Further Studies with Cell-Free Extracts from Mouse Spleen on X-Ray Induced Mortality.*† (22578)

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We have been able to offer evidence(1) in support of a humoral spleen factor effective in the recovery of mice from X-ray-induced mortality. In these studies it was shown that administration of cell-free saline extracts from spleens removed 6 days after exposure of the donor mice to the LD 30 14 days of X rays resulted in a definite and statistically significant reduction of mortality. However, extracts made from spleens of donor animals exposed to the LD 55 14 days and removed at the same time after irradiation failed to show such protective effect. The results obtained with extracts from normal spleens were ambiguous. The ambiguity was partly traceable to a saline effect on X-ray mortality previously described(2).

Studies in which it was possible also to demonstrate the presence of the humoral spleen factor in extracts from normal mouse spleen tissue are here presented.

Methods. A. *Experimental Animals.* Male white Swiss mice of the Institute stock, weighing 25 ± 4 g were used. B. *Irradiation* of the mice took place as previously described in groups of 12 animals(3). The radiation factors were 200 KVP, 25 ma, 0.25 mm Cu + 1.0 mm Al added filter, HVL = 0.8 mm Cu. The X-ray equipment was calibrated prior to each set of exposures.‡ All animals to be used for the evaluation of spleen extracts were exposed to 425 r/air which dose represents, under the conditions of this experiment, the LD 78 15 days. (Table I, group A.) C. *Spleen Extracts.* Unirradiated or irradiated mice were killed by dislocation of the neck and the spleens removed immediately. The organs were quick-frozen with dry ice, ground to a fine powder in a mortar cooled with dry ice. Various amounts of 0.9% saline were added resulting in concentrations of one, two, etc., spleens per cc. The mixture remained for extraction in a refrigerator for at least 24 hours and was then centrifuged at 3-5°C at 2,500 rpm for 30 minutes for the removal of grosparticulate matter. The supernatant was decanted into a sterile beaker and passed through a Buchner filter with several layers of filter paper under light vacuum. This filtrate was then passed through a Selas filter of 03 porosity under heavy vacuum, to obtain extracts free from viable cells and bacterial contamination. The extracts were injected intramuscularly in amounts of 0.3 cc daily for 5 consecutive days starting on zero day immediately after irradiation. D. *Control of reproducibility of extraction method* took place by means of micro-Kjeldahl determinations of total protein and NPN content of the extracts, using a standard distillation method(4). E. *Recording and presentation of effects of extracts on X-ray-induced mortality.* The number of dead animals in each experimental group was recorded daily over a period of 20 days. The following groups were used: (a) non-medicated, irradiated animals; (b) saline-treated, irradiated animals; (c) spleen extract-treated, irradiated animals. F. *Evaluation of results.* The number of survivors in the spleen-treated and saline control groups of equal sizes was compared on the 20th postirradiation day. The time of comparison has been selected on the basis of our preliminary studies(1) showing that an effect of spleen extract was not noticeable prior to the end of the second postirradiation week, and that no important changes in mortality rates occurred after this date. This choice was furthermore determined by our observation that at the 20th

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TABLE I. Comparison of X-Ray-Induced Mortality in (A) Non-Medicated, Irradiated Mice; (B) Saline-Treated, Irradiated Mice; (C) Normal Spleen Extract-Treated, Irradiated Mice.

Days after irradiation →	Group A Non-medicated					Group B Saline controls					Group C Spleen extracts				
	0	5	10	15	20	0	5	10	15	20	0	5	10	15	20
											Extr D				
Exp. 1	72*	72	63	14	11	72	71	53	18	15	72	67	47	24	22
											Extr 2D				
2	48	48	36	13	9	48	48	30	7	5	48	48	32	13	12
											Extr 4D				
3	24	24	16	4	3	24	24	21	3	3	24	24	23	5	5
Total animals alive	144	144	115	31	23	144	143	104	28	23	144	139	102	42	39
Mortality, %	0	0	20	78	83	0	1	28	80	83	0	3	29	70	73

* No. of survivors.

postirradiation day, 100% of the survivors of an exposure to 425 r/air were 2 ± 1 animal in 12 groups of 12 mice each. This consistency in the number of survivors among irradiated non-medicated controls appeared advantageous to the study of deviations in the number of survivors caused by medication of irradiated animals.

Results. 1. *Influence of saline injection on mortality produced by standard X-ray dose.* In view of the previously described saline effect on X-ray mortality(2), it appeared necessary to ascertain whether or not injection of a volume of saline equal to that of the spleen extracts would influence the X-ray-induced mortality under the conditions of the present experiment. Comparison of groups A and B of Table I shows that saline did not alter the X-ray-induced mortality significantly.

2. *Effect of extracts from unirradiated mouse spleens on X-ray mortality.* For evaluation of the effect of extracts made from normal mouse spleens, the following preparations were used: 1 spleen/cc, 2 spleens/cc, and 4 baby mouse§ spleens per cc of saline, representing the extracts designated as D, 2D, and 4D respectively. The data obtained are summarized in column C of Table I (Exp 1-3). In each group there were more survivors in the spleen-treated group of animals than in an equally large group of simultaneously observed saline controls. While the differences in each of these groups are not of statistical significance, these observations indicate a definite trend toward higher survival in the spleen-treated animals. Comparison of total experience for groups B and C, comprising 144 spleen-treated and 144 saline controls, shows that there were 39 survivors among the spleen extract-treated animals and 23 among the saline controls. The difference between 39 and 23 is significant since $\chi^2 = 4.6246$; $P = 0.02$ to 0.05.

3. *Effect of extracts from spleens from irradiated donor animals on X-ray mortality.* Donor animals were exposed to the LD 55/14 days which, under the conditions of our experiment, was represented by 410 r/air. Spleens were removed on the 6th and 10th day after irradiation, respectively. The extracts were designated as DX_6/410 r and DX_{10}/410 r respectively, the letter D indicating that one spleen was extracted with 1 cc of saline; X stands for irradiation; the subscript indicates day of removal of spleens from the donor mice exposed to the X-ray dose shown after the slant. The results obtained with these extracts (Exp 4 and 5) are shown in Table II. No difference was found in the number of survivors of either of the 2 spleen-treated groups when compared with their respective saline controls.

4. *Reproducibility of spleen extraction method.* To ascertain the reproducibility of the spleen extracts a determination was made of total protein and NPN content of the extracts. The following averages were found: spleen weight 135 mg (120-160), total protein 975 mg % (710-1,290), and NPN con-

§ *i.e.*, mice 2 to 3 days of age.

TABLE II. Comparison of X-Ray-Induced Mortality in Saline-Treated, Irradiated Mice with That of Irradiated Mice Treated with Spleen Extracts from Irradiated Donor Animals.

Days after irrad. →	Group B Saline controls 0	5	10	15	20	Group C Spleen extracts 0	5	10	15	20
						Extr DX_6/410 r				
Exp. 4	48*	48	36	10	9	48	48	30	13	9
						Extr DX_{10}/410 r				
5	36	36	20	7	7	36	36	23	9	7
Total animals alive	84	84	56	17	16	84	84	53	22	16

* No. of survivors.

tent 54.5 mg % (41.6-70.0). These data indicate a reasonable reproducibility of this relatively crude extraction method. To obtain information on quantitative aspects of the extraction method, similar determinations were made in extracts where 2 spleens were extracted with 1 cc of saline. Two determinations of total protein and NPN in such extracts resulted in averages of 1,850 mg % of total protein (1,800-1,900), and 98.4 mg % of NPN (83.8-113.0). The chemical analysis thus indicates a satisfactory reproducibility of the extraction method also from the quantitative aspect. This view is furthermore supported by the data on baby mouse spleens. Average weight of the baby mouse spleens was only 18 mg, 1/8 the average weight of the adult mouse spleens.

Concentration of spleen in the preparation using 4 baby mouse spleens is about 1/2 that from 1 adult mouse spleen using 1 spleen/cc. Total protein content in the 3 determinations averaged 427 mg% (350-490) and NPN 30 mg % (29.0-31.7). These values are one-half of averages shown for D extracts and further indicate reproducibility of the extraction method. Finally data obtained from the study of extracts made with one spleen from an irradiated animal per cc are given. The average weight of these spleens was 44.7 mg (38-55), roughly 1/3 of the average weight of the unirradiated spleens. Average values for total protein and NPN were 253 mg% (220-270) and 12.9 mg % (5.1-17.2), respectively, about 1/4 the average values found for normal spleens. The definitely lower total protein and NPN content of this preparation in contrast to the preceding ones is noteworthy.

Discussion. In this study the existence of a humoral factor in cell-free saline extracts made from spleens of unirradiated mice has been clearly demonstrated. These results round out our preliminary studies in which the presence of this factor was not demonstrated due to technical difficulties. It appears noteworthy that spleen extracts varying over a wide range in their protein and NPN content offer protection against X-ray doses which would otherwise kill about 78% of mice on the 15th day after irradiation. The significance of the data here presented appears to be enhanced by the fact that spleen extracts made from spleens removed from donor animals exposed to an X-ray dose killing about 55% of the animals within 14 days failed to show a protective effect, in line with our previous observations(1). The failure of these extracts to protect is paralleled by the results of the chemical studies of extracts made from these irradiated spleens which show a total protein and NPN content of about 1/4 that found in equally prepared extracts from normal spleens, while spleen concentration amounted to roughly 1/3.

It would be tempting to assume that the spleens reduced in weight by irradiation to 1/3 of average weight of normal spleens are deficient in this humoral factor. However, such a statement could only be made with certainty if extracts made from irradiated spleens in concentrations equal to those made from normal mice would also show reduction in protective effect. Unfortunately, technical difficulties (irradiation of the very large numbers of donor mice necessary to obtain sufficiently large amounts of extracts) prevented the use of such concentrated extracts from irradiated spleens. The question therefore remains whether the differences between effects of extracts made from spleens of unirradiated and irradiated donor animals are merely due to a quantitative difference in concentration of the extractable factor, or whether the irradiated organ, shrunk to one-third of normal size and consisting chiefly of fibrous tissue, is functionally deficient with respect to elaboration of the humoral factor.

Our previous experiences(1), in which a protective spleen extract was obtained from organs of irradiated animals which were in an active stage of regeneration and reduced to only one-half or less of the average normal spleen size, support the last assumption.

Summary and conclusions. 1. Continuing previous studies, employing improved bioassay methods, the protective effect of cell-free saline extracts made from spleens of normal mice against X-ray-induced mortality in this species has been demonstrated. 2. Extracts made from the spleens of donor animals exposed to an X-ray dose which killed 55% of the donor animals in 14 days failed to show a protective effect, which was in agreement with previous observations. 3. A certain amount of parallelism between the protective effect of various extracts made from normal mouse spleens and the failure to protect of those made from irradiated mouse spleens was found in studies of the total protein and NPN content of such extracts. 4. It appears that within a wide range of concentration of total protein and NPN content such protection can be achieved. 5. However, reduction of average content of total protein and NPN below one-half of lowest average value for normal spleens, ascertained in samples made from spleens of irradiated donor animals, was found to be associated with a lack of protection. Whether this failure is due merely to quantitative or also to qualitative differences in these extracts is not clear.

Since the writing of this paper, an article by Allen *et al.* appeared in *Science,* 1956, v123, 1080, in which postirradiation protection of rabbits with plasma obtained from the splenic vein has been reported. Presence of a humoral spleen factor has thus been indicated in another animal species with a different method.

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Anaphylaxis in the Mouse: Possible Relation of the Schultz-Dale Reaction to Serotonin Release.* (22579)

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The evidence against the release of histamine in mouse anaphylaxis has been summarized(1). The purpose of the experiments reported here is to determine, using the Schultz-Dale technic, whether or not the substance responsible for contraction of the sensitized mouse uterus in the presence of antigen is serotonin (5-hydroxytryptamine).

Materials and methods. The virgin female mice were of the DBA or BALB/c strains, and were over 6 months of age when used. The mice were immunized intramuscularly in the hind legs with alum-precipitated egg white, as previously described(1). This technic of immunization is sufficient to render the mice anaphylactically sensitive to egg albumin as evidenced by the occurrence of a characteristic shock reaction on the intravenous injection of egg albumin(2); and by a contraction of the uterine smooth muscle of the sensitized mouse upon the addition of egg albumin *in vitro,* using the classical Schultz-Dale technic(1). Mice which had not received a prior injection of antigen never reacted by either technic when egg white was

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