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Radioprotective effects of lipid A, liposomes, and liposomes containing lipid A in mice

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Lipid A from Gram-negative bacterial lipopolysaccharide (endotoxin) was incorporated into liposomal membranes and examined as a prophylactic radioprotectant compound in lethally irradiated mice. Splenic hematopoietic activity, resulting in increased numbers of spleen cell colonies, was induced both by lipid A alone or more strongly by liposomal lipid A. Increased survival of lethally irradiated animals was induced to a slight extent by liposomes alone, to a greater extent by lipid A, and at the highest level by liposomes containing lipid A. Under conditions where 100% of untreated or saline-treated animals died of acute radiation syndrome after 20 days, more than 90% of the animals pretreated with liposomal lipid A were still alive 30 days after irradiation. We conclude that lipid A had substantial radioprotectant activity by itself, and the activity was enhanced by incorporation into liposomes. Liposomes alone also exhibited mild radioprotectant effects.

Introduction

It is well established that Gram-negative bacterial lipopolysaccharide (endotoxin) has a substantial ability to stimulate hematopoietic activity [1,2] and also to induce protection against acute radiation syndrome caused by lethal ionizing radiation [3,4]. The antiradiation effects of endotoxin occur at levels of radiation that cause injury to hematopoietic tissue resulting in inhibition of stem cell lymphoid cell proliferation. It is widely thought that endotoxin serves as an immunomodulating agent that stimulates secretion of endogenous cytokines or lymphokines by macrophages, and its is believed that these latter substances are mediators that exert radioprotectant activity [5].

Most of the biological activities of endotoxin can be reproduced by the lipid component of lipopolysaccharide, lipid A [6]. However for radioprotectant effects it has been claimed that the polysaccharide component of lipopolysaccharide is the active moiety, and that lipid A lacks protective activity [7]. Lipid A can be readily incorporated into liposomes and liposomal lipid A has considerable adjuvant activity [8,9]. When liposomes are injected parenterally into animals they accumulate mostly in phagocytic cells of the liver, spleen and bone marrow [10]. It is likely that the enhanced adjuvant activity of liposomes and liposomes containing lipid A occurs because of massive uptake of liposomes by macrophages with resultant stimulation of the macrophages [8,9]. Because of the probable relationship between macrophage stimulation and radioprotectant ac-

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tivity [5], the protective effects of lipid A and liposome-associated lipid A were examined in detail.

In this study we demonstrate that lipid A has a potent ability to stimulate hepatopoiesis in lethally irradiated rodents, and the animals receiving lipid A are protected against acute radiation syndrome. The antiradiaton effects are markedly increased by incorporation of lipid A into liposomes.

Materials and Methods

Experimental animals

CD-1 (ICR) BR female mice (Charles River Breeding Laboratories, Portage, MI), five to six weeks of age, 22–24 g were used. They were quarantined, tested for the presence of *Pseudomonas* and *Proteus* species, and allowed to adapt to the environment for a minimum of 3 weeks before use. The mice were maintained in microisolator cages (Lab Products Inc, Maywood, NJ) at a concentration of not more than 110 cm^2 per mouse. All cages, bedding, laboratory chow (NIH 31 Autoclavable Mouse Ration, Zeigler Bros, Inc., Gardners, PA) and water bottles were sterilized by autoclaving. Distilled water, adjusted to a pH of 2.4–2.6 with hydrochloric acid, and laboratory chow were made available ad libitum. Mice were housed in an air-conditioned facility with a light/dark cycle of 15/9 h. All animal handling, experimental manipulations and transfers were conducted in a biological laminar flow hood.

Irradiation procedures

Twenty, or fewer, mice (control and treated) were placed in a ventilated Plexiglas chamber that had been sterilized by 70% ethanol. The dimensions of the chamber were 28 cm (diameter) by 3.5 cm (height). The chamber with the mice was then placed in a sterilized polyester filter bag and irradiated in a Gamma-cell 40 cesium-137 irradiation unit (Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 1.05 Gy/min (105 rad/min). The mice that had been injected with saline were included as controls in each radiation run of the treatment groups to insure that a lethal dose was given in the survival studies. No 'caging' effects were noted when controls and treatment groups were housed in multiple cages.

Lipid A

The details of the preparation of lipid A are given elsewhere [11,12]. Chloroform soluble lipid A was prepared from *Escherichia coli 0111:B4* lipopolysaccharide (Difco Laboratories, Detroit, MI) by acetic acid hydrolysis to remove the polysaccharide moiety, treated with EDTA to render the lipid A chloroform-soluble, and extracted by the Bligh-Dyer method to further purify the lipid A. When chloroform-solubilized lipid A was to be used in aqueous solution it was dried with nitrogen gas and solubilized with 0.5 ml of 0.5% triethylamine and diluted to the desired concentration with pyrogen-free 0.154 M NaCl.

Liposomes and liposome-encapsulated lipid A

The detailed methods for liposome preparation are given elsewhere [12]. Briefly, liposomes were prepared by drying a mixture of dipalmitoylphosphatidylcholine (DPPC) (Calbiochem-Behring, La Jolla, CA), cholesterol (Calbiochem-Behring) and dicetyl phosphate (K and K Laboratories, Fairview, NJ) in molar ratios of 2 : 1.5 : 0.22 in a pear-shaped flask with a rotary evaporator, with additional drying for 1 h under high vacuum. Liposomes were formed by adding a small amount of 0.5 mm glass beads and 0.154 M pyrogen-free NaCl to the flask and vigorously shaking for 1.5–2 min with a vortex mixer. Liposome-associated lipid A was prepared in the same manner except the lipid A was dried with the liposomal lipids in a molar ratio of 0.02 μmol of lipid A phosphate per μmol of DPPC. All glassware was sterilized in an oven at 180 °C, or autoclaved for 1 h, to remove endotoxin contamination.

Experimental methods

Mice were injected intraperitoneally with 0.2 ml of solutions of lipid A, liposomes, or liposome-associated lipid A before irradiation. The control animals were injected with pyrogen-free saline. The effects of the various inocula on the hematopoietic recovery were evaluated by the endogenous spleen cell assay as described by Till and McCulloch [13,14]. The mice were irradiated with a dose of 7.0 Gy, killed by CO_2 asphyxiation 10 days after irradiation, and the spleens were removed and fixed in Boulin's solution. Spleens were placed between loosely taped 25 by 75 mm

microscope slides and the numbers of splenic nodules were counted with the aid of a dissecting microscope. Survival studies were performed by irradiating the mice with a minimal lethal dose of 8.0 Gy and noting the number of survivors daily for 30 days.

Hematopoietic results were evaluated statistically by a modified logarithmic transformation as described by Smith et al. [15] and the P values were calculated by the Student t-test [16]. For statistical evaluation of survival data one-sided significance tests for survival rate differences (e.g., 25 μg vs. 10 μg of lipid A) were carried out using the normal approximation test (Z test) for comparing two proportions [16]. In order to obtain a more sensitive test for consistent directional difference between doses in survival rates a combined analysis (over the three treatment groups) using the Mantel-Haenszel procedure [17] was used. The effects of either dose or treatment or 30 day survival rate were also examined by fitting log-linear models [17].

Results

Effects of liposomal lipid A on endogenous spleen colony formation

The effects of injecting lipid A, liposomes, and liposomes containing lipid A on endogenous spleen colony forming units (CFU-S) 24 h before 7.0 Gy irradiation of mice is shown in Fig. 1. Lipid A by itself produced a small but significant ($P < 0.005$) increase at all concentrations tested in the CFU-S when compared to the controls injected with saline. However, the formulation consisting of lipid A incorporated into liposomes produced approximately a 4-fold increase in the number of CFU-S at 25 μg of lipid A per mouse. This increase in the CFU-S caused by incorporating lipid A into liposomes was not due to a simple additive effect of liposomes and lipid A because equivalent amounts of liposomes to those used in the liposomal lipid A preparations did not induce increases in the numbers of CFU-S.

Fig. 2 shows that the time of injection of a small dose (5 μg) of liposomal lipid A was a critical factor in the expression of CFU-S. Liposomal lipid A produced the greatest increase in CFU-S if administered 1 to 3 days before irradiation, with a maximum value obtained at 1 day prior to irradiation. An equal amount of lipid A or liposomes alone did not produce any significant increase in the CFU-S over this time period.

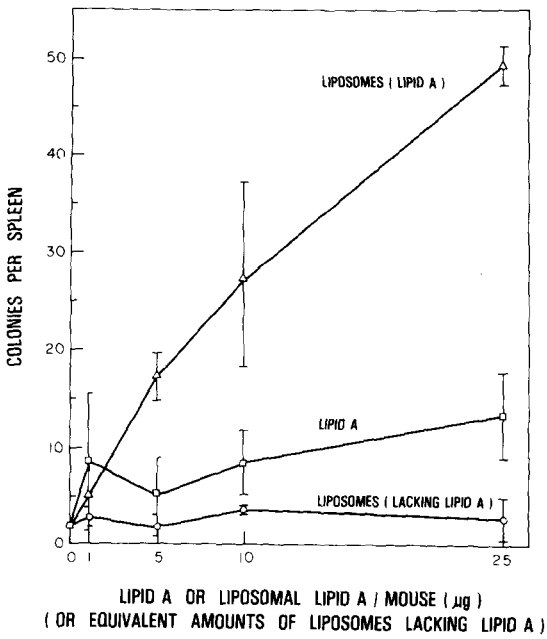

Fig. 1. Effects of incorporating lipid A into liposomes on the endogenous spleen colony forming units in CD-1 female mice. Mice were injected i.p. 24 h before irradiation with 7.0 Gy of γ radiation. Liposomes without lipid A were injected at the same concentrations of liposomes contained in the liposome-lipid A preparations. Data represent the means $\pm$ S.D. of three experiments and each data point represents 16 to 20 mice.

Protection of animals from lethal effects of radiation

Lipid A by itself protected CD-1 mice from the lethal effects of ionizing radiation when it was administered 24 h before irradiation with 8 Gy ($P = 0.014$) (Fig. 3). As shown in Fig. 3, when lipid A was incorporated into liposomes it had more than 2-fold greater effectiveness as a radioprotectant compared to lipid A alone ($P = 0.006$), and more than 90% of the mice survived. Liposomes alone, when examined at the highest concentration tested, also protected 42% of the mice ($P = 0.038$). This radioprotective ability of liposomes alone did not correlate well with hematopoietic activity as measured by the CFU-S assay shown in Fig. 1.

A more detailed analysis of radioprotectant

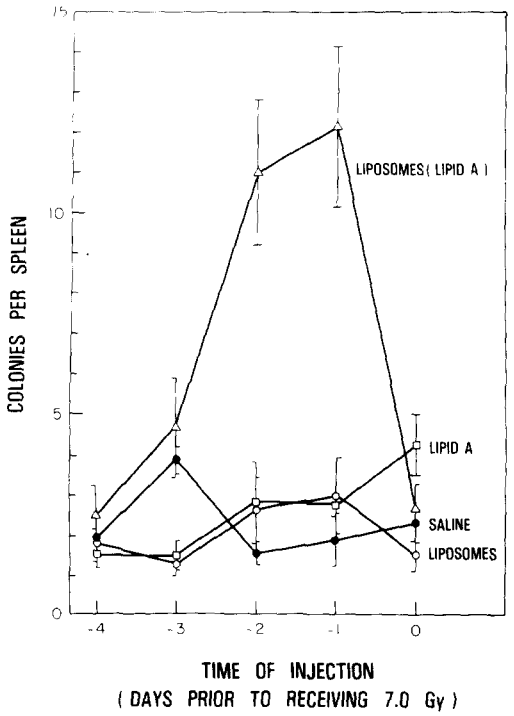

Fig. 2. Effects of time of injection prior to irradiation. Liposomal-lipid A (5 μg) was injected i.p. into CD-1 female mice exposed to 7.0 Gy of γ radiation. Results are the means of three experiments ±1 S.D. Each data point represents 21–24 mice.

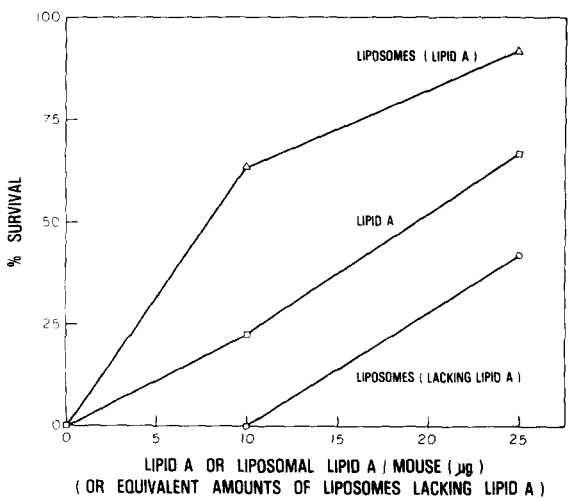

Fig. 3. Protection of CD-1 female mice from a lethal dose of γ irradiation. Mice were injected i.p. 24 h before irradiation with 8 Gy with lipid A, liposomes, or liposomes containing lipid A. Each treatment group originally contained 10–16 mice.

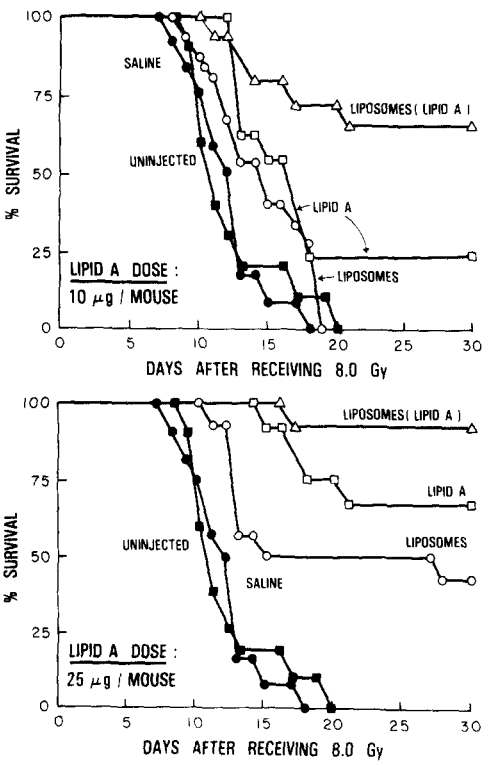

Fig. 4. The 30 day survival profiles of CD-1 mice that had been irradiated with 8.0 Gy of γ irradiation. Mice were injected i.p. with 10 or 25 μg lipid A solubilized with 0.5 % TEA and diluted with saline, 10 or 25 μg of liposomal lipid A, liposomes, equivalent to that of the liposomal-lipid A preparations, without lipid A or saline 24 h before irradiation. Each treatment group originally contained 10–16 mice.

activities is illustrated in Fig. 4 which shows 30 day survival profiles of each of the treatment groups. The uninjected and saline-treated control animals all died within 20 days after receiving 8.0 Gy irradiation. The time interval that elapsed between irradiation and death suggests that death was primarily caused by bone marrow failure. Two different doses of lipid A, 10 μg and 25 μg/mouse, were examined. At the lower dose, liposomes alone did not induce a protective effect, but at the higher dose liposomes alone caused almost 50% survival to occur. Lipid A alone also showed a dose-dependent radioprotectant effect, resulting in almost 67% survival at the higher dose. The strongest radioprotectant effects, resulting in 64% survival at the lower dose and 92% survival at the higher dose, were expressed by liposomes containing lipid A.

Discussion

Liposomes have been proposed as carriers of drugs and other agents for a wide range of purposes. Previous publications have suggested that liposomes can enhance the efficacy of antiradiation drugs under circumstances in which the mechanism of the drug is to serve either as a means to induce tissue hypoxia or as a free radical scavanger [18–20].

When liposomes are injected parenterally into animals they accumulate mostly in phagocytic cells of the liver, spleen, and bone marrow [10]. For certain applications the tendency of liposomes to be taken up by macrophages represents a substantial interfering hurdle. However numerous instances have been identified in which uptake of liposomes by macrophages represents a vital link required for successful biological or therapeutic application [8,9]. Many of the potential clinical uses of liposomes have involved delivery of liposomes to phagocytic cells (Kupffer cells) in the liver [21]. In the present study we have demonstrated the activity of lipid A as an antiradiation compound and the enhancement of that activity by liposomes. Certain toxic properties of lipid A, including neutropenia [22] and *Limulus* lysate coagulation [22,23], are suppressed by liposomes.

Lipid A has numerous biological activities [6] including adjuvant activity [8,9], generation of cytokines and lymphokines [24,25], and stimulation of granulocyte-macrophage colony-forming cells [26]. We now show that lipid A induces splenic hematopoietic activity in lethally irradiated rodents, and also reduces the mortality of rodents exposed to lethal doses of ionizing radiation.

Our conclusion that lipid A induces radioprotective effects contrasts with a previus report that concluded that lipid A lacked protective activity against radiation [7]. However, careful analysis of our data easily resolves the apparent discrepancy. Increased survival was induced by lipid A in our experiments when the compound was administered 1 day, but not 2 days, prior to irradiation. In the previous study protective effects were only examined when lipid A was injected 2 days prior to irradiation [7].

Liposomes lacking A also induced a weak radioprotectant activity by themselves. Parenterally injected liposomes sometimes (but not always) induce a transient stress-induced splenomegaly in rodents [27], and it is possible that this is a phenomenon resulting in nonspecific activation of macrophages. It is likely that radioprotection can be mediated by cytokines or lymphokines secreted by activated macrophages in response to endotoxin [5]. It is therefore possible that other liposomal macrophage-activating substances could also be shown to exhibit radioprotectant activity, and this likelihood will be examined.

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