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Biochimica et Biophysica Acta 1283 (1996) 177–184

BBA Biochimica et Biophysica Acta

Preparation and properties of sterically stabilized hexadecylphosphocholine (miltefosine)-liposomes and influence of this modification on macrophage activation

Reiner Zeisig [a,*], Ines Eue [a], Markus Kosch [b], Iduna Fichtner [a], Dieter Arndt [a]

[a] AG Phospholipids, Max-Delbrück Center for Molecular Medicine, R-Rössle-Str. 10, Berlin-Buch 13122, Germany
[b] University of Münster, Münster, Germany

Received 19 February 1996; revised 7 May 1996; accepted 13 May 1996

Abstract

The aim of the study was to investigate for the first time the preparation, physical properties and macrophage activating effect of sterically stabilized liposomes made from hexadecylphosphocholine (HPC, Miltefosine®) using different poly(ethylene glycol) lipids for coating. We could demonstrate that it is possible to prepare different liposomal vesicle types (MLV, SUV and LUVET) without any problem and with a high stability in buffer (release of hydrophilic marker was < 5% after half a year) and in plasma ($t_{1/2}$ up to several days). The preparation method, including size of polycarbon membrane filter used for the preparation of LUVETs had the main influence on vesicle size and size distribution. The addition of a charged lipid like DCP and different amounts of PEG-lipid up to 10% had no effect on size and stability of PEG-LUVETs. A comparison of activating potency of PEG-HPC-vesicles with commonly used HPC-liposomes was performed with mouse peritoneal macrophages. HPC-liposomes induced a clear release of NO and TNF from mouse peritoneal macrophages especially in a synergistical action with LPS. On the contrary the effect of PEG-liposomes was similar to control cells after a combined activation in vivo/in vitro. The reduced interaction of these liposomes with the MPS was also demonstrated by an unchanged carbon ink uptake after treatment of mice (i.p.) with liposomes prepared with and without PEG-lipid. PEG-HPC-liposomes combine the advantages of HPC, liposomes and PEG-coating, resulting in a promising preparation for treatment of mammary cancers.

Keywords: Hexadecylphosphocholine; Macrophage; Poly(ethylene glycol); Liposome; Mononuclear phagocytic system; LUVET; Stability

1. Introduction

Hexadecylphosphocholine (HPC, Miltefosine®) is the main representative of a new generation of synthetic lipids with a very simple structure. Although HPC has a very simple structure it has been found to have a strong antineoplastic activity in rats bearing MNU-induced, autochthonous mammary carcinomas [1,2]. Recently we could demonstrate that HPC is additional therapeutically active after i.v. or i.p. application in different human mammary carcinomas xenotransplanted to nude mice [3,4].

Beside the superior effect of topical treatment of skin metastasis originated from mammary carcinomas [5] the therapeutic effect following systemic application in human carcinomas was disappointing [6,7]. Because HPC and related alkylphosphocholines are able to form stable liposomes by themselves if cholesterol and a charged component like dicetyl phosphate is added [3], it should be possible to improve the therapeutic index of APC by using it in liposomal form.

A disadvantage for the in vivo use of liposomal preparations is their preferred elimination from circulation by the mononuclear phagocytic system (MPS) [8]. On the basis of several experimental data it is possible to control this uptake by the variation of liposome composition [9–15]. The influence of size [10,11], variation of liposomal con-

Abbreviations: APC, alkylphosphocholine; CF, 6(5)-carboxyfluorescein; CH, cholesterol; DCP, dicetyl phosphate; DPPE, dipalmitoylglycerophosphoethanolamine; DSPE, distearylglycerophosphoethanolamine; EPC, eicosanylphosphocholine; FCS, heat inactivated fetal calf serum; HEPC, hydrated egg yolk phosphocholine; HPC, hexadecylphosphocholine; LPS, lipopolysaccharide; LUVET, large unilamellar vesicle by extrusion technique; MLV, multilamellar vesicle; MPS, monocyte phagocytic system; NO, nitroxide; PBS, phosphate-buffered saline solution; PEG, poly(ethylene glycol); PI, polydispersity index; PT, plateau time; SUV, small unilamellar vesicle; TPC, tetradecylphosphocholine; TNF, tumor necrosis factor.

* Corresponding author. Fax: +49 30 94063213; e-mail: rzeisig@mdc-berlin.de.

PII S0005-2736(96)00090-9

stituents like cholesterol [12,13], surface charge [14], addition of phosphatidylserine [11] or gangliosides [14] are possibilities to influence the uptake of liposomes. Best results have been obtained by the incorporation of phosphoethanolamine derivatives of poly(ethylene glycol) which result in sterically stabilized liposomes (PEG- or Stealth® liposomes) with an enhanced time of circulation in vasculature ([15–18], for review see [8]). Subsequently an increase in drug targeted by liposomes was found in tumor tissue [19,20].

Based on our experience with multilamellar (MLV) and small unilamellar vesicles (SUV) made from HPC or other alkylphosphocholines [3,4,21–24] our intention was now to investigate for the first time the preparation and properties of sterically stabilized HPC-liposomes. Stability and interaction with the phagocytic system in vitro and in vivo were of special interest, as these properties are important preconditions for a successful therapeutic use of these new liposomal preparations.

2. Materials and methods

N-(Methoxypolyethyleneglycol)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, Na salt (PEG_{2000}DSPE) and *N*-(methoxypolyethyleneglycol)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, Na salt (PEG_{2000}DPPE), each with about 45 repetitive ethoxy-units and PEG_{5000}DPPE with about 110 repetitive units were purchased from Sygena (Liestal, Switzerland). HPC was a gift from Dr. Hilgard (ASTA Medica, Frankfurt, Germany) and was stored in a desiccator at room temperature. Hydrated egg-yolk lecithin (HePC, Phospholipon 100H) was obtained from Nattermann Phospholipid (Köln, Germany). Dicetyl phosphate (DCP), cholesterol (CH) and calcein were purchased from Serva (Heidelberg, Germany). CH was recrystallized from acetone prior to use. 5(6)-Carboxyfluorescein (Eastman Kodak Company, Rochester, NY, USA) was purified on Sephadex LH20 [25]. Methyl[^{3}H]thymidine was obtained from Amersham Buchler (Braunschweig, Germany). Sulfanilamide, H_3PO_4, $NaNO_2$ and lipopolysaccharide from *Escherichia coli*, serotype 055:B5 were purchased from Fluka (Buchs, Switzerland), *N*-(1-naphthyl)ethylenediamine dihydrochloride from Sigma-Aldrich (Deisenhofen, Germany); aqueous glutardialdehyde (25%), stabilised with Amberlyst A21 and Triton X-100 from Ferak (Berlin, Germany).

2.1. Preparation and characterisation of liposomes

Vesicles were prepared from APC or HePC, CH and DCP in a molar ratio of 1:1:0.2 (lipid/CH/DCP) by the lipid layer method. For the preparation of PEG-liposomes additional PEG-lipid (5–10 mol%) was added as indicated in tables and figures. The lipid film was hydrated with phosphate-buffered saline solution (PBS, pH 7.4) for MLV preparation and small unilamellar vesicles (SUV) were prepared by repeated sonication from these MLV as reported [3].

SUV or large unilamellar vesicles (LUVET) for determination of stability were obtained from the respective MLV, prepared by hydration of the lipid film with 150 mM CF (PBS) or 50 mM calcein (water). The total amount of encapsulated fluorescence marker was determined measuring the fluorescence intensity using the spectrofluorometer MK 1 (FARRAD Optical, New York, USA) at excitation wavelength of 490 nm and an emission wavelength of 520 nm. Liposomes were destroyed by addition of Triton X-100 (final concentration 2%) and concentrations were calculated from standard curves.

LUVET were prepared by the extrusion technique according to MacDonald et al. [26]. MLV-suspensions (1 mM or 20 mM) was passed repeatedly (usually 19 times) through polycarbon membranes of defined pore size using a LiposoFast™ Basic system (Avestin, Ottawa, Canada).

Liposomes (SUV and LUVET) were separated from free fluorescence marker after preparation by gel chromatography on Sephadex G-75, MLV were washed repeatedly by centrifugation ($20\,000 \times g$) and resuspended in PBS.

The lipid content and liposomal composition of the preparations were determined by high-performance thin-layer chromatography (Automated Multiple Development System and Linomat IV; CAMAG, Muttenz, Switzerland).

Vesicle size determination was performed by dynamic light scattering measurement at an angle of 90 with a Coulter Counter N4 MD model and the AccuComp® System (Coulter Electronics, Hialeah, USA). Mean particle size was calculated by SDP analysis. Size distribution (based on solid sphere weight results) is given as polydispersity index (PI) – varying from 0 (entirely monodisperse) to 1 (completely polydisperse suspension).

2.2. Characterization of liposomal stability

For the characterization of stability in buffer the determination of CF leakage from liposomes was performed by using fluorescence measurements as described previously [21]. Liposomes were stored at 4°C and the release was monitored at least for 6 months.

As a marker for vesicular stability of HPC-liposomes in plasma the release of the fluorescence marker calcein was determined according to Allen and Cleland [27]: Suspensions of MLV, SUV and LUVETs with encapsulated calcein were prepared as described above and finally diluted to a concentration of 2 mM with PBS. Immediately before measurement 50 μl of these suspensions were added to 950 μl of human plasma. This corresponds with concentrations of HPC used for experiments in vivo [4,28]

The fluorescence intensity at $t = 0$ minutes of each sample was defined as F_{start}. The increase in intensity was then followed at 25°C for at least 24 h. For determination

of the total amount of encapsulated marker (F_{final}) finally Triton X-100 was added to the plasma until non increase in fluorescence was observed (25–150 μl).

Stability of liposomes was compared based on time necessary to reach a plateau level with minimum of change. This plateau time (PT) is given as the mean time point of the first time period over 30 min with a change in fluorescence $\Delta F < 1\%/10$ min.

If possible, $T_{1/2}$ is given for the time for release of 50% of marker and was calculated as follows:

$$F(T_{1/2}) = (1/2)(F_{final} - F_{start}) + F_{start}$$

$T_{1/2}$ was then determined from the plot of the release curve at $F(T_{1/2})$.

All determinations were done at least twice.

2.3. Influence on peritoneal macrophages

Activation. Mouse macrophages were derived from the peritoneum of Balb/c mice 4 days after a single i.p. injection of 0.2 ml PBS with 80 μmol/l HPC-MLV or PEG-HPC-MLV by peritoneal lavage. Control macrophages were harvested without any in vivo pretreatment. The viability of macrophages was controlled by trypan blue exclusion and was always > 95%. All cells were cultured in RPMI-1640 medium (Gibco) and supplemented with 2 μmol/ml L-glutamine (Flow Labs), penicillin G (100 units/ml), streptomycin (100 μg/ml, both from Gist-Brocades) and heat-inactivated fetal calf serum, (10% FCS, Gibco). All the reagents except LPS were free of endotoxin contaminations as determined by the *Limulus* amoebocyte lysate assay (purchased from Bio*Whittaker, Walkersville, USA; sensitivity < 0.1 ng/ml).

Test- or control macrophages ($1 \cdot 10^5$ in 200 μl RPMI 1640/well) were incubated in a 96-well-microtiter plate (Costar) in the absence (control) or presence of MLV (100 μM) or LPS (50 μg/ml) for 4 h or 48 h at 37°C in a humidified atmosphere containing 5% CO_2.

TNF assay. Macrophages from mice treated with liposomes before or from untreated mice were cocultered with the indicated reagents for 4 h. Culture supernatants were assayed for TNF activity using the L 929 bioassay [29] and human recombinant TNF (Biozol) for standard as described previously [23].

The mean of TNF-release factor (TR) is given, calculated from TNF, released after treatment with the indicated agents, divided by the TNF released from control (untreated) macrophages. The mean TNF-release of control macrophages was 41.3 ± 28.3 pg/ml.

NO assay. NO secretion from macrophages was determined in supernatants after 4 and 48 h of incubation with 100 mmol/l MLV by measuring the total concentration of NO_2^-, a stable reaction product of NO, according to Ding et al. [30] as reported [21].

The absorbance was measured with a Spectra II Photometer (SLT) at 560 nm. The concentration of NO was calculated from a sodium nitrite standard curve and given in ng/ml. All determinations were carried out in duplicate in at least three independent experiments.

Carbon clearance in vivo. This experiment was used for measurement of the interaction of liposomes with the MPS as described earlier [31]. Briefly, four mice per group received equimolar doses of free HPC, HPC-SUV, PEG-HPC-SUV or physiological saline (for control). India ink was administered i.v. at certain times (for details see Fig. 5). Remaining ink was determined spectroscopically at 650 nm in 25 μl blood samples, taken from the retroorbital venus plexus. The elimination half life is given for India ink ($t_{1/2}$).

3. Results

3.1. Preparation and properties of LUVETS

SUV prepared by conventional sonification (Table 1) show a size of 74 ± 28 nm (PI: 0.31), the diameter for PEG-SUV were found to be e.g. for PEG_{2000}DSPE 86 ± 26 nm (PI: 0.14).

Large unilamellar vesicles were prepared from HPC-MLV containing different PEG-lipids (PEG_{2000}DSPE, PEG_{2000}DPPE and PEG_{5000}DPPE) by repeated extrusion through polycarbon membranes. Size and size distribution determined by dynamic light scattering measurement were compared with HPC-SUV.

In order to obtain optimal results with regard to these properties of liposomal populations, the number of necessary extrusions was found to be between 15 and 19. A further increase of up to 49 passages resulted in a very narrow size distribution with PI of 0.012 ± 0.028 ($n = 6$) and only a slightly reduced mean size of 118 ± 23 nm (found for HPC-LUVETs with PEG_{2000}DSPE).

There was no significant difference between LUVETs (unpublished data) and PEG-LUVETs, neither in size nor in size distribution (Table 1). Size of both vesicle types was only dependent on filter pore size (Fig. 1). The smallest distribution and therefore the most homogeneous vesicle population was obtained by 19-fold extrusion through filter with pores of 200 nm diameter (PI: 0.079) for HPC-liposomes without PEG-lipid (not shown) and through filter of 100 nm pore size (PI: 0.07) for liposomes with additional PEG-PE.

Contrary to HPC-liposomes, PEG-HPC-vesicles were found to be stable even in the absence of DCP (Table 1). The small diameter (about 53 nm) of SUV with long chain length PEG_{5000}PE is remarkable compared to SUV made from the short derivatives or to nonpegylated SUV (between 77 and 98 nm).

It is possible to modify the content of PEG-lipid in the liposomal composition without any problem concerning preparation within 0 to 10 mol%. Size (not shown) was found to be between 120 ± 29 nm and 123 ± 28 (PI

Table 1
Influence of PEG-lipid, dicetyl phosphate and preparation method on vesicle-size and distribution

APC	Type of liposome	PEG-PE	DCP content	Diameter (nm)	PI [a]
HPC	SUV	–	+ [b]	74 ± 28	0.306 ± 0.046
	LUVET	–	+	131 ± 5	0.067 ± 0.030
HPC	MLV	PEG_{2000}DSPE	+	858 ± 204	0.887 ± 0.060
			– [c]	800 ± 206	0.880 ± 0.062
		PEG_{2000}DPPE	+	330 ± 9	0.583 ± 0.005
			–	533 ± 21	0.745 ± 0.031
		PEG_{5000}DPPE	+	546 ± 174	0.748 ± 0.084
			–	604 ± 109	0.769 ± 0.056
HPC	SUV	PEG_{2000}DSPE	+	86 ± 26	0.137 ± 0.022
			–	84 ± 26	0.160 ± 0.022
		PEG_{2000}DPPE	+	98 ± 33	0.104 ± 0.080
		PEG_{5000}DPPE	+	53 ± 17	0.200 ± 0.082
			–	54 ± 17	0.170 ± 0.082
HPC	LUVET	PEG_{2000}DSPE	+	125 ± 29	0.061 ± 0.027
			–	114 ± 27	0.075 ± 0.030
		PEG_{2000}DPPE	+	134 ± 41	0.104 ± 0.080
		PEG_{5000}DPPE	+	114 ± 34	0.338 ± 0.183
TPC	LUVET	PEG_{2000}DSPE	+	106 ± 31	0.141 ± 0.048
EPC		PEG_{2000}DSPE	+	130 ± 36	0.091 ± 0.054

Liposomes were prepared from 1 nM MLV-suspensions (APC/CH/DCP/PEG-lipid with (+) or without (–) DCP) by sonication (12 × 4 min, SUV) or by repeated extrusion (19 times) through polycarbon filter of 100 nm size (LUVETs). Liposomes were characterized by dynamic light scattering.
[a] Unimodal results given as mean diameter ± S.D. ($n = 6$).
[b] APC/CH/DCP/PEG-lipid (1:1:0.2:0.1, molar ratio).
[c] APC/CH/PEG-lipid (1:1:0.1, molar ratio).

0.054–0.075) for LUVETs made from $DSPE_{2000}$PE of 2, 5 and 10 mol%. Therefore we added the PEG-lipid in a molar ratio to HPC of 1:20 for standard preparations. Sterically stabilized liposomes were also prepared from homologeous alkylphosphocholines with a shorter (TPC) or with a longer chain (EPC) without any problem (Table 1).

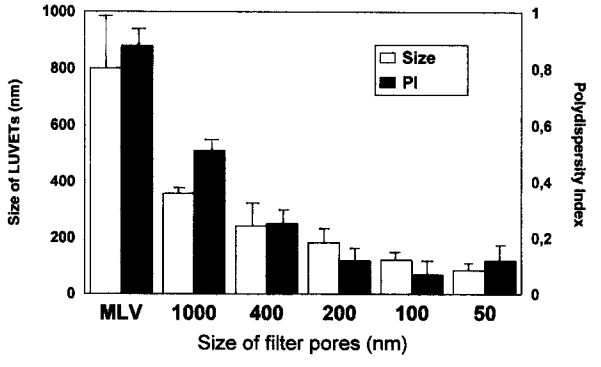

Fig. 1. Influence of filter pore size on vesicle diameter during preparation of PEG-HPC-LUVETs (concn. 1 mM; composition: HPC/Chol/DCP/PEG_{2000}DSPE in a molar ratio of 1:1:0.2:0.1) by repeated extrusion through polycarbonate filter of indicated size, using a LiposoFast system were investigated. Size and size distribution was determined by quasielastic light scattering, using a Coulter Counter Accucomp System. Data representing unimodal results for size, given in nm (open bars) and distribution of vesicle size (closed bars), given as PI-factor (varying from 1 (polydisperse) to 0 (monodisperse)). Data represent the means ± S.D. of three different liposomal preparations.

3.2. Stability of liposomes

The stability of PEG-vesicles in buffer was investigated by the use of 6(5)-carboxyfluorescein as a hydrophilic marker. CF was encapsulated in SUV and the release of marker was determined over a period of 5 to 6 months. Data are given in Table 2. After this time only 6% of encapsulated marker was released from SUV, prepared under addition of DCP. Using liposomes without this charged lipid the release was further decreased to 3.65%.

Physical stability in human plasma was determined

Table 2
Release of 6(5)-carboxyfluorescein from PEG-HPC-SUV in buffer

Liposome preparation	Time (Days)	Release [a] (%)
With DCP	14	2.13 ± 0.65
Absolute content of 6CF:	28	2.53 ± 0.62
74.88 ± 0.95 μg/μmol	63	3.86 ± 0.71
	161	5.93 ± 0.71
Without DCP	7	0.97 ± 0.43
Absolute content of 6CF:	28	1.52 ± 0.68
78.92 ± 9.63 μg/μmol	70	2.19 ± 1.15
	154	3.65 ± 1.99

20 mM SUV (HPC/CH/DCP/PEG_{2000}DSPE, 1:1:0.2:0.1 molar ratio) with encapsulated CF were prepared by sonication (Branson sonifier, 6 × 4 min/20°C) and stored at 4°C. The determination of CF by fluorescence measurement was done at 490/520 nm.
[a] Given are the means ± S.D. of three separate determinations.

using calcein as marker according to Allen [27]. The fluorescence of this marker is independent of pH changes in the range of 6.0–8.5 and circumvent interactions of fluorophore with changing proton concentrations. The release was followed at least for 24 h, as the highest plasma levels of liposomes were found in vivo between 12 and 24 h in different studies [15,32].

Following the leakage of calcein from MLV or SUV during more than 4 weeks in undiluted plasma demonstrated that the release is a process consisting of three phases (Fig. 2). The first, initial phase was found to be a single exponential release turning into a second, the plateau phase. About 15–50% of the marker was released at this time from HPC-liposomes, whereas liposomes made from HePC released lower amounts (Table 3). The release was finished by a repeated enhancement after a longer time of plasma incubation (third phase). In the following experiments the plateau-time (PT) and the concentration of encapsulated marker at this time were used for characterization of liposomal stability.

We found two groups of liposomes. First group involving MLV and LUVET (Table 3, preparations 3–8) showed low PT-values between 0.5 and 2 h. Up to half of the marker (22.4–58.7%) was released during this time. The second group (preparation 9–12) consisted only of SUV of different preparations and was found to have PT-times greater 6 h.

The amount of released marker was generally lower than in the first group. Especially SUV with DCP were very stable after an initial release of 16.1 resp. 23.7% of total marker.

Control liposomes made from hydrated egg yolk lecithin in a similar composition were included in this study (Table 3, preparation 1 and 2). These liposomes were also quite stable, but had very short PT accompanied with a low level of released marker.

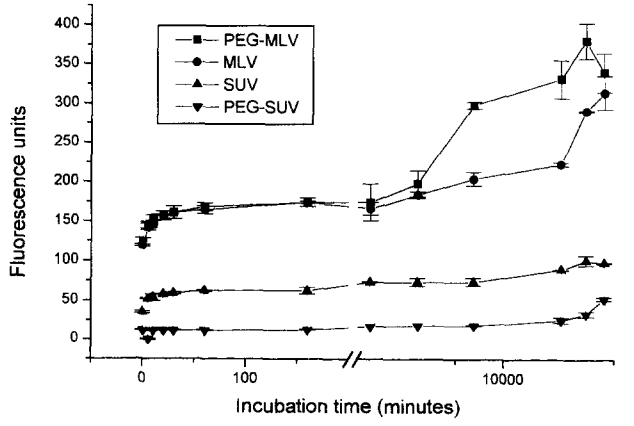

Fig. 2. Release of calcein from different liposomal preparation was determined after incubation in human plasma (concentration 100 μM) by fluorescence measurement at 490 nm excitation wavelength and 520 emission wavelength. Measurements were accomplished every 5 minutes during first hours and finally once every day. Given are the mean fluorescence units of two measurements

Table 3
Stability of HPC-liposomes in human plasma

No	Liposome preparation	PT (in min) [a]	Released calcein (%) [c]	$T_{1/2}$ (d) [b]	Max calcein (mmol/mol) [d]
1	HePC-MLV [e]	10	2.4	> 12	98.2
2	PEG-HePC-MLV [e]	60	5.4	5.7	647.3
3	MLV	30	58.7	≈ 2.5	144.4
4	LUVET [f]	55	26.9	> 1	63.6
5	PEG-LUVET [f]	60	22.4	> 1	86.3
6	PEG-MLV	60.5	35.2	≈ 16.8	244.5
7	PEG-LUVET	100	45.7	0.08	19.1
8	LUVET	110	57.8	0.04	90.3
9	SUV [f]	400	21.7	> 1.08	20.3
10	PEG-SUV [f]	400	32.0	≈ 1.08	38.5
11	SUV	1470	23.7	> 30	114.2
12	PEG-SUV	2980	16.1	> 30	76.8

Liposomes (HPC/CH/DCP, molar ratio 10:10:2; PEG-vesicles with additional 5% $DSPE_{2000}PEG$) with encapsulated calcein were incubated with human plasma to a final concentration of 100 μM. The release of calcein was determined by fluorescence measurements at 490 nm (excitation wavelength) and 520 nm (emission wavelength) after incubation of the liposomal suspensions in human plasma to a final concentration of 100 μM.

[a] Plateau time, time necessary to reach the plateau level of marker release.

[b] Time for the release of 50% of encapsulated marker.

[c] Amount of marker found in liposomes at plateau level (in percent of total encapsulated marker).

[d] Amount of total marker found in liposomes after destruction by addition of Triton X.

[e] Liposomal lipid used was HePC in place of HPC.

[f] Preparations without DCP.

3.3. *In vitro results*

We investigated the macrophage activating properties of PEG-HPC-MLV compared to HPC-MLV with respect to stimulation of NO- and TNF-release.

Whereas HPC-MLV caused a clear increase in NO-release from 80 pg/ml released from control macrophages to 158 pg/ml after stimulation for 48 h in vitro, the liposomes with PEG-PE had no significant influence on NO neither after 4 nor after 48 h of coincubation (Fig. 3).

The TNF content in the supernatant was determined in vitro as a second marker of macrophage activation. Macrophages cocultured in vitro with HPC-MLV or with the classical activator LPS were found to release up to 10-fold (liposomes) or 30-fold (LPS) more TNF than the control macrophages (Fig. 4, left set of bars). PEG-HPC-MLV were again without any effect. The combination of a first in vivo application of liposomes, with a second activation in vitro changed the situation (Fig. 4, bar set in the middle): the HPC-MLV induced release was decreased to the control level, whereas the LPS induced TNF amount was further increased. In contrast, the TNF release from macrophages stimulated in vivo with PEG-HPC-MLV (Fig.

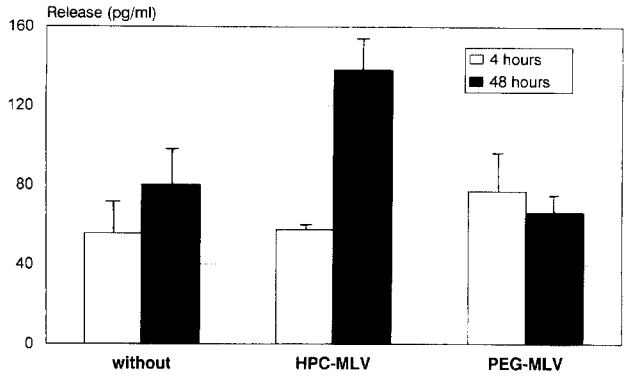

Fig. 3. Stimulation of NO release from mouse peritoneal macrophages was determined in in-vivo pre-treated (80 mM HPC-MLV or PEG-HPC-MLV) or untreated mouse peritoneal macrophages after isolation and culturing ($1 \cdot 10^5$ in 200 μl RPMI 1640/well). The concentration of NO is given in pg/ml. All determinations were carried out in quadruplicate in at least three independent experiments.

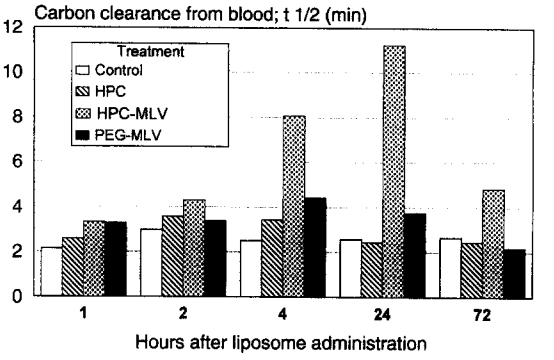

Fig. 5. Carbon clearance from blood circulation in mice after pretreatment with different HPC-liposomes was determined for MPS uptake. Four mice/group were pretreated with HPC, liposomes or physiologic saline i.p. After different times (see diagram), india ink was administered i.v. and ink was determined spectroscopically at 650 nm in 25 μl blood samples, taken from the retroorbital venus plexus. The elimination half life ($t_{1/2}$) in minutes was calculated.

4, right set of bars) gave similar results as the release from macrophages without any pretreatment in vivo.

3.4. In vivo results

The measurement of carbon clearance from blood is a suitable method to assay the function of the mononuclear phagocytic system. In accordance with our expectations the application of HPC-MLV resulted in a clear time dependent reduction in uptake of second vesicles like carbon particle from the blood circulation (Fig. 5). The strongest depression in phagocytic clearance of carbon (highest $t_{1/2}$) was achieved with 'conventional' MLV after 24 h from the administration. The free micellar HPC and the sterically stabilized HPC-liposomes were found to have no influence on the MPS, as the $t_{1/2}$ for elimination of carbon particles was similar to the control group.

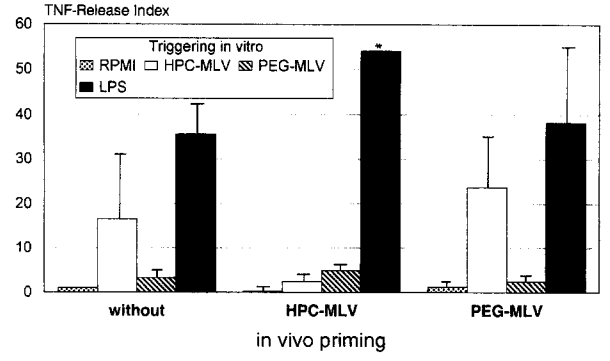

Fig. 4. Production of TNF from mouse peritoneal macrophages was determined on in vivo pretreated (80 mM HPC-MLV or PEG-HPC-MLV) or untreated mouse peritoneal macrophages after coculturing in vitro ($1 \cdot 10^5$ in 200 μl RPMI 1640/well) with 100 mM HPC-MLV, 100 mM PEG-HPC-MLV or LPS (50 mg/ml) for 4 h. TNF determination was done as described in Section 2. TNF-release is given as TNF release factor (TR). All determinations were carried out in quadruplicate in at least three independent experiments. * Levels of TNF were found to be beyond maximum determination limit; thus no S.D. value could be calculated.

4. Discussion

In previous studies [22–24] we have investigated the potency of HPC for macrophage activation to tumoricity. In the present investigation we were interested in the conditions to circumvent the MPS uptake of HPC-vesicles in order to improve the targeting of this cancerostatic compound to tumor site.

The preparation of sterically stabilized liposomes by coating of vesicles with poly(ethylene glycol)-PE, the most common method, was carried out mostly with natural lipids. No experience with PEG-vesicles prepared from HPC has been reported so far. The aim of our study presented here was to investigate the preparation of PEG-HPC-liposomes of a defined size and the comparison of classical HPC-liposomes with PEG-liposomes with regard to their macrophage activating properties.

Preparation of PEG-LUVETs by an extrusion technique [26,33,34] or of PEG-SUV by sonification from HPC-MLV was done without any problem. Whereas the sonification technique resulted in SUV of a more or less homogeneous and defined diameter, the size of extruded vesicles is related mainly to polycarbon filter size. This offers the opportunity to produce liposomes with a defined size open to selection. The vesicle size was related to the pore size, but not similar to it. Filter up to 200 nm pore size produced vesicles with a smaller diameter, pore size of 100 and 50 nm resulted in vesicles with a diameter being clearly greater than the pore size. This was also found with liposomes made from phosphatidic acid, whereas other lipids (phosphocholine or phosphatidylserine) formed LUVETs of diameters distributed below the pore size [26].

Vesicle populations of LUVETs are more homogeneous with respect to size than SUV made by sonication or by French press technique [35]. Especially the use of filter pore sizes of 200, 100 and 50 nm produced liposome preparations with a very narrow size distribution, whereas the larger pore sizes gave broader ones. This effect could be explained by the inhomogeneous character of the MLV population, consisting not only of large vesicles, but also of smaller ones (data not shown), which are then not effected by the extrusion.

In agreement with other data [17], most stable sterically stabilized HPC-LUVETS and SUV were prepared with a PEG content of 5–10 mol%. The PEG-lipid seems to stabilize the HPC-vesicle. Whereas HPC forms stable vesicles only in the presence of cholesterol and a negatively charged component like dicetyl phosphate [3] or 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol [35], PEG-HPC-SUV without a charged component were as stable as liposomes with DCP. We assume that the PEG-chains, surrounding the vesicle, protect them against lipid related charges of other liposomes and reduce the liposome–liposome interactions.

A prerequisite for therapeutical use is the stability of liposomes during storage and in biological fluids. To continue on data from earlier studies [3] we characterized the storage stability of liposomes by measuring the CF leakage from vesicles in buffer (pH 7.4). Size determinations showed first that the release of marker was not accompanied with a shift in size or size distribution (data not shown). Taking into account that the liposomal membrane itself consists of the physiological active lipid, a slow release of encapsulated dye should be a marker for maintenance of the vesicular structure and thus for intact liposomes.

In biological systems HPC-liposomes should be stable at least for 24 to 48 h, because peak plasma concentrations of the HPC [36] and of PEG-liposomes [15,16,32] made from other lipids were found in blood circulation during this time.

Our data for stability obtained at 25°C using calcein as marker are in a good agreement with data resulting from HPC-SUV with encapsulated CF marker at 37°C in human serum [35]. The retention of marker was in both experiments characterized by a fast initial release followed by a longer stable phase at a level of about 30–50% of total amount of encapsulated marker without an additional release. Concerning the $T_{1/2}$ times we characterize the HPC-liposomes as stable, compared to data from other studies. Whereas the stability of liposomes made from saturated natural lipids [27] are in the range of up to several hours we determined $T_{1/2}$ times up to days.

This stability could be explained in part by the high amount of cholesterol necessary to prepare the HPC liposomes. The mixture of the single chain lipid HPC with cholesterol compensate the missing second aliphatic chain usually found in natural lipids and could further explain the observed high retention of encapsulated hydrophilic marker. Cholesterol is well known to enhance the rigidity of the liposomal membrane [9,37,38]. A similar influence was also found with palmitoylphosphatidylethanolamine [39]. On the other hand this does not explain the variation in stability between the different liposomal preparations. Based on the similar liposomal composition the preparation method should have an important influence on the molecular structure of the membrane, resulting in defects or gaps in the bilayer (MLV or LUVET) or in liposomes with a more regular bilayer (SUV). In some reports [40,41] SUV made from other lipids were of a similar stability characteristic or were even stabilized in serum by blood proteins [42].

Besides the high stability of all HPC-vesicles in buffer and in plasma, a superior effect by sterical stabilization was not observed.

Because sterical stabilization should result in a reduced interaction between liposomes and MPS we extended this study to experiments of macrophage activation in vitro and in vivo.

We found in first experiments in vitro that the direct cytotoxic effect of PEG-HPC-liposomes on tumor cells was similar to liposomes without PEG-PE (unpublished data). The next question was directed to the macrophage activating potency of PEG-liposomes. We found in previous studies [22–24] that HPC-liposomes (without PEG) are able to induce a release of the well known mediators for cytotoxic activity – TNF and NO [43,44] – from macrophages. Remarkable is the synergistic effect of these liposomes with lipopolysaccharide, resulting in an enhanced release of the mentioned markers [23,24].

It is known that the coating of vesicles with PEG-derivatives reduces the uptake by the MPS [16,17]. Thus a macrophage activation should be minimized if PEG-HPC-liposomes are coincubated with the cells. Whereas in previous experiments the influence of liposomal composition on macrophage activation compared to control liposomes made from hydrated egg phosphocholine was investigated [23], we compared in this study directly the effect of sterically stabilized liposomes with plain liposomes (control liposomes) of similar composition and size.

Activation of macrophages by PEG-vesicles was diminished as expected compared to HPC-liposomes. This was found both in the coculture in vitro and in experiments with a combined activation in vivo/in vitro. These indicate that the interaction of HPC-liposomes with macrophages is reduced if vesicles are coated by incorporating PEG-PE into the vesicular bilayer. First results in quantifying this direct effect in vitro by the measurement of phagocytic uptake of liposomal HPC confirm this observation (data unpublished).

Additionally, a reduced uptake of PEG-liposomes by the MPS could be demonstrated with the carbon ink uptake experiments in vivo. These data gave indirect evidence for the reduced interaction of PEG-HPC-liposomes with the

MPS and should thus lead to an improvement of antitumor activity by an increasing concentration of the drug at tumor site. Further experiments to confirm these results in vivo are under investigation.

Our results with PEG-HPC-liposomes encourage the hope for a more efficient application form of liposomes made from HPC. A better targeting of HPC to tumor could finally result in an increased therapeutic index of HPC. Experiments are now ongoing to proof this conclusion by determining the organ distribution of free or liposomal HPC with or without sterically stabilization and to test these liposomes on different mammary carcinomas in nude mouse models. Additionally, these 'cytotoxic liposomes' should be an interesting application possibility used for the encapsulation of other antineoplastic drugs. The reduction in MPS uptake obtained by sterically stabilized liposomes opens the way to deliver a combination of two drugs with different modes of action more efficiently to tumor site with an additional or synergistic effects.

Acknowledgements

We thank Anneliese Schütt and Anne-Dorothee Teppke for their excellent technical assistance. This research was supported by grant 24911 from the Deutsche Forschungsgemeinschaft, Germany.

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