Effect of Sub-lethal Total Body X-radiation on Susceptibility of Mice to *Clostridium septicum* Toxin.* (19723)

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Numerous workers have investigated the effect of exposure to large doses of X-radiation on the susceptibility of animals to experimental infection with bacterial(1,2), viral (3,4), and rickettsial(5) agents. It has been found that irradiated animals generally, but not always, developed clinical symptoms or succumbed, when given doses of these viable agents which were too small to lead to the development of clinical disease in non-irradiated control animals. The enhanced susceptibility of irradiated animals has been attributed to the deleterious effects of exposure to large doses of X-rays in bringing about 1) lowered peripheral granular leucocytes and lymphocyte counts, 2) suppression of bone marrow and lymphatic tissue elements, 3) inhibition of antibody production, 4) altered activity of the fixed and wandering phagocytic cells, and 5) altered local tissue immunity. For pertinent reviews, the reader is referred to the papers of Taliaferro and Taliaferro(6), Craddock and Lawrence(7), and Shields Warren(8). The impediment of lymphatic blockade, and of the screening action of liver and spleen has also been held responsible(9).

The use of agents which multiply in the host before and during the period of clinical disease entails a series of complicating factors which make it difficult to deduce from experimental results the possible causes of the enhanced susceptibility of irradiated animals. A bacterial toxin appeared to be a somewhat less complicated tool for the study of susceptibility changes in irradiated animals. The toxin of *Clostridium septicum* was selected for this study because of the practical interest inherent in the problem of gas gangrene in man or animals previously exposed to ionizing radiation. This agent also offered the advantage of relatively rapid action(10), a factor of some importance in the study of the causes of radiation-induced increases in susceptibility in view of the findings that the rate of recovery from radiation damage of different tissues and functions proceeds at dissimilar rates, and that the time lapse between irradiation and infection is a critical factor in general(11).

Materials and methods. Female albino mice 5 to 6 weeks of age, from a single source (Rockland Farms) were used throughout these experiments. The method employed for exposure to X-rays was described in detail elsewhere(12). Briefly, the radiation factors were as follows: 200 KVP, 20 ma, tsd 75 cm, filter 0.25 mm Cu and 1 mm Al, HVL 0.75 mm Cu, output approximately 21 r per minute measured in air.

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In preparing toxin, a medium consisting of proteose No. 3 broth (proteose peptone No. 3, Difco, 20 g; NaCl, 5 g; Na_2HPO_4, 5 g; distilled water, 1,000 ml) was poured over a shallow layer of defatted horse meat in an Erlenmeyer flask and was autoclaved for one hour at 20 lb pressure, cooled rapidly, and inoculated with 5 ml of a 24-hour broth culture of *C. septicum* for each liter of medium. Sufficient sterile 40% glucose solution was added to raise the concentration of glucose in the medium to 0.2% at the beginning of the incubation period, to 1% after 18 hours of incubation, and finally to 2% after 24 to 30 hours of incubation.‡ After 24 hours of incubation

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‡ The gradual increase in glucose concentration was suggested in a personal communication from Dr. H. A. Dettwiler.

TABLE I.

A. LD_{50} of *Clostridium septicum* toxin for irradiated and non-irradiated control mice.

	Mice, dead/total, after inoculation of indicated amount of toxin (ml)												
	.03	.04	.05	.06	.07	.08	.09	.10	.12	.14	.16	.18	.20
X-rayed*	0/3	1/3	1/3	1/3	2/3	2/3	1/3	2/3	3/3	3/3	3/3	3/3	3/3
Control	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	1/3	2/3	2/3	3/3

LD_{50}X (X-rayed): .07 ml
LD_{50}N (Control): .15 ml

B. PD_{50} of specific antitoxin in mice injected with .20 ml of toxin.

	Mice, dead/total, after injection of antitoxin Reciprocal of antitoxin dilution†								
	20	40	80	160	320	640	1280	2560	Normal serum‡
X-rayed*	1/6	1/6	0/6	3/6	3/6	4/6	6/6	6/6	5/5
Control	0/6	0/6	1/6	0/6	0/6	3/6	4/6	4/6	6/6

PD_{50}X (X-rayed): 1.97×10^{-3} ml
PD_{50}N (Control): 5.71×10^{-4} ml

* 350 r total body X-radiation 6 days before administration of antitoxin, and 7 days before injection of toxin, respectively.
† Antitoxin diluted in normal horse serum 1:20. .5 ml aliquots were given intramuscularly.
‡ Normal horse serum 1:20.

at 37°C samples were removed at approximately 2-hour intervals and were titrated for their hemolytic activity against sheep erythrocytes(13). The toxin was harvested by centrifugation and was filtered through a Seitz sterilizing pad when no further increases in the hemolytic activity of the culture were noted. The sterile filtrates were stored at 4°C for no longer than one week and showed little deterioration during this storage period.

Solutions of the dry toxin, as well as dilutions of the filtrates were prepared in proteose No. 3 broth just prior to their use in animal inoculations. Dilutions of antitoxin were prepared in a 2% solution of normal horse serum in physiological saline solution. The toxin was given routinely by the intraperitoneal route (0.5 ml of the various dilutions), while antitoxin was given intramuscularly (0.5 ml).

Since most of the animals which received one or more lethal doses of toxin died within 24 hours after injection, and because generally no deaths occurred later than 48 hours after injection of the toxin, all experiments were terminated 4 days after toxin had been given to the animals.

In titrations of LD_{50} dose of toxin and PD_{50}§ dose of antitoxin, 3 to 4 mice were used for each dose in those experiments in which the dose-increment was kept very small; in other experiments 6 to 8 animals were used for each dose. Both LD_{50} and PD_{50} values were calculated according to the method of Reed and Muench(14).

Experimental. Shown in Table I are a protocol and representative data of LD_{50} and PD_{50} titrations of *C. septicum* toxin and antitoxin in irradiated and in non-irradiated control mice. It may be seen that the LD_{50} dose of this particular lot of toxin was approximately 0.07 ml for mice which had been exposed to 350 r total body X-radiation 7 days before the test, while the LD_{50} for the control animals was approximately 0.15 ml. Additional results of LD_{50} titrations in mice irradiated with 250 or 350 r one week before the test have been summarized in Table II, together with the results of titrations in non-irradiated control mice. It appears that under the conditions of these experiments irradiated mice consistently succumbed to smaller amounts of toxin than did the non-irradiated controls. The ratios of LD_{50} doses for control and irradiated animals ranged from 1.2 to 2.3. The higher ratios were charac-

§ The term PD_{50} (protective dose $_{50}$) is used to denote the smallest amount of anti-toxin which protects 50% of the animals exposed to an arbitrary challenge dose of toxin.

TABLE II. LD_{50} and PD_{50} Doses of *C. septicum* Toxin and Antitoxin in X-irradiated and in Control Mice.

Toxin preparation	LD_{50} for Normal mice	LD_{50} for Irradiated mice	Challenge dose of toxin	PD_{50} for normal mice, ml	PD_{50} for irradiated mice Observed, ml	PD_{50} for irradiated mice Expected,* ml	% difference†
1	.66 mg	.29 mg‡	1 mg	3.71×10^{-3}	8.25×10^{-3}¶	7.75×10^{-3}	+ 6.6
2	.15 ml	.07 ml ‡	.20 ml	5.71×10^{-4}	1.97×10^{-3}¶	1.48×10^{-3}	+24.8
3	.21 "	.17 " §	.25 "	5.35×10^{-4}	8.83×10^{-4}‖	8.56×10^{-4}	+ 3.1
3	.21 "	.17 " §	.40 "	2.08×10^{-3}	2.17×10^{-3}‖	2.52×10^{-3}	—16

* Expected PD_{50} for irradiated mice $= PD_{50}$ for normal mice $\times \frac{\text{challenge dose} - LD_{50} \text{ for irrad. mice}}{\text{challenge dose} - LD_{50} \text{ for normal mice}}$

† % difference $= \frac{(\text{observed} - \text{expected}) \; PD_{50} \text{ for irradiated mice}}{\text{observed } PD_{50} \text{ for irradiated mice}} \times 100.$

‡ Mice challenged 7 days after exposure of the entire body to 350 r.

§ Mice challenged 7 days after exposure of the entire body to 250 r.

¶ 350 r total body x-radiation 6 days before administration of antitoxin, and 7 days before inj of toxin, respectively.

‖ 250 r total body x-radiation 6 days before administration of antitoxin, and 7 days before inj of toxin, respectively.

teristically found when LD_{50} doses for mice exposed to 350 r were compared with similar values for the normal animals.

It may also be seen from the results presented in Table I that more antitoxin was required to protect irradiated mice against an arbitrary lethal dose of toxin than was needed for the protection of control mice which received the same amount of toxin. In this particular experiment the PD_{50} dose of antitoxin for irradiated mice was about 2.9 times greater than the PD_{50} dose for control mice. In similar titrations, summarized in Table II, the ratio of PD_{50} doses for irradiated and for control mice varied from 1.1 to 2.2.

These results indicated that exposure of the animals to X-radiation resulted in injury which increased the susceptibility of the animals to the toxin of *C. septicum,* and that more antitoxin had to be given to irradiated mice than to non-irradiated mice in order to protect the animals from death when an arbitrary lethal dose of toxin was injected.

Discussion. The finding of increased susceptibility to the toxin of *C. septicum* among mice which had previously been exposed to sub-lethal X-radiation is consistent with the observation that previously irradiated animals generally appear to be more susceptible to a great number of different pathogenic agents. That the difference in LD_{50} doses of this toxin for irradiated mice and for non-irradiated control animals was not of the order of magnitude encountered in similar experiments with certain viable bacterial agents(1,2) may indicate that anti-bacterial defense factors are more severely damaged by X-radiation than are antitoxic defenses.

The fact that more antitoxin was required for the protection of previously irradiated mice might have been the result of radiation-induced damage to the factors and mechanisms responsible for the distribution and disposition of parenterally introduced foreign proteins (antitoxin in this case). On the other hand this observation might merely have reflected the greater susceptibility of irradiated mice to the lethal action of toxin. It could be expected that irradiated mice would require more antitoxin for their protection because in their case more of the toxin contained in the challenge dose needed to be neutralized.

It may be seen from Table II that the amount of antitoxin required to protect 50% of non-irradiated control mice against 0.40 ml of a particular preparation of toxin (preparation 3) was 2.08×10^{-3} ml while 5.35×10^{-4} ml of antitoxin sufficed to protect 50% of a similar group of mice against 0.25 ml of the same toxin. Since the PD_{50} dose of antitoxin may be considered to be the smallest amount which neutralizes, *in vivo,* all but one LD_{50} dose of the arbitrary challenging dose of toxin, and since the LD_{50} dose was found to be 0.21 ml of this preparation of toxin, one may cal-

culate that 5.35×10^{-4} ml of antitoxin were required to neutralize 0.25-0.21, or 0.04 ml of toxin. It may then be predicted that mice challenged with the larger dose of toxin (0.40 ml) would require $5.35 \times 10^{-4} \times \frac{0.40-0.21}{0.25-0.21}$, or 2.54×10^{-3} ml of antitoxin as PD_{50} dose. This expected amount of antitoxin approached closely the experimentally observed value of 2.08×10^{-3} ml (a difference of 22%), and therefore appeared to warrant similar calculations of expected PD_{50} values in irradiated mice. These calculations were based on the assumption that irradiated mice, tested under the conditions of these experiments, were as capable of utilizing passively administered antibody as were the corresponding non-irradiated control mice.

It may be seen from Table II that the predicted PD_{50} values differed by no more than 25% from the experimentally observed values. In view of the errors inherent in the calculations of LD_{50} and PD_{50} doses, even the greatest difference between expected and observed values encountered in these experiments appeared to lack significance. The data therefore appear to support the hypothesis that irradiated and non-irradiated control mice do not differ from each other in their ability to utilize antibody.

Though other results obtained in this laboratory(2) indicated that the difference in LD_{50} doses of tetanus toxin for irradiated and non-irradiated mice is, like that of *C. septicum* toxin, only 2- to 3-fold, further data would be required to establish the validity of such a generalization for susceptibility to the action of bacterial toxins. It appears possible, however, that X-radiation may not necessarily result in selective severe damage to antibacterial defenses, but that exposure to sublethal X-ray may rather lead to modifications of host tissues or enzyme systems which might result in the production of a more favorable environment for the propagation and spread of viable agents.

The efficacy of passive serum prophylaxis against *C. septicum* toxin in animals which had received large, though sublethal doses of X-rays is of obvious practical value if these findings were to hold equally true for exposure to other ionizing radiations, and to animals other than mice. It should be noted that the antitoxin was given 6 days after exposure of the mice to X-radiation, and that at this particular post-irradiation time increases in capillary permeability were presumably quite pronounced(15). It is interesting to observe that, in spite of the presumptive "leakage" of antibody from the circulatory system(16), the animals could be effectively protected with amounts of antitoxin which were not greatly in excess of those required for the protection of non-irradiated control mice, and that the difference in the PD_{50} doses for irradiated and control mice could well have been due to the fact that, since irradiated mice succumbed to smaller amounts of (not neutralized) toxin than the controls, a greater proportion of the challenge dose of toxin needed to be neutralized in the case of irradiated animals.

Summary and conclusions. Total body exposure to 250 and 350 r reduced the resistance of mice so that animals injected 7 days after irradiation succumbed to approximately one-half the amount of *C. septicum* toxin which was required to kill non-irradiated control mice. Antitoxin given intramuscularly 6 days after irradiation, and one day before challenge with toxin, protected the life of both irradiated and control mice. Irradiated mice required from 1.1 to 2.2 times more antitoxin for their protection than did control animals. The increased antibody requirement of irradiated mice could be explained by the enhanced susceptibility to toxin of these animals. The results indicated that exposure to severe doses of X-radiation, in spite of its effects on capillary permeability, apparently fails to significantly raise the minimum amount of antibody required for effective antitoxic prophylaxis.

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Effect of Administration of Amino Acids on Circulating Eosinophils and Lymphocytes in Normal and Adrenalectomized Rats. (19724)

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It has been known for some time that glutamic acid is the only amino acid that increases the oxygen consumption when added to nervous tissue *in vitro*(1,2). Weil-Malherbe(3) has made the claim that the therapeutic effect of the administration of glutamic acid in certain diseases of the central nervous system as reported by some investigators(4) was due to the liberation of adrenalin and not to the unique behavior of this amino acid on nervous tissue. (The controversy regarding the therapeutic efficacy of glutamic acid is outside the scope of this paper.) Since it has been shown that adrenalin will produce an eosinopenia and lymphopenia in animal and man(5-8), it was thought that similar effects should be produced by the administration of glutamic acid if the claim of Weil-Malherbe were correct.

A study was made of the effect of the oral administration of various amino acids and water or 0.5% saline on the blood picture of a group of white rats before and after adrenalectomy.

Procedure and methods. Each of a group of 12 male rats weighing approximately 200 g at start of the experiment was given 5 ml of a 5% solution of l-glutamic and l-aspartic acid as their mono-sodium salts, glycine, dl-α alanine and water by stomach tube with a rest period of several days between the administration of each of these constituents. The rats were not fasted overnight. The food was removed from the cages at 9 a.m. before the experiments were started. Water was allowed *ad lib.* The tail of the rat was warmed in water and blood was obtained by cutting the lateral vein of the tail near its distal end with a razor blade. Blood counts were made immediately before and 4 hours after the administration of the test material. A complete series with the various substances was carried out on each rat and the blood eosinophil count was determined by the method of Randolph (9). The complete series was then repeated on the same rats and total white, differential, and total lymphocyte counts were made. After completion of the 2 series the same rats were adrenalectomized and the same tests were repeated. Thus each rat was subjected to 20 experimental tests. Three of the 12 rats were lost as a result of the operation.

The adrenalectomized rats were given 5% glucose in physiological saline solution for drinking and the amino acids were dissolved in 0.5% saline instead of water. The rats were treated, otherwise, in the same manner as before adrenalectomy except that during the