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Toxicity of gamma irradiated liposomes. 2. In vitro effects on cells in culture

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Abstract

In this study, the effects of liposome composition and gamma irradiation on their interactions with cell cultures were studied. The cytotoxicity test and the growth inhibition test clearly revealed toxic effects of liposomes composed of unsaturated phospholipids and gamma irradiation of these preparations enhanced their toxic effects. The murine fibroblast cell-line L 929 was less affected compared to the macrophage cell-line RAW 264 with a higher endocytic capacity. On the other hand, both gamma irradiated and non-irradiated liposomes composed of saturated phospholipids were non-toxic for the cells, and irradiation did not affect their drug delivery properties. Hence, it seems that gamma irradiation is appropriate for sterilisation of these liposomes. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Liposomes; Gamma irradiation; Toxicity; Cell culture

1. Introduction

Gamma irradiation is an alternative sterilisation method for substances which because of their sensitivity towards high temperatures can not be autoclaved. Gamma irradiation is approved for sterilisation of some pharmaceuticals (Woods and Pikaev, 1994; Reid, 1995) and seems therefore to be an alternative sterilisation method for liposomes. One publication (Commission of the European Communities, 1990) has given some guidelines intended for applicants wishing to use ionising radiation in the manufacture of medicinal products. They claim information about the effects of irradiation on the stability of the product and formation of degradation products. In addition, studies concerning toxicological risks caused by the irradiated products should be evaluated.

Albertini and Rustichelli (1993) have summarised the effects of gamma irradiation on liposomes. Unfortunately, peroxidation of

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unsaturated phospholipids (Zuidam et al., 1995) and formation of radiolysis products like lysophospholipids, free fatty acids, phosphatidic acid and different hydrocarbons takes place during gamma irradiation (Tinsley and Maerker, 1993; Stensrud et al., 1996; Zuidam et al., 1996a). The physical stability is affected to a lesser extent (Zuidam et al., 1995; Stensrud et al., 1996; Zuidam et al., 1996a). Zuidam and co-workers published recently a review of possible sterilisation methods for liposomes where they concluded that more studies are necessary before gamma irradiation can be accepted as a safe and convenient sterilisation method for liposomes (Zuidam et al., 1996b).

In another study (Stensrud et al., 1999), the effects of liposome composition and gamma irradiation on the interaction with the hemostatic mechanisms (hemolysis, platelet aggregation and coagulation) in vitro were studied. Non-irradiated liposome suspensions showed no hemolysis of erythrocytes but after irradiation up to 3.1% hemolysis was measured. The degree of hemolysis depended on the concentration and the type of phospholipids incubated with the red blood cells. Negatively charged DSPG-liposomes induced a small but reversible platelet aggregation. These liposomes also affected the coagulation cascade where prolonged coagulation times where measured. After irradiation the coagulation times were progressively prolonged.

In spite of these concerns, gamma irradiated liposomes (15 kGy) made of egg yolk lecithin, cholesterol, and stearylamin (4:3:1) have been administrated in large volumes to patient without any severe side effects (Coune et al., 1983; Sculier et al., 1986). Little changes in the blood parameters was observed in these studies (no hematologic toxicity, no hemolysis or changes in the blood viscosity). During each infusion, a dose related activation of the complement system was observed.

Several studies have been carried out in order to study the interaction of liposomes with cultured cells. Studies performed both with animal cell lines and human cell lines suggest that charged lipids are most toxic (especially positively charged stearylamin), leading to inhibition of cell growth and/or cytolytic effects (Campbell, 1983; Mayhew et al., 1987). Whereas the irradiation induced degradation product LPC is known to have cytolytic and membrane perturbing properties (Weltzien, 1979), the inclusion of phosphatidic acid in the liposomes (egg PC:cholesterol:phosphatidic acid, 4:1:1 w/w) showed minimal toxic reactions in cultured L 1219 cells (Campbell, 1983). So far, no work has been carried out to evaluate the toxicity of gamma irradiated liposomes for cultured cells.

The aim of the present work was to evaluate the effects of liposome composition and gamma irradiation on the interaction with cell cultures. Growth inhibition test and toxicity assays were carried out using two different cell types. The effect of irradiation on the drug delivery properties of liposomes in macrophages was also evaluated using encapsulated clodronate (dichloromethylene bisphosphonate). Whereas high concentrations of free clodronate are required to inhibit the growth of RAW 264 macrophages in vitro, encapsulation in liposomes enhances the potency by a factor of 20–200 (Mönkkönen et al., 1995).

2. Materials and methods

2.1. Materials

Dimyristoyl phosphatidyl choline (DMPC), distearoyl phosphatidyl choline (DSPC), distearoyl phosphatidyl glycerol (sodium salt) (DSPG), and soya phosphatidyl choline (soya PC) were kindly provided by Nattermann Phospholipid, Köln, Germany. Egg L-α-phosphatidyl choline (egg PC) and cholesterol were purchased from Sigma (St. Louis, MO). Clodronate (dichloromethylene bisphosphonate). was obtained from Leiras Pharmaceutical Co. (Tampere, Finland). Dulbecco's modified Eagle's medium (DMEM), 10 000 units of penicillin, streptomycin, and fetal bovine serum were obtained from Gibco (Grand Island, NY). All other chemicals were of analytical grade.

2.2. Preparation and characterisation of liposomes

The liposomes were prepared as follows: chloroform/methanol solutions of the phospholipids were evaporated to dryness in a 250 ml round bottom flask. The phospholipid film was then hydrated above the phase transition temperature with phosphate-buffered saline (PBS) pH 7.4 (pH. Eur.). The hand-shaken liposomes were allowed to swell for two hours above the phase transition temperature (N_2 atmosphere) before extrusion above the phase transition temperature with a Lipex extruder (Lipex Biomembranes Inc., Vancouver, Canada). The liposomes were passed three times through a through a two-stacked 0.6 μm polycarbonate membrane (Nucleopore®, Costar Corp., Cambridge, USA) followed by ten passages through a two-stacked 0.1 μm polycarbonate membrane. The lipid content of the liposomes was measured using the phosphorus assay described by Bartlett (New, 1990). The liposomes (10 mg/ml) were stored in vials under N_2 atmosphere in the refrigerator (2–8°C). Clodronate containing liposomes for drug delivery studies were prepared by reverse phase evaporation method from phospholipid:cholesterol (67:33), having a phospholipid composition of 25:75 mol% DSPG/DSPC and are subsequently referred to by phospholipid content only (Mönkkönen et al., 1995). Clodronate encapsulated in liposomes was analyzed spectrophotometrically (Mönkkönen et al., 1994a).

One day after preparation, liposomes were irradiated at ambient temperature with a ^{60}Co source. The samples were exposed to a dose 25 kGy (15 kGy/h) which was controlled by a dosimeter (type 4034, Harwell). Before carrying out the experiments, the size of the liposome suspension was measured again and the liposome suspension was finally extruded once more to ensure a desired size distribution.

Determination of liposome size was carried out at a 90° angle by photon correlation spectroscopy (PCS) (Coulter N4 MD). Measurements were performed in triplicate using the unimodal model for size distribution. Samples were diluted in PBS and the refractive index and viscosity of pure water were used in the calculations.

The zeta potential of the liposomes was measured using a Doppler-Elecrophoretic Light Scattering Analyser (Coulter DELSA 440®). The liposome suspension was diluted in PBS and analysed at 25°C. The zeta potential (ξ) is deduced from the mobility (U) based on the equation:

$$\xi = 4\pi\eta U/\varepsilon$$

where η is the viscosity of the suspending medium and ε denotes the dielectric constant.

2.3. Cell culture experiments

The growth-inhibitory properties and the cell cytotoxicity of the different liposome suspensions were studied on two cell lines; a murine macrophage cell line, RAW 264 and a murine fibroblast cell line, L 929 as described elsewhere (Mönkkönen et al., 1994b).

In growth inhibition experiments, the cells were grown in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin and incubated in 7% CO_2 atmosphere at 37°C. The cells were counted by a Coulter model ZM counter and diluted to 4×10^4 cells/ml. Cell-suspension containing 100 μl were dispensed into 96-well plates (4×10^3 cells/well) (Nunc, Roskilde, Denmark) and allowed to adhere for 24 h. Quadruplicate wells were treated with 5 μl of the liposome suspension from a semi-logarithmic dilution series (10–1000 μM). On each plate, eight wells were left without cells as blank, four wells were used to obtain the original cell count, and four control wells were treated with 5 μl PBS. After incubation for 46 h, the serum-containing medium was replaced with DMEM (Nargi and Yang, 1993) and the cell viability was evaluated with MTT (Hansen et al., 1989).

In cytotoxicity experiments, cells were plated at 2×10^5 cells/well (200 μl of a 1×10^6 cell-suspension) and allowed to adhere for 2 h. The non-adherent cells were washed away before fresh medium and 5 μl of the liposomes were added to the wells. After incubation for 20 h, the cell viability was assessed with MTT assay.

3. Results

The different liposomal preparations used and the corresponding zeta potentials are shown in Table 1. The diameter and the width of the size distribution (S.D.) of the different extruded liposome suspensions varied from 92 to 103 nm and from 25 to 34 nm, respectively. The size of the liposomes did not change after irradiation ($n = 3$). DSPC-liposomes tended to form aggregates during storage at room temperature, but de-aggregated easily after heating above the phase transition temperature (Tm).

Only soya PC-liposomes in oxygen atmosphere (both non-irradiated and irradiated) showed any cytotoxic effects on the RAW 264 cells (Fig. 1). The EC50 values for the non-irradiated and the irradiated liposomes were 652 and 627 μM, respectively. The EC50 is the effective concentration of lipids (μM) which kills the cells by 50%. On the other hand, no effect was seen on the L 929 cells (Fig. 2). Experiments were partly repeated with another batch of liposomes, and the maximum deviation from the average value was $< 10\%$.

The growth inhibitory properties of the various types of liposomes are shown in Table 2. The maximum deviation from the average value was less than 15% in two separate experiments. Growth inhibition was observed for liposomes composed of unsaturated phospholipids and was more pronounced after irradiation. The liposomes stored and irradiated in nitrogen atmosphere inhibited the growth less than those stored under oxygen atmosphere. L 929 cells were influenced to a lesser extent. In addition, DMPC-liposomes seemed to affect the growth inhibition of RAW 264 cells.

Clodronate encapsulated in gamma irradiated and non-irradiated DSPG/DSPC (25:75 mol%) liposomes showed similar growth inhibition properties on RAW 264 macrophages (Fig. 3). Corresponding non-loaded liposomes did not affect the cell growth (data not shown).

4. Discussion

The uptake of liposomes in cells involves binding of the liposomes to the cell surface followed by endocytosis (Lee et al., 1992). Different cells vary with their ability to take up liposomes and thereby by their sensitivity towards the liposomal formulation (Heath and Brown, 1989–90. It seems possible that at least some of the toxic effects of liposomes are mediated directly at the cell surface, (Mayhew et al., 1980) especially for cells which are not strongly endocytotic. Macrophages (RAW 264 cells) have a higher endocytotic capacity than fibroblasts (L 929). In general, negatively charged liposomes associate more effectively and are more easily taken up by the cells than neutral ones (Hsu and Juliano, 1982; Heath et al., 1985; Mönkkönen et al., 1994b).

Table 1
Zeta potential of different liposome suspensions (PHB, pH 7.4) before and after irradiation 25 kGy[a]

	Zeta potential (mv)			
	0 kGy		25 kGy	
DSPC	−1.8	(1.1–2.5)	−4.9	(4.7–6.1)
DMPC	−1.7	(1.4–1.9)	−4.4	(4.2–5.2)
DSPG	−28.2	(27.6–31.0)	−30.0	(29.1–32.4)
20% DSPG/DSPC	−12.9	(12.0–15.2)	−16.1	(15.0–16.9)
Soya PC (O)[b]	−1.1	(0.8–1.2)	−7.6	(5.2–9.1)
Soya PC (N)[c]	−1.2	(1.1–1.5)	−5.9	(4.7–7.4)
Egg PC (N)[c]	−1.4	(1.1–2.2)	−3.8	(3.1–5.2)

[a] Mean, min, and max values for three batches are reported.
[b] O denotes oxygen atmosphere.
[c] N denotes nitrogen atmosphere.

The non-irradiated as well as the irradiated liposomes proved to be non-toxic to the two cell lines used. Only at very high doses, the unsaturated soya PC-liposomes (oxygen atmosphere) were cytotoxic to the RAW 264 cells. The L 929 cells were not affected at all. In our study we used liposomes in the size range 88–104 nm which the L 929 cells should be capable to take up (Mönkkönen et al., 1994b).

The unsaturated phospholipids inhibited the cell growth and this effect was more pronounced in oxygen atmosphere and after irradiation. Soya PC contains more double bonds than egg PC and inhibited the growth more. It seems that the presence of peroxidised phospholipids are responsible for this effect. The growth of L 929 cells were not inhibited to the same extent, indicating reduced uptake of the liposomes by these cells. Heath et al. (1985) also proved L 929 cells to be less sensitive towards empty liposomes than RAW 264 cells. The liposomes became more negatively charged after irradiation due to the presence of charged degradation products (fatty acids, phosphatidic acid) (Stensrud et al.,

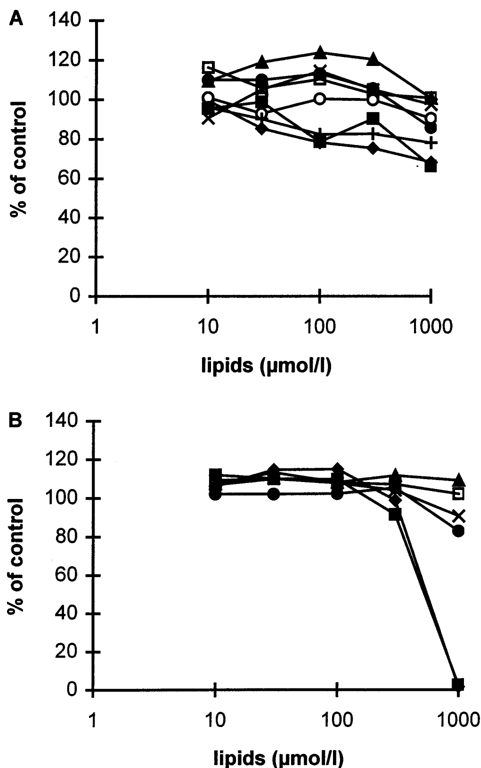

Fig. 1. Effect of various liposome formulations (non-irradiated and irradiated 25 kGy) on the viability of RAW 264 cells (cytotoxicity). A: ◆, DSPC non-irradiated; ■, DSPC irradiated; ▲, DMPC non-irradiated; ×, DMPC irradiated; □, DSPG non-irradiated; ●, DSPG irradiated; ○, 20% DSPG/DSPC non-irradiated; +, 20% DSPG/DSPC irradiated. B: ◆, Soya PC (O) non-irradiated; ■, Soya PC (O) irradiated; ▲, Soya PC (N) non-irradiated; ×, Soya PC (N) irradiated; □, Egg-PC non-irradiated; ●, Egg-PC irradiated.

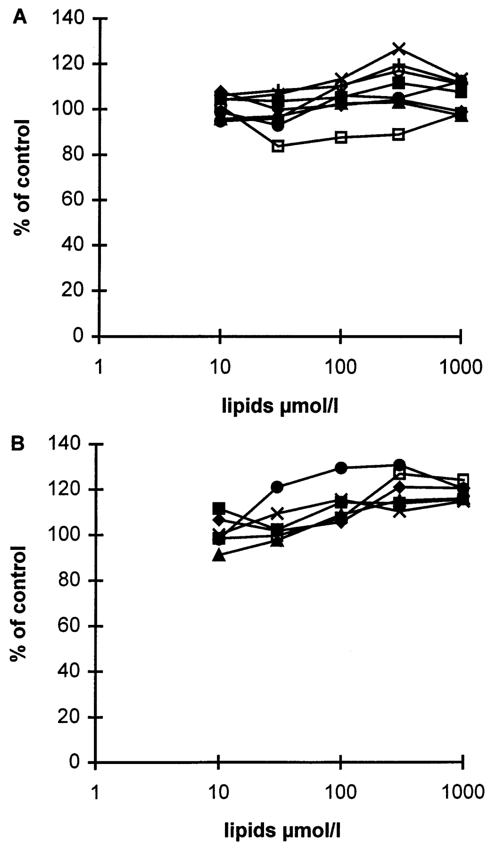

Fig. 2. Effect of various liposome formulations (non-irradiated and irradiated 25 kGy) on the viability of L 929 cells (cytotoxicity). A: ◆, DSPC non-irradiated; ■, DSPC irradiated; ▲, DMPC non-irradiated; ×, DMPC irradiated; □, DSPG non-irradiated; ●, DSPG irradiated; ○, 20% DSPG/DSPC non-irradiated; +, 20% DSPG/DSPC irradiated. B: ◆, Soya PC (O) non-irradiated; ■, Soya PC (O) irradiated; ▲, Soya PC (N) non-irradiated; ×, Soya PC (N) irradiated; □, Egg-PC non-irradiated; ●, Egg-PC irradiated.

Table 2
The growth inhibitory effect of different liposome formulations[a]

	RAW 264 (EC 50 μM)		L 929 (EC 50 μM)	
	0 kGy	25 kGy	0 kGy	25 kGy
DSPC	>1000	>1000	>1000	>1000
DMPC	337	447	>1000	>1000
DSPG	>1000	>1000	>1000	>1000
20% DSPG/DSPC	>1000	>1000	>1000	>1000
Soya PC (O)[b]	191	71	631	204
Soya PC (N)[c]	419	81	>1000	211
Egg PC (N)[c]	574	222	>1000	810

[a] The EC50 is the concentration of lipids (μM) which inhibits growth by 50% ($n=2$).

[b] O denotes oxygen atmosphere.

[c] N denotes nitrogen atmosphere.

1996; Zuidam et al., 1996a; Stensrud et al., 1997). However, the change was limited and it is doubtful that the irradiated liposomes should be taken up more effectively by the cells. Whereas lower concentrations of soya PC (oxygen atmosphere) inhibited growth of the RAW 264 cells, higher concentrations were necessary in order to kill the cells.

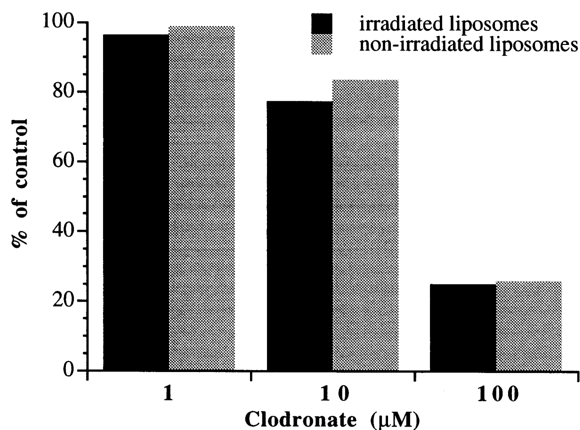

Fig. 3. Effect of clodronate encapsulated in irradiated and non-irradiated liposomes composed of 25:75 mol % DSPG/DSPC on the growth of RAW 264 cells in vitro. The data are from one experiment, similar results were obtained from one parallel experiment.

Of the liposomes composed of saturated phospholipids only DMPC-liposomes showed any inhibitory effects on the phagocytic macrophage cell line RAW 264. The growth inhibition effect was only seen at higher doses of liposomes (EC50 >300 μM). Solid phase vesicles associate with cells to a greater degree than fluid phase vesicles irrespective of surface charge (Szoka et al., 1980). DMPC-liposomes are in the fluid state at the experimental condition (37°C) and further studies are therefore necessary in order to explain this observation. Judged by these toxicity and growth inhibition experiments, liposomes composed of saturated phospholipids seem to be safe for use in drug delivery. Others (Mayhew et al., 1980; Heath et al., 1985; Mönkkönen and Heath, 1993) also report similar observations. Gamma irradiation seems to be an appropriate sterilisation method for these liposomes, because it does not enhance the toxic potential of the liposome suspensions or affects the capability of DSPG/DSPC liposomes to promote intracellular delivery of encapsulated drugs such as clodronate.

5. Conclusion

Liposomes composed of unsaturated phospholipids revealed toxic effects on the cell-line and their toxic effects were increased after gamma irradiation. Liposomes composed of saturated phospholipids did not show any toxic effects, and from safety and drug delivery point of views, gamma irradiation is an appropriate sterilisation method for them.

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