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Glucan: Mechanisms Involved in Its "Radioprotective" Effect

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It has generally been accepted that most biologically derived agents that are radioprotective in the hemopoietic-syndrome dose range (eg, endotoxin, Bacilius Calmette Guerin, *Corynebacterium parvum*, etc) exert their beneficial properties by enhancing hemopoietic recovery and hence, by regenerating the host's ability to resist life-threatening opportunistic infections. However, using glucan as a hemopoietic stimulant/radioprotectant, we have demonstrated that host resistance to opportunistic infection is enhanced in these mice even prior to the detection of significant hemopoietic regeneration. This early enhanced resistance to microbial invasion in glucan-treated irradiated mice could be correlated with enhanced and/or prolonged macrophage (but not granulocyte) function. These results suggest that early after irradiation glucan may mediate its radioprotection by enhancing resistance to microbial invasion via mechanisms not necessarily predicated on hemopoietic recovery. In addition, preliminary evidence suggests that glucan can also function as an effective free-radical scavenger. Because macrophages have been shown to selectively phagocytize and sequester glucan, the possibility that these specific cells may be protected by virtue of glucan's scavenging ability is also suggested.

Key words: radioprotection, macrophages, hemopoiesis, free-radical scavengers

INTRODUCTION

The exposure of mammals to a single whole-body dose of ionizing radiation results in a complex set of symptoms whose onset, nature, and severity are a function of both total radiation dose and radiation quality [3,10,17]. In general, radiation injury can be classified into three syndromes which become evident at progressively higher radiation doses: the hemopoietic syndrome, the gastrointestinal syndrome, and the central nervous system syndrome [3,10,17].

The hemopoietic syndrome occurs at the lowest radiation doses (< 10 Gy) and is manifest by hemopoietic stem cell depletion [8,10,58] and ultimately by depletion of mature hemopoietic and immune cells [2,7,26,29, 42,52], which (whether destroyed directly by the radiation insult or lost naturally through attrition) cannot be regenerated without hemopoietic stem cells. In turn, the loss of mature hemopoietic and immune cells severely impairs antimicrobial immunity, and ultimately death ensues owing to invasive opportunistic infections [5,22,33,53].

Most biological agents previously shown to be specifically radioprotective in the hemopoietic-syndrome dose range (eg, endotoxin, Bacillus Calmette Guerin, *Corynebacterium parvum*, etc), have also been shown to be hemopoietic stimulants [20,31,32,50]. Thus, it has generally been assumed that the "radioprotection" afforded by these agents results from enhanced hemopoietic recovery and, subsequently, from enhanced resistance to microbial invasion. During the past several years we have evaluated the radioprotective properties of glucan, a *Saccharomyces*-derived immunostimulant [14,23], and have shown that it also enhances survival in mice exposed to radiation in the hemopoietic-syndrome dose range [37,39,40]. As with other agents that are radioprotective in this dose range, glucan was shown to significantly accelerate hemopoietic recovery in sublethally irradiated (6.5 Gy) mice [38,39,41]. Specifically, pluripotent hemopoietic stem cells (CFU-s), granulocyte-macrophage progenitor cells (GM-CFC), pure macrophage progenitor cells (M-CFC), erythroid progenitor cells (CFU-e, BFU-e), and peripheral blood cells were all shown to recover more rapidly in glucan-treated mice than in radiation controls. Hemopoietic regeneration also ultimately oc-

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curred in lethally irradiated (9 Gy) glucan-treated mice (this report). However, since at day 15 postirradiation (when most radiation control mice had already died) hemopoietic elements in glucan-treated mice had recovered to less than 5% of normal levels, it appeared that hemopoietic recovery alone was unlikely to account for glucan's ability to radioprotect animals in the first weeks after radiation exposure. Thus, the possibility that early postirradiation glucan may enhance survival by enhancing and/or prolonging the function and/or survival of already existing cell populations important in host defense against microbial invasion (ie, granulocytes and macrophages) was also evaluated.

MATERIALS AND METHODS

Mice

In all experiments, 20- to 25-g C3H/HeN female mice were used. Approximately 3% of all mice entering the AFRRI animal facility were sacrificed for representative quality-control histopathology, bacteriology, and serological viral pathogen screens. While waiting for these results, the remaining mice were quarantined and acclimated to the 6:00 A.M. to 6:00 P.M. light cycle, Wayne Lab Blox rodent chow, and acidified (pH 2.5) water used in the animal facility. Only shipments of healthy mice were released for experimentation.

Glucan

Particulate endotoxin-free glucan, prepared as described by DiLuzio et al [14], was obtained from Accurate Chemical and Scientific Corporation (Westbury, NY). Based on nuclear magnetic resonance, this glucan preparation was reported to be 99.9% chemically pure (N.R. DiLuzio, personal communication), and consisted of 1-3-μm glucan particles suspended in sterile saline. As indicated in the specific experiments, 1.5 mg of glucan (in a 0.5-ml volume) was administered either intravenously (i.v.) via the lateral tail veins, or intraperitoneally (i.p.). Control mice were injected with an equivalent volume of sterile saline or, where specified, a 1.5-mg dose of D-glucose (Sigma Chemical Co., St. Louis, MO) dissolved in sterile saline.

Irradiation

All mice were bilaterally exposed to total-body irradiation administered from the AFRRI cobalt-60 gamma-ray source at a dose rate of 0.4 Gy (1 Gy = 100 rads) per minute. Samples for in vitro free-radical scavenging experiments were exposed to irradiation administered from the same source at a dose rate of ~40 Gy/min. Dosimetry was determined by ionization chambers [49], and all irradiations were performed at room temperature.

Peritoneal Exudate Cell Collection and Macrophage Isolation

Mice were anesthetized with halothane (Halocarbon Laboratories, Inc., Hackensack, NJ) and injected i.p. with 5 ml of calcium- and magnesium-free Hanks' balanced salt solution (HBSS) containing 2 units/ml of preservative-free heparin (Abbott Laboratories, North Chicago, IL). Following gentle massage, the peritoneal lavage fluid was withdrawn through a 20-gauge needle inserted into the cavity, and the cell content of the lavage fluid was determined by counting the cells on a hemocytometer. For macrophage cell isolation, the peritoneal cells were cultured in 35-mm petri dishes (Corning, Corning, NY) in Dulbecco's modified essential medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) at a concentration of 2.5×10^6 cells/ml per dish. Macrophages were allowed to attach for 2 h at 37°C in 5% CO_2. The plates were then washed three times with DMEM to remove the nonadherent cells, and the adherent macrophages used as described for the 5'nucleotidase assay.

Survival Assays

Mice used in survival studies were exposed to 9 Gy of total-body irradiation, and their survival was checked daily for 30 days. Statistical differences between control and glucan-treated mice were determined by the Kruskal Wallis test.

Hemopoietic Cell Assays

The hemopoietic assays used have been described in detail elsewhere [39]. Pluripotent hemopoietic stem cells were evaluated by the spleen colony (CFU-s) assay using 9-Gy irradiated mice as transplant recipients [54]. Twelve 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 a modification [31] of the semisolid agar technique originally described by Bradley and Metcalf [9] and Pluznik and Sachs [43]. Colonies (> 50 cells) were counted after 10 days of incubation in a 37°C humidified environment containing 7.5% CO_2. The cell suspensions used for these assays represented the pool of tissues from 3–12 normal, irradiated, or glucan-treated irradiated mice at each time point. Cells were flushed from femurs with 3 ml of McCoy's 5A Medium (MC5A) 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 MC5A plus 5% HIFBS. The total number of nucleated

cells in each suspension was determined by counting the cells on a hemocytometer.

Bacteriological Assays

To evaluate the occurrence of opportunistic infections in irradiated mice, the liver, spleen, and peripheral blood of animals were monitored for bacterial translocation. Individual mice were halothane-anesthetized, and ~0.5 ml of blood was removed by cardiac puncture. A 0.1-ml sample of blood was then streaked onto two enriched BHI agar plates (DIFCO, Detroit, MI). The liver and spleen of these mice were also aseptically removed, homogenated, and streaked onto two BHI plates. One plate of each tissue was incubated for 48 hours at 37°C and 5% CO_2 to isolate aerobic bacteria. The other plate was incubated for 96 h in an anaerobic jar to isolate anaerobic bacteria. Microorganisms were identified by means of standard criteria [30,51].

Granulocyte Chemiluminescence Assay

Oxidative burst activity was used as an indicator of peripheral blood granulocyte function [27,47,48] and was measured by chemiluminescence [1,28]. To perform these studies, mice were halothane-anesthetized and ~0.5 ml of blood was removed by cardiac puncture and immediately mixed with 1 drop (1 unit) of preservative-free heparin. Twenty microliters of each blood sample was mixed with 200 μl of opsonized zymosan particles suspended in luminol (ZAP, Packard Instruments, Chicago, IL) and light emissions were counted immediately and at 5-min intervals over a 45-min period with the aid of a PICOLITE 6112 Luminometer (Packard Instruments, Chicago, IL). Total white cell counts (Unopettes, Becton Dickinson, Rutherford, NJ) and differential white cell counts were also performed on each blood sample.

Reticuloendothelial-System Carbon Clearance Assay

The rate of removal of colloidal carbon from the circulation was used to measure the phagocytic activity of various macrophage populations comprising the reticuloendothelial system (RES) [24]. To perform this assay, individual mice were injected i.v. with 0.25 ml of diluted colloidal carbon (~160 mg/kg) (C11/143a, Gunther Wagner, Hanover, West Germany), and its removal from the circulation was measured. At 1, 2, 4, 6, 8, and 10 min after carbon injection, ~0.2 ml of blood was removed from halothane-anesthetized mice by cardiac puncture, and 50 μl was immediately dispersed into 4 ml of 0.1% sodium carbonate solution. At the end of the blood collections, the absorbance of each sample was spectrophotometrically measured against a 0.1% sodium carbonate blank at 650 nm. Time (in minutes) was semilogarithmically plotted against absorbance. A regression line was calculated, and the time required for the absorbance to be halved (T1/2) was determined.

Macrophage 5'Nucleotidase (5'N) Assay

Macrophage 5'nucleotidase activity, which has been shown to decrease as macrophage activation increases [34], was used to assess peritoneal macrophage activation in irradiated mice. In this assay, washed dishes of adherent macrophages were lysed with 200 μl of 0.05% Triton X-100 (Sigma Chemical Company, St. Louis, MO) in distilled water, and the cell lysate was used to measure 5'N activity. Specifically, 5'N was assayed with 0.15 mM ^{3}H-adenosine monophosphate (AMP) as substrate in 50 mM Tris buffer (pH 9.0) containing 12 mM magnesium chloride [15]. The specific activity is expressed as nmol AMP hydrolyzed per minute per mg cell protein at 37°C. The protein content of macrophage cell lysates was determined by the Bio-Rad procedure (Bio-Rad Labs, Rockville Center, NY) with bovine gamma globulin as a standard. Triplicate determinations were routinely performed for each assay.

Free-Radical Scavenger Assay

The ability of glucan to act as a free-radical scavenger was assayed by evaluating its reactivity with water radiolysis products in competition with methional as an alternate free-radical trap. In this system, the interaction of methional with free radicals results in the production of ethylene gas. A decrease in ethylene gas production can be interpreted as an agent's ability to compete with methional in the scavenging of free radicals. Ethylene measurements were performed according to the method initially described by Beauchamp and Fridovich [4] and later modified by Pryor and Tang [44]. The radical-assay solution contained 0.1 mM methional (Sigma Chemical Co. St. Louis, MO) in 50 mM phosphate buffer (pH 7.8), and 0.45% sodium chloride. Three milliliters of this solution were pipetted into 10-ml glass reaction vessels, and specified amounts of either D-glucose (Sigma Chemical Co., St. Louis, MO) or glucan were added to each vessel. The vessels were then sealed with Teflon-lined caps and irradiated. After irradiation, 50 μl of gas above each reaction solution was removed with a gas syringe, and the content of ethylene gas was measured by gas chromatography using a Porapac-Q 6-ft column with a Sigma 3B gas chromatograph coupled to a flame ionization detector (Perkin-Elmer, Norwalk, CN). The flow rate was typically 20 ml/min, with the injection chamber maintained at 140°C. Ethylene gas standards were obtained from Scott Speciality Gases (Plumsteadville, PA).

RESULTS

Effect of Glucan on Survival in Lethally Irradiated Mice

Figure 1 illustrates that 1.5 mg of glucan administered i.v. 1 day before a 9-Gy irradiation enhanced survival by 63% ($p < .05$, with respect to i.v. saline or D-glucose controls). This glucan dose and injection route were used to evaluate the effect of glucan on hemopoietic cells, bacterial translocation, granulocyte function, and RES clearance capacity in irradiated mice. However, to perform the 5'N assay in irradiated mice, i.p. injections were used to obtain the number of macrophages needed for this assay more easily. Thus, evaluation of the survival-enhancing effect of i.p. glucan administration was also necessary. In the same figure it can be seen that 1.5 mg of glucan administered i.p. also enhanced survival in otherwise lethally irradiated mice ($p < .05$, with respect to i.p. saline or D-glucose controls). However, i.p. glucan administration was less effective than i.v. administration and enhanced survival by only 26%. As expected, no survival enhancement was observed with either i.v. or i.p. administration of either saline or 1.5 mg of D-glucose.

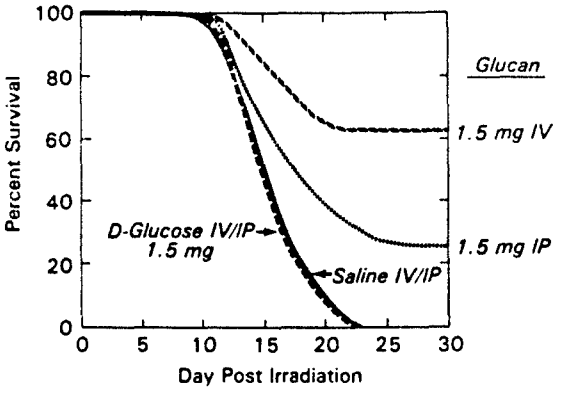

Fig. 1. Effect of route of glucan injection on survival in 9-Gy irradiated C3H/HeN mice. Saline, D-glucose, or glucan was administered 1 day before irradiation, and survival was monitored for 30 days. Data represent cumulative survival data obtained from three separate experiments and a total of 28–33 mice in each treatment group. No difference was observed between i.v. and i.p. saline and D-glucose treatments; thus data from both injection routes were pooled. These groups had a total of 56 and 61 mice, respectively.

Effect of Glucan on Hemopoietic Recovery in Lethally Irradiated Mice

Table 1 describes the bone marrow and splenic CFU-s and GM-CFC recovery observed in mice administered either saline or 1.5 mg of glucan 1 day before a 9-Gy irradiation. Both CFU-s and GM-CFC recovery commenced earlier in glucan-treated mice than in controls. However, hemopoietic activity was not detected in glucan-treated mice until day 13 postirradiation, and even by day 15 postirradiation (when ~65% of control mice were already dead) CFU-s and GM-CFC bone marrow

TABLE 1. Effect of Glucan on Hemopoietic Recovery in Lethally Irradiated Mice (Percent of Normal Control Values)*

	Day postirradiation				
	11	13	15	18	21
Bone marrow					
CFU-s/femur[a]					
Radiation	0	0	0	—[b]	—[b]
Glucan + radiation	0	0	0.15 ± 0.09	0.96 ± 0.16	2.56 ± 0.31
GM-CFC/femur[c]					
Radiation	0	0	0	—[b]	—[b]
Glucan + radiation	0	0.14 ± 0.11	0.49 ± 0.14	0.92 ± 0.26	1.22 ± 0.29
Spleen					
CFU-s/spleen[d]					
Radiation	0	0	0.02 ± 0.02	—[b]	—[b]
Glucan + radiation	0	0.51 ± 0.08	0.68 ± 0.12	12.94 ± 0.96	20.58 ± 2.06
GM-CFU/spleen[e]					
Radiation	0	0	0	—[b]	—[b]
Glucan + radiation	0	1.34 ± 0.22	4.22 ± 0.39	48.49 ± 3.67	262.25 ± 11.33

*Values represent means ± standard errors obtained by averaging data from 3–4 individual experiments.
[a]CFU-s per femur for normal control mice = 1,655.4 ± 55.1.
[b]Not enough animals surviving to test at these time points.
[c]GM-CFU per femur for normal control mice = 6,526.1 ± 118.3.
[d]CFU-s per spleen for normal control mice = 3,314.6 ± 96.5.
[e]GM-CFU per spleen for normal control mice = 1,592.4 ± 77.3.

values were only 0.15% and 0.49% of normal and splenic values were only 0.68% and 4.22% of normal.

Effect of Glucan on Bacterial Translocation in Lethally Irradiated Mice

Figure 2 illustrates the effect of glucan on bacterial translocation in lethally irradiated mice. Bacterial organisms could be detected in the spleens and liver of 10%–13% of both control and glucan-treated mice at 7 days postirradiation. The percent of mice with bacterial flora in these organs increased for both treatment groups through day 11 postirradiation. However, by day 11, significantly fewer glucan-treated mice exhibited splenic and hepatic bacterial flora than did control mice (25%–28% versus 42%–43%). In addition, by day 15 postirradiation, the percent of glucan-treated mice exhibiting bacterial organisms in the liver and spleen had decreased to ~10%, while it had risen to ~70%–80% in control mice. In both glucan-treated and control mice, the percent of animals exhibiting bacterial flora in the peripheral blood was less than that exhibited in the spleen and liver. In the peripheral blood, a bacterial translocation pattern similar to that observed in the spleen and liver was observed on days 11–15 postirradiation (ie, less translocation in glucan-treated than in control mice). However, at earlier time points (days 7 and 9), a greater percent of glucan-treated mice exhibited peripheral blood bacterial flora than did control mice (9%–13% versus 0%–7%). The most commonly observed organisms were *Proteus mirabilis*, *Staphylococcus aureus*, and *Escherichia coli*, and no species difference was observed in the organisms detected in the spleen, liver, or peripheral blood. In some instances, multiple types of bacterial organisms were observed in a single mouse. However, as illustrated in Figure 3, this consistently occurred more frequently in control mice than in glucan-treated mice.

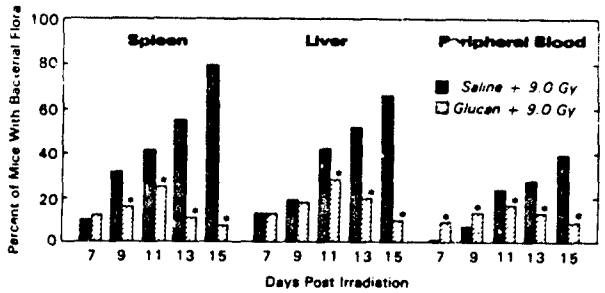

Fig. 2. Effect of glucan on bacterial translocation in 9-Gy irradiated C3H/HeN mice. Saline (control) or glucan (1.5 mg) was administered i.v. 1 day before irradiation. Data from three separate experiments were pooled and represent a total of 31–35 mice in each treatment group at each time point. Statistical differences were assayed by Student's t-test. *p < 0.05, with respect to control mice.

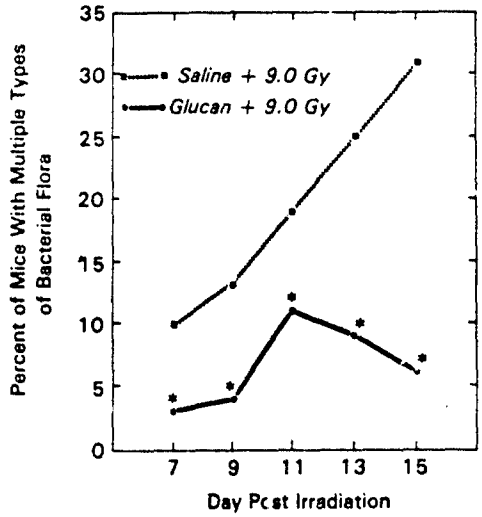

Fig. 3. Effect of glucan on multiple bacterial translocations in 9-Gy irradiated C3H/HeN mice. Saline (control) or glucan (1.5 mg) was administered i.v. 1 day before irradiation. Data from three separate experiments were pooled, and represent a total of 31–35 mice in each treatment group at each time point. Data represent the percent of bacterially infected mice that presented with more than one bacterial species. Statistical differences were assayed by Student's t-test. *p < 0.05, with respect to control mice.

Effect of Glucan on Granulocyte Chemiluminescence in Lethally Irradiated Mice

To evaluate the effect of glucan on granulocyte function, peripheral-blood granulocyte oxidative burst activity was measured by chemiluminescence. As can be seen in Figure 4, no significant differences were observed

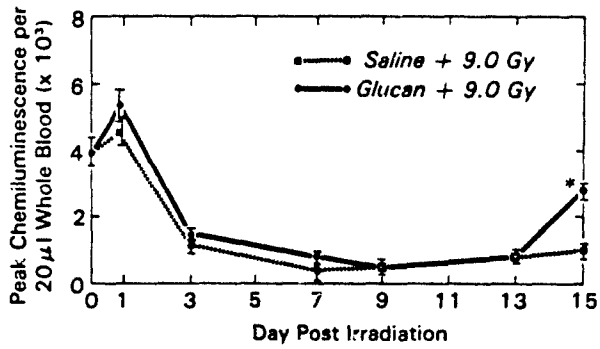

Fig. 4. Effect of glucan on opsonized zymosan-induced peripheral-blood granulocyte chemiluminescence in 9-Gy irradiated C3H/HeN mice. Mice were injected i.v. with saline (control) or glucan (1.5 mg) 1 day before irradiation. Each data point represents the mean ± standard error of data obtained from 8–9 individual mouse blood samples. Statistical differences were assayed by Student's t-test. *p < 0.05, with respect to control mice.

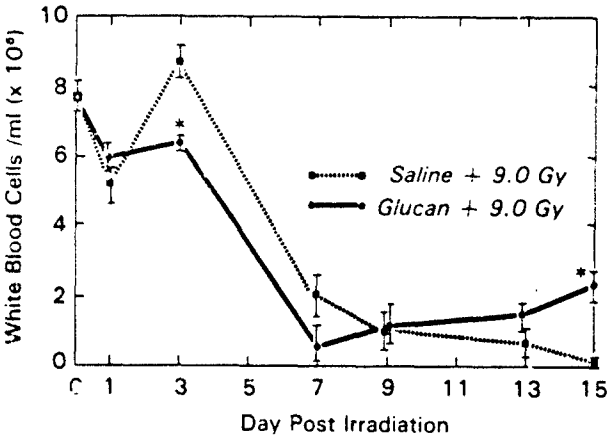

Fig. 5. Effect of glucan on white blood cellularity in 9-Gy irradiated C3H/HeN mice. Mice were injected i.v. with saline (control) or glucan (1.5 mg) 1 day before irradiation. Each data point represents the mean ± standard error of cell counts obtained from 8–9 individual mouse blood samples. Statistical differences were evaluated by Student's t-test. *p < 0.05, with respect to control mice.

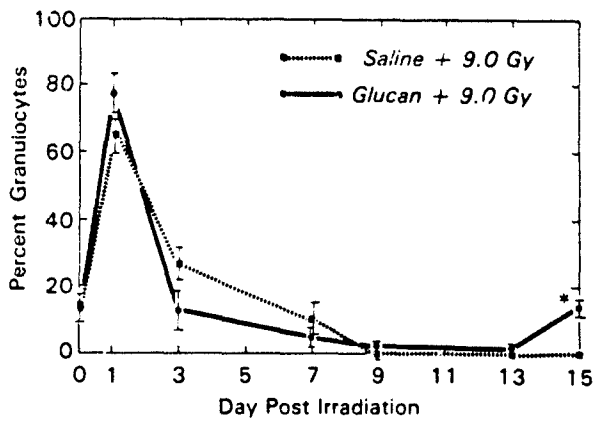

Fig. 6. Effect of glucan on peripheral blood granulocytes in 9-Gy irradiated C3H/HeN mice. Mice were injected i.v. with saline (control) or glucan (1.5 mg) 1 day before irradiation. Each data point represents the mean ± standard error of values obtained from 8–9 individual mouse blood samples. Statistical differences were assayed by Student's t-test. *p < 0.05, with respect to control mice.

between the oxidative burst activity of granulocytes obtained from glucan-treated and control mice until day 15 postirradiation, when the granulocyte response from glucan-treated mice was 140% of that from control mice. Likewise, peripheral-blood white cell numbers and the percent of peripheral-blood granulocytes were significantly elevated in glucan-treated mice 15 days postirradiation (Figs. 5 and 6).

Effect of Glucan on Carbon Clearance in Lethally Irradiated Mice

To evaluate the effect of glucan on RES clearance capacity, mice were assayed for their ability to clear intravenously injected colloidal carbon (Fig. 7). At day 1 postirradiation, glucan-treated mice cleared one-half of the injected carbon in just 6.8 min, compared to 8.2 min in control mice. However, at days 3 and 5 postirradiation, carbon clearance in glucan-treated mice was dramatically slower than in control mice (9.5–9.6 min versus 6.1–6.7 min). This was followed on days 7–11 postirradiation by a period when the T1/2 values of glucan-treated and control mice were approximately equal. However, by days 13 and 15 postirradiation, the carbon clearance T1/2 in control mice had increased dramatically, whereas that in glucan-treated mice had decreased. On day 15 postirradiation, control mice cleared half of the injected carbon in 9.3 min, while glucan-treated mice cleared the same amount in only 5.5 min.

Effect of Glucan on Macrophage 5'Nucleotidase Activity in Lethally Irradiated Mice

To evaluate the effect of glucan on macrophage activation, peritoneal macrophage 5'N activity was assayed. Figure 8 illustrates that macrophages from both control and glucan-treated mice exhibited a rapid and dramatic decrease in 5'N activity (indicative of macrophage acti-

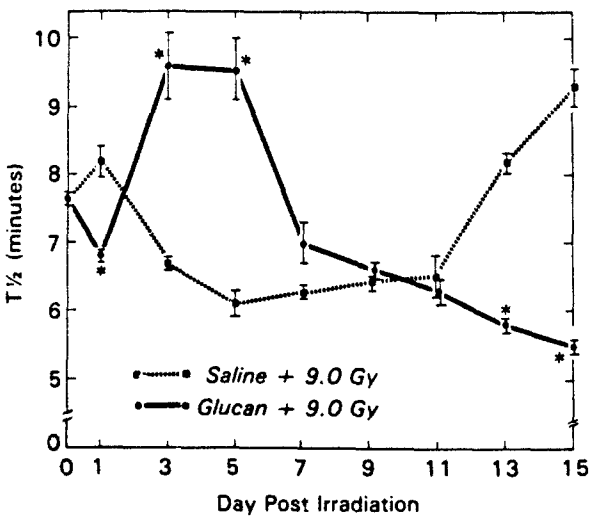

Fig. 7. Effect of glucan on reticuloendothelial-system carbon clearance in 9-Gy irradiated C3H/HeN mice. Mice were injected i.v. with saline (control) or glucan (1.5 mg) 1 day before irradiation. Each data point represents the mean ± standard error of T1/2 values obtained from 7–9 individual mice. Statistical differences were assayed by Student's t-test. *p < 0.05, with respect to control mice.

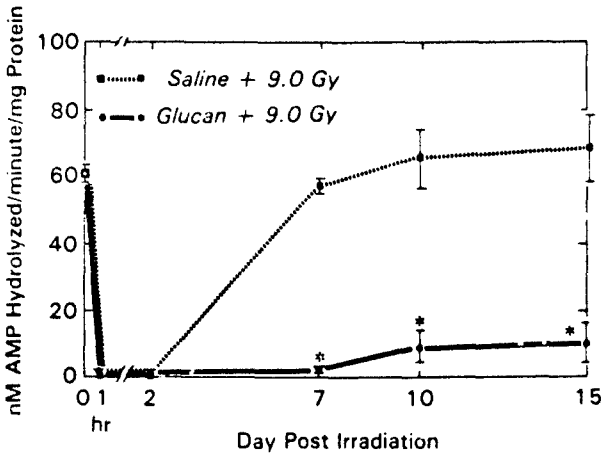

Fig. 8. Effect of glucan on peritoneal macrophage 5'N activity. Mice were injected i.p. with saline (control) or glucan (1.5 mg) 1 day before irradiation. Each data point represents the mean ± standard error of the specific activities from 3–4 separate experiments. In each experiment, peritoneal exudate cells were pooled from 8–10 mice. Statistical differences were determined by Student's t-test. *$p < 0.05$, with respect to control mice.

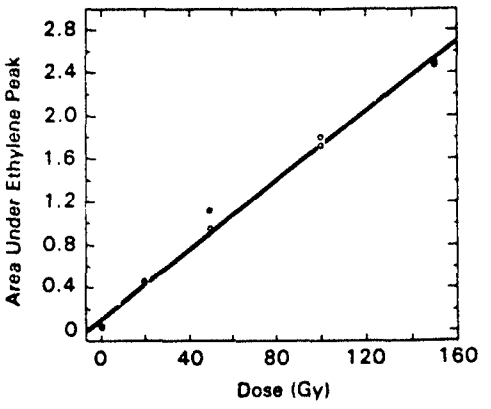

Fig. 9. Effect of cobalt-60 gamma rays on ethylene yields in a 0.1 mM methional/50 mM phosphate buffer solution. The ethylene in a 50-μl aliquot of gas above the reaction solution was analyzed by gas chromatography ~24 hours after irradiation. Each symbol represents an individual sample. Based on least-squares line of fit, $p < 0.0001$.

vation) which persisted through day 2 postirradiation. At these times, 5'N activity was barely detectable in either treatment group. Macrophage 5'N activity in control mice returned to preirradiation levels at days 7, 10, and 15 postirradiation (~60 nmol AMP/min/mg). However, macrophage 5'N activity in glucan-treated mice remained significantly reduced (2–10 nmol AMP/min/mg), indicating macrophage activation through day 15 postirradiation.

Ability of Glucan to Scavenge Radiation-Produced Free Radicals

To address the possibility that once inside macrophages glucan may protect these cells through chemical means (and hence enhance host resistance by enhancing the survival of macrophages), glucan's ability to scavenge free radicals was assayed. A standard curve for the test system used in these experiments, ie, the production of ethylene gas following the irradiation of a methional solution, is shown in Figure 9. In repeated experiments, the dose response for ethylene production from methional was linear over the broad radiation dose range of 0 to 150 Gy. Figure 10 illustrates the effect of glucan or D-glucose (control) on ethylene gas yields in this system. Experiments were performed with a 100-Gy radiation exposure, and data were normalized to the ethylene gas produced by methional after exposure to 100 Gy with no test agent present. These results indicate that glucan is more effective than D-glucose in competing with methional for radiolysis products. Scavenger activity with D-glucose was observed only at D-glucose concentrations greater than 0.083 mg/ml, whereas scavenger activity with glucan was observed at glucan concentrations as low as 0.0075 mg/ml.

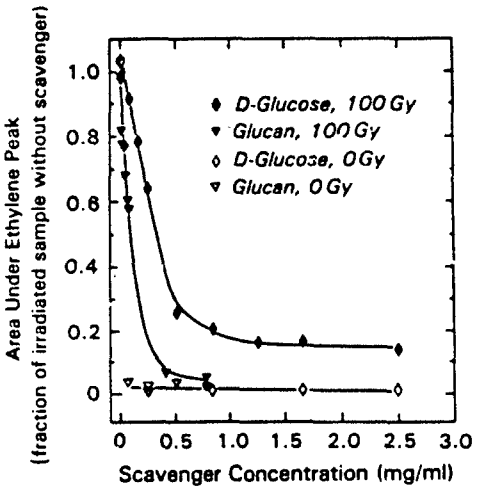

Fig. 10. Effect of glucan or D-glucose (control) on ethylene yields in nonirradiated and 100-Gy irradiated methional solutions. Ethylene in a 50-μl aliquot of gas above each methional-based reaction solution was determined as described in the legend of Figure 9. Data were normalized to the area under the ethylene peak for the 0 Gy or 100 Gy irradiated samples with no test agent present.

DISCUSSION

It has been postulated that most biologically derived agents that are radioprotective in the hemopoietic-syndrome dose range function by enhancing hemopoietic recovery and, hence, by regenerating mature cells necessary for the irradiated host to resist otherwise life-threatening opportunistic infections [5,20,22,31–33, 50,53]. Recently, we demonstrated radioprotection by yet another biologically derived agent, glucan, and reported glucan's ability to not only enhance survival specifically in the hemopoietic-syndrome dose range [37,40], but also to enhance hemopoietic stem cell regeneration in sublethally irradiated mice [38,39,41]. Although there is no doubt that hemopoietic recovery is absolutely necessary for long-term survival following otherwise lethal irradiation [3,10,17], significant hemopoietic regeneration in lethally irradiated glucan-treated mice did not appear to occur early enough to account for the increased survival observed in these animals in the first few weeks postirradiation (Fig. 1; Table 1). Thus, further studies were initiated to evaluate additional mechanisms through which glucan may mediate its radioprotective effects prior to detectable hemopoietic recovery.

The bacteriological studies presented here clearly demonstrated enhanced host resistance to microbial invasion in glucan-treated mice as early as 9–11 days postirradiation (Fig. 2). Not only were glucan-treated mice more resistant to microbial invasion, but in addition they were actually able to arrest the progressive increase in opportunistic infections seen in control mice. This ability to arrest opportunistic infections successfully became clearly apparent after day 11 postirradiation, when glucan-treated mice not only exhibited a progressive decrease in bacterial infections, but also generally presented with single, as opposed to multiple, bacterial species (Fig. 3). Correlated with this increased ability to resist opportunistic infections was the fact that 63% of glucan-treated mice (compared to 0% of control mice) survived the radiation insult (Fig. 1).

The fact that glucan-treated irradiated mice exhibited enhanced host resistance to microbial invasion prior to the detection of significant numbers of new hemopoietic elements prompted investigation into glucan's ability to enhance and/or to prolong the function of mature cell populations present at the time of irradiation. Because both granulocytes and macrophages have been shown to play critical roles in nonspecific host resistance against microbial invasion [21,27], and because these cells have been shown to be relatively radioresistant in comparison to lymphocytes and hemopoietic stem cells [2,7,26, 29,42,52], glucan's effect on these specific cells was evaluated.

Experiments with granulocytes (Figs. 4–6) demonstrated that in both control and glucan-treated mice, granulocyte numbers and granulocyte oxidative burst activity were critically reduced by 1 week postirradiation. In control mice, these parameters showed no signs of recovery prior to death while in glucan-treated mice, both granulocyte numbers and granulocyte oxidative burst activity commenced recovery at day 15 postirradiation. These recoveries, however, occurred too late to explain the enhanced resistance to microbial invasion observed in glucan-treated mice prior to day 15 postirradiation.

While the studies presented in this paper did not suggest that granulocytic mechanisms were involved in glucan's immediate ability to enhance resistance to microbial invasion in irradiated mice, they did strongly implicate the involvement of macrophage mechanisms in this phenomenon. This is perhaps not surprising since, in normal animals, glucan has been shown to enhance macrophage function dramatically [12,13,57] and to increase nonspecific host resistance to a variety of bacterial, viral, fungal, and parasitic infections [for review see reference 12]. Although it appeared that in irradiated mice glucan may also enhance host resistance to infections via macrophage activation, the interpretation of these studies is complicated by the fact that radiation alone can activate macrophages [2,6,7,11,36].

The carbon clearance studies presented in this paper confirmed this phenomenon in that an increased clearance capacity was observed in control mice on days 3–11 postirradiation (Fig. 7). While it was suspected that glucan-treated mice would exhibit an even faster carbon clearance capacity than control mice, on days 3, 5, and 7 postirradiation, they actually cleared carbon less efficiently than controls (Fig. 7). This phenomenon seemed paradoxical; however, it could be explained if following irradiation a RES blockade was established in glucan-treated mice. The glucan used in these experiments was particulate in nature [14] and has been shown to be selectively taken up by macrophages [19]. In addition, even under normal circumstances, macrophages have been shown to phagocytize necrotic lymphocytes and nuclear debris within hours after irradiation [2,25]. Thus, a RES blockade could have resulted in glucan-treated mice owing to the phagocytosis of not only a large number of glucan particles, but also large quantities of radiation-induced debris. Such a RES blockade may also have contributed to the enhanced hemopoietic regeneration ultimately observed in glucan-treated irradiated mice (Table 1) since RES blockade has previously been reported to aid in hemopoietic repopulation in irradiated mice by preventing the phagocytosis of slightly injured, yet still functional, hemopoietic stem cells [35]. Similarly, a RES blockade may have prevented the phagocytic loss of mature functional hemopoietic cells capable of effective defense against microbial invasion in the first few weeks postirradiation. The glucan-induced RES blockade, how-

ever, only persisted through the first week postirradiation. After this time, macrophages in glucan-treated mice appeared to regain their phagocytic capacity. It is known that within a week a large portion of intravenously administered glucan is broken down and metabolized [19]. Thus, the ability of macrophages in glucan-treated irradiated mice to regain their phagocytic activity in the second week postirradiation coincided with a time frame in which most intracellular glucan should have been broken down and metabolized and suggested that once "emptied," glucan-activated macrophages could again commence phagocytosis. By day 9 postirradiation, the clearance capacities of control and glucan-treated mice were identical and at days 11, 13, and 15 postirradiation, the clearance capacity of glucan-treated mice continued to increase while that of control mice progressively decreased. Interestingly, the day 11 time point at which glucan-treated mice began to surpass control mice in their clearance capacity coincided with the time point at which glucan-treated mice also began to arrest microbial invasion by opportunistic pathogens. In addition, at day 15 postirradiation, when opportunistic pathogens were detected in ~80% of the control mice and only ~10% of the glucan-treated mice, glucan-treated mice were capable of clearing carbon twice as rapidly as controls. In support of these RES studies, the 5'N studies presented in this paper also demonstrated that macrophage activation differed in control and glucan-treated mice in the first few weeks postirradiation. In these studies, peritoneal macrophage activation was observed in both control and glucan-treated mice immediately after irradiation (Fig. 8). However, in control mice this activation disappeared by day 2 postirradiation and in glucan-treated mice it persisted throughout the 15-day postirradiation observation period. Thus, both the RES and the 5'N data presented here demonstrated glucan's ability to enhance macrophage function profoundly in irradiated mice, and supports the contention that, in the first few weeks after irradiation, glucan may enhance resistance to opportunistic pathogens (and hence survival) via macrophage-mediated mechanisms.

Although glucan appeared to mediate its radioprotective effects by enhancing and/or prolonging macrophage function, the possibility that even prior to this phenomenon glucan may radioprotect by traditional radical-scavenging mechanisms could not be excluded. On the cellular level, injury following exposure to ionizing radiation has been shown to be attributed to hydroxyl radicals [4], as well as secondarily produced organic radicals [44,56] and hydrogen peroxide reaction products [55], which can result in damage to the cellular DNA, enzymes, and membranes [16,18,45,46]. The possibility that glucan may act to prevent such radiation damage was suggested by the experiments presented in Figure 10 in which glucan's ability to trap free radicals chemically was clearly demonstrated. Since glucan has been shown to be selectively taken up by and sequestered in macrophages [19], once inside these cells glucan may maintain its ability to scavenge free radicals and, thus, selectively protect these cells. If this occurs, it may explain why macrophages in glucan-treated mice function longer and better than macrophages in control mice after irradiation. Studies to determine realistic macrophage intracellular glucan concentrations at the time of irradiation and to elucidate the exact radioprotective potential of glucan once inside purified macrophage cell populations are currently in progress in our laboratories.

In conclusion, the results of these studies suggest the critical role of macrophages (but not granulocytes) in mediating glucan's antimicrobial and hence early survival-enhancing effects in irradiated mice. Because macrophage activation and enhanced macrophage function could be detected as early as 1-24 h after irradiation, it appeared that the macrophages responsible for these responses were "radiation survivors" and did not arise from glucan-induced hemopoietic repopulation which did not become evident until days 13-15 postirradiation. However, from these studies it was impossible to discern if at such later times (eg, day 15 postirradiation), "old surviving," "newly produced," or both types of macrophages were responsible for the responses measured. In spite of this, these studies shed new light on additional mechanisms by which "hemopoietic stimulants" may enhance survival in irradiated mice. Whether additional cellular and/or chemical mechanisms are also involved in glucan's radioprotective effect remains to be determined.

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