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0014-4754/84/111235-07$1.50 + 0.20/0
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Effects of pre- and post-irradiation glucan treatment on pluripotent stem cells, granulocyte, macrophage and erythroid progenitor cells, and hemopoietic stromal cells

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Summary. Glucan, a beta-1,3 polyglucose, was administered to mice either 1 h before or 1 h after a 650 rad exposure to cobalt-60 radiation. Compared to radiation controls, glucan-treated mice consistantly exhibited a more rapid recovery of pluripotent stem cells and committed granulocyte, macrophage, and erythroid progenitor cells. This may partially explain the mechanism by which glucan also enhances survival in otherwise lethally irradiated mice.
Key words. Mice; glucan treatment; Co^{60}-irradiation; stem cells, pluripotent; granulocytes; macrophages; erythroid progenitor cells; hemopoietic stomal cells; hemopoiesis.

Introduction

Glucan, a β-1,3 polyglucose isolated from the inner cell wall of the yeast *Saccharomyces cerevisiae*[1,2] is a potent stimulator of the reticuloendothelial system[3,4] and a dose-dependent modulator of the cellular[5,6] and humoral[7] immune systems and hemopoietic system[8–12]. Specifically, in terms of effects on hemopoiesis, the administration of glucan to normal mice results in an overall increase in the production of pluripotent stem cells and also granulocyte-macrophage, pure macrophage, and erythroid progenitor cells. The enhanced granulocyte and macrophage genesis observed in glucan-treated animals has been associated with glucan's ability to induced the production and/or release of granulocyte-macrophage colony-stimulating activity (CSA)[8,13]. At this time, the mechanisms of glucan's enhancement of other aspects of hemopoiesis are not fully understood.

Because of glucan's profound stimulatory effects on hemopoiesis at the stem cell and progenitor cell levels, its use as a therapeutic agent in cases of hemopoietic depletion induced by drugs and/or radiation has been suggested[10–12]. Recently, it has been reported that glucan induced increased numbers of endogenous pluripotent spleen colony-forming units (E-CFU)[14] and also increased numbers of peripheral blood granulocytes[15] when administered either before or after hemopoietically damaging doses (550–650 rads) of gamma radiation. The purpose of this study was to examine in detail the effects of pre- and post-irradiation glucan treatment on the recovery of hemopoietic stromal cells (HSC), transplantable pluripotent hemopoietic stem cells (CFU-s), granulocyte-macrophage progenitor cells (GM-CFC), macrophage progenitor cells (M-CFC), and erythroid burst and colony-forming cells (BFU-e, CFU-e).

Materials and methods

10- to 12-week-old female $B6D2F_1$ mice were used in all experiments. All mice were quarantined and acclimated to laboratory conditions for 2 weeks before experimentation. Particulate, endotoxin-free glucan was obtained from Accurate Chemical and Scientific Corporation (Westbury, N.Y.) and was prepared according to DiLuzio's modification[2] of Hassid's original procedure[1]. Glucan was diluted in sterile 5% dextrose, and 1.5 mg (approximately 75 mg/kg) was intravenously injected into experimental mice either 1 h before or 1 h after exposure to 650 rads of total-body cobalt-60 radiation.

Normal control (not irradiated and not glucan-treated) and radiation control (irradiated, but not glucan-treated) mice were injected with an equivalent volume (0.5 ml) of sterile 5% dextrose. At 1, 3, 6, 8, 10, 14, and 21 days after irradiation, 2 mice from each group were killed by cervical dislocation, and the bones (2 femurs per mouse) and spleens were removed to be assayed for total nucleated cellularity (TNC), HSC, CFU-s, GM-CFC, M-CFC, BFU-e, and CFU-e.

Cell suspensions. Each cell suspension represented the pool of tissues from 2 mice. Cells were flushed from bones with 3 ml of Hank's Balanced Salt Solution (HBSS) containing 5% heat-inactivated fetal bovine serum (HIFBS). Spleens were pressed through a stainless steel mesh screen, and the cells were washed from the screen with 6 ml of HBSS plus 5% HIFBS. The total number of nucleated cells in each suspension was determined by counting the cells on a hemocytometer.

Hemopoietic cell assays. The hemopoietic assays used have been described in detail elsewhere[11,12]. Bone marrow and splenic hemopoietic stromal cells were cultured according to the method of Wathen et al.[16]. On days 7 and 14, respectively, bone marrow and splenic cultures were fixed in methanol, stained with Mallory's Azure II Methylene Blue, and the number of HSC colonies (> 50 cells) counted. CFU-s were evaluated by the spleen colony assay[17] as previously described[11]. 8 days after transplantation, the recipients were sacrificed and their spleens removed. The spleens were fixed in Bouin's solution, and the number of grossly visible colonies were counted. Committed granulocyte-macrophage hemopoietic progenitor cells (GM-CFC) were assayed by MacVittie's modification[18] of the semi-solid agar technique originally described by Bradley and Metcalf[19] and Pluznik and Sachs[20]. Colonies (> 50 cells) were counted after 10 days of incubation in a 37°C humidified environment containing 7.5% CO_2. Committed pure macrophage hemopoietic progenitor cells (M-CFC) were assayed by the technique described by MacVittie et al.[21]. Cultures were incubated for 28 days at 37°C in a humidified atmosphere containing 7.5% CO_2, before scoring colony formation. Bone marrow and splenic CFU-e and BFU-e were assayed by a modification[22] of the original plasma clot technique described by Stephenson et al.[23]. Clotted CFU-e and BFU-e cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air for 2 days and 7 days, respectively. Plasma clots were then harvested, fixed with 5% glutaraldehyde, and stained with benzidine and giemsa[24]. A CFU-e was defined as an individual aggregate of 8 or more benzidine-positive cells. A BFU-e was defined as a group of many large contiguous clusters of benzidine-positive cells.

Statistical analysis. In each experiment, the normal control values were used to convert radiation control and glucan values into percentages (i.e., percent of normal control). Percent values presented in tables 1–4 represent the means of percent values obtained from at least 3 replicate experiments. Student's 2-tailed t-test was used to determine the statistical difference between mean values obtained from radiation control and glucan-treated mice.

Results

Bone marrow hemopoietic recovery in mice administered glucan 1 h before irradiation is shown in table 1. It can be seen that recovery of the hemopoietic parameters commenced earlier in glucan-treated animals than in animals receiving radiation alone (radiation controls). By 21 days post-irradiation, femoral TNC, HSC, and CFU-e in radiation controls had reached levels comparable to those in glucan-treated mice, while CFU-s, GM-CFC, M-CFC, and BFU-e in glucan-treated mice remained significantly elevated above radiation control levels. Like in the bone marrow, all aspects of splenic hemopoietic recovery commenced earlier in pre-irradiation glucan-treated mice than in radiation controls (table 2). Again, splenic HSC and CFU-e values in the 2 groups of mice were comparable by 21 days post-irradiation, while splenic TNC, CFU-s, GM-CFC, M-CFC, and BFU-e levels in glucan-treated mice remained elevated above radiation control levels. This was true even when splenic CFU-s, GM-CFC, and BFU-e numbers in radiation controls exceeded those of normal control mice (i.e., were greater than 100%). Specifically, although an overshoot in CFU-s, GM-CFC, and BFU-e splenic values was observed in radiation controls by day 21 post-irradiation, the overshoot in glucan-treated mice first became apparent as early as 10 days post-irradiation and remained above radiation control levels through day 21 post-irradiation.

The results of experiments assaying the effects of post-irradiation glucan treatment on bone marrow and splenic hemopoietic recovery are shown in tables 3 and 4, respectively. In all instances except that of bone marrow HSC, hemopoietic recovery again commenced earlier in glucan-treated than in radiation control mice.

Table 1. Bone marrow hemopoietic recovery in B6D2F$_1$ mice given 1.5 mg of glucan at 1 h before 650 rads of whole-body cobalt-60 radiation (% normal control)

Day post irradiation	TNC Radiation control	+ Glucan	HSC Radiation control	+ Glucan	CFU-s Radiation control	+ Glucan	GM-CFC Radiation control	+ Glucan	M-CFC Radiation control	+ Glucan	BFU-e Radiation control	+ Glucan	CFU-e Radiation control	+ Glucan
1	8	18	12	12	0	0	0	0	0	0	0	0	0	0
3	4	4	11	11	0	0	0	2	0	1	0	0	0	0
6	8	8	11	10	0	5	1	5	0	3	0	3	9	13
8	19	28[a]	18	18	1	12[a]	3	16[a]	2	4	1	16[a]	28	51[a]
10	23	58[a]	19	35[a]	3	30[a]	5	35[a]	3	18[a]	4	28[a]	35	83[a]
14	27	69[a]	23	44[a]	5	43[a]	29	50[a]	13	42[a]	14	32[a]	76	86[a]
21	82	81	60	56	22	68[a]	41	92[a]	22	70[a]	57	68[a]	88	96

[a] $p > 0.01$ with respect to the respective time matched radiation control values.

Table 2. Splenic hemopoietic recovery in $B6D2F_1$ mice given 1.5 mg of glucan at 1 h before 650 rads of total-body cobalt-60 radiation (% of normal control)

	TNC		HSC		CFU-s		GM-CFC		M-CFC		BFU-e		CFU-e	
Day post irradiation	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan
1	9	9	2	0	0	0	0	0	0	0	0	0	0	0
3	9	8	2	2	0	0	0	0	0	0	0	0	0	0
6	7	10	4	3	0	2	0	21[a]	0	6[a]	0	0	0	1
8	9	15	10	13	0	4	0	34[a]	0	7[a]	0	2	1	19[a]
10	10	50[a]	13	88[a]	0	60[a]	1	250[a]	1	96[a]	0	180[a]	2	343[a]
14	65	84[a]	41	122[a]	32	190[a]	94	1207[a]	8	316[a]	40	326[a]	410	684[a]
21	76	88[a]	54	50	191	300[a]	744	2503[a]	60	360[a]	121	451[a]	920	918

[a] $p > 0.01$ with respect to the respective time matched radiation control value.

Table 3. Bone marrow hemopoietic recovery in $B6D2F_1$ mice given 1.5 mg of glucan at 1 h before 650 rads of total-body cobalt-60 radiation (% normal control)

	TNC		HSC		CFU-s		GM-CFC		M-CFC		BFU-e		CFU-e	
Day post irradiation	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan
1	8	8	12	6	0	0	0	0	0	0	0	0	0	0
3	4	4	11	9	0	0	0	1	0	1	0	0	0	0
6	8	11	11	10	0	0	1	3	0	1	0	0	9	3
8	19	23	18	13	1	0	3	4	2	2	1	1	28	25
10	23	34[a]	19	14	3	17[a]	5	24[a]	3	12[a]	4	9	35	69[a]
14	27	43[a]	23	19	5	22[a]	29	41[a]	13	26[a]	14	45[a]	76	67
21	82	65[a]	60	45[a]	22	37[a]	41	71[a]	22	52[a]	57	94[a]	88	50[a]

[a] $p > 0.01$ with respect to the respective time matched radiation control value.

CFU-s, GM-CFC, M-CFC, and BFU-e bone marrow contents and splenic TNC, GM-CFC, M-CFC, BFU-e, and CFU-e contents in glucan-treated mice were all significantly elevated over those of radiation controls from days 10 to 21 post-irradiation. However, several other hemopoietic parameters in glucan-treated mice neither remained above nor even equaled those of radiation controls. For example, in glucan-treated mice at 21 days after irradiation, the femoral TNC, HSC, and CFU-e contents and the splenic HSC and CFU-s contents were actually less than those of radiation control mice.

The splenic 'overshoot' phenomenon observed in pre-irradiation glucan-treated mice was again observed in the CFU-s, GM-CFC, M-CFC, BFU-e, and CFU-e post-irradiation glucan-treated mice. Interestingly, the overshoot in CFU-s, GM-CFC, and M-CFC in post-irradiation glucan-treated mice was less dramatic than in pre-irradiation glucan-treated mice, whereas the overshoot in BFU-e and CFU-e in post-irradiation glucan-treated mice was more dramatic than in pre-irradiation glucan-treated mice.

Discussion

Injection of particulate glucan into normal mice has previously been shown to result in increased numbers of bone marrow and/or splenic CFU-s, GM-CFC, M-CFC, BFU-e, and CFU-e[8-12]. The studies presented here demonstrate the ability of glucan to speed bone marrow and/or splenic HSC, CFU-s, GM-CFC, M-CFC, BFU-e, and CFU-e recovery in hemopoietically compromised animals. Specifically, glucan was shown to enhance the recovery of these hemopoietic progenitors when administered either before or after a hemopoietically damaging 650-rad dose of total-body gamma radiation. For example, the most severe depletion of all hemopoietic elements in both radiation control and glucan-treated mice was observed 1–3 days after radiation exposure. However, most hemopoietic elements in glucan-treated mice exhibited a major rebound within 6–14 days after irradiation, while the major rebound in radiation control mice was not observed until 14–21 days after irradiation. It should be pointed out that although both pre-irradiation and post-irradiation glucan treatments significantly enhanced hemopoietic repopulation in comparison to that in radiation controls, the pre-irradiation glucan treatment appeared to be more effective than post-irradiation glucan treatment. These results correlate well with our previous observations that although equal numbers of endogenously arising spleen colonies (E-CFU) were observed in both 1-h pre- and 1-h post-irradiation glucan-treated mice[14], the size of the E-CFU appeared to be larger in mice receiving glucan before irradiation.

In addition it should be noted that the hemopoietic effects of glucan primarily occurred within the first 2 weeks after irradiation, since by 3 weeks post-irradiation hemopoietic values in radiation control and glucan-treated mice in many instances were similar. This was true even in instances when recovery had not yet progressed to normal control values (i.e., 100%), and no explanation for this apparent latent slow down in glucan-induced hemopoiesis is proposed at this time.

It is interesting to note that additional particulate substances (e.g., Zymosan, C. parvum, glass beads, Portland cement) have also been shown to enhance hemopoiesis when administered before and in some cases after sublethal irradiation. However, we do not that feel the enchanced hemopoietic recovery observed in our

Table 4. Splenic hemopoietic recovery in $B6D2F_1$ mice given 1.5 mg of glucan at 1 h before 650 rads of total-body cobalt-60 radiation (% normal control)

	TNC		HSC		CFU-s		GM-CFC		M-CFC		BFU-e		CFU-e	
Day post irradiation	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan	Radiation control	+ Glucan
1	9	14	2	0	0	0	0	0	0	0	0	0	0	0
3	9	7	2	1	0	0	0	0	0	0	0	0	0	0
6	7	9	4	5	0	0	0	2	0	1	0	0	0	0
8	9	11	10	13	0	1	0	5[a]	0	3	0	2	1	1
10	10	21[a]	13	66[a]	0	9[a]	1	68[a]	1	20[a]	0	23[a]	2	103[a]
14	65	100[a]	41	149[a]	32	129[a]	94	1338[a]	8	126[a]	40	468[a]	410	776[a]
21	76	122[a]	54	44[b]	191	159[a]	744	1706[a]	60	198[a]	121	564[a]	920	1515[a]

[a] $p > 0.01$ with respect to the respective time-matched radiation control value; [b] $p > 0.05$ with respect to the respective time-matched radiation control value.

studies is due merely to the particulate nature of glucan, sinced we can easily reproduce these results using a soluble glucan preparation (manuscript in preparation). In addition, previous studies evaluating the hemopoietic radioprotective properties of various other substances have primarily used enhancement of endogenous colony-forming unit (E-CFU) numbers to indicate an agent's radioprotective capacity. Although E-CFU recovery does correlate well with CFU-s recovery, neither the E-CFU nor the CFU-s assays can reveal information about the recovery of the various committed hemopoietic progenitors cells (e.g., GM-CFC, M-CFC, CFU-e, BFU-e). Thus, our study has also demonstrated the radioprotective effects of glucan on these specific progenitor cells as well as on the pluripotent stem cells measured by the E-CFU and CFU-s assays.

Recently we have also shown that the administration of glucan before exposure to lethal whole-body gamma radiation (e.g., 900 rads) is capable of enhancing survival[25]. The exact correlation between glucan's hemopoietic enhancing capabilities and glucan's effects on post-irradiation survival remains to be determined. Since death after irradiation often results from the surgence of opportunistic infections, glucan treatment may provide a critical advantage by early-on stimulating the repopulation of hemopoietic precursors (i.e., CFU-s, GM-CFC, M-CFC) capable of replenishing the granulocytes and macrophages necessary for resisting bacterial invasion. This may indeed be possible since in non-irradiated animals glucan has been shown to profoundly enhance the resistance of animals to a variety of experimental infections[2, 26-28]. Additionally, recent experiments in our laboratory have indicated that glucan-treated irradiated animals present fewer opportunistic infections than do radiation controls (Patchen, Brook, Walker, unpublished observations).

Although much remains to be understood concerning the ability of glucan to enhance the recovery from radiation-induced hemopoietic aplasia, the implications and possible applications of glucan treatment are intriguing.

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0014-4754/84/111240-05$1.50 + 0.20/0
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Short Communications

Isolation and partial characterization of a phytotoxic glycoprotein from culture filtrates of *Rhynchosporium secalis*[1]

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Summary. Rhynchosporium secalis (Oud.) Davis produces a phytotoxic compound with a mol. wt of 275×10^3 which is able to induce chlorotic symptoms in both susceptible and resistant barley leaves. Collectively, the data suggest that the toxin is a glycoprotein. Mild base treatment, by β elimination, indicates that threonine and serine are involved in o-glycosidic linkages with the carbohydrate moiety. Sugar residues occur in the molecule in the ratio of mannose, rhammnose, galactose, glucosamine 13.6:1:1:1.
Key words. Rhynchosporium secalis; barley scald disease; phytotoxin; glycoprotein; phytotoxic; rhynchosporide.

Rhynchosporium secalis is the causal organism of scald disease in barley. A host-selective toxin called rhynchosporoside was isolated from culture filtrates of *R. secalis* and also from infected plants[2]. This toxin was able to reproduce scald symptoms with concentrations as low as 6–12 μg/ml[2]. The original proposed structure of rhynchosporoside was that of a cellobioside linked 2–0 α to 1,2 propanediol and this structure has been recently revised[3]. It turns out that *R. secalis* produces a toxic family of β 1–4 glucosides linked 1–0α to 1,2 propanediol, including the glucoside, the cellobioside, the cellotrioside[3,4] and the cellotetraoside[5]. The 3 lowest polymers of this family of glucosides have affinities for membraneous receptors both in vitro and in vivo[6,7]. The greater affinity is always displayed with the susceptible lines of barley. The compound of greatest affinity to the receptor was the cellobioside of 1,2 propanediol[7]. Preliminary biochemical data have pointed out that among these toxic glycosides the glucoside and the cellobioside have a strong, non-host specific, uncoupling effect on the chloroplastic electron transport, this effect occurring without prior binding to the thylakoids[8].

Thus, even with the great contribution of these toxins to the production of disease symptoms, some events like the overcoming of resistance during the natural process of infection cannot be attributed to the rhynchosporosides. In order to explain this phenomenon we examined the possibility that *R. secalis* produces other toxic molecules of higher molecular weight. Earlier workers claimed that some non-dialyzable compounds from culture filtrates of *R. secalis* were able to induce some of the scald symptoms on barley, oats and wheat[9]. Furthermore, other workers postulated the involvement of high molecular weight compounds, i.e. glycopeptides[4,10,11], in fungal infections of plants.

This paper reports the isolation and purification from culture filtrates of *R. secalis* of a phytotoxic polypeptide. In addition some preliminary data are provided concerning the structure and the biological activity of this phytotoxic compound.

Materials and methods. The seeds of near isogenic lines ATLAS 46 (resistant) and ATLAS (susceptible) were kindly provided by R. Eslick and H. Bockelman, Montana State University. They were grown in potting soil under greenhouse condi-