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Radioprotective Properties of Detoxified Lipid A from *Salmonella minnesota* R595

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In the past, the toxicity of bacterial lipopolysaccharide (LPS) or its principal bioactive component, lipid A, has detracted from their potential use as radioprotectants. Recently, a relatively nontoxic monophosphoryl Lipid A (LAM) that retains many of the immunobiologic properties of LPS has been isolated from a polysaccharide deficient Re mutant strain of *Salmonella minnesota* (R595). The ability of the native endotoxic glycolipid (GL) from *S. minnesota* (R595) as well as dipnosphoryl lipid A (LAD) and nontoxic monophosphoryl lipid A (LAM) derived from GL to protect LPS responsive (CD2F1 or C3H/HeN) and nonresponsive (C3H/HeJ) mice from ^{60}Co γ-irradiation has been studied. Administration of GL, LAD, or LAM to CD2F1 or C3H/HeN mice (400 μg/kg) 24 h prior to exposure provided significant radioprotection. No protection was afforded to C3H/HeJ mice. Experiments were also conducted to determine the relative abilities of GL, LAD, and LAM to stimulate hematopoiesis as reflected by the endogenous spleen colony (E-CFU) assay. Protection was not correlated with the ability of these substances to increase E-CFUs or to induce colony-stimulating activity (CSA).

INTRODUCTION

Endotoxic lipopolysaccharide (LPS) has long been known to protect mice against lethal doses of ionizing radiation (*1, 2*). In addition, LPS elicits numerous immunobiologic responses capable of enchancing host resistance to infectious agents (*3*) or malignant tumors (*4, 5*). Several investigators have chemically or physically altered the structure of native LPS in an attempt to reduce its toxicity while maintaining its beneficial properties (*6–8*). Protection against ionizing radiation has been achieved using LPS derivatives that have been detoxified by alkylation (*9*), emulsification (*10*), radiation exposure (*7*), treatment with chromium ions (*8*), and hydrolysis (*11*). Previous studies using various LPS fragments obtained by sequential hydrolysis suggest that the radioprotective properties of LPS are located principally within the polysaccharide region of the molecule (*11, 12*). Furthermore, when glycolipid or lipid A fractions were

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used in radioprotection experiments, little or no increase in survival was observed (*11, 12*).

In this communication we report some recent observations showing that endotoxin obtained from a polysaccharide deficient Re mutant strain of *Salmonella minnesota* (R595), sometimes referred to as endotoxic glycolipid (GL), markedly increased the survival of CD2F1 and C3H/HeN mice exposed to lethal doses of ^{60}Co γ irradiation. More significantly, it was found that detoxified monophosphoryl lipid A (LAM) obtained from *S. minnesota* (R595), described by Ribi and associates as a potent immunostimulatory agent (*13, 14*), also protected mice from the lethal effects of ionizing radiation. In contrast to the GL parent, the increased survival afforded mice by pretreatment with detoxified lipid A was not correlated with its capacity to enhance the proliferation of endogenous spleen colonies (E-CFU) following sublethal irradiation or to induce plasma colony-stimulating activity (CSA).

METHODS AND MATERIALS

Mice. The male CD2F1 mice were obtained from Harland Sprague Dawley, Indianapolis, IN. The female C3H/HeJ and C3H/HeN mice were purchased from the Jackson Laboratory, Bar Harbor, ME. Mice were acclimated to laboratory conditions for 2 weeks before use. During this time they were quarantined until a random sample was found to be free of histologic lesions of common murine diseases and water bottle cultures (trypticase soy broth) of all animals were found to be free of *Pseudomonas spp.* Mice 10–14 weeks old were used throughout. All animals were maintained on a 6 AM–6 PM light–dark cycle and were allowed a standard laboratory diet (pellets) and acidified water *ad libitum.*

Radioprotectants. The endotoxic glycolipid (GL) from *S. minnesota* (R595) as well as diphosphoryl lipid A (LAD) and the detoxified monophosphoryl lipid A (LAM) from this same organism were obtained from RIBI Immunochem, Hamilton, MO. These substances were routinely dissolved at a concentration of 100 μg/ml in distilled water containing 0.5% triethylamine.

Irradiation. Mice were placed in Plexiglas boxes and irradiated bilaterally with ^{60}Co γ photons [1.17 and 1.33 MeV] at a midline tissue exposure rate of 1.0 Gy/min. The AFRRI Co-60 Facility consists of two planar sources which were raised together to irradiate the mice bilaterally. To measure the midline tissue dose rate, a tissue-equivalent ionization chamber, with calibration traceable to the National Bureau of Standards, was placed midline in a plastic mouse phantom. The variation across the array of mice was measured to be less than 1%, and the uncertainty in the dose measurements is about 5%. In protection experiments mice received 0.1–0.2 ml of GL, LAD, or LAM intraperitoneally. Following exposure survival was monitored for 30 days. Both before and after exposure mice were kept in boxes equipped with microfilter tops to protect them from airborne infections.

In vitro assay of colony-stimulating activity (CSA). Ten micrograms of GL, LAD, or LAM were injected ip into male CD2F1 mice. Three hours later, the animals were anesthetized and bled retroorbitally into heparinized tubes. Each plasma sample was obtained from the pooled blood of three mice. The pooled plasma samples of normal and experimental mice were assayed for their ability to stimulate the growth of bone-marrow-derived granulocyte-macrophage colonies by the double-layer soft agar technique (*15*). CMRL 1066 culture medium for this procedure was prepared as previously described (*16*). Dose-response curves indicated that a 1:6 dilution of test plasma added as 10% (v/v) of total culture volume to the lower layer of agar provided maximum colony-stimulating activity. Colony-stimulating activity was expressed as the number of colonies formed per 1×10^5 murine bone marrow cells. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. Colonies of more than 50 cells were counted after 10 days of incubation. Four separate plasma samples were used to determine each value. Each culture was performed in duplicate.

Endogenous spleen colony assay (E-CFU). Endogenous spleen colony-forming units (E-CFU) were evaluated by the method of Till and McCulloch (*17*). CD2F1 mice were injected with 2.0 μg of GL, LAM, or LAD intraperitoneally 24 h before exposure to 6.5, 7.0, and 7.5 Gy of total body irradiation from the ^{60}Co source. Ten days after exposure the spleens of irradiated mice were removed and fixed in Bouin's fixative, and the number of macroscopic spleen colonies was counted. Two separate experiments were performed involving

five animals per group. Data are expressed as the mean of the number of colonies found on the spleens of 10 animals, ± the standard error of the mean (SEM).

RESULTS

Radiation Protection Experiments

The results presented in Table I which were analyzed using a chi-squared distribution test show that intraperitoneal adminstration of 10–20 μg of endotoxic glycolipid (GL), diphosphoryl lipid A (LAD), or detoxified monophosphoryl lipid A (LAM) conferred significant protection to LPS-responsive CD2F1 and C3H/HeN mice when given 24 h before exposure to a lethal dose of radiation. No significant differences were observed between the two doses employed or among GL, LAD, or LAM ($P > 0.05$) in CD2F1 mice. On the other hand, no protection was afforded by these glycolipids to LPS-nonresponsive C3H/HeJ mice. Although the detoxified LAM increased survival of CD2F1 mice exposed to 10 Gy to about the same extent as the more toxic GL, LAM was found to be more radioprotective than the latter substance in survival experiments using the C3H/HeN mice ($P < 0.005$). Maximum protection using GL or LAM in CD2F1 mice was achieved when these substances were given 24 to 48 h prior to exposure (Fig. 1). Duplicate experiments were performed at each time using a total of 20 mice per group. A two-way analysis of variance (GL or LAM vs time) was conducted on the data presented in Fig. 1. There was no statistical difference between

TABLE I

Radioprotection of Mice Using Endotoxic Glycolipid (GL), Diphosporyl Lipid A (LAD) and Detoxified Monophosphoryl Lipid A (LAM) from *Salmonella minnesota* R595[a]

Mouse strain	Compound	Dose (μg)	No. animals surviving	Percent survival
CD2F1[b]	LAM	10	43/50	86
	LAM	20	15/20	75
	LAD	10	45/50	90
	LAD	20	27/30	90
	GL	10	18/20	90
	GL	20	8/10	80
	Controls	0	0/30	0
C3H/HeN[c]	LAM	20	10/10	100
	LAD	20	3/10	30
	GL	10	4/10	40
	Controls	0	1/10	10
C3H/HeJ[c]	LAM	20	0/10	0
	LAD	20	0/10	0
	GL	10	0/10	0
	Controls	0	0/10	0

[a] Mice injected ip 24 h prior to exposure to ^{60}Co γ radiation.
[b] CD2F1 mice received 10 Gy.
[c] C3H/HeN and C3H/HeJ mice received 9.5 Gy.

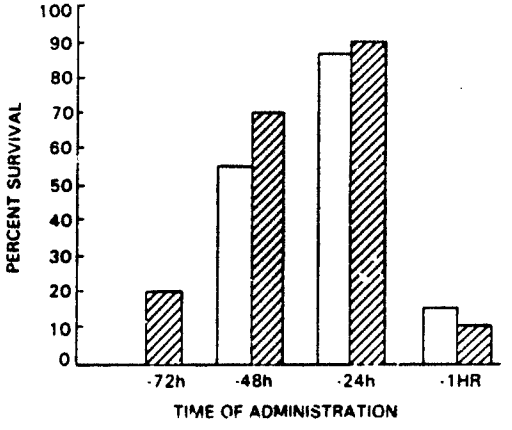

FIG. 1. Effect of time of administration of GL or LAM (10 μg/mouse) on the survival of CD2F1 mice exposed to 10 Gy ^{60}Co γ. Closed bars = GL treated; open bars = LAM treated.

GL or LAM although there was a significant difference over time ($P < 0.01$). Using a Neuman-Keul comparison test, there was no significant difference between radioprotection at 24 to 48 h, but there was a significant decrease for these two times in comparison to treatment 72 h prior to irradiation.

Figure 2 compares the survival of untreated with GLY and LAM treated CD2F1 mice as a function of radiation dose. A minimum of 20 mice were used at each dose of radiation. From these experiments the $LD_{50/30}$ dose for the untreated control mice was found to be 9.39 Gy [95% of confidence limits = 9.29–9.49]. In the case of mice receiving the parent GL, the $LD_{50/30}$ was shifted to 11.28 [10.93, 11.64], or a dose reduction factor (DRF) of 1.20. For LAM the $LD_{50/30}$ was estimated to be 11.0 Gy [10.75, 11.25] or a DRF = 1.17 using a probit analysis.

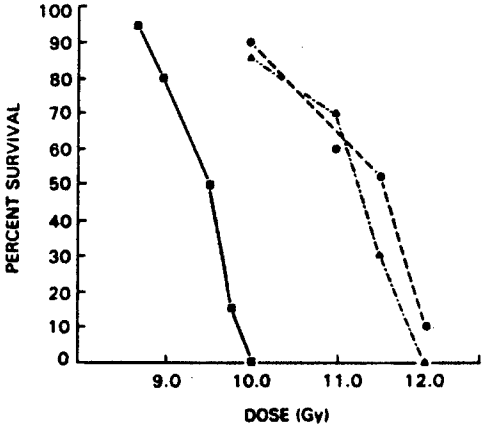

FIG. 2. Effect of ^{60}Co γ-radiation dose on the survival of CD2F1 mice pretreated with GL or LAM. ■, Controls; ▲, LAM-treated mice; ●, GL-treated mice.

Endogengous Spleen Colony Formation (E-CFU)

Table II illustrates the capacity of the three radioprotective glycolipids from *S. minnesota* to stimulate the formation of endogenous spleen colonies (E-CFU) in irradiated CD2F1 mice. Both GL and LAD markedly increased E-CFU content at all three radiation doses studied, while no stimulation of E-CFUs was observed in mice challenged with LAM.

Factors with Colony-Stimulating Activity (CSA)

The effect of GL, LAD, and LAM on the induction of plasma factors (CSA) which stimulate the growth of granulocyte-macrophage colonies *in vitro* was also studied (Table III). Table III was analyzed using a Mann-Whitney test with a Bonfferroni allocation of Type I error ($P = 0.05$). There was no difference in the effects of GL and DP at 3 and 6 h post infection. Both compounds significantly increased the plasma level of CSA at these times. The plasma of mice challenged with LAM contained significantly less CSA than those receiving either GLY or LAD.

DISCUSSION

The radioprotective effect of bacterial endotoxins has been known for over 30 years (*1, 2*). Subsequent studies demonstrated that radioprotection could also be achieved using "detoxified" endotoxin preparations (*7–11*). Collectively these studies suggested that the locus for radioprotection is situated in a region of the endotoxin molecule separate from that responsible for its toxicity. Evidence presented by Nowotny *et al.* (*11*) and by Urbaschek (*18*) has tended to confirm this notion. Using derivatives obtained by mild hydrolysis of *Salmonella minnesota* 1114 endotoxin it was shown that the lipid A component conferred no radioprotection to mice (*11*). On the other hand the nontoxic polysaccharide fraction (PS) obtained from the same hydrolysate retained a radioprotective activity approximately equal to that of the parent endotoxin. In further experiments it was shown that the toxic activities of endotoxin could be selectively neutralized using homologous antiserum directed at the lipid moiety, but that the resulting "immune complex" was fully radioprotective (*12*). These observations also gave support to the notion that the PS region of molecule is the principal active

TABLE II

Effect of GL, LAD, and LAM on E-CFU in Sublethally Irradiated CD2F1 Mice[a]

Radiation dose (Gy)	E-CFUs (d10)			
	Controls	GL	LAD	LAM
6.5	1.2 ± 1.2	14.0 ± 2.6*	8.8 ± 2.2*	0.2 ± 0.2
7.0	0.6 ± 0.4	12.0 ± 3.4*	7.6 ± 2.4*	0.2 ± 0.2
7.5	0.2 ± 0.2	11.2 ± 1.8*	5.0 ± 1.8*	0.0 ± 0.0

[a] Mice received 2 μg of GL, LAD, or LAM 24 h prior to exposure to ^{60}Co γ radiation. Each value is the mean of ten determinations ± SEM.

* $P \leq 0.01$ with respect to control using Student's *t* test.

TABLE III

Effect of GL, LAD, and LAM on Plasma Colony Stimulating Factor (CSA) Levels in CD2F1 Mice[a]

Time (h)	No. of colonies			
	Controls	GL	LAD	LAM
3	0	70 ± 6	57 ± 6	9 ± 4
6	4 ± 2	62 ± 9	44 ± 10	15 ± 5
24	0	10 ± 3	0	0
48	0	0	0	0

[a] Mice received 10 μg GL, LAM, or LAD ip at $t = 0$. Each value represents the mean of four determinations. Errors are ± SEM.

site responsible for radioprotection. Furthermore, endotoxic glycolipid isolated from the polysaccharide-deficient rough mutant of *S. minnesota* (R595) was reported to have a significantly diminished capacity to confer radioprotection (*11, 12*).

In contrast to the literature cited above we have found that relatively low doses (400 μg/kg) of endotoxic glycolipid (GL) from *S. minnesota* conferred significant protection to endotoxin-responsive strains of mice (CD2F1 and C3H/HeN). The magnitude of the dose reduction factor (DRF ≅ 1.2) is approximately the same as that achieved previously using endotoxin from smooth *Salmonella typhosa* (*2*). The results of our studies employing endotoxic glycolipid and diphosphoryl lipid A from *S. minnesota* (R595) suggest that the radioprotective properties of lipopolysaccharide may not be restricted solely to the PS region of the molecule. More significantly it was found that the detoxified monophosphoryl lipid A from *S. minnesota* (R595) was approximately equal to the parent endotoxin in its capacity to confer protection to lethally irradiated mice. This confirms and extends the observations of Ribi and associates who showed that beneficial properties of lipid A can be uncoupled from its toxic properties by removing a single phosphate group from the reducing end of the glucosamine disaccharide unit (*13, 14*).

Although the precise mechanism by which endotoxins promote survival in irradiated animals is not entirely understood, it has been generally accepted that increased survival is associated with their ability to stimulate hematopoietic recovery (*19, 20*) Presumably, this process is initiated by the action of endotoxin on reticuloendothelial macrophages (*21*), triggering the release of humoral factors such as CSA (*15, 22*) that enchance stem cell proliferation.

In this study both the parent glycolipid and the diphosphorlyl lipid A markedly enhanced the formation of E-CFUs in irradiated mice. These substances also induced elevated plasma levels of CSA within 3 h after administration. The detoxified monophosphoryl lipid A (LAM), on the other hand, did not promote the formation of E-CFU in the spleen and was much less effective than either GL or LAD in elevating plasma levels of CSA. Nevertheless, LAM was found to be just as effective as GL or LAD in protecting against lethal doses of ^{60}Co radiation. Previous investigators have also found that the ability of endotoxin to increase hematopoiesis in the spleen does not necessarily correlate with increased survival following irradiation (*23–25*). It is possible that E-CFUs in the spleen are not representative of changes in whole-body

numbers of hematopoietic stem cells (*26*). On the other hand, it is also conceivable that the radioprotective effect of the bacterial glycolipids as well as polysaccharides (PS) used in previous studies may be unrelated to their ability to stimulate hematopoiesis and/or induce CSA. Along these same lines evidence has been reported suggesting that the CSA-inducing component obtained from endotoxin hydrolysates is not the same as the PS component eliciting radioprotection (*11*).

Addison and Berry (*27*) have recently presented evidence suggesting that protection of mice against lethal irradiation with serum mediators elicited by injection of endotoxin is not solely dependent on the presence of CSA alone. Our observations showing that LAM is radioprotective but does not stimulate E-CFUs and is only a weak inducer of CSA suggest that bacterial glycolipids may exert their radioprotective effects by mechanisms other than hematopoietic stimulation. We are currently exploring the possibility that bacterial lipopolysaccharides and glycolipids can elicit the production of endogenous radioprotective factors *in vivo*.

Although the DRF of 1.2 determined in this study for LAM is much lower than those generally observed for aminothiols such as S-2-(3-aminopropylamino) ethylphosphorothioic acid (WR 2721), the application of LAM in combination with radiotherapy offers intriguing possibilities for treating tumors. There has been a great deal of recent interest in using chemical agents to modify the sensitivity of neoplastic vis-a-vis normal cells to obtain a selective advantage in radiation killing of tumors (*28, 29*). It has already been reported that LAM administered in combination with mycobacterial cell wall skeleton (CWS) causes tumor regression (*14*). It is therefore conceivable that LAM could be used to promote radiation-induced killing of neoplasms while simultaneously conferring protection to normal tissues.

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