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Protection Induced by Bacterial Endotoxin Against Whole-Body X-Irradiation in Germfree and Conventional Mice.* (30046)

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A number of naturally produced microbial substances including dextran(1,2), levan(3), zymosan(3,4), bacterial endotoxins(4,5), and Gram-positive antigens(5) have been tested for their ability to protect mammals exposed to whole-body ionizing radiation. The best known of these are the bacterial endotoxins derived from Gram-negative enteric bacteria, and chemically defined as high molecular weight complex polysaccharides(6) which have provided some measure of protection from irradiation in several rodent species(5). Protection measured as increased survival is dependent on time and dose of endotoxin inoculation in relation to time of irradiation, as well as dose and type of radiation(5,7,8).

Recent studies have shown that the composition of the intestinal flora plays a major role in ability of mice to respond to bacterial endotoxins(9,10). Pathogen-free as well as specific pathogen-free (SPF), and germfree mice have been shown to be more resistant to the lethal effects of *Escherichia coli* endotoxin than conventional mice(10,11). Monoinfection with coliform bacteria does not induce susceptibility to endotoxin in ex-germfree animals(12). Prior contact with heat-killed or living bacteria rendered Rockefeller Swiss (NCS) albino mice more susceptible to the lethal effects of endotoxin, especially when that endotoxin was prepared from the infecting bacterial strain(11). Physiological reactions to small doses of endotoxin were essentially prevented in NCS mice previously vaccinated with dead bacteria.

Other evidence indicates that germfree and conventional mice are equally susceptible to the toxic effects of endotoxin from *Salmonella enteritidis* and *S. flexneri*(13). Certain immunological responses to small doses of these endotoxins were found to be similar in both groups of animals. On this basis, there is little difference in type or magnitude of reaction to bacterial endotoxin in animals in constant contact with living Gram-negative bacteria and those denied such contact.

Since the dose of bacterial endotoxin required for radiation protection is small, its effects may be altered according to the number and species of endogenous bacteria liberating endotoxin in the intestinal tract. Conceivably, such an animal may have the capability of immunological neutralization of small amounts of endotoxin rendering that dose partially or totally ineffective. By the same reasoning, germfree mice may show exaggerated responses to endotoxin resulting in a greater degree of radiation protection. To obviate the interference of endotoxin produced by endogenous bacteria and to discern to what degree the presence of Gram-negative enteric bacteria alter these responses, germfree animals are the experimental animals of choice. We report here the results of a comparative study of the alterations in the dose-response curves of conventional and germfree mice exposed to X-irradiation 24 hours after receiving 10 μg of endotoxin.

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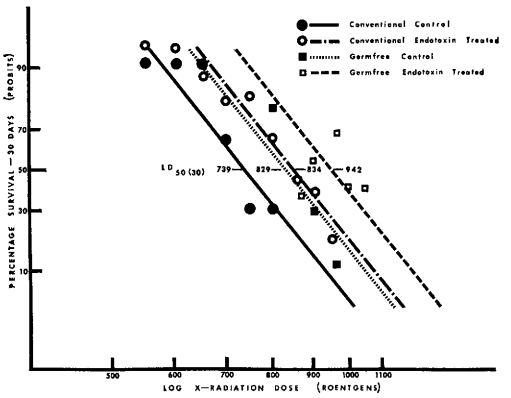

FIG. 1. Dose response curves of conventional and germfree mice exposed to whole-body X-irradiation 24 hr after inoculation with 10 μg bacterial endotoxin and corresponding saline inoculated controls.

Materials and methods. *E. coli* Bactolipopolysaccharide (Difco), produced by the Boivin trichloroacetic acid procedure was prepared as a 50 μg/ml suspension in deionized water, ampouled and autoclaved for 20 minutes at 15 lb pressure and 121°C.

A total of 290 conventional CFW mice of both sexes were maintained on sterilized Purina 5010C Lab Chow and tap water *ad libitum*. These mice were housed 5 to 10 per fiberglass cage and maintained in our laboratory for 3 to 4 weeks before X-irradiation, at which time they weighed 25 to 30 g and were 60 to 70 days of age.

A total of 171 CFW germfree mice of both sexes were maintained on sterilized Purina 5010C Lab Chow and sterile tap water *ad libitum* in Trexler type plastic isolators(14). These mice were housed 4 to 7 per plastic shoe box cage and at irradiation weighed 22 to 28 g and were 75 to 80 days of age. Routine microbiological examinations for germfree status(15) were done at 2-week intervals and at termination of the 30-day observation period.

In these studies 180 conventional and 95 germfree mice were inoculated intraperitoneally with 10 μg (0.2 ml) of the sterilized endotoxin suspension 24 hours before irradiation. Control groups of 110 conventional and 76 germfree mice received 0.2 ml of a 0.9% saline solution intraperitoneally 24 hours before irradiation. Inoculations and irradiations were performed between 10 am and 2 pm.

All animals were maintained in temperature-controlled rooms kept on a 6 am-6 pm light-dark cycle and observations were made at 2-hour intervals during the 30-day period subsequent to irradiation.

The source of X-irradiation was a 260-kvp clinical therapy X-ray machine operated at 240 kv and 15 ma with a filtration of 1.0 mm Al and 0.25 mm Cu at a rate of 35 to 40 r/min and at a skin-target distance of about 75 cm. The irradiation of germfree and conventional mice was carried out under procedures described elsewhere(16).

Results. The data used to plot the dose-response survival curves (Fig. 1) are presented in Table I. From these values, straight lines with a common slope of 11.42 were drawn through each set of points using the method of maximum likelihood estimation (17,18). Calculated chi square values for the testing of linearity of response, parallelism of regression and residual heterogeneity were all less than tabulated values. Presented in Table II are the $LD_{50(30)}$ and fiducial limit estimates of each experimental group. Although there is considerable overlap in the fiducial limits of these lines, treatment curves and the corresponding control curves were determined to be significantly different from each other at the 95% level of confidence by chi square analysis. In addition, there is a statistically significant difference at the same level of confidence between the curves for germfree and conventional controls and between those for germfree and conventional endotoxin-treated mice.

The mean survival time for those groups in which mortality is less than 100% was calculated from the survival times of only those mice that died within the 30-day observation period. There were no significant differences between survival times of endotoxin-inoculated germfree mice and endotoxin-inoculated conventional mice when each group was compared to its appropriate control.

The LD_{0-100} range for control conventional mice was 500 to 850 r and 700 to 1000 r for their germfree counterparts. The LD_{0-100} range for endotoxin-treated conventional and germfree mice was 500 to 1000 r and 800 to 1100 r respectively.

TABLE I. Percentage Survival and Mean Survival Time of X-Irradiated (A) Conventional Mice and (B) Germfree Mice.

		Endotoxin treated (10 μg IP)			Saline controls		
	Dose (r)	Survival ratio, No. survivors/ No. irradiated	% Survival	Mean survival time (days)	Survival ratio, No. survivors/ No. irradiated	% Survival	Mean survival time (days)
A.	500	18/18	100		10/10	100	
	550	15/16	93.75	12.31	9/10	90	12.79
	600	14/15	93.33	11.88	9/10	90	12.98
	650	14/16	87.50	17.01 ± 1.97*	9/10	90	28.65
	700	15/18	83.33	11.01 ± .53	7/11	63.64	16.78 ± 4.72*
	750	13/16	81.25	12.37 ± 1.19	3/10	30	9.39 ± .36
	800	11/17	64.71	13.69 ± 2.41	3/10	30	11.29 ± 1.42
	850	7/16	43.75	14.86 ± 1.92	0/10	0	11.09 ± 1.14
	900	6/16	37.50	15.92 ± 1.02	0/10	0	10.69 ± .67
	950	3/16	18.75	10.89 ± .73	0/10	0	11.05 ± .57
	1000	0/16	0	9.49 ± .43	0/10	0	9.69 ± .73
B.	700	12/12	100		8/8	100	
	800	11/11	100		7/9	77.78	11.88 ± 0
	866	3/8	37.5	14.08 ± 1.23	0/8	0	14.62 ± 2.05
	900	8/15	53.33	13.51 ± 1.35	3/11	27.27	15.24 ± 1.43
	966	6/9	66.67	12.53 ± 2.04	1/9	11.11	12.54 ± .66
	1000	4/10	40	12.00 ± 2.19	0/9	0	13.91 ± 1.68
	1050	4/10	40	12.58 ± .40	0/9	0	11.60 ± .31
	1100	0/10	0	11.52 ± .87	0/8	0	12.35 ± .32
	1150	0/10	0	13.08 ± .68	0/5	0	12.54 ± .55

* ± S.E.

The $LD_{50(30)}$ values as determined by the least-squares method(19) were as follows: 704 r for the conventional saline-inoculated controls and 804 r for the conventional endotoxin-treated mice; 832 r for the germfree saline-inoculated controls and 946 r for the germfree endotoxin-treated mice. These values check well with the $LD_{50(30)}$'s as determined by the maximum likelihood estimation.

Discussion. A 10 μg inoculation of a Boivin type preparation of endotoxin 24 hours before X-irradiation provides protection against X-irradiation in both germfree and conventional mice. Both the calculated and graphic $LD_{50(30)}$ determinations indicated an increase of LD_{50} X-ray dose of approximately 100 r in treated *vs* non-treated germfree and conventional mice. Radiation dose-reduction factors(20) of both germfree and conventional mice calculated either on the basis of the least-squares method or the method of maximum likelihood estimation were almost identical, all values being in the range of 1.13 to 1.15.

TABLE II. Estimates of $LD_{50(30)}$'s and Fiducial Limits Determined by Method of Maximum Likelihood Estimation.

Status of mice	Treatment before irradiation	$LD_{50(30)}$	Fiducial limits
Conventional	Saline	739	682– 810
"	Endotoxin (10 μg IP)	834	794– 886
Germfree	Saline	829	723– 941
"	Endotoxin (10 μg IP)	942	875–1000

Similarity in response to bacterial endotoxin as found in this investigation is consistent with the findings of Landy(13), who noted no difference between germfree and conventional mice in their reactions to toxic and small doses of endotoxins. It appears, then, that the reactions to inoculated endotoxin that confer radiation protection are independent of any reactions to endotoxin released in the intestinal tract by Gram-negative bacteria in the microflora.

Endotoxin is ineffective as a radioprotective drug in the "gut death range" when survival percentage and survival times are compared. Since protection is limited to the "hemopoietic death range," it is suggested that protection is by sparing hemopoietic cells or by activating surviving precursor cells to repopulate hemopoietic centers. The advance in hemopoiesis by endotoxin has been discussed by Smith and coworkers(21) as causally related to radiation protection. The ex-

act mechanism by which endotoxin induces radio-protection is unknown and this is being investigated.

Summary. When conventional and germ-free mice are administered 10 μg of *E. coli* endotoxin intraperitoneally 24 hours prior to whole-body X-irradiation, the $LD_{50(30)}$ X-ray dose is raised to about the same degree for both groups: from 739 r to 829 r for conventional mice and 834 r to 942 r for germ-free mice. For both groups the X-ray dose-reduction factors lie in the narrow range of 1.13 to 1.15.

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Plaque Studies with Certain Group B Arboviruses. III. St. Louis Virus on Chick Embryo Tissue Culture.* (30047)

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Although several group B arboviruses have produced plaques or shown distinct cytopathic effects (CPE) in chick embryo tissue culture (CETC), most reports indicate failure with St. Louis encephalitis (SLE) virus (1,2,3). In studies reported here several strains of SLE virus produced clear cut CPE in CETC monolayers and some strains produced excellent plaques under agar. Using 2 very similar strains recently isolated in Florida, 2 distinct plaque variants were noted in each, one much larger than the other, each producing what appear to be genotypic progeny.

Materials and methods. Virus strains. P-15 from *Culex* mosquitoes of Pinellas County and the first SLE mosquito isolate from Florida(4), and TBH-28, a subsequent human brain isolate(5), were tested in early mouse brain passage. Each had a titer of $10^{8.5}$ TCD_{50} per 0.1 ml in hamster kidney

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