Variations in the Relationships Between Total $^{59}$Fe Uptake and the Uptakes Into Heme and Nonheme Fractions of Spleen and Bone Marrow in Irradiated Mice and Mouse Radiation Chimeras

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A chemical method for the separation of heme and nonheme iron-containing fractions in mouse tissues has been used to study the uptake of $^{59}$Fe into both the heme and nonheme iron fractions in spleen and femoral bone marrow, in order to investigate the possible relationships between the total uptake into spleen tissue and the rate of uptake into the heme fractions. In control mice, only about one third of the $^{59}$Fe is directly associated with heme during the first day after administration, and this fraction decreases as radioactive red cells are released from the spleen into blood. Ten days after X-irradiation of mice, the proportion of $^{59}$Fe in the spleen heme fraction 6 hr after intraperitoneal administration decreased from about 30 to 10% of the total splenic activity as the radiation dose was increased from 500 to 750 rads. A similar alteration in the proportion of $^{59}$Fe in the spleen haem fraction occurred in mouse radiation chimeras as the dose of injected syngeneic bone marrow cells was reduced from $5 \times 10^5$ to zero. Similar results were found in femoral bone marrow. These results indicate that in this system the rate of uptake into whole tissue is not a measure of the uptake into heme; it is necessary, therefore, to use the method involving the measurement of iron uptake into heme, rather than the total tissue uptake, when estimating hemoglobin synthesis or erythropoiesis.

The rate of uptake of $^{59}$Fe from plasma into erythrocytes and erythropoietic tissues has been used by many investigators as a measure of hemoglobin synthesis and also as an indicator of erythropoietic activity.1-3 The uptake of $^{59}$Fe into erythrocytes in irradiated mice, radiation chimeras, and irradiated polycythemic mice that have been treated with erythropoetin is commonly used as a measure of erythropoiesis;3,5 some investigators have also used the uptake of $^{59}$Fe from plasma into mouse spleen as a measure of splenic erythropoiesis and of cell proliferation in spleen colonies after irradiation.6-8

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The use of $^{59}$Fe uptake in studies of erythropoiesis depends on several assumptions, including the assumption that the $^{59}$Fe is taken up mainly into hemoglobin in the system under investigation and not into other molecules. Although this is probably a valid assumption when uptake into erythrocytes is considered, it is known that in mouse spleen there may be a significant uptake into iron stores as well as into hemoglobin, since in polycythemic mice in which little erythropoiesis is occurring, there is still a significant amount of $^{59}$Fe uptake into the spleen.$^9$ However, $^{59}$Fe uptake into erythrocytes gives only a measure of total-body erythropoiesis, and so the measurement of erythropoiesis in specific tissues such as femoral bone marrow or spleen may require the estimation of uptake into heme, rather than the uptake into the whole tissue, which has previously been used.$^6$–$^8$

These studies were therefore undertaken using a chemical method for the separation of heme from nonheme iron to investigate the $^{59}$Fe uptake into spleen and femoral bone marrow, in order to determine whether total uptake gives a reasonable estimate of the uptake into hemoglobin, or whether a significant and variable proportion of the $^{59}$Fe is taken into the iron stores of these tissues. If the latter case occurs, then the measurement of $^{59}$Fe uptake into the chemically separated heme fraction of the tissue should give a much more reliable estimate of erythropoiesis in that tissue.

**Materials and Methods**

The animals were female, albino, specific-pathogen-free CSI mice, weighing 20–25 g, and obtained from Scientific Products Farm, Ash, Kent, England. They were housed four per cage and allowed water (containing 0.5 g/liter sulfadimidine-sodium biphosphate and 8 drops/liter of saturated saccharin solution ad libitum.)

Irradiation was carried out using a G.E. Maximar X-ray unit, operated at 250 kV peak and 12.5 mA, with added filtration of 0.5 mm Cu and 1.0 mm Al. Dose rates were estimated at the center of a wax mouse phantom, using a Baldwin-Farmer secondary-standard dosimeter, and were about 200 rads/min. Mice were exposed singly in a perspex box (3 in. diameter x 1 in. deep). The results are expressed as the mean values obtained from groups of 8 to 15 mice.

The mice were killed 10 days after irradiation, and the spleen placed in Bouin's solution$^6$ to render the colonies visible, or separated into various iron-containing fractions. In certain cases the femora were also removed and the bone marrow cells aspirated; the number of nucleated cells in the left femur was then estimated, and the various iron-containing fractions of the cells from the right femur were investigated.

Control mice were killed at specified intervals after the injection of $^{59}$Fe. The $^{59}$Fe activity was estimated using a Nuclear Enterprises Gammatic well-type scintillation counter.

$^{59}$Fe Administration

$^{59}$Fe in ferric citrate solution was obtained from the Radiochemical Centre, Amersham, England, and diluted in sterile isotonic saline. The mice each received 1 $\mu$Ci of $^{59}$Fe in 0.25 ml solution containing between 0.04 and 0.09 $\mu$g iron; since these mice had serum iron levels of 2 to 8 $\mu$g/ml,$^{10}$ this injection contains less than 1% of the plasma-iron pool and should not alter the iron pool significantly. Iron was injected intraperitoneally, always on the 10th day after irradiation, and at 9 a.m. to minimize any effects of diurnal variations of iron pools and hemoglobin synthesis.$^{11}$ The mice were killed 6 hr later, and the uptake of $^{59}$Fe into various pools (determined as the percentage of the amount injected) was measured.
Administration of Syngeneic Bone Marrow Cells

The recipients received 750 rads total-body radiation and received bone marrow cells within 4 hr. Donor mice were killed by exposure to ether vapor. Their femora were removed and the epiphyses cut off with a scalpel blade. The marrow was aspirated from the medullary cavity using 1 ml of Eagle’s medium in a syringe with a No. 20 needle, and then gently taken up into, and expelled from, the syringe several times to produce a single cell suspension. Known numbers of cells (0, 5 x 10^4, 9 x 10^4, 2 x 10^5, 4 x 10^5) were injected into a lateral tail vein of the lethally irradiated recipient.

Separation of Heme and Nonheme Iron-containing Fractions

This method was developed from the technique of Cole and Paul, and is described in detail in a previous publication. The tissues were homogenized in a Waring blender for 5 min at top speed while suspended in Drabkin’s solution. The homogenate was taken to pH 2 with 2N HCl to separate heme from the hemoglobin molecules, and an equal volume of ethyl methyl ketone added. Heme is extremely soluble in this solvent, and therefore appears in the organic phase, whereas nonheme iron is localized in the aqueous phase.

RESULTS

Unirradiated Spleen

The relative amounts of ^59^Fe activity in the heme and nonheme fractions of the spleen of unirradiated mice at various times after injection are shown in Fig. 1, with the activity expressed as the percentage of the amount administered. Total splenic activity decreased gradually from 6 hr to 30 hr after injection; it then decreased more rapidly. Both the heme and nonheme levels of ^59^Fe decreased with time (Fig. 1A), and the change of the heme activity is presumably caused by the movement of radioactive red cells from the

Fig. 1.—The uptake of ^59^Fe into the spleen of nonirradiated mice during the 3 days following intraperitoneal injection of the ^59^Fe. (A) The distribution of activity between heme and nonheme spleen fractions, with the activity expressed as the percentage of the amount injected: open circles and solid lines, total; open circles and broken lines, nonheme; crosses and solid lines, heme. (B) Percentage of spleen ^59^Fe in the heme fraction. Means and standard errors of the means are shown.
spleen into blood, since a parallel increase occurred in the amount of activity in the blood. The relative rate of decrease in activity with time was more rapid in the heme than in the nonheme layer, since the ratio of heme 59Fe to total spleen 59Fe remained constant, at about 30–35% from 6 to 24 hr, and then decreased to 15% by 72 hr after injection (Fig. 1B). In this time period after injection, the total activity in liver decreases by about 20–30%, presumably due to release of storage iron into blood followed by its reutilization.

Fig. 2.—The uptake of 59Fe into the spleen of irradiated mice 6 hr after the intraperitoneal injection of 59Fe, and 10 days after total-body X-irradiation with 500–750 rads. (A) The distribution of activity between heme and nonheme spleen fractions, with the activity expressed as the percentage of the amount injected: open circles and solid lines, total; open circles and broken lines, nonheme; crosses and solid lines, heme. (B) Number of spleen colonies occurring after irradiation. (C) Percentage of spleen 59Fe in the heme fraction. Means and standard errors of the means are shown.
TOTAL $^{59}$Fe UPTAKE

in other tissues. Similar results, with only 30 to 35% of the $^{59}$Fe associated with heme, were found in femoral bone marrow from these mice 6 hr after intraperitoneal injection of the iron.

A separation of the spleen tissue into fractions containing haemosiderin-, ferritin-, heme-, and transferrin-bound iron showed that by 6 hr after injection, about 80% of the nonheme $^{59}$Fe is localized in ferritin stores.

**Endogenous System**

In mice that had been irradiated, but had not had foreign bone marrow cells administered, the total amount of $^{59}$Fe activity in the spleen, and the amounts in the heme and nonheme splenic fractions, showed no large variations 10 days after irradiation with doses of 250-400 rads and 6 hr after injection of radioiron; with doses higher than 500 rads, the activity in the spleen and in the heme and nonheme fractions decreased progressively, with the total uptake decreasing from 5% (of the amount administered) after 500 rads to 1.6% after 750 rads (Fig. 2A). The amount of $^{59}$Fe in the heme fractions, decreased from 40 to 10% as the dose was increased from 500 to 750 rads (Fig. 2C). It is of interest that this represents a decrease by a factor of 3 in the $^{59}$Fe uptake per unit weight of spleen in the heme fraction, and a slight increase in the nonheme fraction, as the dose was increased from 550 to 750 rads. Parallel experiments involving fixed spleens after 500, 550, 600, 700, and 750 rads showed that in the strain of mice used, the number of spleen colonies decreased progressively from about 20 per spleen after 500 rads to 0-2 per spleen after 750 rads (Fig. 2B), which is similar to the findings of other workers using the endogenous system.

**Exogenous System**

In mice that have been given 750 rads, followed by the injection of foreign bone marrow cells (Fig. 3A), the amount of $^{59}$Fe in the spleen fractions and total spleen increased, apparently non-linearly. If spleens are fixed in Bouin’s solution to make any spleen colonies visible, then the chemical separation procedure will not extract heme, and it is therefore not possible to correlate $^{59}$Fe uptake directly with the number of colonies in a spleen. However, each experimental group was divided and half of the spleens were fixed in Bouin’s solution and the colony number estimated (Fig. 3B), while the other half were separated into different iron-containing fractions. The relationship between $^{59}$Fe uptake and colony number after identical treatments is shown in Fig. 3C; after the administration of 4 x $10^5$ cells, the colonies show confluence. Total splenic activity increased approximately linearly with colony numbers, as found by other workers; the amount of activity in the heme fraction also increased with colony number, but with a power greater than 1 (i.e. the curve is concave upward). This is reflected in Fig. 3D, which shows the proportion of splenic $^{59}$Fe activity in the heme fraction as a function of the number of cells injected. The percentage of activity in heme fraction increased from 11% to 35% with increasing cell dose (and thus with colony number).

In these experiments, the femora were also taken from each mouse, the
number of nucleated cells removed from the cavity of one femur was estimated, and the ratio of heme $^{59}\text{Fe}$ to total $^{59}\text{Fe}$ in the bone marrow cells from the other femur was measured. The number of nucleated cells in the femur was found to increase linearly with the number of nucleated syngenic

Fig. 3.—The uptake of $^{59}\text{Fe}$ into the spleen of mouse radiation chimeras 6 hr after the intraperitoneal injection of $^{59}\text{Fe}$, and 10 days after total-body X-irradiation with 750 rads followed by the intravenous injection of 0 to $4 \times 10^6$ syngeneic bone marrow cells. (A) The distribution of activity between heme and nonheme spleen fractions after different numbers of injected bone marrow cells, with the activity expressed as the percentage of the amount injected: open circles and solid lines, total; open circles and broken lines, nonheme; crosses and solid lines, heme. (B) Spleen colony numbers after bone marrow injection. (C) Distribution of activity between heme and nonheme spleen fractions, at different mean spleen colony numbers: open circles and solid lines, total; open circles and broken lines, nonheme; crosses and solid lines, heme. (D) Percentage of spleen $^{59}\text{Fe}$ activity in the heme fraction.
cells previously injected (Fig. 4A). The percentage of the femoral $^{59}$Fe activity in the heme fraction was found to be much lower than in control animals after low cell doses (Fig. 4B), but after higher cell doses ($2 \times 10^5$ and $4 \times 10^5$), the percentage of activity in femur increased to about 35%, which is of the same order of magnitude as that occurring in spleen after these cell doses.

**Discussion**

The importance of distinguishing between heme and non-heme iron uptake into erythropoietic tissues is clearly demonstrated by the variations in the ratio of heme to nonheme $^{59}$Fe in the spleen of nonirradiated mice at different times after injection, and by the fact that the maximum proportion of heme $^{59}$Fe in the spleen and femoral bone marrow of these CS1 mice is about 30 to 35%.

It is of interest that in each of the experimental situations where relatively high levels of erythropoiesis were occurring (that is, unirradiated mice, mice receiving 500–550 rads, and irradiated mice that had received $2 \times 10^5$ or $4 \times 10^5$ marrow cells), the ratio of heme to total $^{59}$Fe is approximately 1 to 3 in both spleen and femur in each case. It is therefore possible that this is the
normal optimal value of the heme to total $^{59}$Fe level that can be achieved in erythropoietic tissue of CS1 mice, but further work is necessary to define the importance of nonheme iron in erythropoietic cells.

The gradual loss of radioiron from the spleen of the nonirradiated mice can be partially attributed to the movement of labeled red cells into the blood. However, even 2 or 3 days after injection there is a small amount of $^{59}$Fe in plasma, which has probably been released from ferritin stores, and this is available for uptake into the spleen. Some of the nonheme iron in spleen is also probably available for direct uptake into the heme fraction. Thus the heme $^{59}$Fe levels depend on (1) the initial uptake of $^{59}$Fe from blood, (2) the loss of labeled red cells, (3) the later gradual uptake of $^{59}$Fe from blood, (4) the direct movement of iron from nonheme fractions, (5) the number of circulating red cells in the blood in spleen. Similarly, the nonheme iron levels in spleen will depend mainly on (1) the initial and later uptake of $^{59}$Fe from plasma into the ferritin stores (which constitute the main fraction of nonheme iron in spleen), but also on (2) the plasma transferrin $^{59}$Fe level (which is probably relatively insignificant by 6 hr after injection) and (3) the release of iron from iron stores directly into the heme fraction or blood plasma. Consequently, the precise significance of changes in the $^{59}$Fe levels in total spleen and the spleen fractions cannot yet be defined.

The changes in the total uptake of $^{59}$Fe into the spleen of mice irradiated with different doses of X rays, with a decrease in uptake as the dose is increased from 500 to 750 rads, are similar to those found in other strains of mice. The reduction in the amount of $^{59}$Fe uptake into the heme fraction, as the dose increases from 500 to 750 rads, may be associated with the decrease in the number of spleen colonies since their cells are predominantly erythroid. The experiments in which spleens were fixed in Bouin’s to render colonies visible showed that after 500 rads there were on the average about 20 colonies and after 750 rads usually 0 or 1 colony per spleen. However, the observations that both heme and nonheme $^{59}$Fe levels decrease with increasing radiation dose and that the proportion of activity associated with heme decreases from 35% to 12% as the radiation dose is increased from 500 to 750 rads indicate that the total $^{59}$Fe uptake into the spleen of irradiated CS1 mice may not be a good indication of splenic erythropoiesis.

The endogenous system, in which animals received varying doses of radiation, is complicated by the fact that the spleen parameters, such as colony number and $^{59}$Fe uptake, depend on many other parts of the biological system, which will each respond to the different radiation doses. In addition, the amount of noncolony tissue, which will presumably contain some of the nonheme iron, will vary with radiation dose. The exogenous system, in which all recipients receive the same radiation dose, is therefore simpler in many respects; the radiation damage remains constant in each system, and the response is altered by the administration of a specific number of cells. It therefore seemed possible that in this situation the total iron uptake into spleen might give reasonable estimates of relative rates of erythropoiesis, especially since the total uptake had been shown to be proportional to the number of spleen colonies.
The results obtained using the exogenous system were similar to those found by other workers, with the number of spleen colonies increasing with the number of injected nucleated marrow cells, and the total $^{59}$Fe uptake being proportional to the number of colonies. However, the fact that the amount of heme $^{59}$Fe increases nonlinearly as the colony number increases (with the fractional amount of the total activity that is associated with heme increasing from about 12% at low colony numbers to 35% at high colony numbers) cannot be explained by postulating a fairly constant uptake into nonheme iron stores in the spleen, since the level of nonheme $^{59}$Fe also increases with colony number.

Thus the results obtained using unirradiated mice, and irradiated mice with and without administered bone marrow cells, have all indicated that (1) the majority of the radioactive iron is in nonheme fractions and (2) neither the nonheme activity, nor the ratio of heme to nonheme activities, remains constant.

The actual significance of spleen heme and nonheme iron levels depends to some extent on the localization of these iron-containing fractions within the spleen. In some other mouse strains, many of the colonies are mainly erythropoietic in nature, although nonerythropoietic colonies may occur. The correlation observed in these experiments, and by other workers, between colony number and total spleen $^{59}$Fe uptake indicates that a large proportion of the radioactive iron may be taken into colonies; both the dissection of some colony tissue from spleen and autoradiographic studies of spleen indicated that iron tends to be concentrated in these colonies relative to the rest of the spleen. These observations do not exclude the possibilities that certain noncolony areas may concentrate iron or that some colonies may not localize the iron. Nevertheless, if a large proportion of the iron is localized in colonies, then it seems that the erythropoietic colonies take up significant quantities of iron into nonheme sites, since 65% to 85% of the total activity is in their nonheme fractions. The localization of the majority of splenic $^{59}$Fe in colonies thus confirms the experiments of Siracusa, Silini, and Briganti, and of other workers, who have demonstrated that the total splenic $^{59}$Fe uptake can be used to study cell proliferation in spleen colonies of mouse radiation chimeras; however, the presence of a significant proportion of nonheme iron in colonies implies that care must be taken when interpreting the results in terms of splenic erythropoiesis. The presence of nonheme iron in colonies, together with the large and variable uptake of $^{59}$Fe into the nonheme fraction of spleen and bone marrow, indicates that the method involving the subtraction of a constant factor from the iron uptake to allow for the uptake into nonerythropoietic tissue may require modification to render it valid.

The uptake of $^{59}$Fe into red blood cells does, of course, eliminate these difficulties involving nonheme iron, but this system necessarily measures erythropoiesis 2 or 3 days prior to death (instead of several hours) and measures erythropoiesis in the total body rather than in a specific tissue.

Consequently, since the results indicate that total iron uptake into erythropoietic tissue is not a reliable guide to the uptake into heme in that
tissue (and thus cannot be used as an accurate measure of erythropoiesis), the use of the chemical separation technique to measure iron uptake into the heme fraction of tissue should enable more reliable estimates of hemoglobin synthesis and erythropoiesis in specific tissues to be made.

References

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