumor cell coculture. Macrophages were incubated with medium, MDP (100 μg/ml) or MDP + liposomes (POPC/DOPS = 7:3; 0.5 μmol/ml). After 4 h the supernatant with the liposomes was removed and ^{3}H-labeled tumor cells were added together with MDP, liposomes, MDP + liposomes or medium alone. After 48 h ^{3}H-release into the supernatant was determined. Given is the mean percentage of cytolysis of four experiments. Bars, S.D.

sequent 44-h period, when the activator (MDF or LPS) is no longer present (bars D).

The lack of effect of the presence of PS-liposomes during the first 4 h (bars E) cannot be ascribed to an insufficient amount of cell-associated liposomes since, following a 4 h incubation with 0.5 μmol/ml of PC/PS (7:3) liposomes, approx. 1600 nmol of liposomes are taken up by the macrophages per mg cell protein (not shown). As shown in Fig. 5, MDP-induced tumor cell lysis is strongly inhibited when such an amount of PS-liposomes is taken up by the cells.

3.6. Effect of preincubation with PS-liposomes

To further investigate chronological aspects of the inhibitory effect of PS-liposomes, we exploited our earlier observation that macrophages preincubated with MDP for 24 h before addition of tumor cells have lost their tumor cytotoxic state and cannot be reactivated for at least 48 h [9]. In the present study macrophages were preincubated for 24 h with MDP and/or PC/PS-liposomes. After 24 h, the media were removed and tumor cells were added in fresh medium together with MDP (Fig. 8). As we previously found, preactivation with MDP resulted in a steep drop in MDP-induced tumor cell lysis (bar B vs. bar A, $P < 0.001$). The presence of PS-containing liposomes during the preincubation period with MDP, was able to partially prevent this drop (bar D vs. bar B; $P < 0.002$). These data, combined with the data shown in Figs. 6 and 7, indicate that the larger part of the PS effect on the activation process is achieved

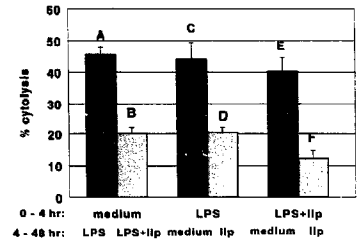

Fig. 7. Effect of PS-liposomes on LPS-induced tumor cytotoxicity; presence of PS-liposomes during the 4-h activation period or the 48-h macrophage/tumor cell coculture. The experiment was performed as described in the legend of Fig. 6 with LPS (100 ng/ml) or LPS + liposomes (POPC/DOPS = 7:3; 0.5 μmol/ml). Given is the mean percentage of a typical experiment in triplicate. Bars, S.D.

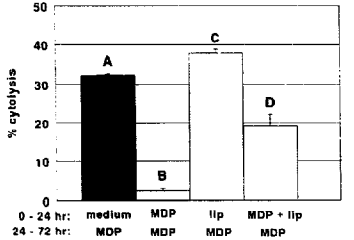

Fig. 8. Effect of PS-liposomes on reactivation of liver macrophages with MDP. Macrophages were incubated with medium, MDP (100 μg/ml), liposomes (POPC/DOPS = 7:3; 0.5 μmol/ml) or MDP + liposomes. After 24 h the supernatant was removed and ^{3}H-labeled tumor cells were added together with MDP. After 48 h ^{3}H-release into the supernatant was determined. Given is the mean percentage of cytolysis of a typical experiment in triplicate. Bars, S.D.

between 4 and 24 h after the addition of MDP to the macrophages.

Liposomes alone for 24 h did not have a significant effect on the cytotoxic activity of the macrophages (Fig. 8, bar C).

Similar results were obtained for activation with LPS (not shown).

4. Discussion

PS-containing liposomes were found to interfere with the development of MDP- and LPS-induced liver macrophage cytotoxicity. Our results suggest that the inhibitory effect of PS-liposomes involves the later stage(s) in the activation process rather than the initial stage (up to 4 h) or the actual cytolytic process. Inhibition is apparently not related to the amount of liposomes taken up by the macrophages, since macrophages incubated for 24 h with liposomes before activation with MDP express full tumor cytotoxicity.

Gilbreath et al. showed that PS-containing liposomes inhibit lymphokine-induced macrophage microbicidal activity against *Leishmania major* [10–12]. They observed, however, no inhibition of lymphokine-induced macrophage tumor cytotoxic activity. In addition, in their studies it was concluded that PS-liposomes interfere with one or more early events in the induction of microbicidal macrophages [10], while we conclude from our observations that PS rather affects later activation events. Observations by Keller et al. [13] may very well explain the apparent discrepancy between the results with lymphokines as described by Gilbreath et al. [10] and our results using MDP or LPS as immunomodulators. Keller et al. showed that lymphokines and bacteria, both of which induce tumoricidal activity, trigger different secretory responses in bone-marrow-derived mononuclear phagocytes. Induction of tumoricidal activity by lymphokines was not associated with an enhanced secretion of IL-6 and PGE2 secretion while heat-killed intracellular bacteria resulted in a markedly enhanced secretion of these products [13]. Similarly, we found that MDP and LPS, both originating from bacterial cell wall material, induce secretion of several cytokines [14].

Although the results presented in Fig. 5 show a good correlation between extent of inhibition and amount of PS taken up by the macrophages, this does not exclude the possibility that the inhibitory effect is accomplished by extracellular PS because high uptake necessarily means high medium concentration as well. Compatible with an extracellular target for the PS effect appears the observation that the amount of PS liposomes taken up during a 24-h incubation is unable to cause any inhibition of activation by MDP during a subsequent 48-h incubation period (Fig. 8, bar C). Also in that case, however, the signal given to the cell by extracellular PS (or a metabolite) would have to be transduced to an intracellular site because the activation induced by MDP or LPS during a 4-h preincubation can still be abolished by PS liposomes added after the activators have been removed (Figs. 6 and 7, bars D). We have, at present, no compelling evidence to favor either an intracellular or an extracellular site of action of PS. Also if a PS metabolite were involved, it could be formed both intra- and extracellularly.

In either case, it is clear that sustained availability of PS is required (Figs. 6 and 7, bars E vs. bars F). This might also explain the direction of the effect of cholesterol; whenever it had an effect on the PS-induced inhibition, it was an enhancement. Cholesterol is known to retard enzymatic degradation of liposomal phospholipids [15].

We have shown previously that the activating potency of LPS towards liver macrophages is diminished 20–100-fold by encapsulating it in liposomes [16]. We attributed this to intralysosomal inactivation of LPS. It is unlikely that the inhibitory activity of PS on LPS-induced cytotoxicity in the present experiments is caused by spontaneous association of the LPS with the liposomes such that it is taken to the lysosomal compartment and inactivated: liposomes present during a 4-h incubation with LPS just slightly reduced the induced tumor cell lysis (Fig. 7, bars C versus E), while the inhibitory effect was clearly manifest when the liposomes were added after the LPS had been removed (Fig. 7, bar D). Monastra et al. [17] showed that liposomes containing PS significantly reduced the endotoxin-induced TNF serum level in mice and rabbits. Also in this study a direct LPS–liposome interaction at the site of injection or in plasma could be excluded.

PS has been shown to possess immunosuppressive

properties. For example, it prevents the primary humoral immune response in mice and in cultured spleen cells it inhibits the response to mitogens activating the T-cell antigen receptor [18]. PS-liposomes reduced nitric oxide synthesis from peritoneal macrophages treated with IFN-γ, IFN-γ/MDP and IFN-γ/TNF-α and reduced the IgG response to bovine serum albumin [19]. Calderon et al. [20] demonstrated that the impaired ability of macrophages from mice bearing D1-DMBA-3 mammary tumors to produce nitric oxide in response to LPS could be ascribed to PS overexpressed/shed by these tumor cells. It has also been shown that orally administered PS suppresses antigen-specific IgM production against sheep red blood cells, intubated intragastrically [21]. In the latter study the authors postulate that PS, which is situated predominantly on the inner surface of the eukaryotic cell membrane, is released after the destruction of cells, thus controlling and limiting inflammation. Fadok et al. [22,23] showed that PS is exposed on the surface of apoptotic lymphocytes and thereby triggers specific recognition and removal of such cells by macrophages. Also the observation that PS-liposomes block the recognition of sickled red cells expressing PS externally by macrophages, led to the hypothesis that macrophages might specifically recognize PS [24,25].

As shown by Gilbreath et al. [11], the inhibition of microbicidal activity was directly influenced by changes in the phospholipid head group as well as by the number of unsaturated bonds in the phospholipid acyl groups. Progressively increasing unsaturation of the acyl moieties optimized the inhibitory effect of the phospholipids. Similarly, we found that inhibition of MDP-induced macrophage activation by DOPS was slightly lower than that caused by brain-PS. A potential involvement of phospholipase A2 activity is suggested by the observation that this enzyme is strongly influenced by the degree of unsaturation of the *sn*-2 fatty acid in the substrate [26] and that activated macrophages display increased levels of phospholipase activities [27]. Bellini and Bruni [28] showed that the inhibitory action on mitogen-induced T-cell activation of PS requires a serum protein which displays phospholipase A activity specifically towards PS. Upon incubation of this protein with PS, lyso-PS and the *cis*-unsaturated fatty acid are released. The latter was found to be mainly responsible for the PS-induced inhibition of mitogen-induced T-cell activation.

We have no satisfactory explanation for our observation on the enhanced uptake of liposomes containing 30 mol% of PS compared to liposomes containing 10 mol% of PS or 30 mol% of DPPG. At present we are studying this phenomenon in more detail.

The results presented in this paper demonstrate the importance of the selection of components for the formulation of carrier liposomes in delivery of immunomodulatory agents. To explain the inhibitory effect of PS, we favor a pharmacological interference of this phospholipid or one of its metabolites with the activation process, probably following internalization and intracellular processing by the macrophages.

Acknowledgements

The authors thank Bert Dontje and Jan Wijbenga for expert technical assistance, and Ciba Geigy for generous supplies of MDP and placebo phospholipids.

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