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ASEPTIC ENDOTOXEMIA IN RADIATION INJURY
AND GRAFT VERSUS HOST DISEASE

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Armed Forces Radiobiology Research Institute
Bethesda, Maryland

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13. ABSTRACT Endotoxemia of intestinal origin may impair physiologic and immunologic functions in individuals subjected to high doses of either ionizing radiation or radiation followed by transplants of foreign immune competent cells capable of producing graft versus host disease (GVHD). The results of the following studies support the contention that aseptic endotoxemia may be due to failure to clear endotoxin from the circulation and detoxify it in the liver of immunosuppressed animals. To determine if intestinal endotoxin may contribute to pathogenesis in immunosuppressed individuals, B6CBF1 mice were exposed to 850 rads of x rays and transplanted with allogeneic CBA spleen cells. Aseptic endotoxemia, detected with the limulus lysate assay, was found at 24 and 72 hours postirradiation in livers of mice irradiated only. Contrary to this, endotoxin was not found in mice on days 5, 7 and 8 after x irradiation. Mice died between 11 and 13 days after radiation exposure at which time bacteria and endotoxin were detected in the liver. In contrast endotoxin was demonstrable on days 1 and 5 in mice undergoing GVHD. Mice receiving allogeneic grafts after 850 rads survived only 7 days, while gram-negative organisms were detected frequently in liver fragments from 24 hours to day 7. By day 4, hepatosplenic localization of I. V. injected ⁵¹ Cr endotoxin was reduced 50 percent in these animals and twofold to threefold increases in endotoxin levels were found in lung, kidney, heart and brain. Hepatosplenic endotoxin concentration was also depressed in mice receiving radiation alone, reaching a low between days 12-14. Endotoxin levels in other organs were not altered. Thus endotoxin capable of modifying physiologic and immunologic functions is present in detectable amounts in the liver of immunosuppressed mice.		

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FOREWORD
(Nontechnical summary)

Many bacteria which reside in the intestines of normal individuals possess a toxic substance called endotoxin, liberated as bacteria decompose. This toxin, as well as the bacteria which contain it, is harmless in normal individuals, because factors within the intestine and the reticuloendothelial system (principally cells within the liver and spleen which engulf foreign substances) protect other more susceptible body tissues from exposure to endotoxin. The following studies were conducted to learn how lethal doses of x rays or x rays followed by grafts of blood cell forming tissue from unrelated animals (which induce an immunologic response termed graft versus host disease) affect the natural equilibrium between mice and endotoxin of intestinal origin.

Endotoxin was looked for in livers of mice which were irradiated only. Although no bacteria were detected in the liver during the first 72 hours after irradiation, endotoxin was found. This endotoxin most likely originated in the gastrointestinal tract. Endotoxin was not found in mice on days 5, 7, and 8 after x irradiation. Mice died between 11 and 13 days after radiation exposure at which time bacteria and endotoxin were detected in the liver. In contrast, endotoxin was demonstrable at days 1 and 5 in irradiated mice undergoing graft versus host disease. Mice receiving grafts of blood forming cells after 850 rads of x rays survived only 7 days while bacteria containing endotoxin were detected frequently in liver fragments from 24 hours to day 7. By day 4, localization of intravenously injected isotope labelled endotoxin in the liver and spleen was reduced 50 percent in these animals

and twofold to threefold increases in endotoxin levels were found in lung, kidney, heart and brain. Endotoxin concentration by the liver and spleen was also depressed in mice receiving radiation alone, reaching a low by day 13. Endotoxin levels in other organs were not altered. Additional tests support the contention that endotoxemia without the presence of bacteria in the same tissues may be due to the failure of the liver of immunosuppressed animals to detoxify endotoxin originating in the gut.

ABSTRACT

Endotoxemia of intestinal origin may impair physiologic and immunologic functions in individuals subjected to high doses of either ionizing radiation or radiation followed by transplants of foreign immune competent cells capable of producing graft versus host disease (GVHD). The results of the following studies support the contention that aseptic endotoxemia may be due to failure to clear endotoxin from the circulation and detoxify it in the liver of immunosuppressed animals. To determine if intestinal endotoxin may contribute to pathogenesis in immunosuppressed individuals, B6CBF1 mice were exposed to 850 rads of x rays and transplanted with allogeneic CBA spleen cells. Aseptic endotoxemia, detected with the limulus lysate assay, was found at 24 and 72 hours postirradiation in livers of mice irradiated only. Contrary to this, endotoxin was not found in mice on days 5, 7 and 8 after x irradiation. Mice died between 11 and 13 days after radiation exposure at which time bacteria and endotoxin were detected in the liver. In contrast endotoxin was demonstrable on days 1 and 5 in mice undergoing GVHD. Mice receiving allogeneic grafts after 850 rads survived only 7 days, while gram-negative organisms were detected frequently in liver fragments from 24 hours to day 7. By day 4, hepatosplenic localization of I. V. injected ^{51}Cr endotoxin was reduced 50 percent in these animals and twofold to threefold increases in endotoxin levels were found in lung, kidney, heart and brain. Hepatosplenic endotoxin concentration was also depressed in mice receiving radiation alone, reaching a low between days 12-14. Endotoxin levels in other organs were not altered. Thus endotoxin capable of modifying physiologic and immunologic functions is present in detectable amounts in the liver of immunosuppressed mice.

I. INTRODUCTION

In mice suffering from radiation injury or graft versus host disease (GVHD), biologically active endotoxins may add a life threatening stress to animals already severely compromised by immune depression. Endotoxemia may play a twofold role in the ultimate lethality. First, small amounts of free endotoxin from the gut pool¹¹ may accumulate aseptically in tissues to levels which enhance damage and alter susceptibility to exogenous or endogenous infection. Second, endotoxin shock may occur subsequent to gram-negative bacterial sepsis.

The possibility of an endotoxin-induced contribution to mortality in irradiation injury or GVHD has been considered by others.^{14, 19} However, no attempt was made to correlate bacteremia with detection of endotoxin. Furthermore, the presence of intestinal microflora has also been implicated previously in GVHD.^{4, 7-9, 16}

The investigations described in this report were designed to determine (1) if endotoxemia is a feature of the radiation syndrome and GVHD; and (2) if endotoxin is derived from either systemic infection or the gut pool. Phagocytic impairments which can influence metabolism of endotoxin in immunologically depressed animals were also evaluated.

II. MATERIALS AND METHODS

Animals. Male CBA (H-2^k) and (C₅₇BL/6 x CBA) (H-2^b x H-2^k) F1, henceforth B6CBF1, mice obtained from Cumberland View Farm, Clinton, Tennessee, were acclimated to laboratory conditions for 5 weeks. When used, they weighed 20-25 g and were 10 to 12 weeks old. All animals were maintained on a 12-hour

light-dark cycle. D and G laboratory pellets and water, pH 2.2, were available ad libitum.

X-ray exposure. Mice were inserted into Plexiglas restrainers and exposed to 850 rads (40 rads/min) from a 300 kVp General Electric Maxitron x-ray generator operated at 20 mA. The skin-target distance was 90 cm. Added filtration included 2.00 mm Al and 1.0 mm Cu. The HVL of the x-ray beam was 1.54 mm Cu. All radiation exposures were performed in the morning.

Spleen cell grafts. B6CBF1 animals destined to undergo GVHD received I. V. injections of 5×10^6 allogeneic CBA spleen cells 2 to 3 hours after irradiation. The manner of preparing the spleen cell suspensions was previously described.¹⁰ Samples of spleen cell preparations were cultivated in thioglycollate to test for the presence of bacteria.

Microbiologic and endotoxin assays conducted with hepatic tissue. Limulus tests for endotoxin were performed as described by Reinhold and Fine.¹² Liver samples were prepared for assay by homogenization in pyrogen-free water (1:1 w/v) and centrifuged at 1000 g for 15 min. The supernatant fluid was adjusted to pH 4.0 ± 0.1 with 0.1 of 50 percent (w/v) anhydrous dibasic potassium phosphate buffer. This preparation was centrifuged at 1000 g for 10 min, after which 0.1 ml of the supernatant fluid was mixed with an equal volume of pyrogen-free water. Half of this preparation was frozen while the remainder (0.1 ml) was mixed with 0.1 ml limulus lysate and incubated at 37°C for 60 min. The formation of a solid clot in the bottom of the tube was accepted as a positive indication of the presence of endotoxin.

Endotoxin cleared by the liver was also detected with the actinomycin-D (AMD) procedure.¹⁷ At 48 hours postirradiation, livers were removed in an aseptic manner and homogenized 1:1.5 in saline. This homogenate was centrifuged at 1000 g for 15 min and 1.2 ml of supernatant fluid mixed 1:1 with AMD (50 $\mu\text{g}/\text{ml}$). Two mice were injected I. P. with 1.0 ml of the AMD-liver preparation from each irradiated animal. The mice were then observed for lethality over a 48-hour period.

For microbiologic assay, a fragment of liver tissue was removed surgically and placed in thioglycollate broth (Difco). Identification of microorganisms was by standard bacteriological methods.

Endotoxin distribution studies. Lipopolysaccharide obtained from Salmonella typhosa 0901 (Difco) was resuspended in pyrogen-free water. For studies of endotoxin distribution in immunosuppressed animals, this preparation was labelled with $\text{Na}_2^{51}\text{CrO}_4$ (New England Nuclear, specific activity 5-500 Ci/g) according to the method of Braude et al.¹ Endotoxin and isotope (10 mg/mCi) were incubated for 24 hours at 37°C, diluted with 5 volumes of saline and dialyzed. One milligram ^{51}Cr labelled endotoxin in 0.5 ml saline was injected I. V. Activities of ^{51}Cr in tissues were determined in a Nuclear-Chicago Ultrascaler II well type gamma counter.

Survival studies. Endotoxin or pyrogen-free water was administered by gavage into mice in 0.5-ml volumes, in the morning. Differences in survival times of mice treated either with water or endotoxin were evaluated statistically with the aid of the Mann-Whitney and Kruskal-Wallis tests.²

III. RESULTS

Detection of endotoxin and bacteria in liver samples. In mice subjected to 850 rads of x irradiation only, aseptic endotoxemia was detected in the liver between 24 and 72 hours (Figure 1). No endotoxin was detected at days 5, 7 and 8. Bacteria (primarily *Proteus mirabilis* and *Escherichia coli*) were cultured from liver fragments only on days 11-13, at which times endotoxin was also detected. Mice given radiation only died during this period. In contrast, endotoxin was detected on days 1 and 5 in livers of mice undergoing GVHD. Mice receiving a bacteria-free suspension of 5×10^6 allogeneic CBA spleen cells immediately after 850 rads survived only 7 days, and gram-negative organisms were detected consistently in liver samples from 24 hours to day 7 (Figure 2).

The detection of aseptic endotoxemia at 48 hours postirradiation was verified by the AMD procedure. Mice sensitized to endotoxin by AMD were inoculated I. P. with homogenized liver obtained from irradiated or nonirradiated animals following which survival times were measured. Most sensitized mice (15/20) inoculated with homogenates from irradiated animals died, whereas negligible mortality followed inoculation with liver homogenates from nonirradiated animals.

Organ uptake of labelled endotoxin. In B6CBF1 mice undergoing GVHD, a 50 percent reduction in hepatosplenic localization of labelled endotoxin injected I. V. occurred by day 4 (Figure 3). Twofold to threefold increases in labelled endotoxin levels were found in lung, kidney, heart and brain. In mice irradiated only, hepatosplenic concentration of ^{51}Cr endotoxin was unaffected until 12-14 days after radiation

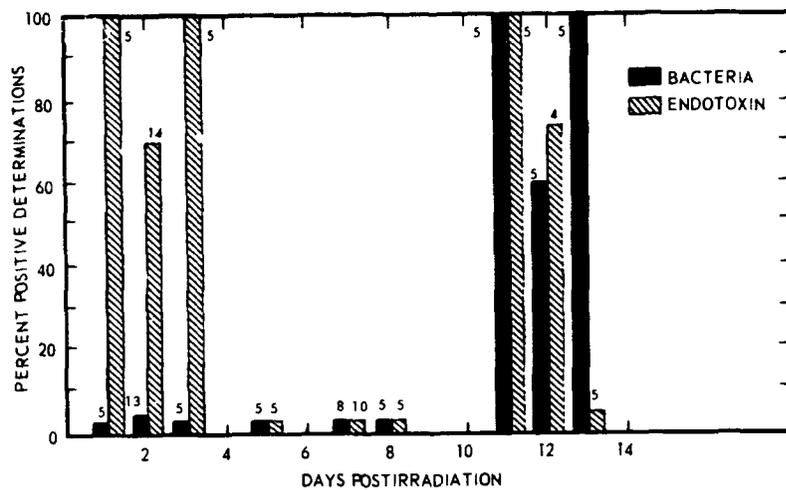


Figure 1. Endotoxin and bacteria detected in livers of mice receiving 850 rads x radiation. Numbers associated with individual bars denote number of samples tested.

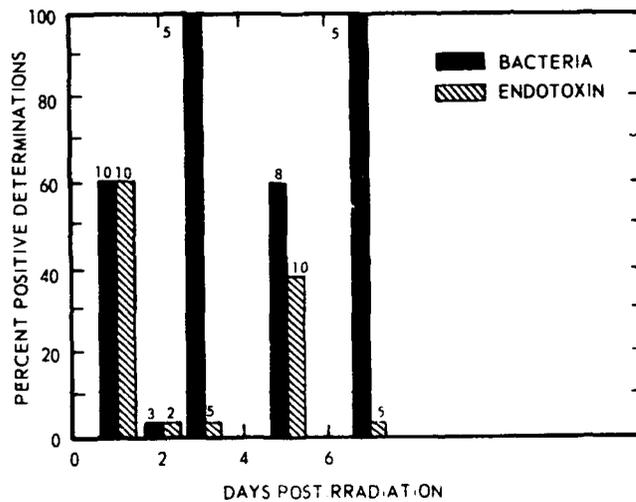


Figure 2. Endotoxin and bacteria detected in livers of mice grafted with 5×10^6 allogeneic spleen cells immediately after receiving 850 rads x radiation. Numbers associated with individual bars denote the number of samples tested.

(Figure 4). Endotoxin levels in other organs were not significantly altered. The apparent enhancement of splenic uptake observed at days 5 and 9 after radiation does not reflect an increase in phagocytic activity. This phenomenon is due instead to the 60 percent weight reduction in spleens of irradiated animals.

Gut permeability to endotoxin. Mice receiving 850 rads of x radiation or undergoing GVHD were given ^{51}Cr labelled endotoxin orally. Hepatic uptake of the radioactive label was monitored for 24 hours after injection, at which time most labelled endotoxin was excreted. Assays of hepatic uptake were made at days 1, 2, 9 and 12 postirradiation and on days 2 and 4 postinitiation of the graft versus host reaction. No uptake of labelled endotoxin was detected in both normal and immunosuppressed animals. In further experiments, peritoneal cavities were rinsed with 2 ml water and samples of the rinse solution assayed for ^{51}Cr . Assays were conducted on normal animals and on those receiving radiation or radiation and foreign immune competent cells 24 hours earlier. Again all tests were negative, indicating no significant escape of endotoxin from the gut.

Peritoneal rinses were also obtained from immunosuppressed animals at intervals corresponding with liver assays (Figures 1 and 2) and tested for endotoxin with the limulus lysate test. These tests were also negative.

Further evidence against significant escape of intestinal endotoxin from the gut after irradiation or during GVHD was obtained by studying survival after endotoxin was delivered by gavage postirradiation. No change in survival was found when mice were given two 2-mg doses of endotoxin on days 1 and 2 or 4 and 6 postirradiation or

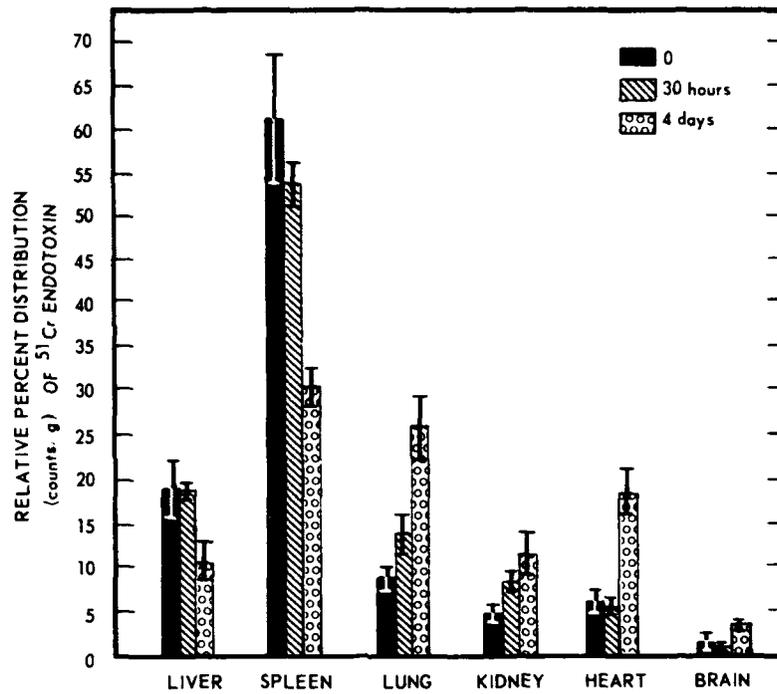


Figure 3. Clearance of ^{51}Cr labelled endotoxin by mice undergoing graft versus host disease

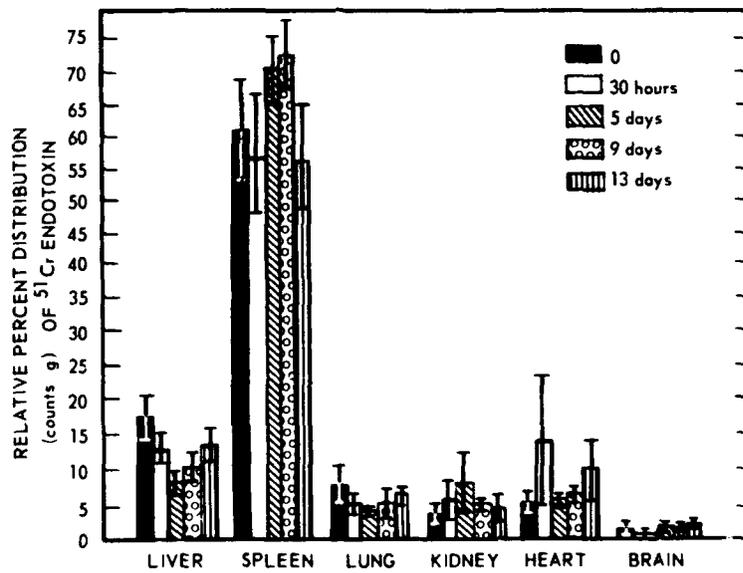


Figure 4. Clearance of ^{51}Cr labelled endotoxin by mice exposed to 850 rads x radiation

on days 0 and 3 of the GVHD. In further studies in which mice received 850 rads of x radiation only, survival times were compared in animals given two injections totaling 0, 2, 4, or 8 mg of endotoxin. These injections were given on days 2 and 5 after irradiation. No differences in survival times were observed among the four groups.

IV. DISCUSSION

For the first time, direct evidence has been obtained that endotoxin is present in livers of irradiated animals and animals grafted with allogeneic cells after irradiation. Furthermore, endotoxin was detected in the livers of these animals with or without the presence of bacteria in that organ. This suggests strongly that aseptic endotoxemia occurs during the first several days after irradiation. This early endotoxemia, presumably of intestinal origin, may contribute to further impairments in endotoxin elimination and reduce the animals' resistance to infection at a later time. These possibilities are presently being investigated.

Negative endotoxin determinations of liver samples obtained from animals between days 5 and 10 postirradiation may imply transitory correction of impairments which permit endotoxemia. Endotoxin detected in livers of immunosuppressed mice could be due to increased escape of endotoxin from the gut.⁵ However, we were unable to confirm this process. Another factor which would permit endotoxin accumulation in the liver is impaired hepatic degradation of this substance.¹³ The latter possibility was not examined in our study.

In contrast to findings in mice suffering radiation injury alone, the presence of endotoxin in the livers of animals undergoing GVHD was often associated with

bacterial infection (Figure 2). Thus, endotoxin detected during GVHD could either be from the gut pool or from infecting microorganisms. Since mice undergoing GVHD did not die until days 6 or 7, bacteria detected shortly after grafting probably represent very low level infections. A positive test for endotoxin, however, requires 10^3 - 10^4 gram-negative microorganisms.⁶ Thus, viable bacteria in tissues may be only partly responsible for endotoxin detected in these studies.

Infections have been reported by others to be a feature common to secondary disease.¹⁸ Hepatic lesions induced by grafted immunocompetent cells may make mice undergoing GVHD more susceptible to random infection than animals receiving radiation alone. Although the liver is relatively radioresistant,³ GVHD is characterized by focal necrosis of hepatocytes and Kupffer cells and Kupffer cell hyperplasia.¹⁵ The lymphoid tissues also exhibit profound cellular necrosis. In addition, intestinal epithelial necrosis during GVHD, as well as impairment of intestinal lymphoid function, could enhance microbial escape into other tissues.

Endotoxin from the gut pool may be more prevalent in tissues of mice undergoing GVHD than our data indicate. Whereas bacterial uptake by the liver may be normal, we found that hepatosplenic localization of labelled endotoxin was reduced more in mice undergoing GVHD than in those irradiated only. Therefore, in animals undergoing GVHD, the liver may not sequester detectable amounts of endotoxin.

Thus, we have established that endotoxin in detectable amounts may be found in the livers of irradiated mice and mice undergoing GVHD. This endotoxin may enhance susceptibility to subsequent infections associated with these immunosuppressed animals.

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