The Elevation of Blood Levels of Zinc Protoporphyrin in Mice Following Whole Body Irradiation

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Elevation of zinc protoporphyrin (ZPP) levels in the blood has served as an indicator of lead poisoning and iron deficiency anemia for many years. We have discovered that sublethal doses of whole body irradiation with x-rays also elevates ZPP 2–3-fold over normal levels. The ZPP level does not begin to increase until days 12–14 postirradiation and peaks between days 18 and 20 before returning to normal levels between days 28 and 35. Increasing the radiation dose delays the onset of the rise in ZPP, but does not affect the magnitude of the elevation. At lethal doses, ZPP elevation is not observed. Neither of the two previously described mechanisms that cause elevations of ZPP, namely iron deficiency and inhibition of ferrochelatase, are responsible for the radiation-induced elevation of ZPP. The elevation of ZPP appears to be correlated with the recovery of the hematopoietic system from radiation injury.

Materials and Methods

Materials

Erythropoietin, step I, was obtained from the Connaught Medical Research Lab., Toronto, Canada. Mesoporphyrin IX was obtained from Porphyrin Products, Logan, UT. WR-2721 [S-2-(d-aminopropylamino)-ethylphosphorothioic acid] was a gift from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. The [55Fe], 11.8 mCi/mg, was obtained as ferrous citrate in saline from ICN. Serum iron, total iron binding capacity, and unbound capacity were determined using the [55Fe] Serum Iron/Iron Binding Capacity Radioassay Kit from Corning Medical and Scientific (Corning, NY). Proteins were determined by the method of Bradford with a kit from Bio-Rad (Richmond, CA). Total serum bilirubin was determined by the direct spectrophotometric method of Meites and Hogg. Hemoglobin concentrations were measured using a Hemoglobinometer (Coulter Electronics, Hialeah, FL), and red blood cells were counted by use of a Coulter Counter.

X-Irradiation of Mice

Mice were irradiated with 250 KVP x-rays at 15 mA using a G.E. Maximap 250 H1 unit with a 1.0-mm aluminum filter and a target-to-object distance of 50 cm. The average exposure rate was approximately 80 R/min (2.06 x 10^2 coulomb/kg/min). In most experiments, the standard dose was 550 rad (5.5 grays). Mice were placed in a circular plastic pan having 10 equal compartments, which was rotated under the beam to insure equal exposure. Unless mentioned otherwise, the mice used in these experiments were C3H/HeJ males, 6–8 wk old, weighing 16–24 g. They were housed 10 or less in a cage and fed and watered ad libitum.

Zinc Protoporphyrin (ZPP) Measurement

ZPP levels were determined using a dedicated hematofluorometer (ZPP Meter-Aviv Associates). Samples of whole blood, collected in a heparinized microcapillary tube, were placed on a Corning brand 25 x 25 mm glass coverslip. One drop of blood (about 25 μl) was sufficient for an assay. The coverslip was inserted into the detector and excited at 420 nm, and the fluorescence of the sample at 594 nm was measured. The data are usually expressed as micrograms ZPP per gram hemoglobin. The readings were taken 10 min after the blood was placed on the coverslip. This time interval allowed a...
uniform layer of red blood cells to settle on the coverslip, thereby eliminating errors due to differences in hematocrit between samples.19,20 The samples were assayed 4 times and the average was recorded. The standard deviation on the 4 determinations was usually ± 0.1 μg ZPP/g Hb.

Zinc protoporphyrin was also determined by extraction from red cells, as described by Smith et al.,21 followed by quantitation with high pressure liquid chromatography (HPLC) by a modification of the method of Bonnett et al.22 The HPLC chromatography was carried out on a 25 cm × 4.6 mm I.D. column packed in our laboratory with Rosil C-18, 5 μm spherical material from Alttech Associates. The injection volume was 25 μl, and the column was developed isocratically with 90% methanol, 10% 1 mM tetrabutylammonium phosphate dissolved in water. The porphyrins were detected with a Kratos fluorescence detector, FS 970.

Treatment With WR-2721 [S-2-[(3-Aminopropylamino)-Ethylphosphorthioic Acid]

The radioprotectant drug (WR-2721) was dissolved in distilled water and administered intraperitoneally to the mice 15 min prior to irradiation, in accordance with the procedures of Yuhas.23 The dosage given was 438 mg/kg. Fifteen minutes after injection, the mice received 500 rad of 250 KVP x-rays.

Determination of Serum Iron, Total Iron Binding Capacity, and Unbound Iron Binding Capacity

Forty mice received 550 rads of x-rays and were killed in groups of five on the indicated days after irradiation. Five unirradiated animals served as controls. The mice were anesthetized; blood samples for ZPP determination and hematocrit determination were taken in microcapillary tubes from the orbital sinus. The mice were then exsanguinated through the left brachial artery using a heparinized needle. Blood was taken in microcapillary tubes from the orbital sinus. The mice were then exsanguinated through the left brachial artery using a heparinized pipette. The blood was centrifuged and the plasma removed and frozen at −80°C until assayed. The plasma iron, the total iron binding capacity (TIBC), and the unbound iron binding capacity (UIBC) were determined using the Corning Serum Iron/Iron Binding Capacity [59Fe] Radioassay Kit. Two 50-μl samples of plasma were taken for each assay. One sample was used for the serum iron determination, and the other for the UIBC determination. The amount of 59Fe retained by the resin during the assay was determined using a gamma counter. The TIBC was calculated from the formula: TIBC = UIBC + serum iron. Three different standards with known TIBC, UIBC, and serum iron levels provided with the kit were used to standardize the results. All three (TIBC, UIBC and plasma iron) were reported in μg iron/dl of serum. A fourth value useful in diagnosing iron deficiency—the percent iron saturation—was calculated by dividing the plasma iron level by the TIBC and multiplying the result by 100. The percent iron saturation indicates the portion of the TIBC that is being used to carry iron in the plasma. An iron saturation value below 16 is indicative of iron deficiency.24

Splenectomy

Splenectomies were performed according to Layendecker25 1 wk prior to irradiating mice with 550 rad. The wound clips were removed just prior to irradiation.

Ferrochelatase Assay

Ferrochelatase was assayed by using a modification of the method of Williams et al.26 Livers and spleens were excised from the mice on the indicated days after irradiation and also from the unirradiated controls. Five milliliters of homogenization buffer (0.25 M sucrose, 0.05 M Tris, pH 7.5, and 0.01% Tween 20) was added to 5 g of spleen or 5 g of liver. The tissues were then homogenized and sonicated at high speed for 30 sec with a Polytron homogenizer (Brinkman Instruments), then cooled with ice for 1 min. The homogenization—sonication was repeated for 30 sec. Samples of blood from irradiated and control animals were prepared by pooling 1 ml of heparinized blood from each animal (minimum of 10 animals) and then removing a 5-ml aliquot from each pooled group. Each 5-ml aliquot of blood was mixed with 1 ml of the homogenization buffer and subjected to the same procedures described for the livers and spleens. The protein concentration of each of the homogenates (liver, spleen, and blood) was determined, and the samples were diluted with homogenization buffer to a concentration of 50 mg protein/ml.

Stock solutions for the assay were made up according to Williams et al.,26 with the exception of the “working” iron solution. The “working” iron solution contained 10 mg ascorbic acid, 8.35 mg FeSO4·7H2O, 10 mg reduced glutathione, and 7.44 μCi of [59Fe as ferrous citrate (specific activity 11.8 mCi/mg) in 3.0 ml of deoxygenated water. The working iron solution also contained 62,000 cpm of [59Fe/25 μl (the amount added to each reaction tube). Further modifications were made to reduce the amount of sample in each assay tube. Each reaction tube contained 50 μl of Tween 20, 250 μl of mesoporphyrin IX (42.5 nmole in 95% methanol), 1.25 ml of 0.3 M Tris, pH 7.5, and 250 μl of homogenate. Plastic culture tubes with caps were used for reaction vials. The reaction mixture, minus the working iron solution, was flushed with N2 and capped. The tubes were incubated at 37°C for 4 min, after which 25 μl of the working iron solution was added and each tube again flushed with N2 before capping. After incubating the tubes at 37°C for 24 min, 125 μg carrier heme was added, and the total heme extracted with ethyl acetate/glacial acetic acid, as described by Williams et al.26 The incorporation of Fe[59] into heme was linear up to 45 min. Samples were counted in a gamma counter.

Studies on the Combined Effects of X-Irradiation and Lead

Lead was administered to 38 mice by placing lead acetate in their drinking water at a concentration of 4 × 10−2 M for 4 wk. For the next 18 days, the lead acetate concentration was increased to 5 × 10−1 M. The degree of lead poisoning was monitored by measuring the ZPP and by assaying blood lead levels via atomic absorption spectroscopy. After 30 of the lead-poisoned mice had been irradiated with 550 rad of x-rays, they were divided into two groups of 15. The first group was continued on lead acetate (5 × 10−2 M), and the second group received normal water. The mice were monitored for ZPP levels and hematocrit, with no more than 50 μl of blood being taken from each mouse per week.

RESULTS

Elevation of ZPP After Irradiation

In the experiments described below, the mice received 550 rad, and the blood ZPP level was then determined at the indicated time postirradiation, as shown in Fig. 1. The average ZPP level of unirradiated C3H/HeJ mice (n = 67) was 1.9 μg ZPP/g Hb, and the average hematocrit was 45. Similar ZPP levels were also found for a different strain, CD-1 Swiss outbred (Charles River Co., Wilmington, MA), and also for Sprague-Dawley rats. There was no difference in the normal ZPP levels between males and females.

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RADIATION AND ZPP ELEVATION

Fig. 1. Elevation of blood ZPP levels following whole body x-irradiation. C3H/HeJ male mice received 550 rad of 250 KVP x-rays. On the indicated days, the hematocrit (—) and ZPP (—) concentrations were measured. On days 5–16, there were 20 mice per data point, and on days 18–24, there were at least 5 mice per point. The control value, day 0, is the average of 67 mice. Standard error bars for each data point are provided.

Following whole body irradiation, ZPP levels remained constant at about 1.9 μg ZPP/g Hb through day 7, when they rose slowly through day 16 to 2.8. At day 16, the ZPP level rose rapidly, reaching a peak of 4.3 on day 20 postirradiation. The ZPP then decreased at about the same rate with which it had increased. ZPP levels returned to normal by day 35.

Also shown in Fig. 1 are the hematocrit values, which decreased on day 7 and dropped to the lowest point on day 13 or 14. The sharp rise in ZPP (days 17–21) occurred at the same time that the hematocrit rose (days 17–20).

In order to confirm that ZPP became elevated during the postirradiation period, we used an independent assay for ZPP that involved extraction of porphyrins from red cells followed by HPLC. The results confirmed that ZPP became elevated after irradiation and reached a peak on day 20.

Effect of Radiation Dose on ZPP Elevation

The elevation of ZPP was not observed at doses of 300 and 400 rad, but was seen at a dose of 450 rad. After receiving 400 rad, mice were examined for ZPP every 2 days (5–10 mice/data point) up to day 24 postirradiation, with no apparent change in ZPP levels from those of unirradiated controls (not shown). This finding indicates the existence of a threshold below which radiation does not induce ZPP elevation. The elevation of ZPP was not observed in C3H/HeJ mice or rats exposed to lethal x-ray doses.

Having determined the lower limit below which ZPP was not elevated and the upper limit set by hematopoietic death, we focused our attention on the effect of radiation dose between these limits. As shown in Fig. 2, when mice received 450, 500, or 600 rad of x-rays, the ZPP rose to the same level regardless of dose, but the onset of the elevation of ZPP was delayed at the higher doses of radiation. In a different strain of mice, CD-1, the threshold below which ZPP elevation did not occur was 600 rather than 450 rad. In rats, the threshold was also 600 rad.

Comparison of Serum Bilirubin and ZPP Levels Following Irradiation

Bilirubin absorbs at 420 nm, and its fluorescent emission slightly overlaps that of ZPP. The fluorometer used in these studies has a much greater sensitivity for detecting ZPP than for bilirubin, as its window is set on the ZPP emission peak; at this wavelength, there is very little overlap by the emission from bilirubin.

However, we felt that it was necessary to eliminate the possibility that the elevation of ZPP was a spurious result that was actually due to bilirubin elevation. To rule out this possibility, 40 mice received 550 rad of x-rays and were then killed, 5 each at days 1, 2, 3, 7, 10, 14, 16, and 21 postirradiation. Twenty-eight unirradiated mice were used as controls. The mice were anesthetized and exsanguinated using heparinized pipettes, and hematocrit and ZPP determinations were made for each mouse. A sample of the red cells was washed with saline to remove bilirubin, and the ZPP continued to remain elevated (data not shown). Serum was collected and the bilirubin concentration was determined using the direct spectroscopic method of Meites and Hogg. The normal bilirubin level in C3H/HeJ male mice, as determined from the 28 unirradiated controls, was 0.30 mg/dl.

Bilirubin levels approached zero, with an average of 0.15 mg/dl, on day 2 and remained low through day 7. These levels returned to 0.38 mg/dl by day 10 and
continued to rise, reaching a peak of 1.06 mg/dl on day 10 before decreasing to normal levels by day 21. The elevation of ZPP could not be a spurious consequence of bilirubin elevation, as ZPP was still elevated to twice the control level on day 21, at which time bilirubin had dropped to normal.

**Hematologic Parameters Following Irradiation With 550 Rad of X-Rays**

Various hematologic parameters were compared with ZPP values at the indicated times after irradiation (Table 1). Initially, the ZPP slowly increased, and the red cell count, hematocrit, and hemoglobin concentration decreased. After 16 days, these parameters began to rise. On about day 14, the ZPP level began to increase. The ZPP concentration per red cell can be calculated from the MCH, which expresses the average hemoglobin concentration (in picograms) per red cell. This value for unirradiated mice was approximately $34 \times 10^{-18}$ g ZPP/RBC. The values for days 16, 19, and 21 postirradiation were 57, 85, and $90 \times 10^{-18}$ g ZPP/RBC, respectively. The value of red blood cells (MCV) decreased until day 16, when the MCV then increased to a value of 73 cu mm by day 21. These high MCVs are indicative of a population of newly synthesized red cells (young red cells have larger volumes than older cells), and the increase in ZPP concentration per cell indicated that the elevation of ZPP at 16 days postirradiation was due to elevated ZPP levels in young, newly synthesized cells as the hematopoietic system recovered from radiation damage. Between days 21 and 24 postirradiation, the red cell count increased from $5.18 \times 10^{10}$ RBC/cu mm to $7.44 \times 10^{10}$, with a corresponding decrease in ZPP from 3.7 to 2.4 g ZPP/g Hb. The sharp decrease in ZPP following day 20 might be the result of diluting out high ZPP-containing red cells by cells containing lower amounts of ZPP.

To determine if the elevated ZPP levels might simply be the result of increased reticulocyte numbers or a response to erythropoietin, 6 mice were treated with 5 U of erythropoietin given i.p. These mice exhibited an increased reticulocyte count from 1% on day 0 to 7% on day 3 posttreatment; however, no change in the ZPP level was observed. Similarly, mice that were maintained under hypoxic conditions for 14 days and monitored routinely for ZPP levels and hematocrit showed hematocrits increasing from 45% to 75% without any change in ZPP.

**Table 1. Hematologic Parameters in C3H/HeJ Male Mice After Receiving 550 rad of X-Rays**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>0</th>
<th>7</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>19</th>
<th>21</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZPP (g/g Hb)</td>
<td>1.9 ± 0.1</td>
<td>2.1 ± 0.2</td>
<td>2.2 ± 0.3</td>
<td>2.2 ± 0.5</td>
<td>2.7 ± 0.5</td>
<td>3.2 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>3.7 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Percent hematocrit</td>
<td>50 ± 2</td>
<td>38 ± 1</td>
<td>32 ± 1</td>
<td>25 ± 1</td>
<td>21 ± 1</td>
<td>17 ± 1</td>
<td>17 ± 1</td>
<td>28 ± 2</td>
<td>42 ± 1</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>15.4 ± 0.7</td>
<td>12.6 ± 0.2</td>
<td>10.9 ± 0.7</td>
<td>9.0 ± 0.3</td>
<td>7.6 ± 0.3</td>
<td>7.2 ± 0.4</td>
<td>10.4 ± 0.8</td>
<td>12.6 ± 0.5</td>
<td>12.7 ± 0.2</td>
</tr>
<tr>
<td>RBC $\times 10^6$</td>
<td>8.66 ± 0.45</td>
<td>7.02 ± 0.18</td>
<td>6.35 ± 0.14</td>
<td>5.07 ± 0.14</td>
<td>4.33 ± 0.19</td>
<td>3.94 ± 0.19</td>
<td>4.70 ± 0.36</td>
<td>5.18 ± 0.20</td>
<td>7.44 ± 0.37</td>
</tr>
<tr>
<td>MCV (cu mm)</td>
<td>58 ± 1</td>
<td>54 ± 1</td>
<td>50 ± 1</td>
<td>50 ± 2</td>
<td>48 ± 2</td>
<td>44 ± 1</td>
<td>59 ± 4</td>
<td>73 ± 2</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>MCH (fl)</td>
<td>17.7 ± 0.4</td>
<td>18.1 ± 0.9</td>
<td>17.3 ± 0.9</td>
<td>17.8 ± 0.5</td>
<td>17.7 ± 0.8</td>
<td>16.0 ± 0.4</td>
<td>22.5 ± 1.2</td>
<td>24.3 ± 0.6</td>
<td>17.2 ± 0.8</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>30.6 ± 0.4</td>
<td>33.3 ± 0.9</td>
<td>34.0 ± 3.7</td>
<td>35.6 ± 1.4</td>
<td>36.6 ± 1.3</td>
<td>40.8 ± 1.1</td>
<td>40.1 ± 1.1</td>
<td>33.5 ± 1.7</td>
<td>29.7 ± 0.6</td>
</tr>
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</table>

Elevation of ZPP is a diagnostic indicator of iron deficiency as well as of lead poisoning. Therefore, it was important to determine the status of iron metabolism in the irradiated mice. Radiation, by halting erythropoiesis, accelerating the loss of red blood cells, damaging the intestines, inhibiting the synthesis of liver and serum proteins, and, later, during the recovery phase, accelerating erythropoiesis, has the potential for perturbing iron metabolism. Thus, it was necessary to assess whether iron deficiency was responsible for ZPP elevation following whole body irradiation. The ZPP level and four diagnostic indicators of plasma iron levels were determined and compared in mice on the indicated days after 550 rad x-irradiation. The four diagnostic indicators of iron metabolism measured were the serum iron level, TIBC, UIBC, and percent iron saturation. The serum iron is the actual quantity of iron (mostly bound to transferrin) in serum. The TIBC reflects the levels of transferrin in the serum and is the total amount of iron that could be carried in the serum if all transferrin-iron binding sites were saturated. The UIBC is the amount of transferrin not bound to iron and is the TIBC minus the serum iron level. The percent iron saturation is the serum iron/TIBC x 100. This value drops below 16% in iron deficiency anemia. As shown in Fig. 3, the percent iron saturation never dropped below 50% during the course of these experiments.

The ZPP for these animals is also shown in Fig. 3. The ZPP peaked on day 14, then dropped slightly, but remained elevated during the next 7 days. The hematocrit in this experiment (not shown) dropped to its lowest point on day 14 and rose to control levels between days 16 and 21. Between days 14 and 16, the serum iron rose from 400 µg/dl to 745, and the TIBC...
jumped from 460 to 775 µg/dl. With the sharp increase in erythropoiesis after day 16, the serum iron levels, TIBC, and percent iron saturation dropped, and the UIBC increased. During iron deficiency anemia, the serum iron and percent iron saturation increase and the TIBC decreases. The exact opposite occurs in iron poisoning, lead poisoning, hemolytic anemia, and sideroblastic anemias, in which serum iron and percent iron saturation increase and the TIBC increases. Shortly prior to and during the time that ZPP levels were rising, serum iron, TIBC, and percent iron saturation all increased, thereby ruling out the possibility that iron deficiency anemia induced by whole body irradiation was the cause of ZPP elevation in irradiated mice.

**Effects of Radiation on Ferrochelatase Activity**

ZPP becomes elevated following lead poisoning due to inhibition of ferrochelatase. It was therefore necessary to determine whether ferrochelatase was inactivated to such a degree following whole body irradiation that ZPP became elevated. Ferrochelatase activity was measured in spleen, liver, and red blood cells. The specific activities of ferrochelatase on days 9, 18, and 20 postirradiation are shown in Table 2. The data shown in Table 2 indicated that ferrochelatase activity was not reduced after irradiation, but, in fact, was increased. Spleen and liver ferrochelatase rose during the postirradiation period. In red blood cells, ferrochelatase is found in the reticulocyte membrane and is absent at the erythrocyte stage. The absence of activity in the blood on day 9 is due to the absence of reticulocytes. Ferrochelatase activity was elevated over control levels on days 18 and 20 postirradiation in all three organs tested. Therefore, the inhibition of ferrochelatase clearly cannot be an explanation for the radiation-induced elevation of ZPP.

**Effect of the Radioprotectant Compound, WR-2721, on Radiation-Induced Elevation of ZPP**

WR-2721 (438 mg/kg) was administered i.p. 15 min prior to 550 rad of x-rays. In WR-2721-protected animals, ZPP did not differ appreciably from control values. The hematocrit in the WR-2721-treated animals dropped to its low value of 31 on day 12. In untreated animals, the hematocrit dropped to 16 on day 13. In both groups of animals, the spleen weights reached their lowest points of 22 mg and 28 mg, respectively, on day 7 postirradiation in the unprotected and in the WR-2721-protected animals. Regeneration was faster in the protected mice than in the unprotected animals. Spleen weight peaked in the protected mice on day 16 to 105 mg, whereas in the unprotected animals, the peak spleen weight (250 mg) was on day 20.

**Splenectomy Delays Radiation-Induced Elevation of ZPP**

Mice were splenectomized and, 1 wk later, received 550 rad of x-rays. The ZPP and hematocrits were measured on the days postirradiation indicated in Fig. 4. The hematocrits in both the splenectomized and unsplenectomized irradiated mice decreased to similar low points—13% and 16%, respectively. The recovery of erythropoiesis from this low point, as indicated by
the return of the hematocrits to normal levels, was much slower in splenectomized mice (24 days) than for the unsplenectomized animals shown in Fig. 1 (10 days).

The rate of increase of ZPP is also much slower in the splenectomized mice, indicating a correlation between recovery of ZPP elevation with recovery of erythropoiesis. Both the slower increase in ZPP and the restoration of the normal hematocrit levels in the splenectomized mice emphasize the importance of the spleen in the rapid recovery of erythropoiesis following radiation. One other interesting observation to be noted in comparing Figs. 1 and 4 is that the rate of decrease of ZPP after it reaches its peak value is similar to its rate of increase. In the experiment summarized in Fig. 1, a rapid rise in ZPP between days 16 and 20, followed by an equally rapid decrease from days 20 to 24, was observed. This relationship between the rates is also evident with the splenectomized mice.

**Combined Effect of Lead and X-Irradiation**

Mice were poisoned with lead and divided into two equal groups. Group I continued to receive lead acetate in the drinking water, and group II was removed from the lead regimen. One-half of the mice in each group was irradiated with 550 rad, and the ZPP levels in all four groups were monitored. At the time of irradiation, the average blood lead levels were 2.8 ppm compared to 0.16 ppm in controls. Figure 5A shows the results for unirradiated mice that continued to receive lead. The ZPP rose slowly over a 35-day period, with the hematocrit remaining essentially unchanged. In the irradiated mice that continued to receive lead, the hematocrit dropped, reached a minimum, and then began to rise (Fig. 5B). The ZPP began to rise at this time, as was previously seen in Fig. 1, but the maximal ZPP reading was higher than was caused from radiation alone. Thus, the effect of irradiation and lead on ZPP elevation appears to be additive.

In the mice placed on a lead-free regimen and not irradiated, the ZPP dropped slowly during the course of the experiment from a slightly elevated value and returned to normal (Fig. 5C). In the irradiated lead-poisoned mice that were put on a normal regimen (Fig. 5D), the typical postirradiation spike in ZPP was observed, but it came a bit later (as did the recovery of erythropoiesis) than occurred in mice that had never been exposed to lead (Fig. 1).

**DISCUSSION**

Prior to this report, the only conditions reported to cause an elevation of ZPP were iron deficiency anemia and lead poisoning. We have demonstrated a third condition that results in an elevation of ZPP in mice and rats, namely, whole body x-irradiation. There was a threshold radiation dose below which ZPP elevation did not occur. The ZPP elevation varied both between strains of mice and between species. The lowest effective dose in C3H/HeJ mice was 450 rad, and in both CD-1 mice and Sprague-Dawley rats, it was 600 rad.

There was a correlation between the recovery of the hematopoietic system and the elevation of ZPP. This was clearly indicated by experiments in which we caused the rise in the hematocrit after irradiation to be delayed by splenectomy, lead poisoning, or changing x-ray dose. In all cases, the elevation of ZPP was correlated with the rise in hematocrit.

The elevation of ZPP was not observed after injec-

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**Fig. 5.** The combined effects of lead poisoning and x-irradiation on the elevation of ZPP. C3H/HeJ male mice were lead poisoned, as described in the text, prior to receiving 550 rad x-irradiation. (A) The results with unirradiated lead-poisoned mice that continued to receive Pb\(^{2+}\); (B) irradiated animals that continued to receive lead-containing water; (C) the unirradiated mice; and (D) irradiated mice placed on normal water. The ZPP levels shown are based on averages of 4 or 5 animals per data point. The ZPP and hematocrit levels of all sets of animals were followed at various times postirradiation. Blood samples, 30–40 μl, were obtained from each animal every 6 days. By staggering several groupings, samples could be obtained every 2–3 days. The ZPP levels are represented by (■■■) and the hematocrits by (○—○). A solid line in C shows the ZPP levels in non-lead-poisoned unirradiated controls.
tion of erythropoietin, indicating that the synthesis of ZPP may not occur inadvertently during elevated hematopoiesis, but that it occurred only during recovery of the hematopoietic system from chemical, radiation, or nutritional insult. A major difference between the elevation of ZPP during recovery from radiation damage and lead poisoning was the appearance of a “spike” in the radiation response that has not been reported in lead toxicity studies. In splenectomized mice, the spike was not as acute, both on the upward and downward sides, indicating that the spleen may be involved either with the production of a cohort of cells with high levels of ZPP, with the removal of this cohort, or both. As shown in Table 1, a 44% increase in the red cell count occurred between days 21 and 24 postirradiation. The sharp decrease in ZPP during this same time could be accounted for if the red cells produced during this time had less ZPP than cells present between days 16 and 20. The cells with reduced ZPP levels would dilute out the high ZPP-containing cells, resulting in an overall decrease in the “average” ZPP level.

Changes in bilirubin, hemoglobin, and hematocrit have been reported to cause false elevations of ZPP when measured by a dedicated hematofluorometer. However, as we report here, these changes were not responsible for the radiation-induced elevation of ZPP. One other artifact considered was free protoporphyrin, which has a fluorescence peak at 632 nm. Elevation of free protoporphyrin occurs in iron deficiency and lead poisoning and in certain genetically induced hemolytic anemias. Rabbits receiving a single injection of lead acetate showed an initial increase in free protoporphyrin and ZPP. Elevation of free protoporphyrin also occurs with increased erythropoiesis in response to hypoxic conditions. The hematocrit in mice maintained under hypoxic conditions increased to 75 without any change in ZPP (T.L. Walden, and W.R. Farkas, unpublished results). Prototype hematofluorometers for ZPP detection, described earlier in the literature, had wider fluorescence detection windows and picked up free protoporphyrin as well as ZPP. The hematofluorometer used in these studies was specifically dedicated to the quantitation of ZPP. The elevation of ZPP was confirmed by an independent assay using HPLC.

Measurements of the serum iron, total iron binding capacity, and the percent iron saturation following irradiation indicated that iron deficiency was not the explanation of radiation-induced ZPP elevation. ZPP becomes elevated during lead poisoning due to inhibition of ferrochelatase; however, radiation did not diminish ferrochelatase. The ZPP elevation resulting from the combined treatments of irradiation and lead poisoning was additive. A dose of 900 rad given to rats has been reported to cause an uptake of zinc by spleen mitochondria. That observation may be significant, as ferrochelatase is a mitochondrial enzyme. The concentration of zinc may be important in the formation of ZPP, because duck erythrocytes contain only one-third as much zinc as do human erythrocytes, but lead-poisoned ducks accumulate free protoporphyrin rather than ZPP. Our attempts to increase ZPP levels in normal mice by daily injections of zinc and/or by placing zinc in the drinking water did not succeed (data not shown).

The biologic significance of elevated levels of ZPP are, for the most part, unknown. It is known that the ZPP complex is unable to transport oxygen because it is diamagnetic. During the period of rapid recovery of erythropoiesis from radiation damage, the formation of ZPP may be preferable to the accumulation of free protoporphyrin. Hemin and ZPP are able to stimulate and maintain protein synthesis in both intact reticuloocytes and cell-free hemolysates. Hemin and other metalloporphyrins (ZPP has not been tested) can bind to the hemin-controlled translational repressor (HCR), inhibiting it and preventing the phosphorylation and inactivation of elongation factor 2. Protoporphyrin can also bind to and inactivate the HCR, but, unlike hemin and ZPP, it cannot simulate or maintain protein synthesis.

ZPP affects the activity of several enzymes, including heme oxygenase and biliverdin reductase. Injection of ZPP at a dose of 50 μmole/kg body weight caused a 50% decrease in heme oxygenase activity 16 hr after injection in rats. Heme oxygenase catalyzes the breakdown of heme to bilirubin. ZPP is not degraded by the enzyme and acts as a competitive inhibitor. ZPP also affects other enzyme systems, as it caused an 18% increase in oxygen consumption in a rat liver homogenate, whereas protoporphyrin had no effect. Hemin, as an inhibitor, and protoporphyrin, as an activator, have been proposed as a regulatory system for guanylate cyclase. Possibly, ZPP has some as yet undiscovered role in regulating the hematopoietic system during recovery from chemical, radiologic, or nutritional insults. These studies indicate that damage to the hematopoietic system and subsequent recovery are responsible for ZPP elevation. If true, then bone marrow suppressive drugs should elicit a similar effect. Studies showing elevation of ZPP by hematotoxic drugs are now in progress.

The magnitude of the elevation of ZPP due to lead poisoning is very species-dependent. In humans, ZPP elevation during lead poisoning is much more extensive...
than in mice and rats. If this is also true of the ZPP elevation due to radiation, it may be a useful indicator of the status of the hematopoietic system in patients being treated with radiation and hematopoietic drugs. Finally, it is unlikely that a blood sample with an elevated ZPP discovered during a screen for lead poisoning would have come from a subject who had received high doses of radiation. Nevertheless, it should be kept in mind that factors other than lead poisoning and iron deficiency can cause elevated ZPP.

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The elevation of blood levels of zinc protoporphyrin in mice following whole body irradiation

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