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**STIMULATED HEMOPOIESIS AND ENHANCED SURVIVAL
FOLLOWING GLUCAN TREATMENT IN SUBLETHALLY AND
LETHALLY IRRADIATED MICE**

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Abstract — Hemopoietic effects of the reticuloendothelial agent glucan were assayed in normal mice and in mice hemopoietically depleted by exposure to ^{60}Co radiation. In normal mice, glucan administration increased the content of bone marrow and splenic transplantable pluripotent hemopoietic stem cells (CFU-s), committed granulocyte-macrophage progenitor cells (GM-CFC), and pure macrophage progenitor cells (M-CFC). Erythroid progenitor cells (CFU-e) were increased only in the spleen. In sublethally irradiated mice (650 rads), glucan increased the number of endogenous pluripotent hemopoietic stem cells (E-CFU) when administered either before or after irradiation. The most pronounced effects were observed when glucan was administered 1 day before, 1 h before, or 1 h after irradiation. In addition, the administration of glucan before lethal irradiation (900 rads) enhanced survival. The most significant results were seen when glucan was administered 1 day prior to irradiation. The possibility of using agents such as glucan to enhance hemopoietic reconstitution and prevent septicemia following chemotherapy and/or radiotherapy is discussed.

Glucan, a B-1,3 polyglucose, has been shown to modulate reticuloendothelial and immune responses. For instance, it has been shown that macrophage proliferation, phagocytosis, adherence, and lysozyme synthesis as well as primary and secondary antibody responses, cell-mediated immune responses, anti-tumor responses, and anti-bacterial, fungal, viral, and parasitic responses are enhanced following glucan administration (Riggi & DiLuzio, 1961; Woolees & DiLuzio, 1963; DiLuzio, 1967; DiLuzio, Pisano & Saba, 1970; Kokosis, Williams, Cook & DiLuzio, 1978; DiLuzio, Williams, McNamee, Edwards & Kilahama, 1979; Reynolds, Kastello, Harrington, Crabbs, Peters, Jemski, Scott & DiLuzio, 1980; Cook, Holbrook & Parker, 1980; DiLuzio, 1983). In addition, glucan has been shown to alter hemopoietic proliferation and differentiation. Specifically, increased numbers of not only granulocyte-macrophage (GM-CFC), pure macrophage (M-CFC), and erythroid (CFU-e and BFU-e) progenitor cells but also pluripotent hemopoietic stem cells that give rise to these progenitors (CFU-S) have been observed following glucan administration (Burgaleta & Golde, 1977; Patchen & Lotzova, 1980; Patchen & MacVittie,

1983a). The multiple effects of glucan on the reticuloendothelial, immune, and hemopoietic systems thus make glucan an intriguing candidate for therapeutic use in instances of radiation-induced and/or chemical-induced hemopoietic and immune depression.

The purposes of this study were to (a) delineate the temporal hemopoietic responses elicited by a glucan dose known to stimulate reticuloendothelial and immune responses in normal mice, (b) determine the feasibility of using glucan to enhance recovery from radiation-induced hemopoietic depletion, and (c) determine the feasibility of using glucan to enhance survival in irradiated mice.

EXPERIMENTAL PROCEDURES

Mice

In all experiments, 10-12 week old female C₃H/HeN mice (Charles River Laboratories, Willmington, MA) were used. Animals were maintained on a 6 a.m. to 6 p.m. light-dark cycle. Wayne Lab Blox and hyperchlorinated water were

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available *ad libitum*. All mice were quarantined and acclimated to laboratory conditions for at least 2 weeks before experimentation. During this time, the mice were examined and found to be free of lesions of murine pneumonia complex and oropharyngeal *Pseudomonas* sp.

Glucan

Particulate, endotoxin-free glucan (glucan-P) was obtained from Dr. N. R. DiLuzio (Tulane University School of Medicine, New Orleans, LA) and was prepared according to DiLuzio's modification (DiLuzio *et al.*, 1979) of Hassid's original procedure (Hassid, Joslyn & McCready, 1941). Sterile saline was used to dilute glucan-P to either 3.0 mg or 0.8 mg per ml. Mice were then i.v. injected via the lateral tail veins with a 0.5 ml volume of the glucan preparations yielding either 1.5 mg or 0.4 mg per mouse. Normal control mice (not irradiated and not glucan-treated) and radiation control mice (irradiated but not glucan-treated) were injected with an equivalent volume of sterile saline.

Cell suspensions

Each cell suspension represented the pool of tissues from three mice. Cells were flushed from femurs 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.

Irradiation

Bilateral total-body irradiation administered from the AFRR1 ⁶⁰Co source at a dose rate of 40 rads/min was used in all radiation experiments.

Spleen colony-forming unit (CFU) assays

Spleen colony-forming units (CFU) have been shown to arise from the clonal proliferation of pluripotent hemopoietic stem cells. Exogenous spleen colony-forming units (CFU-s) were evaluated by the method of Till & McCulloch (1961). Recipient mice were exposed to 900 rads of total-body irradiation in order to completely eradicate endogenous hemopoietic stem cells. Three to 5 h later, 5×10^5 bone marrow or 5×10^5 spleen cells were i.v. injected into the irradiated recipients. Ten days after transplantation, the recipients were

ethanized and their spleens removed. The spleens were fixed in Bouin's solution, and the number of grossly visible spleen colonies were counted. Endogenous spleen colony-forming units (E-CFU) were also evaluated by a method of Till & McCulloch (1963). Mice were exposed to a 650 rads dose of total-body irradiation in order to only partially ablate endogenous hemopoietic stem cells. Ten days after irradiation, the spleens were removed and fixed in Bouin's solution. Then the spleen colonies formed by the proliferation of surviving endogenous hemopoietic cells were counted.

Granulocyte-macrophage colony-forming cell (GM-CFC) and macrophage colony-forming cell (M-CFC) assays

Hemopoietic progenitor cells committed to the granulocyte-macrophage lineage (GM-CFC) were assayed by MacVittie's modification (1979) of the semi-solid agar technique originally described by Bradley & Metcalf (1966) and Pluznik & Sachs (1965). The upper agar-medium mixture for cell suspensions consisted of equal volumes of 0.66% agar and double-strength supplemented CMRL 1066 medium. The CMRL 1066 was supplemented with final concentrations of 10% HIFBS, 5% trypticase soy broth, 5% heat-inactivated horse serum, antibiotics and L-asparagine (30 µg/ml). The agar-medium mixture for the lower feeder layer consisted of equal volumes of 1.0% agar and supplemented double-strength CMRL 1066. Both pregnant mouse uterine extract (PMUE) (2.5% v/v) and mouse L-cell-conditioned medium (LCM) (13% v/v) were added to each 1 ml feeder layer as sources of colony-stimulating activity (CSA). Colonies (>50 cells) were counted after 10 days of incubation in a 37°C humidified environment containing 7.5% CO₂. Hemopoietic progenitor cells committed only to the macrophage lineage (M-CFC) were assayed by the technique described by MacVittie & Provaznik (1978). Double-layer agar cultures were prepared as in the GM-CFC assay except that no CSA was initially incorporated into feeder layers. This initial absence of CSA eliminated the proliferation of GM-CFC. Two days after the cells were originally plated, CSA was added by incorporating PMUE and LCM into a mixture of supplemented CMRL 1066 containing a final concentration of 0.33% agar, and pipetting 0.5 ml of this mixture on top of the previously cultured cells. Cultures were incubated for an additional 25 days at 37°C in a humidified atmosphere containing 7.5% CO₂, before scoring colony formation.

Erythroid colony-forming-unit (CFU-e) assay

Bone marrow and splenic hemopoietic progenitor cells committed to the erythroid lineage (CFU-e) were assayed by a modification of the original CFU-e plasma clot technique described by Stephenson, Axelrad, McLeod & Shreeve (1971). Each ingredient was either reconstituted or diluted with supplemented alpha medium (SAM) (Weinberg, McCarthy, MacVittie & Baum, 1981). Briefly, to make 1 ml of the plasma clot suspension, the following were mixed: 0.1 ml cells (5×10^6 nucleated cells), 0.3 ml HIFBS, 0.1 ml 25% beef embryo extract, 0.1 ml 10% bovine serum albumin, 0.1 ml (0.02 mg) L-asparagine, 0.1 ml 10^{-3} M 2-mercaptoethanol, 0.1 ml erythropoietin (Ep), and 0.1 ml of 37°C bovine citrated plasma. Immediately, 0.1 ml of the mixture was pipetted into each of six microtiter wells. Step III anemic sheep plasma (Connaught Labs, Inc., Swiftwater, PA) was used as the source of Ep. Bone marrow and splenic CFU-e clot suspensions contained 0.25 and 0.5 units Ep/ml, respectively. Control clots contained SAM in place of Ep. After incubation at 37°C in a humidified atmosphere containing 5% CO_2 in air for 2 days, plasma clots were harvested, fixed with 5% glutaraldehyde, and stained with benzidine and gentian (McLeod, Shreeve & Axelrad, 1979). A CFU-e was defined as an individual aggregate of eight or more benzidine-positive cells.

Survival studies

Mice used in survival studies were exposed to 900 rads of total body irradiation, and their survival was checked daily for a period of 30 days.

RESULTS*Temporal hemopoietic effects of glucan-P in normal mice*

Figures 1-5 illustrate the temporal effects of 1.5 mg of glucan-P on bone marrow and splenic cellularity, CFU-s, GM-CFC, M-CFC, and CFU-e responses in normal mice. Except for bone marrow CFU-e, the absolute numbers of these various hemopoietic elements all increased following glucan-P administration. Peak splenic responses were consistently observed 5 days after glucan-P treatment. The bone marrow CFU-s response also peaked on day 5 post-treatment; however, peak marrow GM-CFC and M-CFC responses did not occur until day 11 post-treatment. The bone marrow hemopoietic responses were also much less dramatic

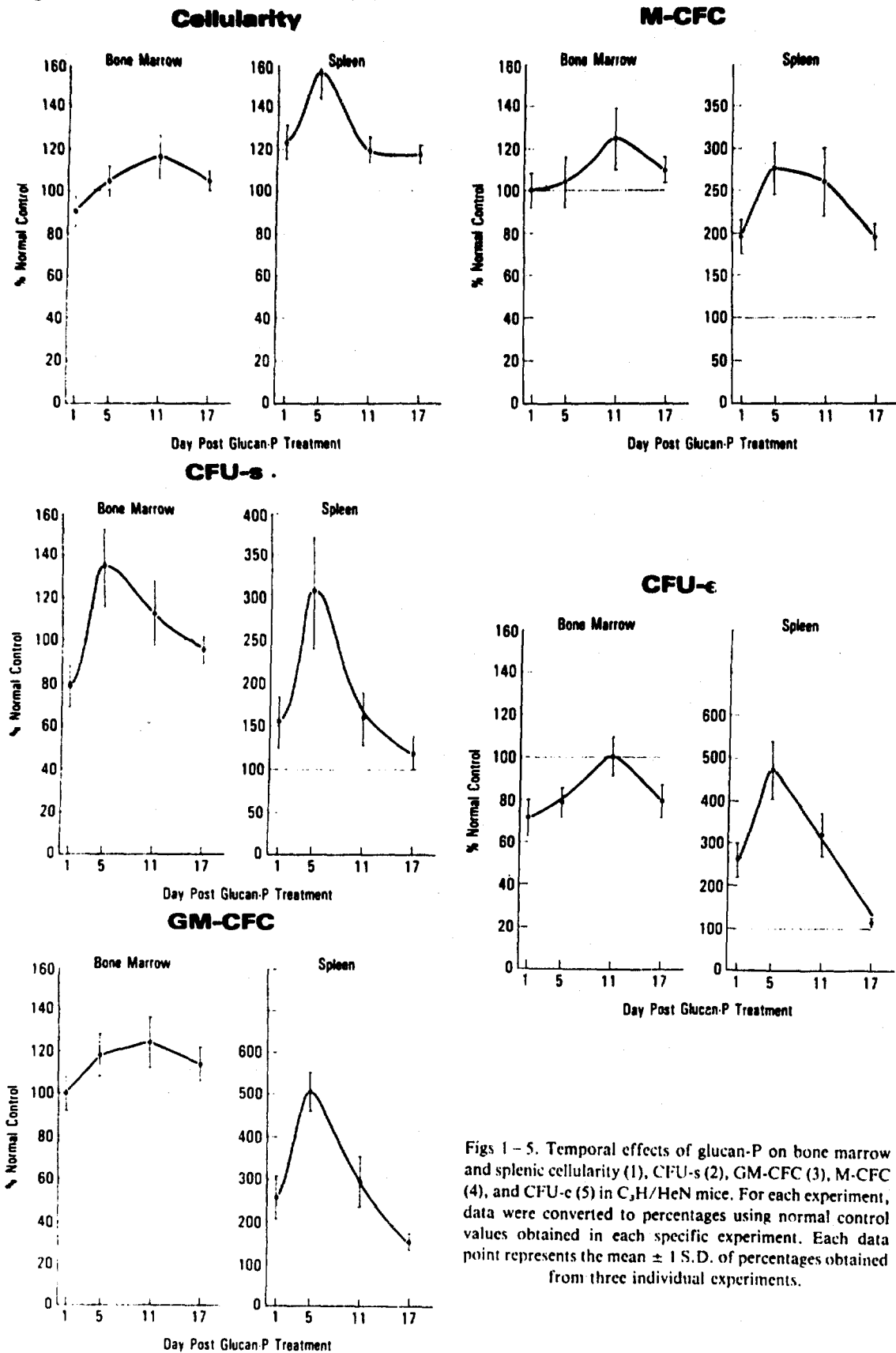
than the splenic hemopoietic responses, e.g. 115-135% of normal control values in the bone marrow vs 270-500% of normal control values in the spleen. Interestingly, bone marrow CFU-e values never rose above normal control values and, in fact, were in most instances lower than control values. This erythropoietic depression, however, was only temporary, and by 30 days post-treatment, hematocrit values in glucan-P-treated mice had returned to normal control values (Table 1).

Glucan-P's effect on enhancing recovery from radiation-induced hemopoietic depletion

The endogenous spleen colony assay was used to screen glucan-P for its ability to enhance hemopoietic pluripotent stem cell recovery when administered before or after a hemopoietically compromising dose of irradiation. Table 2 presents data from experiments in which glucan-P was administered at 17, 11, 5 and 1 days or 1 h before or after a 650 rads dose of ^{60}Co irradiation. Because an enhanced E-CFU response was anticipated, both a 1.5 mg and a 0.4 mg glucan-P dose were used in order to insure that discrete countable E-CFU colonies could be obtained. Glucan-P was quite effective at enhancing E-CFU numbers even when administered as long as 17 days before irradiation. However, as the interval between glucan-P treatment and irradiation was shortened, the E-CFU response became more pronounced, with almost equally dramatic effects being observed with glucan-P administered at either 1 day or 1 h before irradiation. Although not as pronounced as preirradiation glucan-P treatment, postirradiation glucan-P treatment was also quite effective at enhancing E-CFU colony formation. In fact, glucan-P given 1 h after irradiation was almost as effective at enhancing E-CFU colony formation as was glucan-P administered 1 h before irradiation.

Glucan-P's effect on survival of irradiated mice

Because of glucan-P's ability to enhance hemopoietic recovery when administered both before and after irradiation, the ability of this agent to also enhance survival in otherwise lethally irradiated animals was next investigated. Based on the E-CFU results, glucan-P was administered either 1 day or 1 h before or 1 h after irradiation. As illustrated in Fig. 6, 1.5 mg of glucan-P administered 1 day before an otherwise lethal dose of irradiation significantly enhanced survival. Thirty days postirradiation, 51% of the 1.5-mg glucan-P-treated mice were alive. Although the protocol for these studies included only



Figs 1-5. Temporal effects of glucan-P on bone marrow and splenic cellularity (1), CFU-s (2), GM-CFC (3), M-CFC (4), and CFU-c (5) in C₃H/HeN mice. For each experiment, data were converted to percentages using normal control values obtained in each specific experiment. Each data point represents the mean \pm 1 S.D. of percentages obtained from three individual experiments.

Table 1. Hematocrit values in glucan-P treated mice

Hematocrit value % of normal control	Day post glucan-P treatment							
	1	5	11	17	30	60	90	120
	102±3	98±2	83±3	89±4	99±3	101±3	100±2	102±3

Table 2. Effect of pre- and post-irradiation glucan-P treatment on E-CFU

Time of glucan-P injection (days)	Pre 650 rads					Post 650 rads				
	17	11	5	1	1 h	1 h	1	5	11	17
E-CFU (% radiation control value*)										
0.4 mg (n = 24)	174±29	261±45	306±52	590±90	548±66	539±60	408±48	141±48	1	1
1.5 mg (n = 24)	326±26	442±45	990±110	1503±177	1448±174	1355±139	447±95	260±35		

*Radiation control E-CFU values for -17, -11, -5, -1, -1h, +1h, +1, and +5 days were 1.9±0.2, 2.0±0.3, 2.1±0.4, 1.9±0.4, 2.2±0.6, 1.7±0.7, 2.3±0.7 and 10.0±1.8, respectively.

*Not quantifiable due to confluent colony growth.

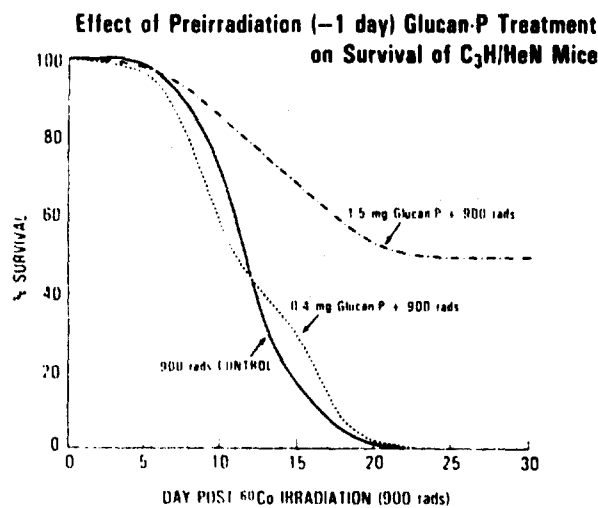


Fig. 6. Effects of two doses of Glucan-P on 30 day survival of C₃H/HeN mice when injected 1 day before 900 rads of cobalt-60 radiation. Percent survival is based on cumulative data obtained from 5 - 15 individual survival studies. Radiation control data are based on survival of 201 mice, 1.5 mg glucan-P data are based on 83 mice, and 0.4 mg glucan-P data are based on 56 mice.

a 30 day survival, 49% of these irradiated mice treated with 1.5 mg of glucan-P actually exhibited long-term survival and were ultimately euthanized 12 - 13 months after irradiation. By contrast, the lower 0.4 mg glucan-P dose was not effective at

enhancing survival in similarly irradiated mice. Similar to radiation controls, none of these mice survived beyond 21 days postirradiation. Figure 7 illustrates the survival effects of glucan-P administered 1 h before lethal irradiation. As can be

seed, survival in mice receiving 0.4 mg of glucan-P did not differ from radiation controls. However, survival in mice receiving the 1.5 mg glucan-P dose was slightly prolonged, even though long-term survival (i.e. ≥ 30 days) was not different from that of radiation controls. Although 1.5 mg glucan-P treatment did exhibit some positive effects on survival when administered prior to irradiation, it was not effective when administered after irradiation (Figure 8). In fact, when administered postirradiation, glucan-P actually induced death more rapidly than in radiation controls. This effect was dose-dependent, with the higher 1.5 mg glucan-P dose being more detrimental than the lower 0.4 mg glucan-P dose.

DISCUSSION

Agents capable of regulating (and in particular stimulating) hemopoietic proliferation and differentiation at the stem cell level have a variety of potential benefits. One such benefit would be to reduce lethality attributed to the hemopoietic syndrome following irradiation and/or chemotherapy. Because reticuloendothelial and immune reconstitution are critically important in combating the secondary septicemia associated with

hemopoietic depletion, (Hammond, Tompkins & Miller, 1954; Benacerraf, 1960; Cronkite & Bond, 1960; Collins, 1979; Broerse & MacVittie, 1984) we specifically evaluated the hemopoietic effects of glucan-P in the dose range that has been shown to elicit enhanced reticuloendothelial and immune responses.

These studies have clearly demonstrated several effects of glucan-P on normal murine hemopoiesis and on hemopoiesis and survival in irradiated mice. First, our results concerning the temporal effects of 1.5 mg of glucan-P on splenic and bone marrow CFU-s, GM-CFC, M-CFC and CFU-e clearly confirm previous studies suggesting that particulate glucan, in a dose-dependent manner, can enhance hemopoiesis in normal mice (Burgaleta & Golde, 1977; Patchen & Lotzova, 1980; Patchen & MacVittie, 1983a,b). In the studies presented here, stem and progenitor cell hemopoietic responses were evident as early as 1 day post glucan-P treatment, usually peaked on days 5-11 post-treatment, and had either returned to or were declining toward normal control values by 17 days post-treatment. Bone marrow erythropoiesis was the only hemopoietic response not enhanced. However, the tremendous increase in splenic erythropoiesis apparently compensated for the decreased bone marrow erythropoiesis, since only a transient anemia was apparent in glucan-P-treated mice.

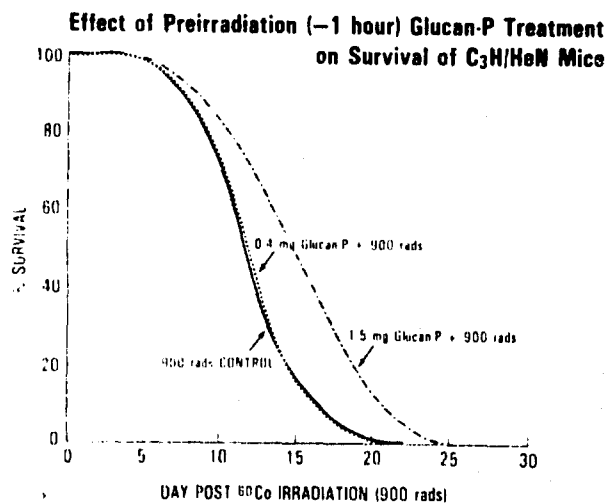


Fig. 7. Effects of two doses of glucan-P on 30 day survival of C₃H/HeN mice when injected 1 h before 900 rads of cobalt-60 radiation. Percent survival is based on cumulative data obtained from 4-15 individual survival studies. Radiation control data are based on survival of 201 mice, 1.5 mg glucan-P data are based on 59 mice, and 0.4 mg glucan-P data are based on 54 mice.

**Effect of Postirradiation (+1 hour) Glucan-P Treatment
on Survival of C₃H/HeN Mice**

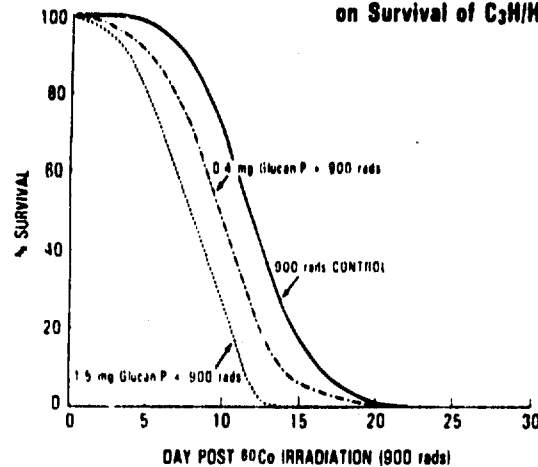


Fig. 8. Effects of two doses of glucan-P on 30 day survival of C₃H/HeN mice when injected 1 h after 900 rads of cobalt-60 radiation. Percent survival is based on cumulative data obtained from 3 - 15 individual survival studies. Radiation control data are based on survival of 201 mice, 1.5 mg glucan-P data are based on 67 mice, and 0.4 mg glucan-P data are based on 30 mice.

Second, the ability of glucan-P to enhance hemopoietic recovery in irradiated mice was also demonstrated. In particular, it was shown that glucan-P could significantly increase pluripotent hemopoietic stem cell numbers (as measured by the E-CFU assay) in partially hemopoietically depleted mice if injected either before or after irradiation. The most dramatic increases were observed with glucan-P administered either 1 day before, 1 h before, or 1 h after irradiation. In any given experiment, glucan-P treatment 1 day before irradiation was slightly more enhancing than treatment 1 h before irradiation, and treatment 1 h before irradiation was slightly more enhancing than treatment 1 h after irradiation.

Finally, the fact that glucan-P administration could significantly influence survival following higher (lethal) doses of irradiation was also demonstrated. Specifically, 1.5 mg of glucan-P, administered 1 day before irradiation, significantly increased long term survival (i.e. ≥ 30 days), and the same dose of glucan-P administered 1 h before irradiation marginally increased short-term survival (i.e. < 30 days). However, when glucan-P was injected 1 h post-irradiation, not only was survival not increased, but it actually decreased compared to radiation controls.

Over the years a variety of other substances have also been shown to alter survival in irradiated mice (Smith, Smith, Andrews & Grenan, 1955; Smith,

Alderman & Gillespie, 1957; Boggs, Marsh, Chervenick, Cartwright & Wintrobe, 1968; Mori & Nakamura, 1970). The substances used have been as diverse as ground glass, carbon particles, non-species related serum, and endotoxin. Almost all these substances produced the best "radioprotective" effect in mice when administered 1 day before irradiation. However, in addition to the timing of the injection, the route of injection was also shown to influence survival results. The survival results we have obtained with i.v. 1 day glucan-P administration in general, correlate with previous studies utilizing various other intravenously administered substances. Even with respect to post-irradiation glucan-P treatment decreasing survival (rather than increasing or not changing survival), a similar phenomenon has been observed with post-irradiation i.v. injection of endotoxin. For example, if either glucan-P or endotoxin are injected into mice 2 - 3 days after 900 rads of irradiation, all animals die almost immediately (Smith *et al.* 1957; Patchen, unpublished observations). Since our glucan-P preparation was endotoxin-free (as determined by the Limulus Lysate procedure), our decreased survival could not have been due to endotoxin contamination in our glucan-P. Rather, we suspect that the decreased survival seen with post-irradiation administration of glucan-P and endotoxin is a more general phenomenon probably inducible by other

agents as well. Interestingly, we have also been working with a different glucan preparation (glucan-E, a soluble B-1, 3 glucan), which is capable of producing the same dramatic survival-enhancing effects as glucan-P or endotoxin when administered 1 day before irradiation, but does not decrease survival (and, in fact, is even slightly radioprotective) when administered after irradiation (Patchen *et al.*, unpublished results). Studies to further evaluate the mechanisms of the differential effects of pre- and post-irradiation glucan-P treatments on survival are currently being conducted in our laboratory.

A variety of mechanisms have been hypothesized to explain the capabilities of numerous "radioprotective" agents (e.g. ground glass, carbon particles, endotoxin, etc.). Most of these agents are "radioprotective" only in the hemopoietic syndrome radiation dose range. Thus, it is not surprising that hemopoietic protection and/or enhanced hemopoietic reconstitution followed by enhanced resistance to endogenous pathogens have been shown to occur following administration of these agents. Since the E-CFU assay measures post-irradiation recovery of endogenous pluripotent hemopoietic stem cells (i.e. the cells from which all other mature cells comprising the blood, immune, and reticuloendothelial systems ultimately arise), a direct correlation has been suggested between an agent's hemopoietic-enhancing potential (as measured by the E-CFU assay) and its survival-enhancing potential in the hemopoietic syndrome radiation dose range (Smith, Budd & Cornfield, 1966; Kinnamon, Ketterling, Stampfli & Grenan, 1980). Based on the differences we observed in E-CFU numbers and survival data produced by 1 day, 1 h, and 1 h glucan-P treatment, our data also seem to suggest such a correlation. However, in spite of the fact that

1 day glucan-P treated mice did exhibit slightly higher endogenous hemopoietic stem cell numbers than 1 h glucan-P treated mice, and 1 h glucan-P treated mice did exhibit slightly higher endogenous hemopoietic stem cell numbers than 1 h glucan-P treated mice, all three treatment groups exhibited extremely elevated E-CFU numbers. Thus, it seems unlikely that the survival effects induced by these three glucan-P treatments would be so different if increased pluripotent hemopoietic stem cell numbers *alone* were responsible for enhanced survival. Instead, it may be possible that in mice treated with

glucan-P 1 day before irradiation, pluripotent hemopoietic stem cells have enough of an opportunity to not only proliferate, but also to differentiate into progenitors capable of giving rise to the mature functional elements of the reticuloendothelial and immune systems necessary to contend with the surge of endogenous pathogens that secondarily induce death 10-20 days post-irradiation in the 900 rads dose range (Hammond *et al.*, 1954; Benacerraf, 1960; Cronkite & Bond, 1960; Collins, 1979; Broerse & MacVittie, 1984). In fact, it has been shown that recovery of the differentiated progenitor cells that do give rise to granulocytes and macrophages is more accelerated in mice treated with glucan-P 1 day before irradiation than in mice treated with glucan-P just 1 h before or 1 h after irradiation (Patchen, 1983; Patchen, MacVittie & Wathen, in press).

In spite of the uncertainties concerning the mechanisms by which glucan-P mediates its effects, it is apparent that glucan-P is a potent hemopoietic stimulant in normal mice and in radiation-depleted mice and that glucan-P administered 1 day prior to otherwise lethal radiation in the hemopoietic syndrome dose range can significantly enhance survival. In addition, when compared to other historical "radioprotectors", glucan-P has the advantages of being nontoxic, nonpyrogenic, and ultimately it can be metabolized to glucose and utilized nutritionally as a food source (Diluzio, 1983).

Glucan-P's hemopoietic and survival-enhancing capabilities, coupled with its ability to nonspecifically stimulate resistance to a variety of bacterial, viral, and fungal infections make glucan-P a primary candidate for use in instances of radiation-induced and/or chemical-induced life-threatening hemopoietic depletion.

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