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RADIATION-PROTECTION AND RECOVERY FROM RADIATION INJURY IN ENDOTOXIN-TREATED MICE: HEMATOPOIETIC RECOVERY AND SENSITIVITY TO A SECOND RADIATION EXPOSURE

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ABSTRACT

The present data confirm and extend our earlier protection studies in mice and show that the protective effect of endotoxin was additive with the protection afforded by hypoxia. The mechanism of endotoxin-protection is thought to involve stimulation of hematopoietic recovery, and the primary objective of the present study was to examine the process of hematopoietic recovery in endotoxin-protected mice. Pursuant to this, both hematological studies and split-dose recovery studies were conducted. The rationale was that if endotoxin accelerated hematopoietic recovery (at the level of the stem cell), the endotoxin-treated mice should show an accelerated rate of return toward normal radiosensitivity. That is, mice given endotoxin before a sublethal exposure to radiation (428 R) should subsequently have higher LD_{50}'s than control animals which were not given endotoxin before the conditioning exposure.

Hematologic studies showed that endotoxin-treated mice had more circulating granulocytes than did controls during the second and third weeks after exposure to 428 R. Of particular interest was the observation that the circulating granulocyte counts in endotoxin-treated animals were higher during the second week than during the third week after 428 R. That is, the granulocytic recovery in terms of numbers of circulating cells, was somewhat transient in character and may be an

"abortive rise". Hematopoietic recovery was also evaluated by determining the number of granulocytes which could be mobilized by endotoxin after 428 R. During the second week greater numbers of granulocytes were mobilized in endotoxin-treated (before 428 R) than in control mice, thus suggesting an earlier production and accumulation of granulocytes in the bone marrow. Therefore, in a general sense, the present hematologic studies confirm and extend the earlier finding by others that granulocyte recovery occurs earlier in endotoxin-treated than in control mice.

The split-dose recovery studies showed that at 3, 5, 9, and 14 days after 428 R, the LD_{50}'s were essentially the same in the endotoxin-treated and control groups. However, at 1 day after 428 R, the LD_{50} of endotoxin-treated animals was significantly higher than that of the controls. This finding at 1 day may be related to a residual protective effect of endotoxin rather than an accelerated recovery from radiation injury per se. In both endotoxin-treated and control animals, 50% recovery from the injury produced by 428 R occurred by ~ 4 days.

Although the endotoxin-treated animals showed evidence of accelerated hematopoietic recovery, as estimated by numbers of circulating and mobilizable granulocytes, no overall increase was observed in the rate at which they returned toward a normal radiosensitivity. The possibility is discussed that in endotoxin-treated mice, the extent of granulocytic recovery is not necessarily indicative of a sustained increase in the rate of hematopoietic stem cell repopulation. The split-

dose recovery data are discussed in terms of the general question of rate(s) of recovery from radiation injury in the mouse.

SUMMARY

The Problem:

Radiation damage to the hematopoietic system is of great importance in animals given midlethal exposures. To a great extent the amount of damage to the hematopoietic system determines if an animal will live or die. Therefore, in any radiation situation involving personnel exposure, hematopoietic parameters such as leucocyte counts will most likely be used to assess radiation injury and the probability of survival. Also, after an initial sublethal exposure, the extent of recovery of the hematopoietic system should, on theoretical grounds, influence the response to a second exposure. One object of the present study was to determine the extent to which leucocyte counts could be correlated with the sensitivity of mice to a second radiation exposure. We have evaluated the radio-protective effects of endotoxins and the relationship between hematopoietic recovery and the rate at which the mouse returns toward a normal radiosensitivity after a conditioning exposure.

The Findings:

Both Pseudomonas polysaccharide (PP) and Typhoid-Paratyphoid vaccine (TAB) increased survival of irradiated mice, and the protective effect differed in two strains of mice. The protective effects of PP and hypoxia were additive and the composite protection ratio was 1.7.

TAB was used in an attempt to accelerate hematopoietic recovery in sublethally irradiated mice. Although the numbers of circulating and

mobilizable granulocytes were greater in TAB-treated animals than in controls during the second post-irradiation week, the TAB-treated animals did not show a more rapid recovery from radiation injury as evaluated by the split-dose technique. The numbers of circulating granulocytes may be of value in predicting the outcome of a radiation episode in terms of survival or death, but the number of granulocytes does not necessarily predict sensitivity to a second radiation exposure.

INTRODUCTION

Recovery from radiation injury has been studied in this laboratory using several animal species (1-6). The method used to evaluate recovery was that of split-dose technique which involves determining the LD_{50}'s for groups of animals at different times after a sublethal conditioning exposure. In general, as the injury produced by the conditioning exposure is repaired or recovered, the LD_{50}'s increase toward normal. When recovery from X- or γ radiation injury is evaluated by the split-dose technique and $LD_{50/30}$ is the end point, it has been inferred that it is principally repair or recovery of the hematopoietic system, at some functional level, which is involved.

We are interested in the relationship between radiation-protection, split-dose recovery, and recovery of the hematopoietic system. In the present experiments, bacterial endotoxin has been used in an attempt to evaluate these relationships. Earlier studies have shown that endotoxins increased survival in several animal species (7-16), and the present data extend our earlier studies and deal also with the question of the combined protective effects of endotoxin and hypoxia. Although the mechanisms of endotoxin-protection is not clearly understood, and many factors may be involved, acceleration of hematopoietic recovery has been observed and is thought to play an important role (13, 17, 18).

The primary objective of the present experiments was to examine hematopoietic recovery in endotoxin-treated mice, and to attempt to

relate hematologic recovery and radiation sensitivity after a sublethal conditioning exposure. The rationale was that if endotoxin accelerated hematopoietic recovery at the level of the "stem cell", the split-dose recovery in endotoxin-treated mice should show an accelerated rate of return toward normal radiosensitivity.

The hematologic data indicate that granulocytic recovery, in terms of numbers of circulating or mobilizable cells, is accelerated in endotoxin-treated mice. This finding is generally consistent with the earlier report of Smith, et al. (13, 17). The split-dose recovery data, however, do not show a sustained increase in the rate at which endotoxin-treated mice return toward normal radiosensitivity. Speculation is offered concerning the influence of endotoxin on granulocytic recovery and concerning the possible relationship between granulocytic recovery, split-dose radiosensitivity, and changes in the "stem cell" population(s). The split-dose recovery data are discussed in terms of the general question of rate(s) of recovery from radiation injury in the mouse.

MATERIALS AND METHODS

The animals used were either CF#1 or LAF_1 female mice 90-120 days old. The animals were held 10/cage on wood shavings and given standard laboratory chow and acidified (pH2.6) tap water ad libitum. Before acidification the water bottles of CF#1 mice were screened for Pseudomonas by routine methods; also some heart's blood and liver irradiated decedents of both strains were cultured. These bacteriological studies indicated that Pseudomonas was vertually absent from these mice. Survival was recorded for 30 days after irradiation.

The radiation source was a Westinghouse 250 kvp machine operated at 15 ma; the filtration consisted of 0.5 mm Cu and 1.0 mm Al; the HVL was 1.4 mm Cu. The CF#1 mice were placed on a rotating turntable in lusteroid tubes and exposed at ~ 30 R/min at a TSD of 92 cm. Based on lithium fluoride dosimetry with abdominal implants, the R to rad conversion factor for this exposure arrangement was 1.2.

In other experiments involving hypoxia, CF#1 mice were placed in a gassing chamber (11 x 17 x 29 cm) divided into individual 6 x 6 x 10 cm compartments; the exposure was at 100 R/min without rotation and the TSD was 51 cm. The R (measured in air without the gassing chamber) to rad conversion factor was 1.5. Hypoxia at the time of irradiation was produced by exposing the animals to an atmosphere of 95% nitrogen and 5% oxygen. The effluent oxygen concentration from the gassing chamber was monitored with a Beckman C-2 Oxygen Analyzer.

The $LD_{50/30}$'s and other statistics were computed from regressions of normal equivalent deviates (probit minus 5) of percent mortality on the radiation exposure in R and also on the natural logaritim of the radiation exposure in R. The regressions were calculated with an IBM 704 computer using a USNRDL program based on probit analysis (19) as adapted to a computer by Aitchison and Brown (20). The use of the exposure rather than logarithm of the exposure did not significantly influence the LD_{50}'s.

The experimental method used to estimate recovery was the split-dose technique. Animals were first given a conditioning exposure and sub-

sequently divided into groups and given graded exposures to determine the $LD_{50/30}$ at different times after the conditioning exposure. The extent of remaining injury in percentage of the conditioning exposure is calculated from the expression, $\frac{LD_{50_o} - LD_{50_t}}{D_c} \times 100$ in which LD_{50_o} is the initial $LD_{50/30}$ and LD_{50_t} is the median lethal dose determined at time, t, after the conditioning dose, D_c. The amount of recovery in R is calculated from the expression, $D_c + LD_{50_t} - LD_{50_o}$.

In some of the radiation-protection and recovery experiments, mice were injected either intravenously (tail vein) or intraperitoneally with 0.2 ml of Typhoid-Paratyphoid vaccine (TAB) 24 hours before irradiation. The TAB was a commerical preparation containing 3×10^8 killed organisms per 0.2 ml; this dose produced no signs of acute toxicity in the mice.

In other experiments involving radiation-protection or granulocyte mobilization, 50 µg of Pseudomonas polysaccharide (PP)[a] was injected intravenously in a volume of 0.05 ml. In granulocyte mobilization experiments, tail blood samples were taken at various times after injection. Mice were bled a maximum of 3 times with an interval of at least two days between bleedings. Total white blood cell counts were made on a Coulter Electronic Particle Counter Model A with a probe having a 100 micron aperture; the aperture current setting was 5 and the threshold setting was 20. Differential leucocyte counts were made by standard methods.

[a] The Pseudomonas polysaccharide used in these experiments was PIROMEN; this material was generously supplied by Flint Eaton and Company.

RESULTS

The effects of Typhoid-Paratyphoid vaccine (TAB) and of Pseudomonas polysaccharide (PP) on 30 day survival are shown in Table 1. Both substances given 24 hours before irradiation significantly increased LD_{50}'s of CF#1 and IAF_1 mice. Earlier protection data indicated that based on mortality at a single radiation exposure (dose) level, intraperitoneal injection was as effective as intravenous injection in reducing mortality. The data in Table 1 confirm this point by showing that the LD_{50} increase in CF#1 mice is not materially influenced by these two routes of injection. The data show that TAB conferred greater protection to IAF_1 than to CF#1 mice, and that TAB was somewhat more effective than PP in the IAF_1.

The protective effects of hypoxia, PP, and a combination of the two procedures are shown in Table 2. The LD_{50} increase produced by hypoxia was four times greater (61%) than that for PP (15%). The combined procedures, that is, PP 24 hours before irradiation and hypoxia during irradiation, increased the LD_{50} by 87%. In all the aforementioned protection studies, the slopes of the exposure-response curves for protected and control animals did not differ significantly.

Hematologic studies were conducted with TAB-treated (24 hours before irradiation) and control CF#1 mice exposed to 428 R; this exposure is ~ 2/3 of the LD_{50}. In two experiments, the number of circulating granulocyte mobilization was also evaluated. Fig. 1 shows the post-irradiation

granulocyte and agranulocyte counts in the two groups. The most striking feature in this figure is the rise in the granulocyte count which occurred between 7 and 10 days in the TAB groups. During this period, the TAB animals showed a transient rise which exceeded the pre-irradiation count; during this same period, the granulocyte counts in control animals remained depressed. The transient character of the rise in the TAB group is shown by the fall which occurred at 13 and 15 days. In this experiment, the granulocyte counts in the TAB group were significantly lower at 13 and 15 days than at 7 and 10 days (Table 3).

A qualitatively similar picture of post-irradiation granulocyte changes was observed in the second experiment (Table 4). The rise in circulating granulocyte counts at 8 and 10 days in TAB-treated animals, although lower than in the first experiment, was quite apparent. Although the count at 14 days fell to 60% of the 10 day count, the two counts do not differ significantly. In this second experiment, the mobilization response was evaluated in TAB-treated and control animals at 3, 8, 10, and 14 days after exposure to 428 R. The number of granulocytes mobilized in response to endotoxin may indicate earlier bone marrow activity and therefore may be a better index of granulopoietic recovery than is the number of circulating cells. At each of the times, the increase in the absolute number of granulocytes (relative to the pre-injection count) was greater in the TAB-treated than in the control animals. The mobilization ratios (count at 12 hours/the pre-injection count) varied somewhat at the different times after irradiation (control range 1.2 - 3.5; TAB-treated

range 1.4 - 2.7). At any given time after irradiation the ratios for TAB-treated and control mice were probably comparable, although questions may be raised about the 14 day point. For comparison, the granulocyte mobilization in non-irradiated mice is shown in Fig. 2; the granulocyte mobilization ratio for normal animals was 2.5.

Split-dose studies were conducted to determine the influence of TAB on the rate of return toward normal radiosensitivity. TAB was given 24 hours before the conditioning exposure to 428 R. Control animals were given the conditioning exposure without prior TAB treatment. Protection controls were also used in some experiments. Some of the animals injected with TAB, usually about 20, were exposed to 700 R (~ $LD_{85/30}$) to verify that TAB was in fact increasing 30 day survival; non-treated control animals were also exposed to 700 R. The mortality in the TAB groups exposed to 700 R ranged from 5 to 2[illegible] which was generally consistent with the expected protection ratio of ~ 1.2[a]. At various times after the conditioning exposure the LD_{50}'s were determined for both treated and control groups. The data in Table 5 show that TAB given 24 hours before the conditioning exposure significantly influenced the LD_{50} only at 1 day after the conditioning exposure. At other times the LD_{50}'s were virtually identical in the TAB and control groups. The amount of injury remaining from the conditioning exposure was calculated from each LD_{50} (see Methods) and the decrease in injury as a function of time is

[a]Earlier studies had shown the protection afforded by TAB and PP were similar and the protection ratio for a dose of 50 μg PP was 1.24 (12).

shown on linear coordinates in Fig. 3. The 100% injury in the figure is based on the conditioning exposure of 428 R. This figure shows a marked difference in remaining injury at 1 day between the TAB and control animals. The initial rapid decline in injury is what might be expected if TAB were to accelerate the rate of return toward normal radiosensitivity. However, this trend was not sustained, and at subsequent times the injury was comparable in the two groups. In connection with this figure, a question may be raised as to the level of initial injury in the TAB group. In using 428 R as the 100% value for the TAB animals, we have assumed that TAB does not act as a "dose reducer" thereby decreasing the level of initial injury (21). However, if one chooses to assume a lower initial injury, using dose reduction factors of 11 to 20%[b], the initial injury would be ~ 385 to 345 R, respectively. The net effect of assuming a lower initial injury would be to increase the estimate of the injury remaining at the various times in the TAB group. The figure would then convey the impression that recovery occurred somewhat more slowly in TAB than in control animals. The point is that if TAP either decreased the extent of initial injury and/or accelerated recovery, a sustained increase in the rate of return or an earlier return toward normal radiosensitivity would be expected; this was not observed.

The LD_{50}'s in Table 5 are based on regression computations utilizing the natural logarithm of the radiation exposure. LD_{50}'s have also been calculated using exposure on a linear scale, but those values are not shown. The exposure scale (log vs. linear) had a negligible effect,

[b]This range is based on the present and earlier protection studies with CF#1 mice (12).

~ 10 R, on the LD_{50} values. When plotting the exposure on a log scale one assumes a lognormal distribution of radiosensitivity; when plotting exposure on a linear scale, one assumes a normal distribution of radiosensitivity. We have considered both distributions in computing coefficients of variation (CV) for TAB-treated and control mice. The CV is an index of the heterogeneity of population response or the variability of individual responses within the population. The CV's in Table 5 show that the variability of radiation response was greater in the split-dose LD_{50}'s than in the single exposure LD_{50}'s. Also, in the single exposure LD_{50} determinations the CV was higher in the TAB-treated than in the control mice. It is likely that, in addition to irradiation, any stress or treatment to which animals are subjected will increase the CV.

The split-dose LD_{50} data are considered from a different point of view in Fig. 4. This figure is a linear plot of the $LD_{10/30}$'s, $LD_{50/30}$'s, and $LD_{90/30}$'s determined for control animals at various times after the conditioning exposure to 428 R. The purpose of this figure is to illustrate and compare the time-dependent radiosensitivity of the more radiosensitive (LD_{10}) and the less radiosensitive (LD_{90}) elements of the population in comparison with the animals of median radiosensitivity (LD_{50}). The values shown at zero time are theoretical and represent the exposure which must be added to 428 R to produce 10, 50, or 90%; these values were based on the single exposure regression of mortality response on exposure. We have also estimated the zero time values by subtracting 428 R from each exposure group involved in the single exposure LD_{50}

determinations; therefore exposures of 600 or 700 R would be 172 and 272 R, respectively. The 30 day mortality observed following 600 or 700 R was assigned to the lower exposures, and an LD_{50} was computed. The computed values for 10, 50, or 90% mortality were within 5 R of the values obtained by the other method described above.

Fig. 5 is relevant to the question of recovery rate(s) in the CF#1 mouse. This semi-logarithmic plot of the remaining injury is based on the LD_{50}'s determined at various times after the conditioning exposure. Two recovery points are not included in this figure; the two hour point in control animals and the 1 day point in TAB animals. Curve A is a least-squares fit to the observed points, and curve B is eye-fitted. The Y intercept should predict the initial injury produced by the conditioning exposure. The intercept of curve A is somewhat greater than the conditioning exposure of 428 R; whereas, curve B is forced through the Y intercept at that level of initial injury.

DISCUSSION

As radiation-protectants, endotoxins or bacterial vaccines are by no means as effective as the classical chemical protectants such as the sulfhydryl compounds (21, 22). However, in mice, endotoxins do have a temporal advantage in that survival is increased when the material is given within 48 hours before or 24 hours after midlethal exposure (8, 10). The principal interest in endotoxins relates not to their efficacy as protectants but to their mechanism of action. As Smith has stated, the histocompatibility problems associated with transplantation of foreign

hematopoietic tissues could be obviated if hematopoietic recovery could be "induced" in the irradiated animals' own hematopoietic system (13). In the general sense, the endotoxin-treated mouse behaves as if hematopoietic recovery had, in fact, been "induced" (13, 17, 18).

We have extended the earlier protection studies, but the primary objective of the present experiments was to examine granulocytic recovery in endotoxin-treated mice, and to attempt to relate this to radiation sensitivity following a sublethal conditioning exposure to radiation. The split-dose recovery data are of particular interest and permit some speculation relating to repopulation of the hematopoietic system and the rate(s) at which mice return toward normal radiosensitivity after a sublethal conditioning exposure.

Radiation-Protection

The present data show that TAB and PP significantly increased the LD_{50}'s of both CF#1 and IAF_1 mice. However, the protection afforded IAF_1 mice, ~ 35% increase in LD_{50}, was greater than in CF#1 mice, ~ 12%. An indication of a strain effect was noted earlier in that BUB mice were protected by lower doses of PP than were CF#1 mice (11). Moreover, it appears that the protection ratio has changed over a period of time in the CF#1 mice, while the LD_{50} has remained essentially constant (21, 11). In earlier protection studies, an LD_{50} increase of ~ 25% was observed (12), but in the present experiments, the increase was only ~ 12%. At present, the basis for the variation in protection ratios is unknown, but Pseudomonas appears not to be involved.

In LAF_1 mice, TAB produced a greater increase in LD_{50} than did PP. This may be related to a dosage differential rather than to a specific attribute of TAB. Using PP we have found that over the range of 2 - 100 ug, the extent of protection is influenced by the endotoxin dose (12).

The protective effects of PP and hypoxia were studied in CF#1 mice. The LD_{50} increase produced by hypoxia was greater, 61%, than that for PP, 15%. When animals were given PP 24 hours before irradiation and hypoxia during irradiation, the LD_{50} increase [illegible]% which indicates that the procedures are directly additive in their protective effects. Such additivity should be expected since endotoxin, although producing marked vascular changes (23), probably does not produce a sustained hypoxia.

Granulocytic Recovery

Mice given TAB 24 hours before exposure to 428 R show an acceleration of granulocytic recovery in terms of the numbers of circulating granulocytes and in terms of the number of granulocytes mobilized in response to an injection of endotoxin. In a general sense, the present data confirm the earlier findings of Smith, et al. They interpreted their data to indicate "endotoxin-induction of hematopoietic recovery" whether the endotoxin was given before or after irradiation (13, 17).

Implicit in the in concept of "induction of hematopoietic recovery" is the idea that the initial injury to the hematopoietic system is comparable in endotoxin-treated and control groups (13). That is, endotoxin does not decrease the damage in terms of the surviving number of stem cells or maturing cells. Although no mechanism has been proposed to

account for the "induction of hematopoietic recovery" (13, 17), this might occur if endotoxin were to produce (a) an increase in the rate of cell division in a stem cell compartment with proportionately more cells moving into a dividing and maturing compartment, (b) a preferential shunting of cells to the dividing and maturing compartment, perhaps at the expense of repopulation of the stem cell compartment, or (c) a shorter sojourn in the dividing and maturing compartment. If operative, any or all of these factors will produce a temporal advance of hematopoietic recovery which might account for the protection observed when endotoxin is given either before or after irradiation.

However, with endotoxin given before irradiation another factor may be important. This factor is the "state" of the hematopoietic system at the time of irradiation. An injection of endotoxin may produce a "shift in relative numbers of cells in various compartments of the marrow, the effect of which is to <u>increase</u> the number of stem cells and/or cells in dividing and maturing compartments. Studies of the time-dependency of endotoxin-protection are generally consistent with this idea (10). Evidence for an increased surviving number of proliferative cells has been obtained in endotoxin-treated mice using the numbers of colony-forming units as the end point (24). An increase in the number of surviving stem cells or other proliferative cells could decrease the time by which the cell counts approach normal. Therefore, when endotoxin is given before irradiation it is not necessary to assume comparable initial injury and only induction <u>per se</u> as the effect of endotoxin in the hematopoietic system.

However, when endotoxin is given during the first day after irradiation, the survival of mice and hamsters is increased, and an acceleration of hematopoietic recovery is also observed (8, 13). The mechanism by which post-irradiation treatment with endotoxin influences the hematopoietic system is not clear, but perhaps in this situation there is some direct stimulation of cells in the dividing and maturing and/or the stem cell compartments as was described above. Endotoxin given within the first day after irradiation probably does not elicit all the responses which are produced by giving endotoxin before irradiation.

Another factor which may be relevant to the question of the influence of endotoxin on hematopoietic recovery is the transitory rise in granulocyte counts which occurs during the second week in the mice given TAB 24 hours before 428 R. During this rise phase, the number of circulating granulocytes in the TAB animals exceeded that in the controls by a factor of 3 - 6. Although the granulocyte counts in the TAB animals approximated pre-irradiation levels during part of the second week, the counts subsequently fell below pre-irradiation levels at days 13 and 15. Between days 18 and 21, the granulocyte counts in both groups approximated pre-irradiation levels. Therefore, during the second post-irradiation week, which may be a very critical period insofar as survival is concerned, the TAB animals have more circulating and mobilizable granulocytes than do controls. In our earlier studies with TAB-protected dogs a greater "abortive" rise was observed than in the mouse (14). There is considerable advantage in using dogs to evaluate the influence of endo-

toxin in granulocyte recovery. The dog's differential leucocyte count is reversed as compared with a mouse. The dog has 8 to 10,000 granulocytes/mm^3 rather than 1500 to 3000/mm^3 as in a mouse; therefore, changes in the numbers of circulating cells can be more readily quantitated in the dog. Also, the normal process of granulocytic recovery after irradiation (in terms of circulating cells) occurs later than in the mouse. This permits a more clear separation of an abortive rise and what might be considered as normal granulocytic recovery. In dogs given TAB before exposure to 360 R (~ $LD_{65/30}$) mortality was significantly decreased, and a transient rise in granulocytes occurred during the second week (14). The nadir in granulocyte counts occurred during the third week in both TAB-treated and control dogs. After the rise period during the second week, there was little difference in the granulocyte counts between the treated and control dogs. Although comparatively few dogs were involved in the initial dog study (14), subsequent experiments involving more dogs have confirmed this general pattern.

Therefore, the question is whether an increased number of granulocytes during the second week indicates (a) regeneration of the hematopoietic system, (b) an accentuation of the abortive rise not accompanied by accelerated hematopoietic regeneration, or (c) an accelerated hematopoietic regeneration which is accompanied by an accentuated abortive rise. The rise appears to be radiation-dose dependent and may be the result of abortive regeneration in the stem cell compartment of the marrow (25, 26). Therefore, the rise in the number of circulating granulocytes may be attributable to the surviving fraction of precursor cells which complete

only a few divisions before death. In the dog and possibly in the mouse, the principal effect of endotoxin pre-treatment may be to increase the extent of the abortive rise. On the other hand, if endotoxin treatment produced an increased surviving fraction of bone marrow stem cells, one might expect both an accentuation of the abortive rise and an earlier return to pre-irradiation granulocyte levels. This same pattern would also be expected if the initial injury were equivalent in the treated and control groups, and endotoxin were to "induce" hematopoietic recovery. On the basis of the present hematologic data derived from mice, it is not possible to exclude these alternate hypotheses. Studies are continuing in both mice and dogs given endotoxin before or after irradiation which may permit a better understanding of the influence of endotoxin on the hematopoietic system.

Radiosensitivity After a Conditioning Exposure

In view of the role ascribed to the hematopoietic system in determining radiation responses in the midlethal exposure range (26), it might be expected that an observed increase in the rate of granulocytic recovery reflects an accelerated rate of bone marrow regeneration and might produce a sustained increase in the rate at which the radiosensitivity (LD_{50}) of mice returns toward normal. In the present studies, no sustained increase in the rate of recovery was observed. Between 3 and 14 days after the conditioning exposure, the LD_{50}'s and therefore extent of recovery were similar in the TAB-treated and control animals. However, at 1 day after the conditioning exposure, the LD_{50} for the TAB

group was significantly higher than that for the control group. This higher LD_{50} in the TAB group at 1 day might be attributable to an accelerated rate of recovery, but it more likely indicates a residual protective effect of the endotoxin given 48 hours before the 1 day LD_{50} determination. Endotoxin given 48 hours before irradiation is known to increase survival (7, 8, 10).

In connection with the split-dose data, it may be of some interest to attempt to interrelate the LD_{50} at 1 day in the TAB animals and the state of their hematopoietic system at that time. These comments are entirely speculative and relate to the control systems which influence the stem cell compartment. If we assume that the split-dose LD_{50}'s are related to the numbers of stem cells present at any given time, the possibility exists that the granulocyte rise during the second week and the increased LD_{50} observed at 1 day after the conditioning exposure are both related to the number of stem cells that were present 1 day after the conditioning exposure. The LD_{50} at 1 day was 152 R higher in the TAB group than in the control group which might indicate a greater number of stem cells in the TAB group. However, at 3 days, the LD_{50}'s of the TAB and control groups were similar, as perhaps were the numbers of stem cells. Also, the LD_{50} for the TAB animals was lower at day 3 than at day 1. The decreased LD_{50} at day 3 might indicate a decrease in the stem cell population in the TAB animals between day 1 and day 3. This could occur if, under "differentiation pressure", a certain fraction of stem cell progeny were totally diverted to various dividing and maturing

compartments at the expense of the stem cell compartment. Perhaps when the size of the stem cell compartment is above some finite number, relatively more cells may be committed to dividing and maturing compartments than when the stem cell population is below some finite number. When the stem cell population is below this hypothetical "critical size" relatively few cells might move into dividing and maturing compartments and the principal activity in the stem cell compartment may be toward restoration of that compartment. This might account for the dose dependence of the abortive rise noted in some species (26), and for the "abortive" rise and radiosensitivity in TAB-treated mice. However, this speculation is based on the assumption that in a split-dose system, the LD_{50} at any given time is related to the size of the stem cell population which, as will be mentioned later, may not be a valid assumption.

One further inference may be drawn from the present split-dose and hematologic data. The data show that after day 1, the LD_{50}'s for TAB and control animals were quite similar in spite of the large difference in the number of circulating granulocytes during the second week. Therefore, since TAB increases 30 day survival, the granulocyte recovery may be of value in the prognosis of survival following acute radiation but does not necessarily predict greater survival following a second radiation exposure.

Rate(s) of Recovery from Radiation Injury

The split-dose technique has been used extensively to measure recovery from radiation injury in mice, recovery being defined as the

return toward normal radiosensitivity following a conditioning exposure. For reviews, see Sacher (27), Storer (28), and Krebs and Brauer (29). Mole (30) and Kallman and Silini have described early cyclic alterations in the radiosensitivity of mice which may be related to radiation-induced synchronization in the cell population which determines radiosensitivity (31). One of these early fluctuations in radiosensitivity probably accounts for the recovery observed in our non-treated animals at two hours after the conditioning exposure. The LD_{50}'s at two hours and at 1 day are identical and indicate 10% recovery.

In mice, recovery curves are usually considered to be adequately fitted by an exponential function (27, 32); that is, the logarithm of percent remaining injury plotted against time after the conditioning exposure produces a straight line. Since mice recover comparatively rapidly, and the dose-response curves at certain times may be shallow, it is difficult to precisely define small changes in radiosensitivity over short periods of time. These difficulties give rise to a certain amount of scatter of the observed points around an exponential recovery curve, and this scatter is usually attributed to "biological variation". However, these deviations may not result from biological variation alone, and the goodness of fit by an exponential function has been questioned for mice (28, 30, 31), and deviations from exponentiality have been reported for sheep and swine (4) and perhaps for the dog (6).

The question of recovery rate is complex, and as Kallman and Silini have shown (31), the inferences drawn pertaining to rate can be markedly

influenced by the coordinates and the parameters which are plotted. Time after a conditioning exposure is usually on a linear X axis; but on the Y axis, which may be either linear or logarithmic scale, either the percent remaining injury, LD_{50}, or rads of injury which are recovered may be plotted. The present recovery data have been shown on both logarithmic and linear ordinates using different parameters on the Y axis. The data, no matter how plotted, indicates that, based on median population sensitivity, the CF#1 mouse recovers from 50% of the initial injury by ~ 4 days. On a logarithmic ordinate, the Y intercept of the fitted exponential curve exceeds the expected initial value of 428 R. This may be wholly fortuitous and the single exponential, curve A in Fig. 4, regardless of the intercept, may be a satisfactory fit to the observed points. On the other hand, if a curve is drawn through the observed points and forced to a Y intercept of 428 R, the result is curvilinear, especially over the first three days. This suggests either an initially different exponential rate producing a recovery half time of ~ 6 days, a linear rate with recovery of ~ 40 R/day, or a more complex function. On the other hand, since cyclic alterations in radiosensitivity may persist for 48 to 72 hours (31); a single recovery rate estimate which includes that period may be relatively meaningless and would not be applicable to subsequent changes in radiosensitivity. Excluding changes in radiosensitivity at less than 24 hours, the impression of a net linear recovery over the first three or perhaps five days may also be gained from Figs. 4 and 5. These figures also indicate that after five days, the recovery

rate may change. In any event, the possibility exists that the radiosensitivity may not change at a constant rate throughout the entire recovery phase.

In connection with recovery rate, one conventionally deals with a rate estimate based on the median radiosensitivity of a population at any given time, that is, the LD_{50}. In split-dose recovery studies, one is dealing with a population's distribution of "basic" radiosensitivity and of recovery potential, both of which would influence their response to a second radiation exposure. That is, elements of a population undoubtedly have different basic radiosensitivities and recover at different rates. Although the LD_{10} and LD_{90} are relatively insensitive estimates of radiosensitivity, a qualitative impression of differences in radiation responses of the population may be gained from Fig. 4. The rate of change in radiosensitivity appears to be different based on comparison of the more susceptible (LD_{10}) and the more resistant (LD_{90}) animals, but such a conclusion is not statistically valid due to the inherent inaccuracy in measuring LD_{10} and LD_{90}. Nevertheless, these data do suggest that the time by which the least and most radioresistant elements of the population recover from 50% of the initial injury may differ by a factor of 2.

In connection with population radiosensitivity, we have observed a great deal of variability in radiation response in the present split-dose studies; whereas, in single exposure LD_{50} studies, the variability is less. This is shown by the coefficients of variation presented in

Table 5. One index of variability is the LD_{10} to LD_{90} exposure range, that is, the slope of the exposure response curve. In terms of the LD_{10} to LD_{90}, this range for the single exposure LD_{50} is ~ 135 R. In all the split-dose LD_{50}'s the range was greater, as is shown in Fig. 4. At 9 days the range was ~ 400 R, but at other times the ranges were of the order of 275 to 300 R. Although not presented here, we have computed slopes for these exposure-response curves assuming both a lognormal and a normal distribution of radiosensitivity. With exposure on either log or linear scale, the slopes of the curves for the split-dose LD_{50}'s are more shallow than the slope of the single exposure curve. At no time does the use of the log as compared with the linear exposure scale materially influence the LD_{50} (differences range from 1 to 17 R), but, as would be expected, the LD_{10} and LD_{90} are more affected. The overall shape of the curves in Fig. 4 is not changed by the use of the log or linear scale; in fact, using the linear scale the separation of the LD_{10}'s and LD_{90}'s is somewhat greater than is shown in the figure. We are currently attempting to determine which exposure scale gives the better fit to the data and we are evaluating the statistical significance of the changes in slope.

A factor which may have bearing on the observed variability is the strain of mouse. The CF#1 is not an inbred mouse and less variability might be expected in a highly inbred strain. However, in terms of coefficients of variations observed in determinations of acute LD_{50}'s, the 8% for the CF#1 is only slightly greater than the 6 to 7% in the inbred

IAF_1 mouse. Although the statistical treatment of the CF#1 slope data is not complete, we feel that in "biological terms" the slopes of exposure-response for split-dose experiments are significantly more shallow than the slope for a single exposure.

We are extending the split-dose studies with mice with the intention of obtaining better estimates of recovery rates(s) and determining the influence of size of the conditioning exposure on recovery rate. At present, it is difficult to relate observed changes in radiosensitivity to changes in the size per se of a stem cell population. This difficulty involves the rapidity with which mice recover as contrasted with estimates of generation times for cell populations (24). Other studies have been conducted with X- and neutron-irradiated mice to determine the relationship between recovery and repopulation of colony-forming units in the femoral bone marrow and in the spleen (33). These findings will be presented separately.

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TABLE 1

PROTECTION RATIOS IN CF#1 AND LAF_1 MICE

Strain-Treatment	$LD_{50/30}$ (R)[a]	Protection Ratio[b]	Number of Mice
CF#1 TAB[c] Intraperitoneally	723 (689-762)[d]	1.12	109
CF#1 TAB Intravenously	712 (662-749)	1.10	60
CF#1 Non-Injected Controls	646 (632-659)	--	164
LAF_1 PP[e] Intraperitoneally	939 (928-95.4)	1.32	179
LAF_1 TAB Intraperitoneally[c]	987 (969-1017)	1.39	100
LAF_1 Non-Injected Controls	707 (691-723)	--	130

[a] These exposures may be converted to rad dose in the abdomen by a factor of 1.2; see Methods.

[b] The protection ratio is the LD_{50} for the treated animals, the LD_{50} for the controls.

[c] 0.2 ml of TAB (3×10^8 organisms) injected 24 hours before irradiation.

[d] The 95% confidence interval is shown in parentheses.

[e] 50 µg of (PP) injected 24 hours before irradiation.

TABLE 2

RADIATION PROTECTION IN CF#1 MICE BY HYPOXIA AND PSEUDOMONAS POLYSACCHARIDE (PP)

Treatment	$LD_{50/30}$ (R)[a]	Number of Mice	Protection Ratio Relative to Non-Treated	Protection Ratio Relative to Hypoxia	Protection Ratio Relative to PP
None	516 (492-534)	138	--	--	--
Hypoxia[b]	831 (776-868)	207	1.61	--	--
PP[c]	596 (570-616)	120	1.15	--	--
Hypoxia and PP	964 (933-988)	104	1.87	1.16	1.62

[a] These exposures may be converted to rad dose in the abdomen by a factor of 1.5; see Methods.

[b] Hypoxia during irradiation.

[c] 50 μg of (PP) injected 24 hours before irradiation.

TABLE 3

GRANULOCYTE COUNTS IN TAB-TREATED AND CONTROL MICE AFTER EXPOSURE TO 428 R

Days After 428 R	Granulocytes/MM^3[a]	
	TAB[b]	Controls
0	2152 (1627-2627)	2152 (1677-2627)
1	1274 (1085-1464)	1564 (1392-1736)
3	294 (234-354)	541 (467-613)
5	1040 (833-1246)	--
6	--	474 (400-548)
7	3164 (2800-3528)	--
9	--	582 (412-751)
10	2934 (2262-3606)	--
12	--	445 (281-607)
13	1382 (1113-1650)	--
15	1389 (1091-1687)	589 (409-769)
18	1905 (1477-2332)	1796 (1357-2234)
21	1749 (1455-2043)	2086 (1611-2562)
23	2979 (2503-3456)	--
24	--	2614 (2309-2920)
25	2868 (2392-3344)	--
26	--	3025 (2491-3560)
27	3321 (2801-3841)	--
28	--	2560 (2112-3008)
30	2350 (1990-2710)	2556 (2172-2739)
33	1974 (1712-2237)	2725 (2207-3242)
42	1973 (1671-2276)	1850 (1541-2159)

[a] The control mean was based on a group of 20 mice; the other means were based on groups of 10; counts within parentheses indicate the 95% confidence interval.

[b] TAB given 24 hours before 428 R.

TABLE 4

MEAN GRANULOCYTE COUNTS BEFORE AND AFTER MOBILIZATION IN TAB-TREATED AND CONTROL ANIMALS SAMPLED AT VARIOUS TIMES AFTER 428 R[a]

Counts Relative to Mobilization	3 Days		8 Days		10 Days		14 Days	
	TAB[c]	Control	TAB	Control	TAB	Control	TAB	Control
--	248 (185-311)	367 (284-451)	1716 (1161-2270)	525 (438-611)	1976 (1470-2470)	339 (241-437)	1185 (814-1556)	440 (253-618)
12 Hours PM[b]	466 (341-593)	446 (340-553)	3992 (2985-4999)	1237 (693-1781)	2726 (1933-3520)	513 (348-678)	3258 (2240-4275)	1501 (821-2182)
24 Hours PM[b]	390 (224-557)	256 (150-363)	2135 (1569-2701)	718 (478-958)	1900 (1390-2409)	451 (297-605)	1901 (1397-2405)	920 (535-1305)
Mobilization Ratio (Based on 12 Hour Counts)	1.87	1.22	2.33	2.36	1.38	1.51	2.74	3.41

[a] 8 mice/group; the mean of three pre-irradiation counts was 2111 (1775-2447) granulocytes; counts within parenthesis indicate the 95% confidence interval of the mean.

[b] Post-mobilization with 50 µg of PP.

[c] TAB given 24 hours before 428 R.

TABLE 5

$LD_{50/30}$'S DETERMINED AT VARIOUS TIMES AFTER THE CONDITIONING EXPOSURE (428 R) IN TAB-TREATED AND CONTROL CF#1 MICE

Time After Conditioning Exposure	TAB-Treated LD_{50}	(95% C.I.)	Number of Mice	Coefficient of Variation[a] Log-normal	Normal	Controls LD_{50}	(95% C.I.)	Number of Mice	Coefficient of Variation[a] Log-normal	Normal
0	--	--	--	--	--	219[b]	(153-285)	164	.25	.24
2 Hours	--	--	--	--	--	257	(231-284)	124	.38	.31
1 Day	410	(375-454)	259	.55	.39	258	(243-275)	342	.43	.43
3 Days	346	(322-371)	336	.49	.53	335	(313-355)	279	.38	.49
5 Days	478	(455-507)	173	.23	.21	471	(450-490)	347	.22	.24
7 Days	569	(482-603)	133	.19	.22	585	(505-621)	139	.26	.28
14 Days	614	(589-630)	140	.15	.15	611	(581-638)	126	.18	.18
--	723	Single Exposure LD_{50} (689-782)	109	.18	.18	646	Single Exposure LD_{50} (632-659)	164	.08	.08

[a] The coefficient of variation (CV) is an index of the variability of the radiation response of individuals in the population. Assuming a lognormal distribution of radiation sensitivity, the CV is determined from the following equation taken from Aitchison and Brown (20); $CV = (e^{\sigma^2} - 1)^{1/2}$ where σ is the reciprocal of the slope of the computed regression of N.E.D. (see Methods) on the natural logarithm of the exposure. Assuming a normal distribution of radiation sensitivity, $CV = \sigma/LD_{50}$.

[b] This value was computed by subtracting 428 R from each exposure group used in the single exposure LD_{50} determination.

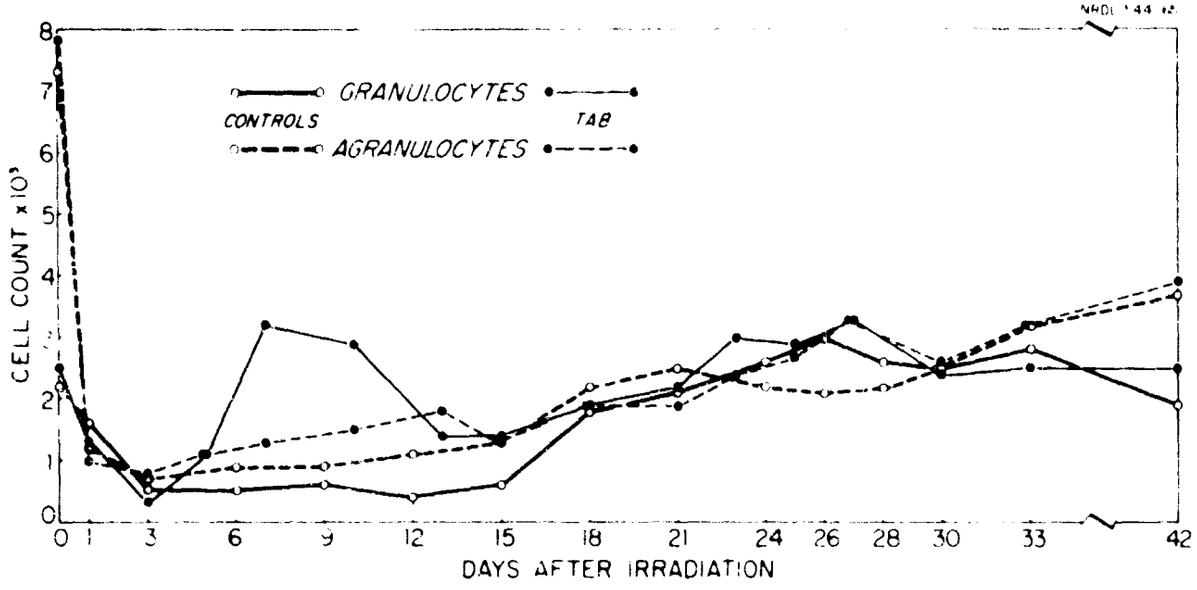

Fig. 1. Leukocyte counts per cubic millimeter in TAB-treated and control mice following exposure to 428 R.

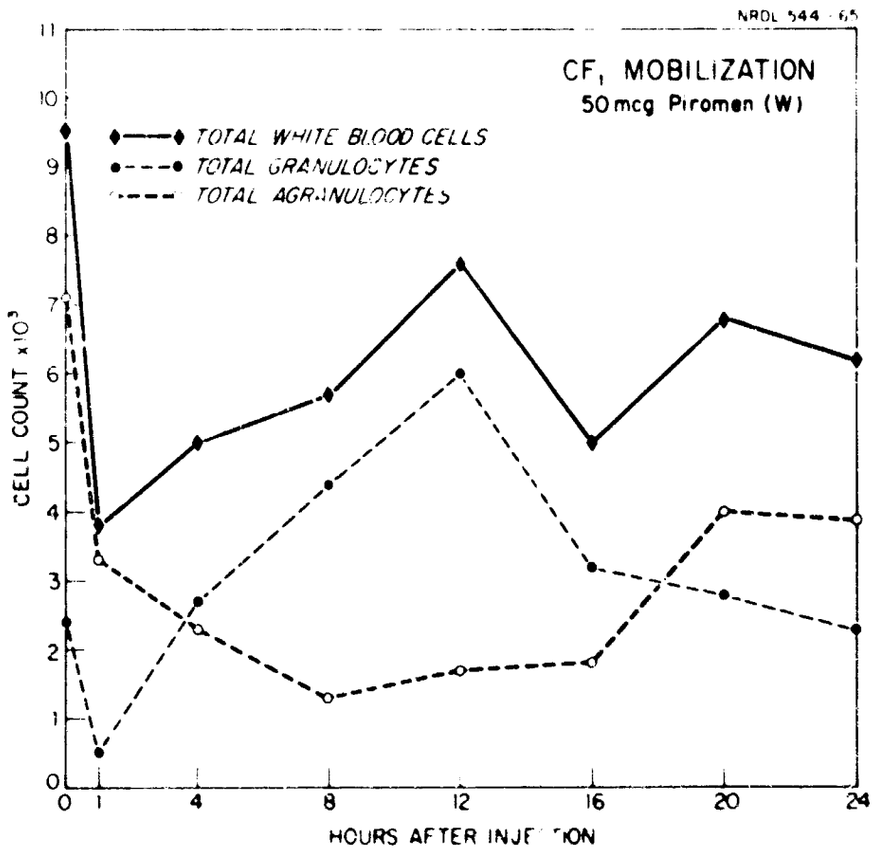

Fig. 2. Leucocyte counts per cubic millimeter in non-irradiated mice injected intraperitoneally with 50 µg of PIROMEN.

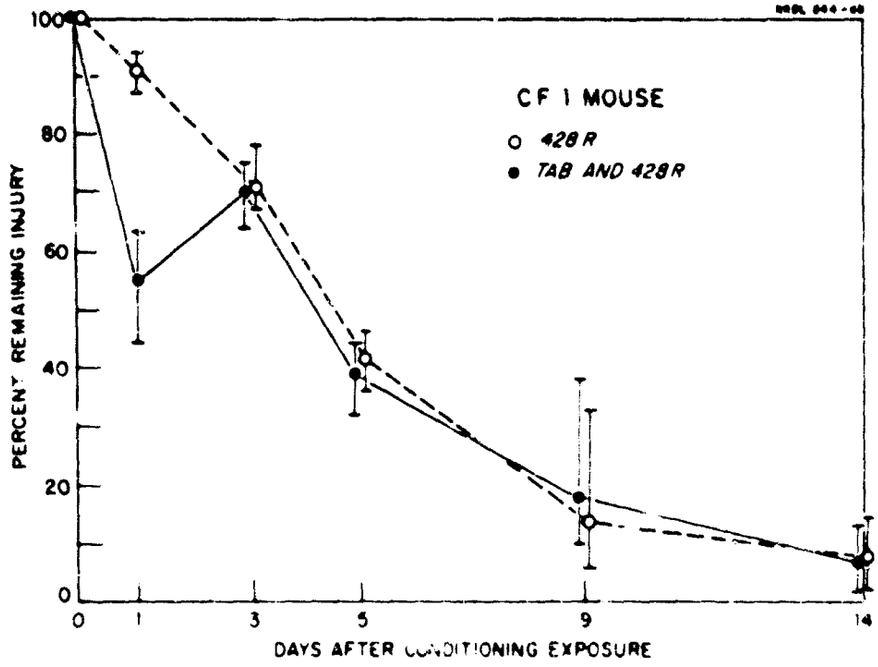

Fig. 3. Percent injury remaining at various times after the conditioning exposure in TAB-treated and control animals. The 100% injury represents the injury produced by the conditioning exposure of 428 R.

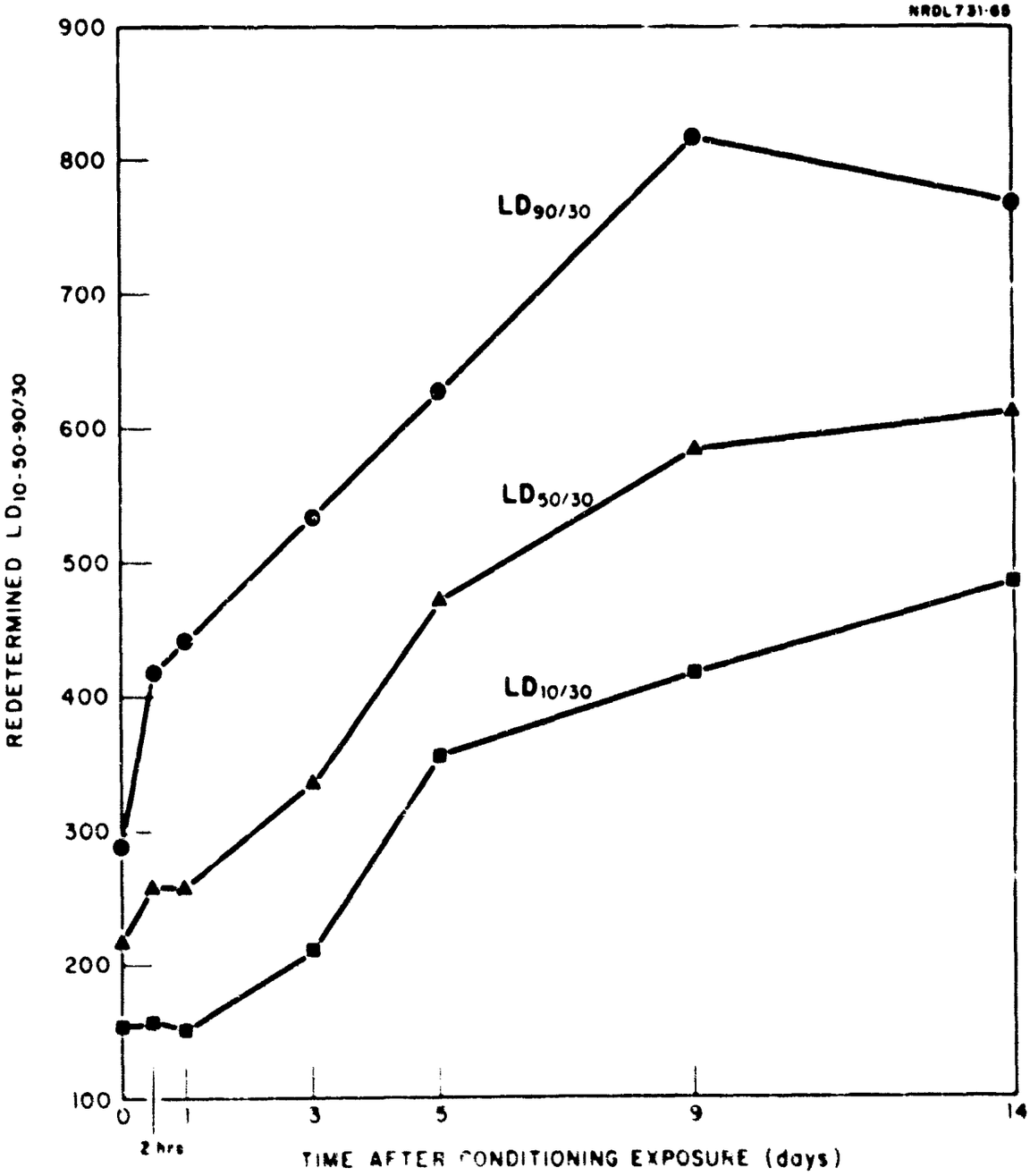

Fig. 4. LD_{10}, LD_{50}, LD_{90} at various times after the conditioning exposure of 420 R. The $LD_{50/30}$ for normal animals is 640 R.

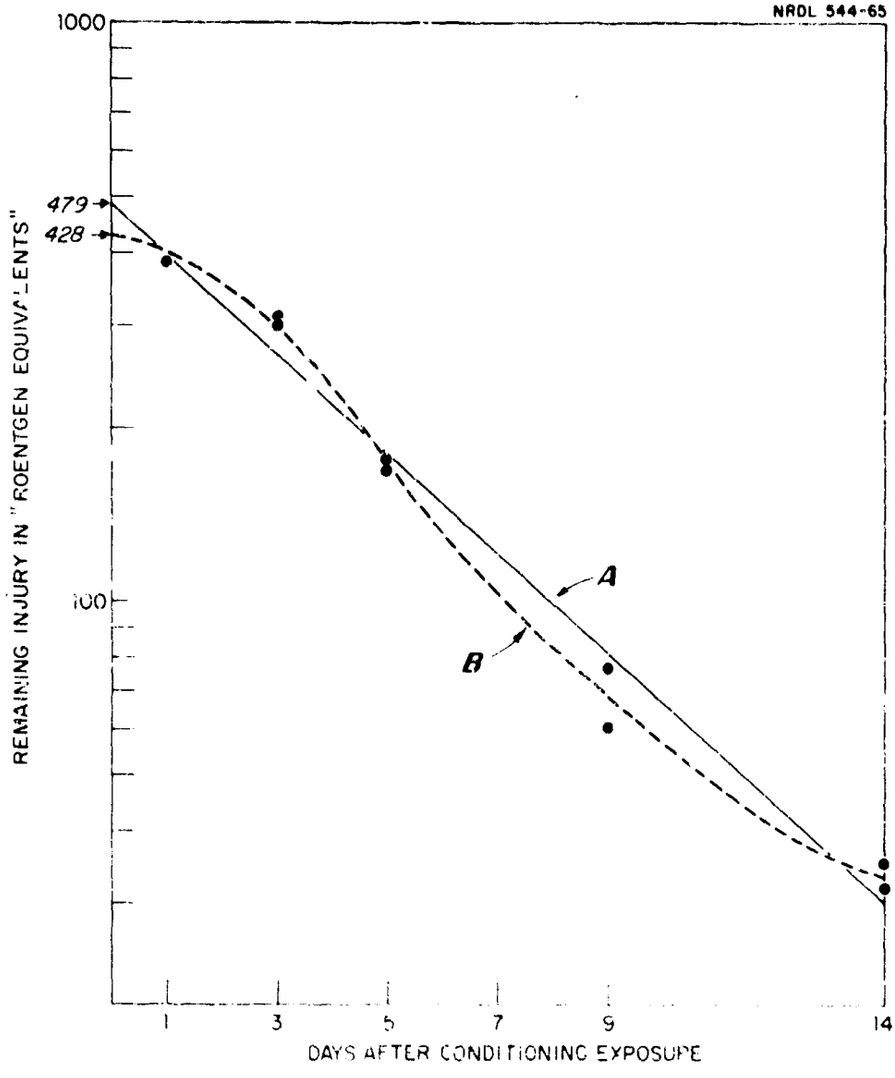

Fig. 5. Remaining injury at various times after a conditioning exposure of 420 R. The two points at each time after three days are based on PAB-treated and control animals. Curve A is a least-squares regression and curve B is eye-fitted.

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13 ABSTRACT The present data confirm and extend our earlier protection studies in mice and show that the protective effect of endotoxin was additive with the protection afforeded by hypoxia. The mechanism of endotoxin-protection is thought to involve stimulation of hematopoietic recovery, and the primary objective of the present study was to examine the process of hematopoietic recovery in endotoxin-protected mice. Pursuant to this, both hematological studies and split-dose recovery studies were conducted. The rationale was that if endotoxin accelerated hematopoietic recovery (at the level of the stem cell), the endotoxin-treated mice should show an accelerated rate of return toward normal radiosensitivity. That is, mice given endotoxin before a sublethal exposure to radiation (428 R) should subsequently have higher LD_{50}'s than control animals which were not given endotoxin before the conditioning exposure.

Hematologic studies showed that endotoxin-treated mice had more circulating granulocytes than did controls during the second and third weeks after exposure to 428 R. Of particular interest was the observation that the circulating granulocyte counts in endotoxin-treated animals were higher during the second week than during the third week after 428 R. That is, the granulocytic recovery in terms of numbers of circulating cells, was somewhat transient in character and may be an "abortive rise". Hematopoietic recivery was also evaluated by determining the number of granulocytes which could be mobilized by endotoxin after 428 R. During the second week greater numbers of granulocytes were mobilized in endotoxin-treated (before 428 R) than in control mice, thus suggesting an earlier production and accumulation of granulocytes in the bone marrow. Therefore,
(Abstract continued on another page)

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in a general sense, the present hematologic studies confirm and extend the earlier finding by others that granulocyte recovery occurs earlier in endotoxin-treated than in control mice.

The split-dose recovery studies showed that at 3, 5, 9, and 14 days after 428 R, the LD_{50}'s were essentially the same in the endotoxin-treated and control groups. However, at 1 day after 428 R, the LD_{50} of endotoxin-treated animals was significantly higher than that of the controls. This finding at 1 day may be related to a residual protective effect of endotoxin rather than an accelerated recovery from radiation injury *per se*. In both endotoxin-treated and control animals, 50% recovery from the injury produced by 428 R occurred by ~ 4 days.

Although the endotoxin-treated animals showed evidence of accelerated hematopoietic recovery, as estimated by numbers of circulating and mobilizable granulocytes, no overall increase was observed in the rate at which they returned toward a normal radiosensitivity. The possibility is discussed that in endotoxin-treated mice, the extent of granulocytic recovery is not necessarily indicative of a sustained increase in the rate of hematopoietic stem cell repopulation. The split-dose recovery data are discussed in terms of the general question of rate(s) of recovery from radiation injury in the mouse.

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Endotoxin Recovery from radiation injury Hematopoietic recovery Granulocyte mobilization						

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