

Histopathologic Effects of Soluble Glucan and WR-2721, Independently and Combined in C3H/HeN Mice (43497)

WILLIAM H. BAKER,*¹ JAMES B. NOLD,[†] MYRA L. PATCHEN,* AND WILLIAM E. JACKSON*

Armed Forces Radiobiology Research Institute,* Bethesda, Maryland 20889-5145 and Hazelton Laboratory,[†] Department of Pathology, Madison, Wisconsin 53707

Abstract. Soluble glucan, an immunomodulator, and Walter Reed (WR)-2721, a radioprotectant, increase postirradiation survival when administered before and after exposure, respectively. Combined, these agents act synergistically through WR-2721's ability to spare hematopoietic stem/progenitor cells from radiation injury and glucan's ability to subsequently stimulate spared cells to proliferate. In this study, the histopathologic effects of WR-2721 (200 mg/kg, ip) and glucan (250 mg/kg, iv), at doses capable of increasing survival in lethally irradiated mice, were evaluated in unirradiated and irradiated female C3H/HeN mice. After treatment, whole body weights and wet organ weights of liver, spleen, and kidney, as well as gross and histologic changes in these and other tissues, were monitored on Days 1, 4, 7, 11, 15, 21, and 28. Morphometric studies of splenic white and red pulps were also performed. Soluble glucan, with or without WR-2721, in unirradiated groups, was associated with splenomegaly, transient morphometrically determined perturbations of white and red pulp areas, and histologic alterations of white pulp. In irradiated mice, splenic weight loss was initially dampened in glucan groups and accompanied by morphologic and histologic changes similar to those seen in unirradiated counterparts. The subsequent rebound of splenic parameters in irradiated mice was limited to WR-2721-treated mice and was associated with hematopoietic reconstitution. Glucan, with or without WR-2721, in unirradiated groups was associated with transient hepatomegaly and associated histologic changes. Similar changes in irradiated animals were seen only in the combined treatment group.

[P.S.E.B.M. 1992, Vol 201]

Survival following lethal exposure to γ -radiation in mice can be enhanced by both immunomodulators and aminothiols radioprotectants (1-4). Glucan, a β -1,3-polyglucosepolymer isolated from the inner wall of the yeast *Saccharomyces cerevisiae*, is one such survival-enhancing immunomodulator (3, 5). Glucan-mediated survival enhancement is correlated with enhanced and/or prolonged macrophage function, as well as with accelerated hematopoietic regeneration (5-9). Walter Reed (WR)-2721 (ethiofos, S-2(3-aminopropylamino)ethylphosphorothioic acid), on the other

hand, is a traditional sulfhydryl radioprotectant (10, 11). The mechanisms by which WR-2721 protects cells from radiation-induced lethality include free radical scavenging, hydrogen atom donation, and induction of hypoxia (1). In addition to the ability of glucan and WR-2721 to individually enhance survival, synergistic survival enhancement can be obtained with the agents used in combination. Specifically, glucan and WR-2721 used individually and in combination have resulted in dose-reduction factors (radiation dose lethal for 50% of treated animals divided by radiation dose lethal for 50% of control animals) of 1.08, 1.37, and 1.52, respectively (1).

Although beneficial in enhancing survival, these agents are not without side effects. Particulate forms of glucan have been reported to induce hepatosplenomegaly, granuloma formation, enhanced susceptibility to endotoxin, and microembolization (12-16). Severe granuloma formation, enhanced susceptibility to endotoxin, and microemboli have not been reported after

¹ To whom requests for reprints should be addressed at Defense Nuclear Agency, Armed Forces Radiobiology Research Institute, Bethesda, MD 20889-5145.

Received September 17, 1991. [P.S.E.B.M. 1992, Vol 201]
Accepted March 25, 1992.

0037-9727/92/2012-0180\$3.00/0
Copyright © 1992 by the Society for Experimental Biology and Medicine

administration of more recently produced soluble glucan preparations. However, a recent preclinical safety evaluation demonstrated splenomegaly and mononuclear infiltrates in the liver after intravenous injection of 1000 mg/kg of soluble glucan over an extended period (16). Such an extremely high dose of glucan, however, is well above the levels needed to enhance survival in irradiated mice. High doses of WR-2721, as well as inducing performance decrements (17), have produced renal tubular epithelial degeneration and necrosis in lymphoid tissues (spleen, lymph nodes, and thymus) (18). The doses of WR-2721 used for radioprotection are below the levels that produce such lesions. Histologic evaluation of combined WR-2721 and glucan has not been reported.

The present study investigates the histopathologic effects of soluble glucan and WR-2721 alone and combined. This study was done in unirradiated and irradiated female C3H/HeN mice using drug doses previously shown effective at promoting hematopoietic recovery and enhancing survival following exposure to lethal doses of ^{60}Co radiation.

Materials and Methods

Mice. C3H/HeN female mice (approximately 20 g) were purchased from Charles River Laboratories (Raleigh, NC). Mice were maintained in an accredited American Association for Accreditation of Laboratory Animal Care (AAALAC) facility in Micro-Isolator cages on hardwood-chip contact bedding and were provided commercial rodent chow and acidified water (pH 2.5) *ad libitum*. Animal rooms were maintained on a 12:12-hr light:dark cycle at $70^{\circ}\text{F} \pm 2^{\circ}\text{F}$, $50\% \pm 10\%$ relative humidity, and at least 10 air changes per hour of 100% conditioned fresh air. Upon arrival, all mice were tested for *Pseudomonas* and quarantined until test results were obtained. Only healthy mice were released for experimentation. All animal experiments were approved by the Institute of Animal Care and Use Committee before performance.

WR-2721 and Glucan. Stock WR-2721 was obtained from Dr. David Davidson (Walter Reed Army Institute of Research, Washington, DC) and kept frozen at -20°C until use. Immediately before each use, the appropriate amount of WR-2721 was weighed out and dissolved in sterile pyrogen-free saline. Exposure of the material to light was minimized. Mice were injected intraperitoneally with 200 mg/kg of WR-2721 in a volume of 0.5 ml. This dose of WR-2721 has been shown to have minimal side effects in mice (18). Glucan used in these studies was a soluble endotoxin-free preparation purchased from Tulane University School of Medicine, New Orleans, LA. Preparation of this glucan (which has sometimes been referred to as glucan-F) has been described previously by DiLuzio *et al.* (19). Mice were injected intravenously in the lateral tail vein with

250 mg/kg of glucan in a volume of 0.5 ml. In combined treatments, mice were administered WR-2721 30 min before irradiation and glucan 60 min after irradiation. Unirradiated mice were injected with glucan approximately 90 min after WR-2721, thereby simulating protocols used in irradiated mice. Control mice were injected intraperitoneally or intravenously with 0.5 ml of normal saline.

Irradiation. Mice were placed in plastic containers and exposed bilaterally to 9.0 Gy total body gamma rays at a dose rate of 0.4 Gy per min in a ^{60}Co source. Dosimetry was determined by ionization chambers as described previously (20).

Experimental Design. Mice were randomly assigned to the following treatment groups: control (CON), glucan (GLU), WR-2721 (WR), combined WR-2721 and glucan (WR/GLU), radiation control (RC), radiation + glucan (RG), WR-2721 + radiation (WR/R) and combined WR-2721 and glucan with interposed radiation exposure (WR/R/GLU). On Days 1, 4, 7, 11, 15, 21, and 28 after treatment, six CON mice and five mice from each treatment group were sacrificed by cervical dislocation, and total body weights and wet organ weights for the liver, left kidney, and spleen were recorded. Samples of the following tissues were then collected and immersed in 10% neutral buffered formalin: brain, bone marrow (left femur), lung, thymus, heart, liver, left kidney, spleen, adrenal glands, stomach, small intestine, urinary bladder, mesenteric lymph node, and pancreas. After adequate fixation, tissues were trimmed, dehydrated in graded alcohols, cleared, embedded in paraffin, cut at $6\ \mu\text{m}$, and stained with hematoxylin and eosin for qualitative and morphometric microscopic examination. Slides were labeled and coded, and morphometric and qualitative evaluations were performed by a boarded pathologist without knowledge of which treatment group was being examined.

Morphometry. Area measurements of the spleen and splenic substructures were obtained by planimetry, using a computer-assisted semiautomated image analysis program (Bioquant IV; R & M Bionetics, Nashville, TN) in conjunction with a digitizing tablet and microscope equipped with a fixed-arm reflector tube. The digitizing tablet was calibrated in the metric system, and corrective factors for each microscope objective lens were entered into the computer program. The splenic areas measured included the total splenic area and white pulp area. The red pulp area was calculated by subtracting the white pulp area from the total splenic area. Percentages of the white and red pulp areas were calculated using planimetric area measurements (21). These percentages were subsequently used to determine the component weights of the white and red pulps.

Qualitative Evaluation. Grading of hematopoietic cellularity in splenic red pulp sinusoids and bone mar-

row was done by scoring cellularity numerically: 0 (severe hypocellularity), no foci of hematopoiesis to occasional hematopoietic clusters over several high power fields ([HPF], $\times 400$ magnification); 1 (mild), 1–2 clusters of hematopoietic cells/HPF; 2 (moderate), 3–5 hematopoietic clusters/HPF; and 3 (confluent), >5 hematopoietic clusters/HPF. Megakaryocytes in 2 HPF of bone marrow and splenic red pulp were counted and averaged/HPF. Lymphoid cellularity in the white pulp was scored as: 0, severe lymphoid depletion; 1, moderate lymphoid depletion; 2, mild lymphoid depletion; and 3, normal lymphoid cellularity. Additionally, white pulp foci of single cell necrosis were counted in 3 HPF/spleen and averaged/HPF. Liver lesions were scored 0 (no lesions), no cellular foci within the section; 1 (minimal), 1–10 foci/10 \times field; 2 (mild), 11–20 foci/10 \times field; and 3 (moderate), >20 foci/10 \times field.

Statistics. Before statistical analysis was done, a log transformation was made on body and organ weights, and an arcsine transformation was used on percentages. The *t* test was then used to compare these data. Comparisons of scored data were made using the Mann-Whitney *U* test. *P*-values of <0.05 were considered significant.

Results

Effects of WR-2721, Glucan, and Combined Treatment in Unirradiated Mice. *Effects on body weights and splenic weights.* No significant differences in body weight were observed. All groups displayed total body weight gain patterns similar to CON mice, with net weight gain recorded for each group (Table I). Splenic weights of CON and WR mice were similar, with the exception of Days 4 and 28, when WR values were significantly less (Fig. 1A). Elevated splenic weights observed in GLU and WR/GLU mice throughout the study were significantly greater than weights of CON and WR mice.

Quantitation of splenic white and red pulp areas and weights. Significant differences in white and red pulp areas between groups were limited to the first 11 days. As demonstrated in white pulp to red pulp ratios (W:R ratios) (Table II), GLU and WR/GLU white pulp areas were significantly elevated above CON on Day 1 and decreased to levels significantly less than CON by Day 4. Subsequently, W:R ratios of all groups remained near or slightly below unity, excluding Day 11, when the WR/GLU white pulp area approached a level significantly greater than CON, WR, and GLU. Distinctive microscopic changes noted in GLU and WR/GLU white pulps on Day 1 are described below.

The white pulp weights of the WR group remained below CON weights throughout the study; however, differences were not significant (Fig. 2A). WR/GLU and GLU white pulp weights were significantly greater than CON within the first 24 hr, but decreased by Days 7 and 11, respectively, to values not significantly different from CON or WR. Subsequently, white pulp weights in WR/GLU and GLU increased on Days 11 and 15, respectively, to levels significantly above CON and WR weights and remained elevated thereafter.

CON and WR red pulp weights remained at a consistent level, with a significant difference noted only on Day 28, when WR red pulp weight was reduced. GLU and WR/GLU red pulp weights on Day 1 were not significantly different from CON or WR; however, by Day 4, they were markedly increased and remained significantly elevated above CON and WR weights throughout the study (Fig. 3A).

Gross and microscopic changes in the spleen. On gross examination, GLU and WR/GLU spleens were enlarged throughout the study. Microscopic qualitative changes associated with increased proportions of white pulp in GLU and WR/GLU spleens consisted of accumulations of a large cell population at white pulp peripheries, and an admixture of a remnant population

Table I. Body Weights in Grams of Unirradiated and Irradiated Mice

Treatment Group	Days after treatment						
	1	4	7	11	15	21	28
Unirradiated groups							
CON	22.8 \pm 0.8	22.9 \pm 1.40	24.3 \pm 0.7	23.5 \pm 0.7	22.0 \pm 0.8	24.1 \pm 0.7	25.5 \pm 0.7
GLU	21.5 \pm 0.7	21.7 \pm 0.80	22.5 \pm 0.6	23.3 \pm 0.4	23.3 \pm 0.4	24.1 \pm 0.7	25.3 \pm 1.2
WR	21.3 \pm 1.0	21.9 \pm 0.40	22.5 \pm 0.6	23.7 \pm 0.6	23.0 \pm 0.8	24.5 \pm 0.9	24.1 \pm 0.4
WR/GLU	20.9 \pm 0.6	20.7 \pm 0.03	21.7 \pm 1.4	22.3 \pm 0.7	23.8 \pm 1.0	22.9 \pm 1.2	24.6 \pm 0.9
Irradiated groups							
RC	22.1 \pm 0.5	22.3 \pm 0.5	22.2 \pm 0.6	19.8 \pm 0.3	17.1 \pm 0.5	NS ^a	NS
RG	20.3 \pm 1.2 ^b	21.4 \pm 0.3	20.2 \pm 0.4 ^{b,c}	17.0 \pm 0.5 ^c	15.8 \pm 0.4	NS	NS
WR/R	23.5 \pm 0.5	21.9 \pm 0.7	22.1 \pm 0.5	22.3 \pm 0.9 ^{c,d}	22.9 \pm 0.7 ^{c,d}	22.9 \pm 0.9	24.4 \pm 0.9
WR/R/GLU	21.1 \pm 0.7 ^b	20.9 \pm 0.5	19.9 \pm 1.0	21.8 \pm 1.0 ^d	23.5 \pm 0.5 ^{c,d}	23.0 \pm 1.2	23.7 \pm 0.5

^a NS, no survivors.

^b Significantly different from WR/R.

^c Significantly different from RC.

^d Significantly different from RG.

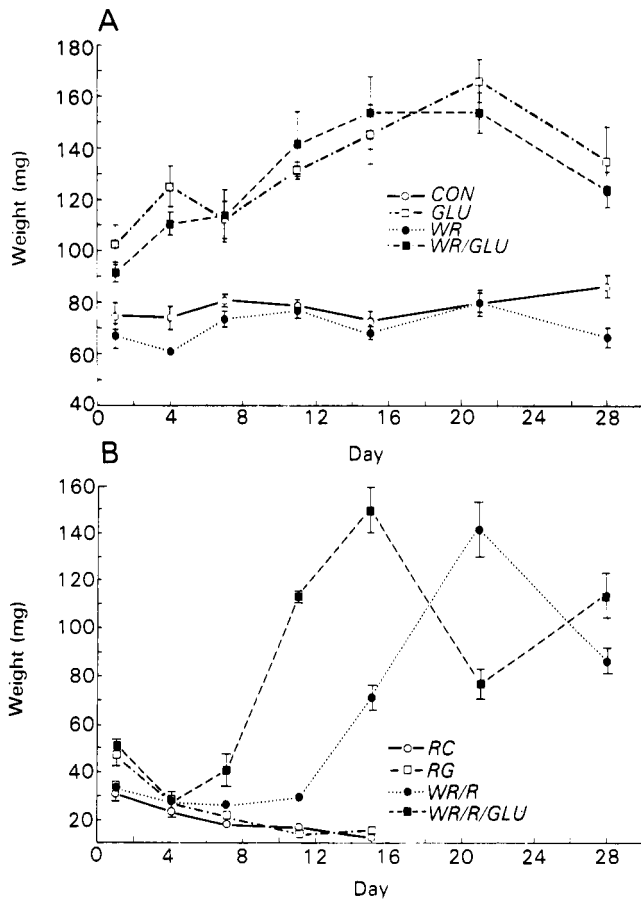


Figure 1. Effects of GLU and WR individually and in combination on splenic weight of (A) unirradiated and (B) irradiated mice.

of small lymphocytes and foci of single cell necrosis in inner periarteriolar sheath regions. Accumulations (Fig. 4A) consisted of coalescing aggregates of numerous cells 15–20 μm in diameter interpreted light microscopically as large lymphocytes and macrophages. By Day 4, cellular accumulations in GLU and WR/GLU groups decreased, and the proportion of small lymphocytes increased, coincident with the decrease in white pulp

areas. Beyond Days 7 and 11, respectively, WR/GLU and GLU white pulps were essentially normal, with only occasional large cell aggregates observed on Days 7 and 11. Regardless of these changes, WR/GLU and GLU white pulp cellularities were judged abundant (3.0).

Single cell necrosis within the white pulp consisted of karyorrhectic and pyknotic debris within the interstitium or within macrophages (tingible body macrophages) (Fig. 4). GLU and WR/GLU spleens displayed significantly greater numbers of foci of necrosis than CON or WR spleens on Day 1, with foci in WR/GLU spleens being more numerous than in GLU spleens. After Day 1, there was a marked decrease of necrosis in both glucan-treated groups to levels lower than that of the CON group.

Splenic red pulp cellularity, assessed by judging relative quantities of erythropoietic aggregates, megakaryocytes, and granulopoietic foci, was abundant (3.0) for all groups on all sample days, excluding GLU spleens on Days 1 and 4 and WR/GLU spleens on Day 1 (all moderate). In general, decreased cellularity in GLU and WR/GLU spleens was attributed primarily to decreased hematopoietic tissue characterized by individual clusters of erythrocytic and myelocytic cells. This contrasted with the coalescing to confluent aggregates of hematopoietic cells noted in CON and WR spleens. Additionally, sinusoids adjacent to these islands of hematopoiesis were engorged with numerous red blood cells (congestion). On subsequent sample days, all groups displayed comparable cellularities, and congestion was resolved. There was no significant difference in the numbers of megakaryocytes/HPF among treatment groups or between treatment groups and CON. Prominent foci of granulopoiesis, occasionally noted subjacent to the splenic capsule and along trabeculae, were not associated with a particular treatment group or time frame.

Effects on liver. Liver weights in all groups in-

Table II. White to Red Pulp Ratios of Unirradiated and Irradiated Mice

Treatment group	Days after treatment						
	1	4	7	11	15	21	28
Unirradiated groups							
CON	0.67	0.93	1.02	0.64	0.72	0.81	0.75
GLU	1.30	0.59	0.61	0.44	0.85	0.87	0.87
WR	0.43	0.63	0.87	0.62	0.70	0.74	0.90
WR/GLU	1.20	0.50	0.54	1.06	0.94	0.88	1.03
Irradiated groups							
RC	0.31	0.26	0.34	0.24	0.10	NS ^a	NS
RG	0.59	0.26	0.31	0.27	0.14	NS	NS
WR/R	0.37	0.27	0.36	0.38	0.27	0.21	0.33
WR/R/GLU	0.64 ^b	0.22	0.30	0.22	0.16	0.41	0.32

^a NS, no survivors.

^b Significantly different from RC and WR/R.

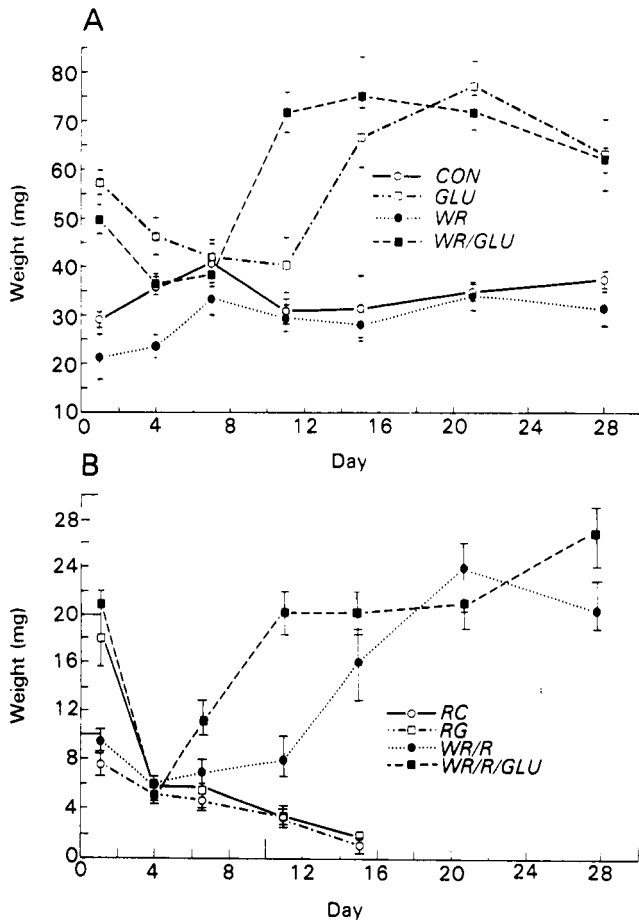


Figure 2. Effects of GLU and WR individually and in combination on splenic white pulp weight of (A) unirradiated and (B) irradiated mice. Each point represents the mean product of individual splenic weights and percentage of white pulp area, derived morphometrically from histologic sections.

creased over the first 21 days, with a slight decrease in treatment groups by Day 28 (Fig. 5A). Throughout the study, WR and WR/GLU liver weights were comparable to CON and GLU, respectively. GLU weights were significantly greater than CON and WR weights on Days 7, 11, 15, and 21 and Days 11, 15, and 21, respectively; WR/GLU weights were significantly greater than CON and WR weights on Day 15 and on Days 4 and 15, respectively.

On gross examination, livers from both groups of glucan-treated mice displayed occasional, ≤ 1 mm in diameter, light tan foci subjacent to the organ capsule. These foci were most prominent on sample Days 11–21. Microscopic changes within the liver of WR, GLU, and WR/GLU groups were inflammatory in nature, with lesions in WR mice limited to normal background lesions noted in CON animals. Histologic changes in GLU and WR/GLU groups were comparable with initial minimal acute to subacute hepatitis, building to mild-to-moderate granulomatous hepatitis by Day 21 and initiating regression by Day 28 (Fig. 6A). Day 1 lesions consisted predominantly of randomly scattered

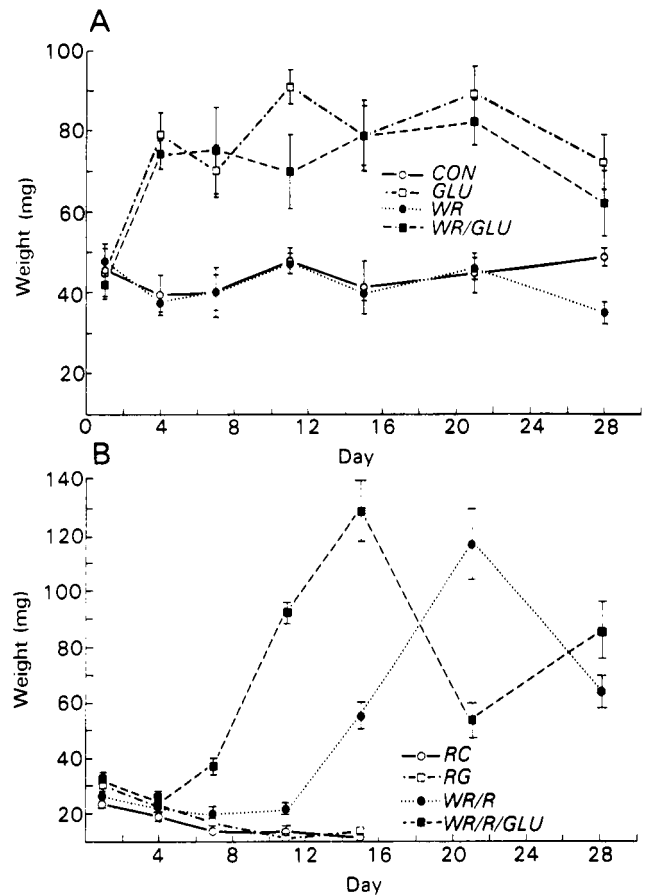


Figure 3. Effects of GLU and WR individually and in combination on splenic red pulp weight of (A) unirradiated and (B) irradiated mice. Each point represents the mean product of individual splenic weight and percentage of red pulp area, derived morphometrically from histologic sections.

40–60- μ m diameter foci of neutrophils with an occasional macrophage. By Day 4, macrophages were the principal cell type admixed with neutrophils, lymphocytes, cellular debris, and infrequent degenerating hepatocytes (Fig. 7, inset). Infrequently, cellular debris was also evident in sinusoids and central veins. By Day 7, involvement was still minimal, but a slight increase in the number and size of foci was evident. There were also noticeable accumulations of mononuclear cells, macrophages, and lymphocytes, directly subjacent to the endothelium of larger efferent vessels (Fig. 7). Portal regions, for the most part, were spared, except in several sections in which there was occasional minimal subacute pericholangitis. By Day 11 and continuing to Day 15, foci were larger (100–200 μ m at greatest dimension) and consisted of macrophages, lymphocytes, and increased numbers of degenerating and necrotic hepatocytes. The number and severity of subendothelial foci increased, and additional foci were present beneath the liver capsule (Fig. 7). On Day 21, inflammatory changes were at a maximum, based on number and cumulative area of foci; however, there was a minimal decrease in

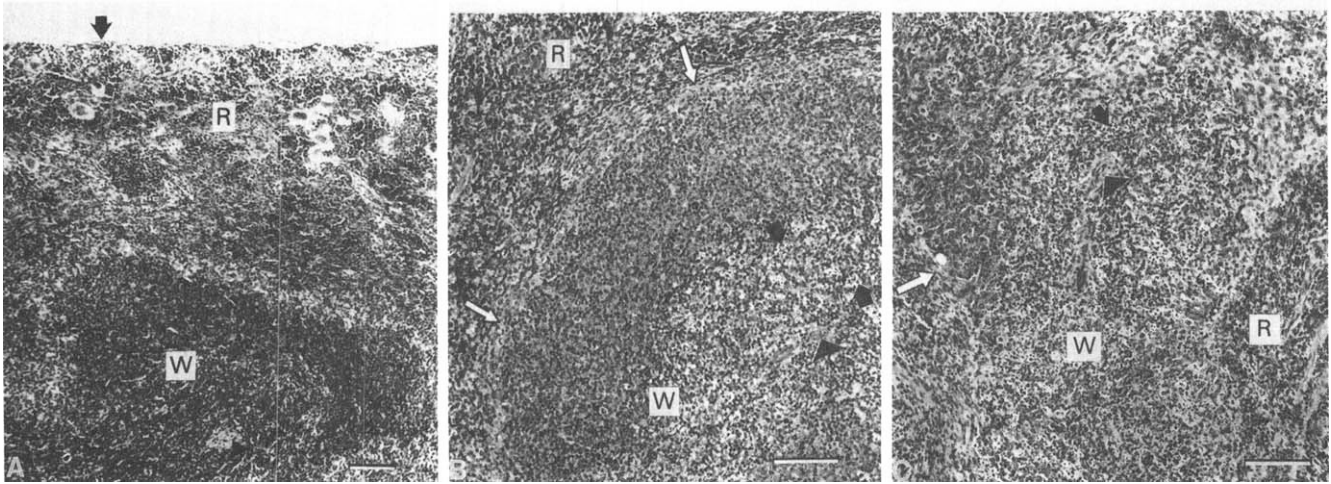


Figure 4. (A) Normal splenic white (W) and red (R) pulp, capsule (arrow), and central artery (arrowhead). (B and C) White and red pulp interface (white arrows) with peripheral mononuclear cells and central necrosis (black arrows) on Day 1 after glucan treatment in (B) unirradiated and (C) irradiated animals. The white pulp central artery is at the arrowhead (bars, 100 μ M).

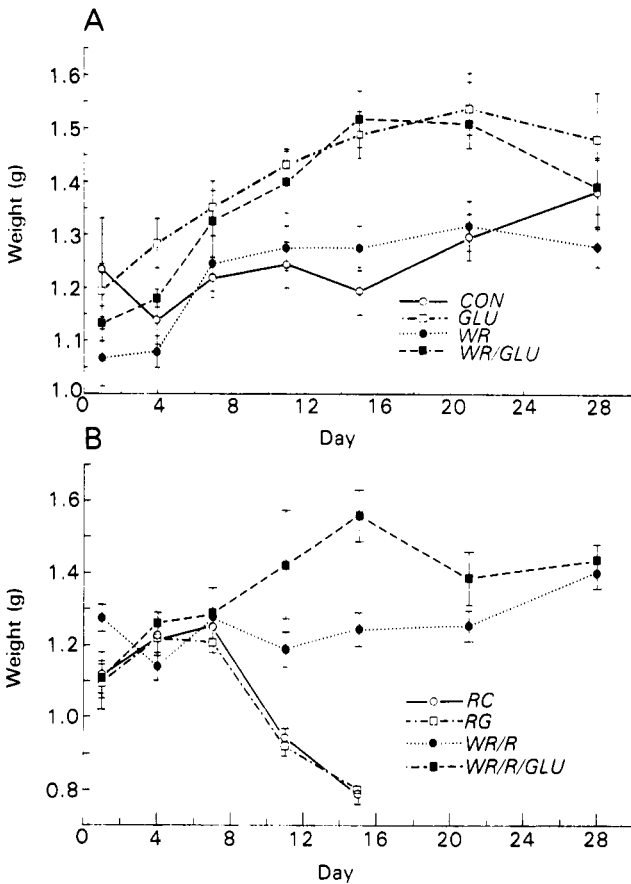


Figure 5. Effects of GLU and WR individually and in combination on hepatic weight of (A) unirradiated and (B) irradiated mice.

the cellularity of foci with a preponderance of macrophages, fewer neutrophils and lymphocytes, and occasional normal hepatocytes. By Day 28, marked involution of lesions was noted by a decrease in size and number of foci and reestablishment of normal hepatic architecture.

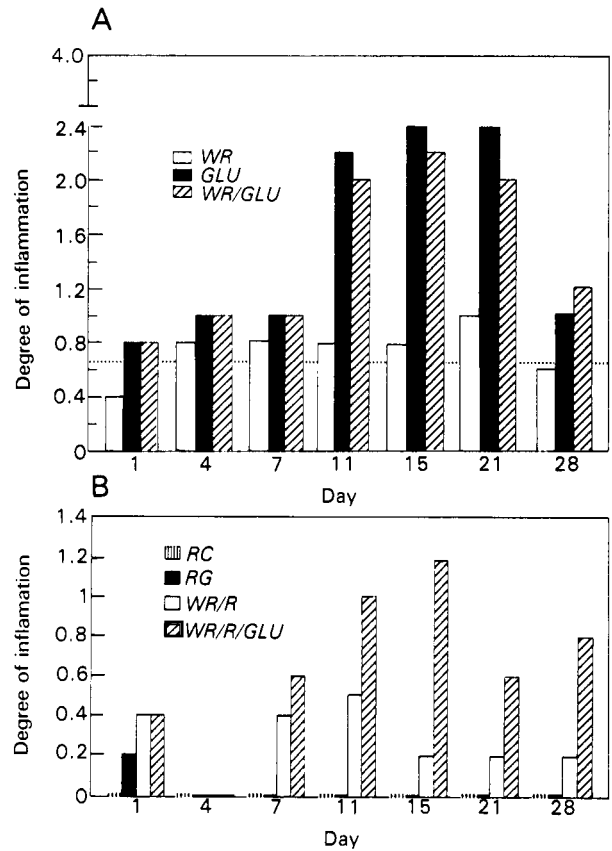


Figure 6. Hepatic inflammation in treated mice. The horizontal dashed line in (A) represents the average background lesions observed in controls (mean = 0.64). The lesions were graded as follows: 1, minimal; 2, mild; 3, moderate; and 4, severe.

Effects on kidney. Significant differences in kidney weight were not observed. Microscopic findings were limited to normal background lesions.

Other tissues examined. Other tissues examined included bone marrow, mediastinal lymph node, thy-

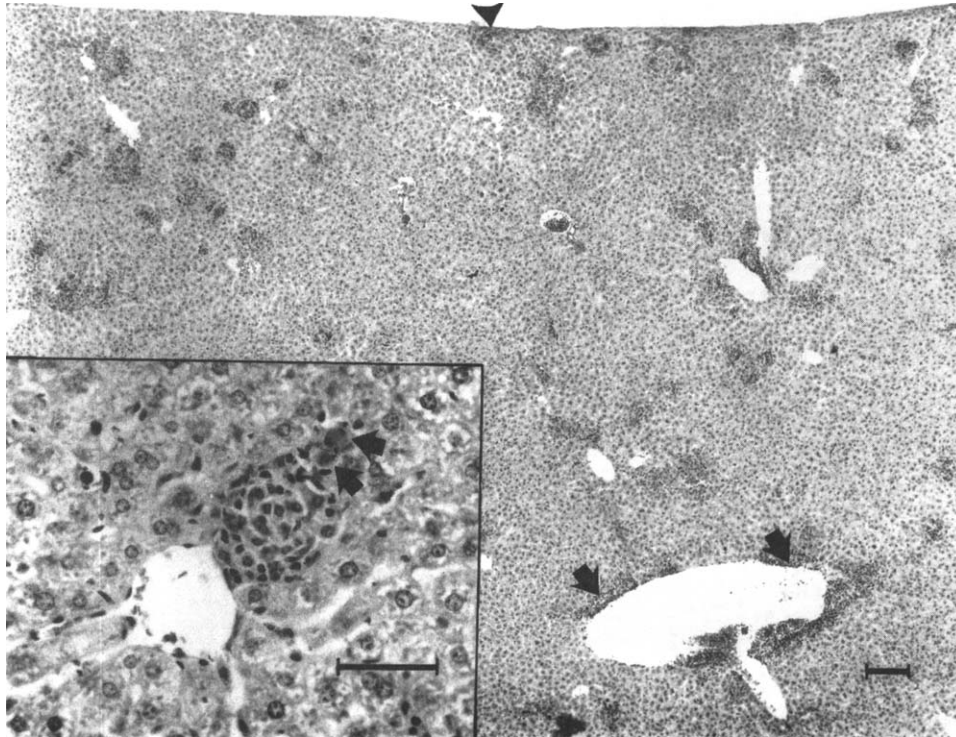


Figure 7. Liver from a WR/GLU-treated mouse 11 days after treatment. Subcapsular (arrowhead) and subendothelial (arrows) lesions consist primarily of admixtures of macrophages, lymphocytes, and neutrophils (bar, 150 μ M). Inset: Subendothelial centrolobular lesion at Day 4, consisting of macrophages, lymphocytes, and two degenerating hepatocytes (arrows) (bar, 50 μ M).

mus, and lung. Cellularity of bone marrow for all treatment groups was judged abundant (3.0) on all sample days except for Day 1 of the WR/GLU group, in which cellularity was moderate (2.0) with mild congestion. Lymph node and thymic morphologies and cellularities were normal. Lung fields were within normal limits.

Effects of WR-2721, Glucan, and Combined Treatment in Irradiated Groups. *Effects on body and splenic weights.* RG and WR/R/GLU body weights were less than WR/R and RC weights for the first 7 days, with sporadic significant differences noted on Days 1 and 7 (Table I). Subsequently, WR/R and WR/R/GLU body weights increased minimally during the balance of the study, whereas RC and RG weights decreased significantly by Day 15. There were no survivors in the RC and RG groups beyond Day 15. Splenic weights in RG and WR/R/GLU groups on Day 1 were comparable and both were significantly greater than those of RC and WR/R groups (Fig. 1B). By Day 4, splenic weights in all groups were equal. Subsequently, RC and RG splenic weights decreased by Day 15, whereas WR/R/GLU and WR/R splenic weights increased significantly above RC and RG groups by Days 11 and 15, respectively. Rebound and recovery patterns of WR/R/GLU and WR/R splenic weights were similar, but approximately 4 to 5 days out of synchronization, apparently due to a 4- to 5-day ad-

vanced recovery in WR/R/GLU mice. Intergroup comparison of WR/R/GLU and WR/R splenic weights of Day 15 with Day 21, respectively, and likewise of Day 21 with Day 28, did not reveal significant differences.

Quantitation of splenic white and red pulp areas and weights. Red pulp predominated in all groups throughout the experiment. Significant differences in splenic compartments were noted on Day 1, with the WR/R/GLU white pulp area significantly greater than the WR/R and RC areas (Table II). Although not statistically significant, there was a similar increase on Day 1 in RG white pulp. Microscopic changes seen in RG and WR/R/GLU white pulps are described below. After Day 1 perturbations, W:R ratios of all groups remained relatively static, with slight decreases noted in the RG and RC groups on Day 15.

RG and WR/R/GLU white pulp weights on Day 1 were comparable, and weights of both were significantly greater than those of the RC and WR/R groups (Fig. 2B). By Day 4, RG and WR/R/GLU weights decreased to levels similar to RC and WR/R groups. Subsequently, WR/R and WR/R/GLU white pulp weights increased from their Day 4 nadir to levels significantly greater than RC and RG weights by Day 15 and remained at levels comparable to their respective Day 15 weights for the remainder of the study. Red pulp weights in RC and RG groups decreased to Day 15 (Fig. 3B). Initial decreases in WR/R and WR/R/GLU

GLU red pulp weights with nadirs at Days 7 and 4, respectively, were followed by increases to levels significantly greater than RC and RG weights by Days 11 and 7, respectively.

Gross and microscopic changes in spleens. By Day 4, all spleens were smaller than normal. WR/R and WR/R/GLU spleens displayed one to several approximately 1-mm diameter colonies that were directly subjacent to and that slightly elevated the splenic capsules. On microscopic examination, these colonies consisted of clusters of erythrocytic and myelocytic elements. Beyond Day 4, RC and RG spleens remained small, whereas WR/R and WR/R/GLU spleens continued to grow to approximately twice normal size as colonies increased in size and number and eventually reached confluency. Confluency was noted by Days 15 and 11 for WR/R and WR/R/GLU spleens, respectively.

Light microscopic changes were as follows: RC and WR/R groups on Day 1 displayed hypocellularity of white (1.0 and 1.8, respectively) and red pulps (0.0 and 1.0, respectively), characterized by a decrease in density of small lymphocytes with mildly scattered single cell necrosis in the white pulp and loss of the bulk of the usual dense hematopoietic elements within the red pulp.

On Day 1, RG and WR/R/GLU groups displayed hypocellularity of red pulp (0.0 and 2.2, respectively) similar to that of RC and WR/R groups. RG and WR/R/GLU white pulps, with cellularities of 1.8 and 2.0, respectively, displayed reactive and degenerative changes as described earlier in GLU and WR/GLU spleens. Necrosis in RG and WR/R/GLU white pulps was sufficiently intense to lower cellularity indexes of the white pulp, even in the face of peripheral expansion (Fig. 4B). Megakaryocyte counts on Day 1 for all groups (RC, 8.3; RG, 8.2; WR/R, 10.1; and WR/R/GLU, 6.3) were comparable.

On Day 4, white pulps of all groups were hypocellular and resembled RC and WR/R white pulps of Day 1 (RC, 1.2; RG, 1.0; WR/R, 1.0; and WR/R/GLU, 1.0). Large cell populations noted in the RG and WR/R/GLU groups on Day 1 were no longer present. Red pulps in all four groups were hypocellular (RC, 0.2; RG, 1.0; WR/R, 2.0; and WR/R/GLU, 1.2); one WR/R spleen was hypercellular with diffuse hematopoiesis. RC and RG red pulp cellularity was concentrated in occasional foci of hematopoiesis, whereas WR/R and WR/R/GLU cellularity was judged slightly higher due to more numerous and larger foci. Megakaryocyte counts on Day 4 in all groups (RC, 1.9; RG, 1.5; WR/R, 2.7; and WR/R/GLU, 1.9) were significantly lower than Day 1 levels.

RC and RG groups on Days 7, 11, and 15, with exceptions noted below, displayed hypocellular white (RC, 1.0, 1.6, and 0.3, respectively; RG, 1.0, 1.0, and

1.5, respectively) and red (RC, 0.0, 0.0, and 0.3, respectively; RG, 0.2, 0.4, and 1.5, respectively) pulps, with reduced numbers of lymphocytes in the white pulp and fewer hematopoietic colonies and no megakaryocytes in the red pulp. One RC spleen on Day 15 displayed a microscopically detectable focus of hematopoiesis in the red pulp. In the RG group on Days 7, 11, and 15, there were one, two, and two spleens, respectively, that displayed several prominent colonies.

WR/R and WR/R/GLU white pulps demonstrated partial re-establishment of small lymphocyte populations by Day 15. From Days 7 to 15, red pulp displayed enlargement of hematopoietic colonies with confluency in three of five WR/R/GLU spleens by Day 11 and in all five WR/R spleens by Day 15. Megakaryocyte counts were at their nadirs in both groups by Day 7 (0.0 and 0.1, respectively), with slight increases noted by Day 15 (0.1 and 0.3, respectively).

On Days 21 and 28, WR/R and WR/R/GLU white pulps displayed a normal complement of small lymphocytes, and red pulps in all spleens were confluent. WR/R/GLU and WR/R megakaryocyte counts rebounded by Day 21 (2.2 and 0.7, respectively) and reached near normal levels by Day 28 (5.6 and 5.9, respectively).

Effects on liver. Liver weights of all groups on Days 1, 4, and 7 were similar with the exception of the WR/R group, with mean weights significantly heavier than WR/R/GLU on Day 1 and lighter by Day 4 (Fig. 5B). By Day 11, RC and RG liver weights decreased significantly below WR/R and WR/R/GLU weights. From Days 7 to 28, WR/R and WR/R/GLU weights displayed slight increases with significant differences between WR/R and WR/R/GLU noted only on Day 15.

On gross examination, no remarkable changes were noted in any of the four groups. Histologic changes in RC and RG livers on Day 1 consisted of occasional minimally dilated pericentrilobular sinusoids (congestion), with and without minimal vacuolar changes in neighboring hepatocytes. Additionally, in RG livers, there were normal background changes as noted in normal, unirradiated control mice (Fig. 6B). Inflammatory changes were not observed in the RC group or in the RG group beyond Day 1. From Day 4 to Day 11, dilation of sinusoids was progressively aggravated to severe and moderate proportions in RC and RG groups, respectively. By Day 11, vacuolar change of hepatocytes within these regions was mild and moderate in RG and RC, respectively. Additionally, RG livers at this time displayed marked necrosis of centrilobular hepatocytes and dissociation of hepatic plates, with occasional single cells or rafts of hepatocytes in sinusoids. These latter changes were not observed in RC mice until Day 15.

Histologic changes in WR/R and WR/R/GLU livers on Day 1 consisted of minimal congestion, with

minimal vacuolar change in WR/R hepatocytes adjacent to congested areas. By Day 4, congestion was not present in the WR/R group, but was still minimal in WR/R/GLU livers and remained so through Day 15. On Days 4 through 15, there were remarkable mitotic figures within hepatocytes in both groups. Such a change was noted in RC and RG groups only on Days 4 and 7. Excluding occasional clusters (3–10 cells) of mononuclear cells within sinusoids on Days 15 and 21, no other hepatic lesions beyond background (degree of inflammation ≤ 0.65) (1) were noted in the WR/R group. These clusters were interpreted as foci of extramedullary hematopoiesis. In WR/R/GLU livers, inflammatory change was the key feature from Days 7 through 28. By Day 7, there was minimal to mild, subacute hepatitis with multifocal, random to centrilobular (10–50 cells) admixtures of lymphocytes, macrophages, and occasional neutrophils within focally expanded sinusoidal spaces. In several foci, there were adjacent degenerating or necrotic hepatocytes. By Day 15, the inflammatory process had developed maximally, presenting primarily a mild to moderate granulomatous hepatitis with increased numbers of multifocal and coalescing aggregates of macrophages, lymphocytes, and neutrophils. Occasional degenerating or necrotic hepatocytes, as well as prominent multinucleated giant cells, were present in some foci. By Days 21 through 28, the inflammatory process was in regression, and aggregates were less cellular. Reduced cellularity in these foci was at the expense of lymphocytes and neutrophils, with remaining cells often consisting exclusively of macrophages.

Effects on kidney. Kidney weights for the WR/R and WR/R/GLU groups did not significantly differ during the study, and on Days 1, 4, and 7, there were no significant differences in kidney weights for all groups. By Day 11, RG kidney weights were significantly below weights of the other groups, and by Day 15, kidney weights of both RC and RG groups decreased to equivalent levels, significantly different from the WR/R and WR/R/GLU groups.

Microscopically, RC kidneys appeared essentially normal for Days 1 through 11. By Day 15, there was occasional minimal, focal, single cell necrosis of cortical tubular epithelium in all samples. RG kidneys were essentially normal, with the exception of incidental focal changes in one Day 7 (minimal, focal, cortical tubular epithelial necrosis) and in one Day 15 (minimal, focal, interstitial fibrosis with glomerular atrophy) preparation. The WR/R kidneys, as in the groups above, displayed focal, cortical, tubular epithelial necrosis; however, in WR/R kidneys, there was also mild epithelial regeneration. On Day 15, one of five WR/R kidneys displayed a focal wedge-shaped lesion whose base abutted a depressed renal capsule and whose apex extended to the pelvis. The lesion consisted of multiple mildly

dilated tubules lined by regenerating, plump, basophilic epithelial cells and surrounded by minimal fibrous connective tissue admixed with accumulations of lymphocytes. This lesion is characteristic of a repairing infarct. The remaining renal tissues of this group were essentially normal. The WR/R/GLU kidneys did not differ in presentation of incidental lesions. However, on Day 15, two of five WR/R/GLU kidneys displayed focal, infarctive lesions undergoing repair (Fig. 8). Differing from the infarctive lesions noted in the WR/R kidney, numerous tubules in these two kidneys contained basophilic hyalin material, interpreted as hyaline casts. Other than these changes, the WR/R/GLU kidneys were essentially normal.

Other tissues examined. Bone marrow cellularity of all groups by Day 1 was severely decreased. The cellularity present consisted of stroma, numerous polymorphonuclear cells, and red blood cells (hemorrhage). The bone marrow of RC and RG groups remained moderately to severely hypocellular through Day 15, even though occasional foci of blast cells were observed on Days 4 and 11, respectively. Hemorrhaging subsided by Day 15 and Day 11 in RC and RG groups, respectively. By Day 7, the WR/R and WR/R/GLU marrow cavities were normocellular and consisted predominantly of polymorphonuclear cells with scattered foci of blast cells.

Thymic responses after irradiation consisted of severe necrosis of cortical thymocytic populations, with maintenance of a population of small thymocytes in the medulla within the first 24 hr. Full repopulation of the thymic cortex was noted in sections of the RC, WR/R, and WR/R/GLU groups by Day 11. Cortical thymocyte recovery in the RG group was not observed.

Lymph nodes from all groups displayed severe lymphocytolysis on Day 1, with clearance of necrotic debris by Day 4. WR/R and WR/R/GLU nodes gradually regained normocellularity around Day 15, whereas RC and RG nodes remained hypocellular.

Discussion

Dosage levels and combinations of WR-2721 and soluble glucan used in our study have been demonstrated capable of affecting survival in C3H/HeN female mice exposed to otherwise lethal doses (9–15 Gy) of ^{60}Co radiation (1). At these doses, toxic effects of the two agents, as measured by any overt physiologic signs, were reportedly minimized. Histopathologic effects of WR-2721 and glucan administered individually have been reported previously (12, 15, 18, 19, 22, 23), but their effects, when given in combination at doses producing the previously noted dose-reduction factors, have not been described in irradiated or unirradiated animals.

In our study, survival responses in irradiated groups were comparable with those of a previous study (1).

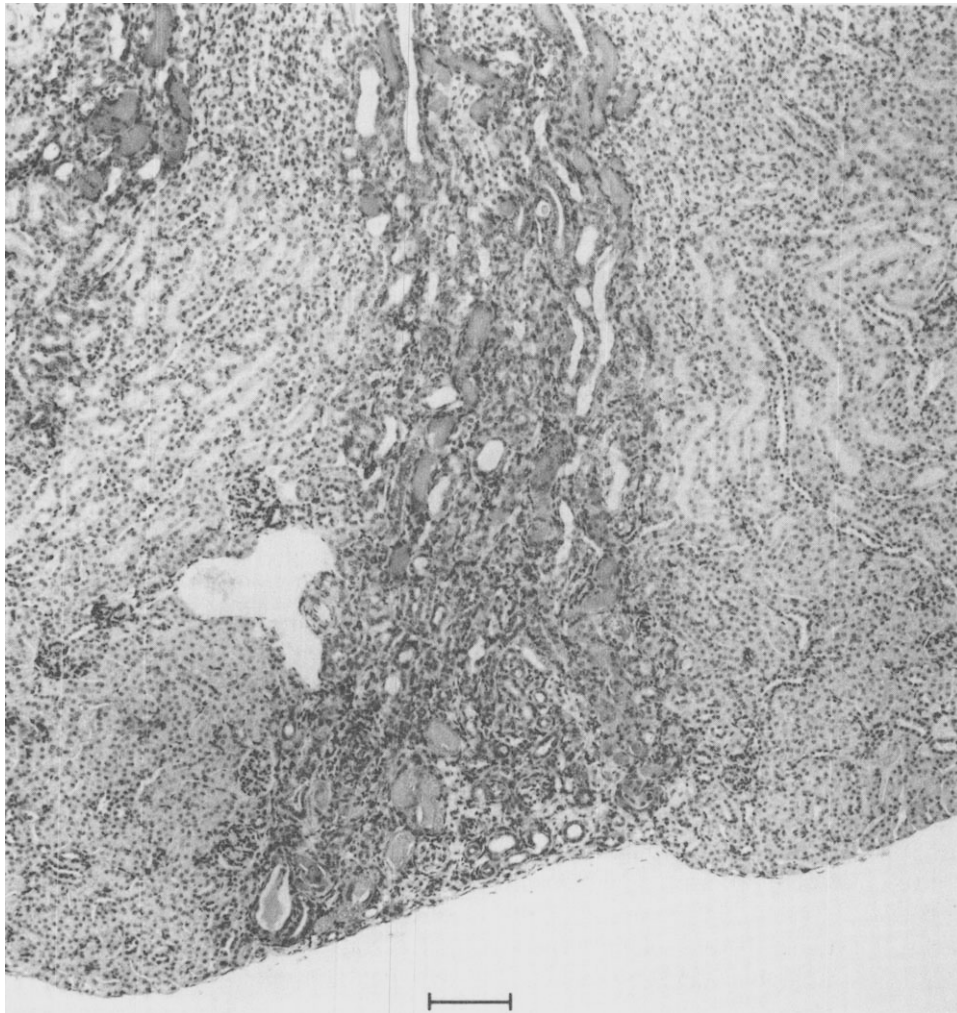


Figure 8. Renal lesion in a WR/R/GLU-treated mouse (Day 15) displaying a resolving infarct consisting of mildly dilated cortical tubules lined with regenerating epithelium and variably filled with hyaline casts and surrounded by minimal accumulations of lymphocytes.

The dose of WR-2721 used in this experiment (200 mg/kg ip) is substantially below reported doses at which lethality, transient decreased body weights, and histologic changes have been observed (9, 18). Our mice did display slightly decreased locomotor activity several hours after treatment, a change reported previously (17).

The glucan used (250 mg/kg iv) was a soluble preparation. Unlike particulate glucan preparations, soluble glucan administration generally has not been associated with hepatosplenomegaly or granuloma formation (19), although prolonged treatment with 1000 mg/kv iv has produced hepatomegaly and liver lesions (16). Splenomegaly was also reported at this high dose, as well as at a low dose of 40 mg/kg, but splenic lesions were absent in both instances.

In our study, body weights of irradiated and unirradiated groups were similar for the first 7 days. Subsequently, WR/R and WR/R/GLU body weights remained comparable to unirradiated groups, whereas

RC and RG groups displayed significant weight losses. Of note here is the sparing effect of WR-2721 in irradiated groups.

Soluble glucan alone has produced mixed splenic responses in unirradiated mice ranging from no increases in weights to significant increases of 44% (16). Attendant histologic lesions were either not observed or not described in previous studies (12, 16, 19). In our study, multiple parameters in irradiated and unirradiated spleens changed significantly after treatment with glucan alone or combined with WR-2721. Twenty-four hours after treatments, splenic weights in unirradiated WR/GLU and GLU mice were significantly greater than in WR and CON mice (37%), and although splenic weights of all irradiated groups were significantly less than CON at this time, a similar relationship was noted in irradiated counterparts WR/R/GLU and RG when compared with WR/R and RC (51% and 53%, respectively). Also common to all glucan-treated groups on Day 1 were significantly greater white pulp

areas and white pulp weights, relative to their non-glucan-treated controls. Finally, microscopic changes within splenic white pulp on Day 1 consisted of marked accumulations of a population of large cells at follicular peripheries and central single cell necrosis (lymphocytolysis). Lymphocytolysis in irradiated groups was more prominent, a characteristic attributed to the additive effects of irradiation and glucan treatments, both independently associated with lymphocytolysis and observed in independent control groups RC and GLU. Early histologic changes in spleens of our mice suggest a transient change within the first 24–72 hr following a single dose of soluble glucan. Similar histologic changes have been noted in C3H/He mice 24 hr after a single intravenous injection of lipopolysaccharide (24). Having assessed only light microscopic changes, we speculate that the cellular accumulations represent admixtures of lymphocytes, macrophages, and, perhaps, dendritic cells (25, 26) activated directly by exposure to glucan and/or activated indirectly by cytokine release from other primary activated cells (25).

Beyond Day 1, continued elevation in unirradiated WR/GLU and GLU splenic weights to Days 7 and 11, respectively, was associated with increased red pulp areas. This glucan-stimulated expansion of red pulp coincides with significant increases in splenic cellularity in general and specifically in splenic red pulp hematopoietic stem and progenitor cells reported previously in soluble, glucan-treated C3H/HeN female mice (6). Subsequently, elevated WR/GLU and GLU splenic weights were accompanied by comparable increases in white pulp areas to proportions approximately equal to red pulp, with recovery in WR/GLU spleens four days in advance of GLU spleens. Ultimate recovery of WR/R/GLU and WR/R splenic weights to levels comparable to or greater than CON spleens was attributed to radioprotective effects of WR-2721 and accompanied by reconstitution of white and red pulps. An estimated 4-day advanced recovery of WR/R/GLU splenic weights was ascribed to glucan and supported by previously documented advanced increases of splenic cellularity and splenic granulocyte-macrophage colony-forming cells in similarly treated C3H/HeN female mice (1). Advanced changes noted in combined treatment groups (WR/GLU and WR/R/GLU) are apparently due to a synergy between WR-2721 and glucan.

Hepatomegaly, as assessed by increased liver weight, was associated with glucan treatment. Previously reported increases in rats and mice (0–13% of control) given soluble glucan were not significant (12, 16), but increases in hepatic weights in our unirradiated (GLU and WR/GLU) and irradiated (WR/R/GLU) groups (15–31% of control) were significant. Increasing and decreasing liver weights within GLU, WR/GLU, and WR/R/GLU groups were directly associated with the onset of and the increase and decrease in severity

of microscopic hepatic lesions. We attribute maintenance of normal liver weights in WR/R mice to the radioprotectant effects of WR-2721, the greater weights in GLU and WR/GLU mice to the phlogistic effects of glucan, and greater weights in WR/R/GLU to glucan's phlogistic effects potentiated by WR-2721.

Microscopic liver lesions were not observed in rats (12) or mice (16, 19) given 10 mg/kg and 200 mg/kg of soluble glucan intravenously, respectively. Hepatic lesions were reported, however, in mice receiving 1000 mg/kg iv (16). In our study, lesions apparent as early as Day 4 were at times difficult to distinguish from normal background changes. Not until around Day 11 were they readily identifiable. This may explain in part the absence of liver lesions 4–5 days after single or multiple doses, as reported in earlier studies (12, 19). Further explanation may be found in the use of lower dosages and different animal species/strains in the studies (12, 16, 19).

The nature of glucan-induced infiltrates and proliferations within our preparations is consistent with previous observations independent of the form of glucan used (16, 23, 27). This was not the case with distribution of our lesions, the majority of which were either random or centrilobular. Similarly, lesions in mice treated with extremely high doses of soluble glucan, 1000 mg/kg, were represented photographically as random (16). However, several studies using particulate glucan in rats or mice reported a predominance of periportal lesions (27, 28). The difference in distribution may be due to physical properties of particulate versus soluble glucan.

Significant renal changes were limited to irradiated groups. Specifically, decreases in kidney weights were noted in RG and RC groups by Days 11 and 15, respectively, and attributed to radiation. Although WR/R and WR/R/GLU kidneys did not display weight losses, they did demonstrate significant microscopic changes in the form of infarcts. These lesions are, in part, compatible with previously reported toxic responses to WR-2721 (18). In addition to the reversible changes of tubular epithelial necrosis and proteinaceous cases reported in that study (CDF₁ female mice given 540 mg/kg of WR-2721 iv), our mice displayed chronic, irreversible renal lesions. These lesions were apparently potentiated by the added stress of lethal irradiation in that our mice received only 200 mg/kg ip, a dose judged nontoxic to CON and WR/GLU mice. This observation supports the caveat extended by authors of the above-cited study warning of potential toxicologic changes at low doses of WR-2721 in debilitated patients.

In conclusion, treatment with soluble glucan (250 mg/kg), whether combined with WR-2721 (200 mg/kg) or not, was associated with transient hepatosplenomegaly with accompanying histologic changes in unirradiated

radiated mice. In lethally irradiated mice, radiation-induced splenic weight loss was dampened in glucan groups on Day 1 and accompanied by morphometric and histologic changes similar to those seen in unirradiated counterparts. Subsequent rebounds of irradiated splenic weights were associated with hematopoietic reconstitution in WR-2721-treated groups, a change that occurred 4 days earlier in the combined treatment group. Hepatic lesions, similar to those noted in unirradiated groups, were observed only in the combined treatment group (WR/R/GLU), in which glucan-induced phlogistic changes were potentiated by the radioprotective effects of WR-2721. Additionally, in the WR/R and WR/R/GLU groups, limited observations suggest that irreversible renal lesions, not present in unirradiated WR-2721 recipients, may be induced with WR-2721 when followed by lethal radiation exposure. Further study is needed to verify the combined effects of WR-2721 and irradiation on renal tissues.

This work was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Work Units 00132 and 00176. Research was conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

The authors wish to thank Brian Solberg for technical assistance in the preparation of tissues and M. Greenville for editorial assistance.

The senior author was a resident in the Department of Veterinary Pathology at the Armed Forces Institute of Pathology during the initial phase of this study and wishes to thank the department for its support.

1. Patchen ML, MacVittie TJ, Jackson WE. Postirradiation glucan administration enhances the radioprotective effects of WR-2721. *Radiat Res* 117:59-69, 1989.
2. Patchen ML, MacVittie TJ. Stimulated hemopoiesis and enhanced survival following glucan treatment in sublethally and lethally irradiated mice. *Int J Immunopharmacol* 7:923-932, 1985.
3. Patchen ML, MacVittie TJ. Comparative effects of soluble and particulate glucans on survival in irradiated mice. *J Biol Response Mod* 5:45-60, 1986.
4. Patchen ML, MacVittie TJ, Solberg BD, D'Alesandro MM, Brook I. Radioprotection by polysaccharides alone and in combination with amino thiols. *Adv Space Res* 12(2-3):233-248, 1992.
5. Patchen ML, MacVittie TJ. Use of glucan to enhance hemopoietic recovery after exposure to cobalt-60 irradiation. *Adv Exp Med Biol* 155:267-272, 1982.
6. Patchen ML, MacVittie TJ, Wathen LM. Effects of pre- and postirradiation glucan treatment on pluripotent stem cells, granulocyte, macrophage and erythroid progenitor cells and on hematopoietic stromal cells. *Experientia* 40:1240-1244, 1984.
7. Patchen ML, MacVittie TJ. Hemopoietic effects of intravenous soluble glucan administration. *J Immunopharmacol* 8:407-425, 1986.
8. Patchen ML, DiLuzio NR, Jacques P, MacVittie TJ. Soluble polyglycans enhance recovery from cobalt-60 induced hemopoietic injury. *J Biol Response Mod* 3:627-633, 1984.
9. Patchen ML, D'Alesandro MM, Brook I, Blakely WF, MacVittie TJ. Glucan: Mechanisms involved in its "radioprotective" effect. *J Leukocyte Biol* 42:95-105, 1987.
10. Yuhas JM. Biological factors affecting the radioprotective efficiency of S-2[3-aminopropylamino]ethylphosphorothioic acid (WR-2721). LD₅₀₍₃₀₎ doses. *Radiat Res* 44:621-628, 1970.
11. Sigdestad CP, Grdina DJ, Connor AM, Hanson WR. A comparison of radioprotection from three neutron sources and ⁶⁰Co by WR-2721 and WR-151327. *Radiat Res* 106:224-233, 1986.
12. Bowers GJ, Patchen ML, MacVittie TJ, Hirsch EF, Fink MP. A comparative evaluation of particulate and soluble glucan in an endotoxin model. *Int J Immunopharmacol* 8:313-321, 1986.
13. Way CF, Dougherty WJ, Cook JA. Effects of essential fatty acid deficiency and indomethacin on histologic, ultrastructural and phagocytic responses of hepatic macrophages to glucan. *J Leukocyte Biol* 37:137-150, 1985.
14. Riggi SJ, DiLuzio NR. Hepatic function during reticuloendothelial hyperfunction and hyperplasia. *Nature* 193:1292-1294, 1962.
15. Deimann W, Fahimi HD. Induction of focal hemopoiesis in adult rat liver by glucan, a macrophage activator. *Lab Invest* 42:217-224, 1980.
16. Williams DL, Sherwood ER, Browder IW, McNamee RB, Jones EL, DiLuzio NR. Pre-clinical safety evaluation of soluble glucan. *Int J Immunopharmacol* 10:405-414, 1988.
17. Landauer MR, Davis HD, Dominitz JA, Weiss JF. Dose and time relationship of the radioprotector WR-2721 on locomotor activity in mice. *Pharmacol Biochem Behav* 27:573-576, 1987.
18. Palmer TE, Glaza SM, Dickie BC, Weltman RH, Greenspun KS. Toxicity studies on the radioprotective agent WR-2721 in CDF₁ mice and beagle dogs. *Toxicol Pathol* 13:58-65, 1985.
19. DiLuzio NR, Williams DL, McNamee RB, Edwards BF, Kitahama A. Comparative tumor-inhibitory and anti-bacterial activity of soluble and particulate glucan. *Int J Cancer* 24:773-779, 1979.
20. Schulz J, Almond PR, Cunningham JR, Holt JG, Loevinger R, Suntharalingam N, Wright KA, Nath R, Lempert D. A protocol for the determination of absorbed dose from high energy photon and electron beams. *Med Phys* 10:741-771, 1983.
21. Weibel ER. *Stereological Methods: Vol. 1, Practical Methods for Biological Morphometry*. London: Academic Press, p9, 1979.
22. Deimann W, Fahimi HD. Hepatic granulomas induced by glucan. An ultrastructural and peroxidase-cytochemical study. *Lab Invest* 43:172-181, 1980.
23. Yamada M, Naito M, Takahashi K. Kupffer cell proliferation and glucan induced granuloma formation in mice depleted of blood monocytes by strontium-89. *J Leukocyte Biol* 47:195-205, 1990.
24. Groeneveld PHP, Koopman G, van Rooijen N. The effects of LPS on the cellular composition of the splenic white pulp in responder C3H/He and non-responder C3H/HeJ mice. *Virchows Arch [B]* 49:183-193, 1985.
25. Steinman RM, Kaplan G, Witmer MD, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers and maintenance *in vitro*. *J Exp Med* 149:1-16, 1979.
26. Sherwood ER, Williams DL, McNamee RB, Jones EL, Browder IW, DiLuzio NR. Enhancement of interleukin-1 and interleukin-2 production by soluble glucan. *Int J Immunopharmacol* 9:261-267, 1987.
27. Deimann W, Fahimi HD. The appearance of transition forms between monocytes and Kupffer cells in the liver of rats treated with glucan. *J Exp Med* 149:883-897, 1979.
28. Pospisil M, Tkadlecek L, Netikova J, Pipalova I, Viklicka S, Kozubik A, Vacha J, Jary J. Interstrain differences in the responsiveness of mice to glucan with respect to hematological effects and manifestations of late damage. *Exp Pathol* 33:27-36, 1988.