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Gram-negative bacterial infections are a major cause of death in individuals whose resistance has been compromised by wounds, irradiation, burns and other stresses. The capability of circulating blood cells to ameliorate gram-negative infections is considered to be a critical mechanism in survival. The hypothesis that platelets and granulocytes play a significant role in mediating the lethal consequences of endotoxemia associated with gram-negative infections was evaluated by

20. ABSTRACT (continued)

studying responses to endotoxin challenge in mice made leukopenic and thrombo-cytopenic by irradiation. Sensitivity to endotoxin entering the circulation from the intestine was increased in animals deficient in granulocytes and platelets. Alterations in blood enzyme levels measured during endotoxemia were different in unirradiated and irradiated mice. The administration of 0.4 mg $ZnCl_2$ or 5 mg cortisone acetate prior to challenge with a lethal dose of <u>Salmonella typhosa</u> endo-toxin provided significant protection against the toxin in unirradiated mice, where-as only cortisone protected the irradiated animals. Failure of zinc to protect irradiated animals may be due to the absence of leukocytes and platelets. This phenomenon requires further study. Protection obtained with zinc may be due to attenuation of membrane related activities of platelets and granulocytes. Zinc protection against endotoxin challenge in unirradiated mice correlated with high blood levels of the ion. Administration of zinc increases the number of circulating leukocytes available at the time of endotoxin challenge, but cortisone promotes a later recovery of leukocyte numbers. Plasma glucose was elevated in zinc-treated mice, but this elevation did not correlate with survival. Cortisone pre-vented increased levels of plasma urea ordinarily associated with endotoxemia. Zinc may enhance early damage to hepatocytes in endotoxin-challenged animals. Cortisone reduced late damage in the liver. Cortisone apparently protects sites of endotoxin action other than blood components. Zinc, in contrast, appears to enhance survival primarily through action on circulating blood cells. Therefore, we believe that platelet and granulocyte transfusions may be useful in combatting endotoxemia and can be used as a postirradiation treatment.

PREFACE

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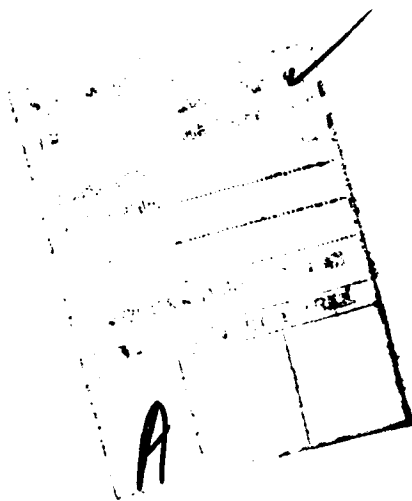
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INTRODUCTION

It is well known that hepatic Kupffer cells concentrate large amounts of endotoxin and release mediators of parenchymal cell function. [1,2,28] Certain blood cells may, like the Kupffer cells, have clearance and regulatory functions during the endotoxin syndrome. Granulocytes and platelets have a strong affinity for endotoxin[35] and were implicated as the cells capable of protecting animals from endotoxin poisoning. [11,12] Irradiated animals are extremely sensitive to endotoxin[15,33] and this sensitivity can be correlated with leukopenia and thrombocytopenia. [39] Granulocytes concentrate and degrade endotoxin[4] and platelets enhance plasma clearance of the toxin. [12] Both of these cell types contribute to regulation of intravascular clotting. [3,22,25]

There is also evidence that the endotoxin-induced inflammatory reactions known as the localized and generalized Shwartzman phenomena depend upon granulocytes and platelets for their expression. [27] Apparently, these reactions are linked to the degranulation of these cells and to the release of mediators which initiate cascades which cause coagulation changes, kinin production, vasomotor disorders and necrosis. There is also evidence that leukocyte-released agents are important in the pathogenesis of endotoxic shock. [21,30-32] For example, infusion of lysosomal enzymes into dogs causes hypoperfusion which leads to shock and death. [20] When platelets interact with endotoxin, they release inflammatory mediators, serotonin, prostaglandins and cathepsin A which can also contribute to vasomotor disturbances. [29] These enzymes and other vasoactive agents released by peripheral blood cells can enhance endotoxemia by causing passage of intestinal endotoxin into the peritoneal cavity. [14]

Mice treated with zinc chloride were protected against death due to endotoxin apparently through the ion's ability to stabilize biological membranes. [34] In this report evidence is presented that zinc protects by virtue of its effect on leukocytes and platelets. This and other evidence to be described below indicates that these cells are intimately associated with the endotoxin syndrome.

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MATERIALS AND METHODS

Mice. Male B6CBF1 mice, 10-14 weeks of age and varying in weight from 22-28 g, were obtained from Cumberland View Farms, Clinton, Tennessee, and used throughout these experiments. Animals were maintained in the manner described previously.[38]

Irradiation. Mice were placed in Plexiglas containers and exposed bilaterally to 1000 rads ^{60}Co radiation (45 rads/min) between 9 and 11 a.m. Radiation was supplied by a 10,000 Ci whole-body irradiator. Mean time to death of these animals was 14 days. All experiments in this report were conducted 7 days after irradiation, when mice are granulocytopenic and thrombocytopenic.[39]

Antibiotic treatment of the gastrointestinal tract. Mice were provided 4.0 mg/ml each of bacitracin and neomycin in their drinking water as described by van der Waaij and Sturm.[36] These animals were kept in sterile cages in a horizontal laminar air flow unit providing 100 linear ft/min air flow.[17] The antibiotic treatment process was begun a week prior to subsequent manipulations and continued throughout the duration of the experiment. Fecal pellets from antibiotic-treated mice were cultivated in thioglycollate broth (Difco). If no growth was obtained, the mice were considered to be decontaminated. This does not imply that the animals were germfree.

Treatments. Zinc chloride (A.C.S. certified, Fisher) (0.4 mg) was administered intraperitoneally (i.p.) in 0.2 cm^3 sterile saline 60 min prior to challenge with endotoxin unless stated otherwise.[34] Cortisone acetate (5 mg) was also administered in a 0.2 cm^3 volume 60 min prior to challenge with endotoxin but was injected subcutaneously (s.c.).[19] Salmonella typhosa endotoxin (Difco) was administered i.p. as a saline suspension between 8 and 11 a.m. Doses of endotoxin used produced 70 percent or greater mortality in 48 hours in unirradiated or irradiated control mice. Much smaller doses of endotoxin were required to achieve mortality in irradiated animals.

Blood studies. Blood was obtained by retro-orbital bleeding into heparinized tubes. Blood obtained from three mice was pooled to make each sample.

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Standard clinical chemical assays were used to determine plasma levels of serum glutamic oxalacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), glucose and urea. A Coulter counter was used to perform total white blood cell counts. Platelet and granulocyte numbers were determined microscopically. Assay procedures for determining the lysosomal enzymes β-glucuronidase, cathepsin B1 and cathepsin D have been described elsewhere.[13]

Statistics. Significance of differences in responses among experimental groups was evaluated with the Student's "t" test. Differences were considered significant if $p \leq 0.05$.

RESULTS

Treatment with cortisone or ZnCl2 prior to challenge with endotoxin. When administered prior to challenge with endotoxin, zinc chloride[34] or cortisone acetate[19] affords significant protection against death. The efficacy of these treatments in mice made leukopenic and thrombocytopenic by irradiation was compared to that in unirradiated animals (Table 1). Both agents reduced mortality following challenge of unirradiated mice with endotoxin. In irradiated mice, however, only cortisone had any value as a protective agent.

Table 1. Percent Mortality (48 hours) in Irradiated[a] and Unirradiated Mice Treated with Cortisone or ZnCl$_2$ Prior to Injection with Endotoxin

Treatment:	Unirradiated + Endotoxin			Irradiated + Endotoxin		
	Saline	Cortisone[b]	ZnCl$_2$[b]	Saline	Cortisone	ZnCl$_2$
% Mortality (48h)	57(14)[c]	4(24)	30(10)	65(23)	24(25)	88(24)

[a] Mice were irradiated with 1000 rads ^{60}Co at 45 rads/min. Irradiated mice were challenged i.p. with 0.2 mg of Salmonella typhosa endotoxin, but unirradiated animals received 0.8 mg of endotoxin.

[b] Zinc (0.4 mg i.p.) or cortisone (5 mg s.c.) were not lethal to irradiated or unirradiated mice when given without endotoxin.

[c] Number in parentheses refers to the number of animals in that experimental group.

Correlation of blood zinc levels with survival following challenge with endotoxin. Zinc may be ineffective in irradiated mice because its protective action possibly is due to effects on cell membranes of leukocytes and platelets. This hypothesis was strengthened by the observation that protection afforded by zinc correlated with a time when the plasma level of zinc was at, or near, its maximum (Figure 1).
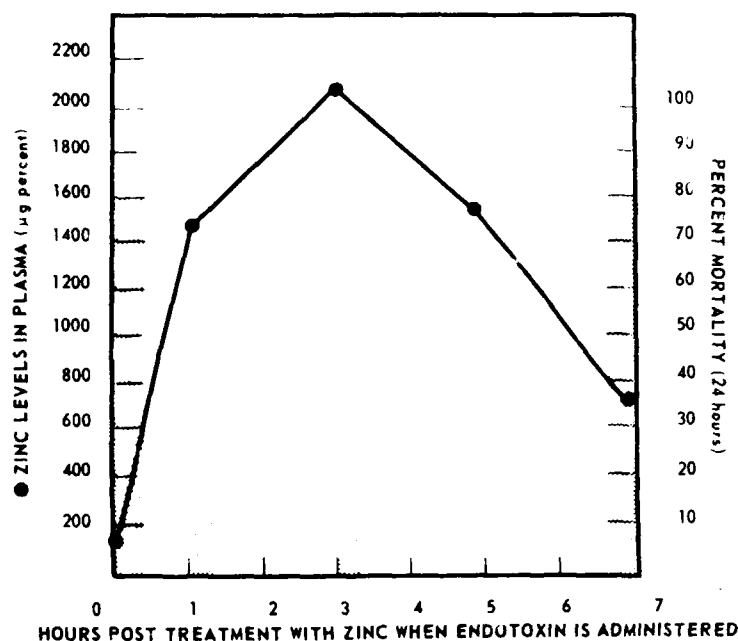
Figure 1. Plasma zinc levels and percent mortality following challenge with endotoxin

Leukocytes and platelets in mice treated with cortisone or ZnCl$_2$. Further studies were conducted in vivo to determine the possible nature of zinc action in blood. Ninety minutes after injection of zinc into mice (Table 2), total leukocyte counts were elevated over those seen in saline treated animals. When endotoxin was injected into mice 60 min after treatment with zinc, and blood samples were taken 30 min later, the total number of leukocytes was elevated. Granulocytes and platelets, although higher than in saline treated mice also challenged with

Table 2. Cortisone- and Zinc-Induced Alterations of Mouse Blood Chemistries and White Blood Cell Numbers from Normal Mice

	175.0 ± 5.1	142.3 ± 19.4	259.0 ± 41.2[b]	465.0 ± 133.1[b]
			(3)	(4)
	111.3 ± 11.5	112.5 ± 9.6	154.0 ± 45.3	310.5 ± 132.3[b]
		(4)	(3)	(3)
	20.7 ± 4.0	15.8 ± 2.1	25.3 ± 1.8	17.3 ± 1.5
		(4)	(3)	(3)
White Blood Cells	-----	2904 ± 843.5	3446.8 ± 906.4	5973.5 ± 133.4[b]

a. Blood is expressed as mm³ and SGOT or SGPT are in Reitman-Frankel units. White blood cells are expressed as cells per mm³. Five samples were used to obtain each blood cell value shown. Values are expressed as mean ± standard deviation. Numbers in parentheses represent the number of samples used to obtain the mean values shown. Blood from three mice was pooled for each sample. Samples were obtained 90 minutes after treatment described.

b. Significantly deviated from control values (p ≤ 0.05).

endotoxin, were not significantly elevated (Table 3). By 5 or 20 hours after endotoxin challenge, total leukocyte levels were comparable to saline treated animals.

The membrane-stabilizing action of cortisone[5,24] could also make this agent useful in protecting blood cells from endotoxin. Unlike zinc, cortisone

Table 3. Numbers of Circulating Blood Cells in Mice Treated with Saline, Zinc, or Cortisone Before Challenge with Endotoxin

Time After Endotoxin (h)	Numbers of circulating blood cells[a]				
	White blood cells			Granulocytes	Platelets (x1000)
	0.5h	5h	20h	0.5h	0.5h
Saline	1319 ± 286	2333 ± 673	3089 ± 1049	343 ± 227	1393 ± 101
Cortisone	2150 ± 659[b]	3361 ± 792[b]	4042 ± 888	667 ± 360	1633 ± 233
Zinc	2571 ± 546[b]	2415 ± 399	2765 ± 1393	781 ± 614	1698 ± 237

a. Expressed as mean ± standard deviation per mm³. Five samples were used to obtain each mean.

b. Significantly deviated from values in saline group (p ≤ 0.05).

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treatment alone had no effect on total white blood cell counts (Table 2). Thirty minutes after injection of endotoxin into mice, cortisone treated animals had leukocyte, platelet and granulocyte numbers comparable to those seen in animals treated with zinc (Table 3). Furthermore, in cortisone treated animals, total leukocyte numbers rose to higher values at 5 and 20 hours than at 30 min after injection of mice with endotoxin.

Urea in endotoxin-challenged mice treated with cortisone or $ZnCl_2$. Uremia has been previously associated with endotoxemia and may contribute to mortality during the syndrome.[37] Treatment with cortisone blocked the endotoxin-induced uremia seen in saline treated, unirradiated animals (23.4 ± 2.8 mg% versus 95.1 ± 3.2 mg%). Slight elevations of urea were seen in animals treated with zinc (114.2 ± 7.1 mg%) compared to mice treated with saline.

Glucose in endotoxin-challenged mice treated with cortisone or $ZnCl_2$. Alterations of glucose levels may be important to the pathophysiology of endotoxemia. Treatment with cortisone muted the early hyperglycemic response observed 30 min following injection of endotoxin into irradiated or unirradiated mice (Table 4). Treatment with zinc produced a marked hyperglycemia in these two groups of mice.

Table 4. Glucose Levels[a] in Mice Treated with Saline, Cortisone or Zinc Chloride Prior to Challenge with Endotoxin[b]

Time, post-endotoxin	Irradiated[c]		Unirradiated		
	0.5h	5h	0.5h	5h	20h
Saline	258.8 ± 21.5	124.5 ± 19.6	328.4 ± 39.7	113.3 ± 16.7	40.8 ± 11.1
	(4)	(4)	(5)	(4)	(4)
Cortisone (5 mg)	218.5 ± 21.2^d	116.3 ± 30.3	282.8 ± 13.9^d	134.8 ± 50.3	33.5 ± 3.0
	(4)	(4)	(4)	(4)	(4)
Zinc (0.4 mg)	338.8 ± 35.4^d	89.8 ± 4.9^d	442.0 ± 77.0^d	115.5 ± 20.2	88.2 ± 4.4^d
	(4)	(3)	(5)	(4)	(5)

[a]Glucose levels are expressed as mg%. Values are expressed as mean ± standard deviation. Numbers in parentheses represent the number of samples used to obtain the mean values shown. Blood from three mice was pooled for each sample.

[b]Irradiated mice were challenged i.p. with 0.2 mg of Salmonella typhosa endotoxin. Unirradiated mice were challenged with 0.75 mg of the toxin.

[c]Mice were irradiated with 1000 rads ^{60}Co at 45 rads/min.

[d]Significantly deviated from control values ($p \leq 0.05$).

Transaminase levels in endotoxin-challenged mice treated with cortisone or ZnCl$_2$. Cortisone may alleviate endotoxin damage in the liver (Table 5). Presumptive evidence for this type of action is the significantly reduced activity of SGOT and SGPT seen in cortisone treated mice 20 hours after injection with endotoxin. In contrast to cortisone, zinc may injure the livers of mice subsequently challenged with endotoxin. This possibility is based on the elevated SGPT activities seen 30 min after endotoxin injection. At 30 min and at 5 hours postendotoxin challenge, SGOT activities were also elevated over values obtained in saline and cortisone treated animals.

Table 5. Transaminase Levels[a] in Mice Treated with Saline, Cortisone or Zinc Chloride Prior to Challenge with Endotoxin[b]

| Time After Endotoxin | UNIRRADIATED MICE | | | | | |
| | SGOT | | | SGPT | | |
	0.5h	5h	20h	0.5h	5h	20h
Saline	136.4±81.0	130.3±14.9	354.5±16.8	63.3±24.0	60.3±14.9	185.0±21.2
	(5)	(4)	(4)	(4)	(4)	(4)
Cortisone	73.2± 7.9	141.3±26.9	295.0±30.0[c]	63.0±12.0	74.7± 8.4	61.7±12.3[c]
	(5)	(3)	(4)	(4)	(4)	(4)
Zinc	238.4±51.0[c]	175.5±33.1[c]	353.2±22.3	140.0±40.8[c]	105.8±45.8	211.7± 3.5
	(5)	(4)	(4)	(4)	(4)	(4)

[a]Enzyme levels are expressed in Weitman-Frankel units. Values shown are as mean ± standard deviation and numbers in parentheses represent the number of samples used to obtain mean values. Blood from three mice was pooled for each sample.

[b]Mice received 0.75 mg of the Salmonella typhosa endotoxin (Difco).

[c]Significantly deviated from control values (p≤ 0.05).

Evidence for altered regulation of metabolic responses to endotoxin in irradiated mice. Activity of plasma β-glucuronidase, a lysosomal enzyme indicative of lysosomal release, and two metabolically important lysosomal enzymes, cathepsins B1 and D, was assayed in plasma (Figure 2). For all three enzymes the responses seen 20 hours following endotoxin inoculation were significantly different between irradiated and unirradiated mice. Plasma levels of β-glucuronidase were increased eightfold ($\sim$800 percent) when normal mice were

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challenged with endotoxin. The rise in plasma β-glucuronidase elicited by endo-
toxin was muted in irradiated animals. Cathepsin B was depressed slightly (13
percent) in mice irradiated only. In unirradiated mice, endotoxin induced a
greater depression of plasma cathepsin B1 than in irradiated mice (300 percent
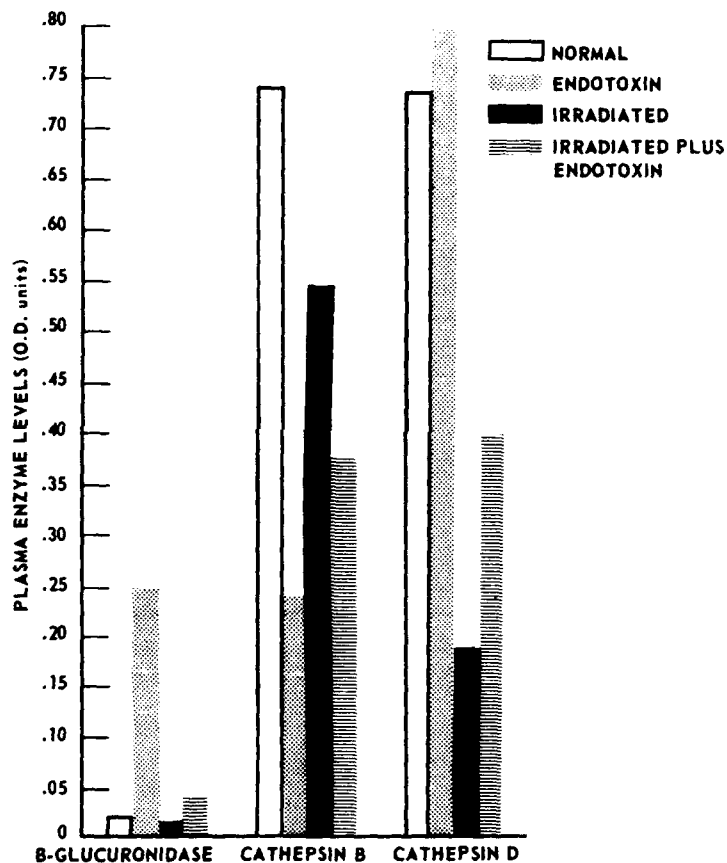versus 30 percent).

Figure 2. Plasma enzyme responses in irradiated and unirradiated mice
challenged with endotoxin

The general pattern of a lessened response seen with β-glucuronidase and
cathepsin B1 following endotoxin challenge in irradiated mice was not observed
for cathepsin D. This enzyme was depressed 370 percent in irradiated mice.

When endotoxin was administered, plasma cathepsin D was unaltered in unirradiated mice, but the enzyme was increased 110 percent in irradiated mice.

Contribution of intestinal endotoxin to the endotoxin syndrome. The possible contribution that leakage of intestinal endotoxin from the gut could make to mortality in animals already suffering from endotoxemia was evaluated (Table 6). This was accomplished by comparing survival of mice whose intestinal flora had been reduced with antibiotics to survival obtained with conventional animals. Apparently, leakage of endogenous endotoxin does not contribute significantly to mortality in unirradiated mice challenged with endotoxin. In contrast, irradiated animals that had been treated with oral antibiotics were much more resistant to endogenous challenge than conventional irradiated mice.

Table 6. Effect of Oral Antibiotic Treatment on Survival of Irradiated[a] and Unirradiated Mice Challenged with Endotoxin[b]

	CONVENTIONAL		ANTIBIOTIC TREATED	
	Unirradiated[c]	Irradiated[d]	Unirradiated	Irradiated
Percent Mortality (48 h)	80 (20)[e]	65(17)	65(20)	28(18)

[a]Mice were irradiated with 1000 rads ^{60}Co at 45 rads/min.

[b]Salmonella typhosa lipopolysaccharide (Difco).

[c]Unirradiated mice were challenged i.p. with 0.8 mg endotoxin.

[d]Irradiated mice were challenged with 100 µg endotoxin.

[e]Numbers in parentheses refer to the number of mice in each group.

DISCUSSION

We have obtained evidence that circulating blood cells contribute significantly to the outcome of endotoxemia. This conclusion is based in part on the finding that zinc may protect mice challenged with endotoxin through action on blood components. Furthermore, the reduction of platelets and leukocytes in

irradiated animals appears to increase sensitivity to intestinal endotoxin and could contribute to alterations seen in blood enzyme levels during endotoxemia.

Zinc action on blood components is indicated by several of our findings described in this report. Protection from endotoxin correlated with high levels of zinc in the plasma. More importantly, if mice were made leukopenic and thrombocytopenic by irradiation, zinc was ineffective in protecting them against endotoxin. In unirradiated mice zinc increased peripheral white blood cell counts before and during early endotoxemia.

The mechanism by which zinc may affect blood cell populations may be related to the fact that zinc can contribute to membrane stability. [8] We have previously reported evidence for such an action of zinc on lysosomal membranes in vivo. [34] Zinc may be essential for normal function of cells involved in inflammation. Both leukocytes[6] and platelets[26] are particularly rich in zinc, which can functionally inactivate them. [7] For example, zinc can inhibit release of enzymes from polymorphonuclear leukocyte lysosomes;[6] block migration, phagocytosis and other membrane-related activities of peritoneal macrophages; inhibit histamine release from mast cells; and obviate aggregation of platelets and their release of serotonin. [9] Recently, zinc was shown to inhibit release of certain leukocytic endogenous mediators from rabbit peritoneal exudate cells in vitro (P. Z. Sobocinski and C. A. Mapes, unpublished data).

The effects of zinc on granulocytes and platelets during the initial phase of endotoxemia could be critical to the ultimate survival or death of the challenged mouse. A characteristic of animals made tolerant to endotoxin is that they respond to challenge with a less marked leukopenia[16] and release of vasoactive agents which among other actions can increase intestinal permeability to endotoxin. [14]

The importance of peripheral blood cells in protection against endotoxin entering the systemic circulation from the intestine is indicated by our studies in conventional and antibiotic treated irradiated animals. After challenge with endotoxin, additional toxin can escape from the gut and enter the circulation via

the lymphatics[18] or the peritoneal cavity. [10] In unirradiated animals, sufficient leukocyte and platelet levels may be present to protect the host against endotoxin leaving the gut. Irradiated mice, however, do not possess this line of defense. For this reason, irradiated mice may profit more from prior elimination of the intestinal source of endotoxin through antibiotic treatment than unirradiated mice.

Potentially harmful changes in enzyme levels were seen in irradiated mice challenged with endotoxin. Lysosomal enzymes contribute to endotoxin toxicity[19,23,25] and the hazard posed by these enzymes is compounded if the capacity to remove or inhibit them is lost. Whether cathepsin B1 and D responses, as well as the lack of an increase in β-glucuronidase after challenge with endotoxin, are related to the absence of peripheral blood cells is difficult to substantiate. Mouse leukocytes are rich in β-glucuronidase and cathepsin D, but poor in cathepsin B1 (S. L. Snyder, unpublished data). The inability of endotoxin to elicit a substantial increase in β-glucuronidase could reflect the leukopenia of irradiated mice. Changes in cathepsin levels are, however, more difficult to interpret. It is conceivable that the changes in cathepsin levels could be due, in part, to changes in the levels of leukocyte-produced inhibitors of their activity.

The pronounced hypoglycemia which occurs late after challenge with endotoxin could be critical during endotoxemia. Since zinc caused hyperglycemia in both irradiated and unirradiated mice challenged with endotoxin, we believe that hypoglycemia is probably not the major factor in ultimate mortality.

Like zinc, cortisone has actions on blood components and at other sites. Since cortisone protects irradiated mice from endotoxin, it is probable that it exerts a major beneficial effect at sites other than blood cells. Cortisone protection may be related to the significant reduction in transaminase and urea levels and recovery of leukocyte levels we observed in mice undergoing endotoxemia.

The results obtained in our studies are consistent with the idea that cortisone increases survival of mice undergoing endotoxemia by virtue of its

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protective effects at sites other than the blood cells. Zinc protection may be more specialized, affecting only those cells found in the blood. If this is the case, then the importance of blood cells in the endotoxin syndrome is apparent.

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