Spleen Desoxyribonucleic Acid Content as an Index of Recovery in X-radiated Mice Treated with Spleen Homogenate*

LEONARD J. COLE AND MARIE ELLIS

From the biochemical standpoint, perhaps the most striking effect of ionizing radiations on living systems involves a defect in the metabolism of the nucleic acids. Thus, one can cite a large number of observations in the literature (1, 18, 14, 16, 28) which state that the incorporation of a variety of labeled precursor substances (inorganic phosphate, formate, NaHCO₃, glycine) into tissue desoxyribonucleic acid (DNA) is markedly inhibited following the exposure of laboratory animals to x-radiation. The inhibition appears to be most pronounced in tissues with high DNA renewal rates (1@) and rapid cell turnover, such as spleen, bone marrow, intestinal mucosa, and thymus. It is of interest, furthermore, that this inhibitory effect of ionizing radiations is rather specific, since under conditions where the turnover rate of DNA is suppressed the rate of incorporation of labeled glycine into protein (1) and that of inorganic P into phospholipid are relatively unaffected (87). Lutwak-Mann (18) has shown that the concentrations of nucleic acid in rat bone marrow and in spleen fall precipitously 8–4 days after whole-body exposure to 500 r x-radiation—a sublethal dose. The change in DNA was found to be greater than that in the ribonucleic acid. By 14 days following irradiation the concentration of DNA in the spleen had returned to practically normal levels.

As shown in previous work from this laboratory (9), a single postirradiation injection of spleen homogenate into otherwise lethally x-radiated LAF₁ mice affords them marked protection against death by radiation. Radiation recovery following administration of spleen homogenates is accompanied by marked hypertrophy of the spleen, presumably reflecting renewed cell division and regeneration in this tissue (6). Furthermore, the studies of Jacobson et al. (15) on spleen shielding in x-radiated mice indicate that survival from acute radiation injury is correlated with hematopoietic recovery. Since there is considerable evidence in the literature (21, 26) for the concept that the average DNA content per nucleus (of resting diploid cells) is constant within the same species, and that new formation of DNA is associated with active cell division (2), it seemed worth while to attempt to utilize the DNA content of the spleen as a biochemical criterion of recovery in x-radiated animals treated with spleen homogenate.

MATERIALS AND METHODS

Female LAF₁ mice (14–16 weeks old) in groups of 40 were exposed to a single dose of 740 r total-body x-radiation, as described previously (9). The radiation factors were: 250 kVp, 15 ma, HVL 1.5 mm. Cu; filter of 0.5 mm. Cu plus 1 mm. Al; TSD, 100 cm.; and dose rate approximately 27 r/min, measured in air with a Victoreen r-meter.

The homogenates were prepared from spleens of young (7–10-day-old) LAF₁ mice in a modified salt-sucrose medium, as described previously (7), except that glucose was added to the medium at a final concentration of 1 mg/ml. One half of the irradiated mice received a single intraperitoneal injection of homogenate, equivalent to 60 mg. of wet weight spleen; the remaining irradiated animals were each injected with equivalent amounts of homogenate which had been heated in a water bath at 60° C. for 20 minutes. The protective factor in spleen has been shown to be labile under these conditions (8). In one of the experiments a number of the irradiated control mice received an injection of salt-sucrose solution instead of heated spleen homogenate. A total of 170 mice were irradiated in these experiments; of these, 60 mice were set aside for determination of 30-day survival. The remaining mice were sacrificed at various time intervals following irradiation. The spleens were removed, weighed individually in a weighing bottle, and analyzed for DNA content.

The analytical method employed was a modification of the Schneider procedure (22), in which ice cold 0.14 m NaCl was
used as the preliminary wash solution (to remove the soluble non-DNA nucleotides) instead of cold dilute trichloroacetic acid (TCA). The weighed spleen was transferred to a chilled Potter-type glass homogenizer and thoroughly homogenized—while the entire assembly was immersed in a cracked ice water bath—with 2 to 3 ml. of 0.14 M NaCl solution. The homogenate was then transferred quantitatively to a 10-ml graduated centrifuge tube, and the volume made up to 10 ml. with 0.14 M NaCl. The suspension was then centrifuged at 1300 g for 10 minutes in a refrigerated centrifuge (5°C). The supernatant fluid was decanted and discarded. The centrifugal residue was resuspended in 10 ml. of fresh, cold 0.14 M NaCl, centrifuged as before, and the supernatant again discarded. The washing process was repeated twice again. Five or 10 ml. of 5 per cent aqueous TCA was now added to the final 0.14 M NaCl washed centrifugal residue of the spleen, and the nucleic acid was extracted by heating in a 90°C water bath for 15 minutes, according to the method of Schneider. After cooling, the hot 5 per cent TCA extract was separated by centrifugation and its DNA concentration determined by the diphenylamine procedure of Dische (10), with 4 ml. of the TCA extract and 8 ml. of the diphenylamine reagent. The developed color was read at 660 mμ in a Coleman Junior Spectrophotometer. A standard DNA curve was prepared. A sample of bovine thymus DNA (Worthington Biochemical Co.) containing 6.99 per cent phosphorus was used as a standard for the analyses. On the assumption that all the P in the sample was DNA-P, the standard contained 70.6 per cent DNA.

The use of cold 0.14 M NaCl as the preliminary wash solution in the above procedure is based on the knowledge that DNA is present in the nucleus in a protein-bound state and, as such, is insoluble in 0.14 M NaCl (11, 20). Furthermore, the ribonucleoproteins and many of the cytoplasmic proteins, as well as the nucleotides, are soluble in 0.14 M NaCl. The use of the 0.14 M NaCl washing procedure, therefore, makes possible the removal of soluble nucleotides, proteins, and ribonucleic acids, and the separation of insoluble protein-bound DNA by a relatively mild and 'physiological' procedure. The thoroughly washed nuclear residue which contains the protein-bound DNA can then be subjected to analysis for DNA.

RESULTS

The data of Chart 1 illustrate the relationship between total DNA content per spleen and spleen weight in normal nonirradiated mice. The linear function obtained would appear to be an expression of the fact, as stated by Thomson et al. (25), that the DNA content of a tissue provides a chemical measure of the number of cells in that tissue.

The time course of the changes in spleen DNA content in irradiated mice treated with spleen homogenate (as compared with control irradiated mice injected with heat-inactivated spleen homogenate) is presented in Chart 2 and in Table 1.

The marked depression in spleen DNA levels following lethal whole-body x-radiation is noteworthy, as well as the reversal of this depression in mice treated with spleen homogenate. As soon as 4 hours after irradiation, the total DNA content of the spleen decreased from a value (in nonirradiated mice) of 1,963 ± 76 μg to 463 ± 70 μg. By 3 days after irradiation the DNA level was down to 220 μg. (a depression of 88 per cent from the normal value), and this low level persisted in

CHART 1.—Relationship between total DNA content/spleen and spleen weight in normal LAF1 mice.
the control irradiated mice until the 9th day, by which time all the mice in this group were dead. The time course of DNA levels in the irradiated mice which had been treated with spleen homogenate was, however, quite different. Although the spleen DNA levels were practically identical in both groups of irradiated mice during the first 4 days postirradiation, the DNA values in the spleen homogenate-treated animals showed a definite and significant reversal of the depression on the 6th day; by the 9th day following irradiation the spleen DNA content in this group of mice exceeded that of the nonirradiated mice. The relatively large fluctuations in DNA values among individual animals in the recovery phase and the return to normal values by 30 days are worthy of note.

Data from a separate experiment (Exper. 1) in which the mice were first sacrificed on the fifth postirradiation day are also presented in Table 1. Again, the results are characterized by the depression of DNA values (as low as 148 ± 1.48 μg/spleen) in the control irradiated mice, until death, and by the marked recovery in DNA levels, beginning at the 6th day, in the spleen homogenate-treated group.

**Changes in DNA concentration.**—In view of considerations relative to the amount of DNA per cell and the fact that the spleen comprises a heterogeneous population of different cell types, it was of interest to investigate the changes in spleen DNA concentration during recovery from acute radiation injury. To this end the data of Experiments 2 and 1, expressed in μg DNA/mg spleen (wet weight), are given in Table 1, and the values for individual mice are plotted in Chart 3. It is apparent that exposure to 740 r elicits a marked depression in DNA concentration in the spleen—from a level of 19.7 ± 0.35 μg/mg in normal nonirradiated mice to a value of 8.5 ± 0.57 μg/mg 24 hours after irradiation. In the control irradiated mice receiving heat-inactivated spleen homogenate, the spleen DNA level tends to move to still lower values with time, and these depressed values persist until death. In the irradiated mice receiving spleen homogenate, the depression in DNA concentration during the first 4 days following exposure to radiation parallels that of the control irradiated animals; by the 6th day, however, the spleen DNA concentration attains values as high as 15 μg/mg; and by the 14th day the values have equaled or exceeded those for nonirradiated mice of the same age.

**Relative changes in spleen DNA content and spleen weight.**—A comparison of the changes in spleen DNA content with those of spleen weight was of interest, since the spleen weight response in whole-body irradiated mice has been used as a

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<th>Time after irradiation</th>
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<tr>
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<td>Total DNA/ spleen (μg/mg)</td>
<td>DNA concentration (μg/mg)</td>
<td>Total DNA/ spleen (μg/mg)</td>
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<td>Exper. 1:</td>
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<td>5</td>
<td>148 ± 14</td>
<td>5.67 ± 0.33</td>
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<td>6</td>
<td>271 ± 15</td>
<td>6.68 ± 0.67</td>
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<td>7</td>
<td>245 ± 11</td>
<td>7.37 ± 0.78</td>
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<td>295 ± 7</td>
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<td>244 ± 16</td>
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<td>291 ± 20</td>
<td>6.39 ± 0.55</td>
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<tr>
<td>1</td>
<td>404 ± 23</td>
<td>8.52 ± 0.13</td>
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<td>4</td>
<td>227 ± 27</td>
<td>7.60 ± 0.60</td>
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<td>6.67 ± 1.07</td>
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Results are given ± S.E. (three or four spleens/point). * One sample. † Mean of two values. ‡ Sixteen spleens analyzed.

TABLE 1

**EFFECT OF TREATMENT WITH SPLEEN HOMOGENATE ON DNA LEVELS OF THE SPLEEN IN X-RADIATED MICE (740 r)**

<table>
<thead>
<tr>
<th>Time after irradiation</th>
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<tbody>
<tr>
<td></td>
<td>Total DNA/ spleen (μg/mg)</td>
<td>DNA concentration (μg/mg)</td>
<td>Total DNA/ spleen (μg/mg)</td>
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Results are given ± S.E. (three or four spleens/point). * One sample. † Mean of two values. ‡ Sixteen spleens analyzed.
biological dosimeter (5). The data on spleen weight from the experiments described above are presented in Chart 4. The response of spleen weight in the control and in the spleen homogenate-treated mice presents a time course qualitatively similar to that observed with spleen DNA content. However, important quantitative differences in these end-points are worthy of note. First, during the involution phase following irradiation (days 1–5), the spleen weight is reduced from the normal by a factor of 4–5, whereas the DNA content per spleen during this phase decreases by a factor of 10. During the recovery period, likewise, the response in terms of total DNA per spleen is more marked than that of spleen weight. Thus, on day 9, the ratio of the means of spleen weights in the spleen homogenate-treated mice, to the control mice is 3.6, whereas the corresponding ratio of the means for total spleen DNA content is 10.6.

Relationship between spleen DNA content and survival.—The survival data of both experiments are given in Table 2. The x-ray dose used was lethal to all the control mice within 11 days; excellent protection, particularly in Experiment 2, was afforded by injection of the spleen homogenate. The reasons for the unusually large number of late deaths (e.g., beyond 20 days) in the mice treated with spleen homogenate in Experiment 1 are not immediately apparent.

On the supposition that the mice used for 30-day survival determination and those sacrificed for spleen DNA analysis are representative of the same homogeneous population, it can be seen that total DNA content of the spleen on day 7 or 8 provides a chemical measure of recovery of this hematopoietic tissue which is correlated with survival. Thus, on day 8, the spleen DNA levels in the mice receiving spleen homogenate were already much higher (up to 1600 µg DNA/spleen) than those for the control irradiated mice (329

**TABLE 2**

<table>
<thead>
<tr>
<th>Exper. no.</th>
<th>Treatment</th>
<th>Survival at 80 days (no./total)</th>
<th>Survival at 30 days (no./total)</th>
<th>Mean survival time of non-survivors (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Spleen homogenate</td>
<td>15/16</td>
<td>8/16</td>
<td>21</td>
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<tr>
<td></td>
<td>Heat-inactivated spleen</td>
<td>0/15</td>
<td>0/15</td>
<td>11</td>
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<tr>
<td></td>
<td>homogenate</td>
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<tr>
<td>2</td>
<td>Spleen homogenate</td>
<td>10/10</td>
<td>19/19</td>
<td>10</td>
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<tr>
<td></td>
<td>Heat-inactivated spleen</td>
<td>0/10</td>
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<td>homogenate</td>
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In the latter group, furthermore, the DNA level never exceeded 400 μg/spleen. In experiment 1, one out of four of the spleen homogenate-treated mice, sacrificed on the 8th day, showed a spleen DNA value (200 μg) corresponding to a control value. Since the 50-day survival among the spleen homogenate-treated mice in this experiment was 50 per cent, it is reasonable to infer that this low spleen DNA value is indicative of the lack of recovery.

Further evidence for the relationship between spleen DNA content and survival was provided by splenectomy data. One spleen homogenate-treated mouse and one control were splenectomized under ether anaesthesia on the 9th day following irradiation. The spleen DNA content of the former animal was 3,280 μg.; the DNA concentration was 19.0 μg/mg; the corresponding values in the control mouse were 249 μg and 4.4 μg/mg, respectively. The treated mouse tolerated the surgery well and was alive and to all appearances normal at 30 days; the control animal died within 24 hours following splenectomy. Two additional mice of the irradiated spleen homogenate-treated group were splenectomized on the 15th post-exposure day. The values for spleen DNA content in these mice were 2,500 μg. and 2,520 μg.; the DNA concentrations were 18.6 and 17.7 μg DNA/mg.

**DISCUSSION**

It is evident from the data presented that a single injection of spleen homogenate in mice exposed to whole-body x-radiation, 100 per cent lethal to control mice, elicits a profound regeneration of the spleen, in terms of DNA content, concomitant with survival of these animals. The recovery phenomenon is further characterized by a delayed appearance first manifest at approximately 6 days after treatment. In contradistinction, no recovery of the spleen DNA content occurs in control irradiated mice injected with heat-inactivated spleen homogenate or with salt-sucrose solution, and none of the latter two groups of control mice survives beyond 10 days following irradiation.

The data will be discussed from two general aspects: (a) time course of spleen DNA content concomitant with radiation-induced involution and regeneration of the spleen; (b) use of the spleen DNA content as an end-point for assaying the radiation protection activity of spleen and bone marrow preparations.

1 Survival of this animal after splenectomy indicates that recovery of other hematopoietic sites—probably bone marrow—had also taken place by the 9th day in the spleen homogenate-treated group.

The results of numerous analytical determinations of tissue DNA in the literature have firmly established the concept of the constancy of DNA content per nucleus in resting diploid cells. Although there have been some conflicting experimental data on the time relation of DNA formation to the stages of the mitotic cycle, the bulk of experimental evidence in the literature indicates that a doubling of the DNA content per nucleus precedes the morphological phenomenon of cell division (24). Furthermore, the data of Stevens et al. on relative mitotic rates in liver and intestinal mucosa (24), of Brues et al. on the incorporation of inorganic P into resting and regenerating liver (4), and that of Bendich (2) on the incorporation of C into nucleic acids of regenerating liver support and are consistent with the hypothesis that DNA new formation is associated with cell division.

Interpretation of the data on spleen DNA presented in this paper must be made in light of the fact that spleen tissue is comprised of different cell types, varying in nuclear:cytoplasmic volume ratio. The rapid and drastic decrease in spleen DNA content during the involutional phase following whole-body x-radiation would appear to be a resultant of cell depletion (3) and of the cessation of new formation of DNA (17). The fact that the relative decrement in DNA content per spleen is greater than that of spleen weight (reflected in the decreased spleen DNA concentration) could be accounted for by a differential loss, from the spleen, of cells possessing relatively large nuclear:cytoplasmic volume ratios, e.g., the small lymphocytes. If differential loss of erythrocytes from the spleen occurred as part of the picture, this would have the effect of increasing the DNA concentration since mature erythrocytes are practically devoid of DNA.

Lavik and Harrington (17) observed a gradual decrease in DNA-P concentration of rat spleen during the first 4 days following an LD₅₀ dose of inorganic P. They have discussed this finding from the standpoint of the loss of DNA-rich cells from the spleen resulting from irradiation. It is of interest that the mean concentration of DNA in normal rat spleen found by these workers is lower by a factor of 3 than the corresponding values in normal mouse spleen, as determined here.

The recovery phase, heralded by the rise in spleen DNA content and concentration, may be explained to a large extent on the basis that the newly formed spleen cells possess a small cytoplasmic mass relative to that of the nucleus, i.e., the restoration of small lymphocytes and relatively mature leukocytes to the cytoarchitecture of
the regenerating spleen in the group treated with spleen homogenate. However, it is also evident
that the regeneration of splenic tissue during this phase represents a dynamic growth process in
volving the elaboration of new cells which is accompanied by a net synthesis of DNA.

Marston et al. (19) have investigated the time
course of recovery in the peripheral leukocyte
count in N.I.H. strain mice exposed to 475 r x-
rays (an LD90 dose) and treated with mouse spleen
homogenate. A parallel depression of the leukocyte
count in the mice treated with spleen homogenate
and in the control mice up to the 7th day was ob-
served. Between the 7th and 9th day, the leuko-
cyte levels in the mice treated with spleen homoge-
nate showed marked recovery, “while in the ir-
radiated controls the counts usually remain below
1,000 per cu. mm. for 14 days.” The time sequence
of changes in spleen DNA content observed here
likewise shows a parallel depression in the treated
and control groups for the first 5 days. Significant
recovery in DNA levels, however, was seen by the
6th postirradiation day. It is of considerable in-
terest that the recovery of the level of DNA in
the spleen precedes that of peripheral leukocyte
count—presumably reflecting the DNA require-
ment for resumption of leukopoiesis. It is note-
worthy also that the earliest recovery from body
weight loss in x-irradiated mice treated with spleen
homogenate does not become manifest until the
8th day following treatment (6)—thus following by
2 days the spleen DNA index.

It appears, then, that the measurement of
spleen DNA level provides a biochemical index of
recovery from whole-body x-radiation damage,
and one which is rather more sensitive than that of
spleen weight. Admittedly, it is easier to weigh
the spleen than to determine its DNA content.
However, it should be noted that the wet weight
of a tissue is the resultant of a number of con-
stituents, such as water, ribonucleic acid, protein,
lipid, etc., whose concentrations are subject to
considerable variation, even in the absence of
changes in cell numbers (25). Furthermore, in the
spleen, the considerable contribution of red blood
cell mass to its wet weight adds an additional sig-
ificant variable. The DNA content of the spleen,
on the other hand, provides a measure of one spe-
cific end-point—an end-point which furthermore
uniquely reflects changes in the number of cells
in a tissue and in the cell population. The present
results also suggest the use of the spleen DNA
end-point as a biochemical assay for radiation
protection activity of spleen homogenates and
fractions thereof. Such an assay, even if semi-
quantitative, would be superior in many respects
to the all-or-none criterion of mortality.

SUMMARY

1. The time course of total DNA/spleen and
spleen homogenate concentration in LAF3 mice following
whole-body exposure to 740 r x-rays (an LD90
dose) and the effect of spleen homogenate treat-
ment on this end-point have been studied. At 24
hours postirradiation, the DNA content/spleen
decreased from a normal value of 1,963 ± 70 µg.
After 3 days the DNA level had declined further to
229 µg., and this low level persisted in the con-
trol, irradiated mice until death (9th day). DNA
concentration was likewise depressed from normal
values of 19.7 ± 0.35 µg/mg to levels of 8 µg/mg
until death.

2. A single postirradiation injection of spleen
homogenate into the irradiated mice elicited a
profound regeneration of the spleen in terms of
DNA content, concomitant with survival of these
animals. The recovery phenomenon is charac-
terized by reversal of the depression in total DNA
content/spleen, which is, however, not manifest
until the 6th day; by the 9th day the DNA total DNA
values exceed those of normal mice. Further evi-
dence for the relationship between spleen DNA
content and recovery was provided by DNA
analyses on splenectomized mice which had re-
ceived spleen homogenate treatment.

3. The magnitude of the response in DNA
content/spleen, both during involution and in the
recovery phase, was greater by factors of 2 and 3,
respectively, than the spleen weight response.
Recovery of the spleen DNA in x-irradiated mice
receiving spleen homogenate precedes that of
peripheral leuocoeyte count and body weight loss.
The results indicate that the DNA level of the
spleen provides a sensitive biochemical index of
recovery following x-radiation exposure. It is sug-
gested that this end-point be employed as a bio-
chemical assay for the spleen radiation protection
factor.

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