Effects of Radiation on Radioiron Uptake in Mouse

Erythrocytes in vitro

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ABSTRACT

The in vitro iron uptake by erythrocytes was examined as an indicator of radiation effects on erythropoiesis in mice. Erythrocyte suspensions were incubated with $^{59}$Fe, previously bound to serum for 3 hours at 37°C. The changes of the iron uptake within 48 hours following X-irradiation of 25 R and 50 R showed biphasic patterns. The reduction of the uptake at 18–20 hours after irradiation correlated exponentially with the exposure doses from 25 R to 200 R.

In animals irradiated with 200 R, the recovery from the radiation-induced depression of the iron uptake by the in vitro method was delayed by two days from that estimated by the in vivo iron uptake studies. Since the in vitro uptake is mostly due to young reticulocytes present in the erythrocyte suspension, the delay of two days in recovery is likely to be attributed to the time required for erythroblasts in bone marrow and spleen to mature and be released to blood as reticulocytes. The in vitro iron uptake reflects the reticulocyte counts, and can be a simple and reliable indicator for radiation-induced disturbances of erythropoiesis.

INTRODUCTION

In the course of the experiments on the recovery of erythropoiesis in mice exposed to sublethal radiation, we found that there was an abortive rise of radioiron uptake in erythropoietic tissues on the 4th to 5th day after irradiation. To clarify whether this rise in erythropoietic tissues is attributed to the increase of erythrocyte precursors which mature and eventually appear in circulation, it would be desirable to observe the change in the number of immature erythrocytes newly released into circulation. In the clinical examination, the percentage of reticulocytes is usually used to assess the rate of released immature erythrocyte. However, since

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the examination carried out at the time when reticulocytes counts are decreased, it is very difficult to obtain an accurate count as well as age distribution of reticulocytes by the ordinary smear method.

It is well known that iron bound to serum protein (transferrin) is transferred to immature erythrocytes (reticulocytes) in vitro.\(^2\) Klein and Cavaliere reported that the decrease in in vitro uptake of iron by erythrocytes after irradiation of ducks was attributed to the decrease in the number of reticulocytes\(^3\). Belcher and Courtenay also observed that the changes in the rate of iron uptake by circulating reticulocytes of rat after irradiation was correlated with changes in their number and age distribution\(^4\).

The present study was undertaken to determine whether the radioiron uptake of erythrocytes in vitro could be used for one of the indicators of radiation effects on erythropoiesis in mice.

MATERIALS AND METHODS

Animals. Adult male CF\(^\#\)/Nrs and C57BL/6J/Nrs mice, furnished by Technical Service Division at the Institute, were used throughout this study. Age of mice ranged from 70 to 90 days old. All mice were housed individually in plastic cages in prior to and the entire period of the experiment. Pellet diet and water were given ad libitum.

Radiation. The mice received whole-body X-irradiation at a dose rate of 92 R/min in air from an X-ray machine operated at 200 KVP and 20 mA; the HVL was 1.2 mm Cu. Every exposure was monitored by a Radocon Model 601 dosimeter.

Radioiron uptake in vitro. Radioiron in the form of \(^{59}\)FeCl\(_3\) (specific activity was approximately 70 μCi/μg) was diluted in 1 per cent sodium citrate and the pH was adjusted to 6.2 by adding a few drops of 0.1 N HCl. Several lots of serum to be bound to \(^{59}\)Fe were previously collected from unirradiated healthy mice of both strains and kept in deep freeze until the day of the experiment. The serum was incubated at room temperature for one hour with the \(^{59}\)Fe solution at its activity of 2.5 μCi/ml. For electrophoretic analysis, an aliquot of each \(^{59}\)Fe bound serum was applied to a cellulose acetate membrane strip and run for one hour at 250 volt using Veronal buffer (pH: 8.6, μ: 0.01). After drying, the strip was cut into 5 mm wide segments. Radioactivity of each segment was measured. In all the samples of serum, the radioactivity was found only at the region of transferrin. For the preparation of erythrocyte suspension, heparinized blood was collected from each mouse by cardiac puncture and washed with 10 volumes of balanced salt solution by centrifugation. Then about 30 per cent suspension of packed cells were prepared by adding balanced salt solution. The number of erythrocytes in the suspension was determined by the ordinary method with a hemocytometer. One ml. of the erythrocyte suspension was transferred into duplicate test tubes and 0.2 ml. of \(^{59}\)Fe bound serum was added. The mixture of the erythrocytes and \(^{59}\)Fe was incubated at 37°C for 3 hours with gentle agitation. The strain of mice from which the serum was
obtained was identical to that from which the erythrocytes were obtained in each experiment. After the incubation, the tubes were kept in an ice bath and the radioactivity of the mixture in each tube was counted in a well-type scintillation counter. Then, the erythrocytes were repeatedly washed with cold balanced salt solution until the activity of the washed erythrocyte reached a constant value. Three washings were usually sufficient. Finally the packed and washed erythrocytes were hemolysed by exposure to an appropriate volume of 0.1 N HCl and brought to the initial volume with distilled water. Then the radioactivity remaining in the hemolysate was counted.

In a base-line experiment, the erythrocyte suspension from a pooled samples was incubated with 0.2 ml of 59Fe bound serum at various cell concentrations. The rate of iron uptake by erythrocytes was constant at the concentrations from $0.5 \times 10^9$ to $3.2 \times 10^9$ (Fig. 1), so that the iron uptake could be calculated in terms of the percentage of remaining activity in the hemolysate per 1000 million erythrocytes.

Radiorion uptake in vivo. Groups of irradiated mice, consisted of at least 8 mice per group, were killed at daily intervals. Five hour before sacrifice, the mice were injected intravenously with 0.3 $\mu$Ci of 59Fe. After they were sacrificed by chloroform anesthesia, spleen and hind limbs were cleaned of adhesive tissues and placed in test tubes and the radioactivity was counted. The iron uptake by whole body bone marrow of each mouse was calculated by using the ratio of hind limb bone marrow to whole body bone marrow. This ratio was previously estimated from the radioactivity in bones by following procedures. Five hours after 59Fe injection, mice were exsanguinated completely by perfusion with saline injecting into the left ventricle of heart. After removal of skin and viscera, the carcasses were boiled in water for 15 min. Boiled carcasses were then incubated in 1 per cent papain in saline overnight at 37°C. The undigested tissues were removed and the bones were washed completely with water. The radioactivities of both whole bones and hind limb bones were separately counted. The loss of activity in hind limb bones during these procedures was negligible. Thus the ratio of hind limb bone marrow to whole body bone marrow was calculated by dividing the count of hind
limb bones by that of whole bones. The mean value of this ratio for CF#1 mice was 0.19. The iron uptake by total erythropoietic tissues was obtained by adding the value of spleen to that of whole body bone marrow.

Reticulocyte counts. A few drops of blood sample from heart were taken into a test tube containing 1 ml. of 0.4% brilliant creosyl blue in saline with sodium citrate. The blood and the stain were mixed well in the test tube and the mixture was allowed to stand for 10 min. Then the mixture was centrifuged for 10 min and the excess of the stain was decanted. The blood was then mixed again and used to prepare films on glass slides. The percentage of reticulocytes in 3000-4000 cells examined under an oil-immersion objective was determined.

RESULTS

The time-course of the iron uptake by erythrocytes within the first 48 hours after irradiation of animals were determined on C57BL mice. The erythrocyte suspensions were prepared from the pooled blood samples of 5 mice which were exposed to either of 25 R, 50 R, 100 R or 200 R or radiation at the given time before being sacrificed. An initial increase in the iron uptake appeared at 6 hours after irradiation of 25 R. At all other doses, the iron uptake remained at control level until 6 hours, and then decreased gradually. The first nadir was found at 18 hours. At doses of 25 R and 50 R, there was a rise reaching a maximum at 36 hours. At doses of 100 R and 200 R, no significant rise was found during the observation period of 48 hours. It seems, however, that the early changing patterns after irradiation were principally biphasic (Fig. 2).

![Graph showing iron uptake over time](https://academic.oup.com/jrr/article-abstract/14/1/9/919596/Effects-of-Radiation-on-Radioiron-Uptake-in-Mouse)

**Fig. 2.** Changes in the iron uptake *in vitro* by erythrocytes within 48 hours after the graded doses of X-irradiation. Each circle indicates the mean of duplicate determinations on pooled sample from five C57BL mice (compared to the determinations on unirradiated control mice). Shaded area indicates the variations among 10 determinations on unirradiated control mice.
The effects of radiation dose on the iron uptake were then determined on both CF1 and C57 BL mice at the time when the first nadir was found, 18-20 hours after irradiation. The results of five experiments were shown in Fig. 3. The results were expressed as the percentage of the iron uptake in the unirradiated control simultaneously determined in each experiment. There is a wide variation in the uptake observed in different samples of same dose group. This variation may be due in part to the variation in 59Fe bound serum between experiments. Despite the wide variation, the iron uptake decreased with the increase of radiation dose. Subsequently, the iron uptakes were studied by using the single lot of 59Fe bound serum in C57 BL mice. The results were shown in Fig. 4. The dose relation of the reduction in the iron uptake appeared to be exponential.

In order to detect the abortive rise as found during the recovery of the iron uptake by erythropoietic tissues, the change in the iron uptake by erythrocytes in vitro was followed daily up to 9 days after irradiation of animals with 200 R. In this experiment, CF1 mice were used, because the abortive rise during the recovery of erythropoietic tissues was prominent in CF1 mice. The result of change in the iron uptake by erythrocytes was shown in Fig. 5. The changing pattern was quite similar to that seen in the iron uptake by erythropoietic tissues at 5 hours after administration of 59Fe, but the magnitude of the rise in the iron uptake by erythrocytes was greater than that found in erythropoietic tissues and the time to reach a maximum was delayed about 2 days.

The changes in the iron uptake by erythrocytes after irradiation of CF1 mice

![Graph](https://example.com/graph.png)

**Fig. 3.** Radiation dose dependency on the iron uptake in vitro by erythrocytes at 18-20 hours after irradiation of animals. Each circle indicates the mean of duplicate determinations on pooled sample from 5 mice. Results of 5 different experiments are expressed as the percentage of simultaneous determinations on unirradiated control mice in each experiment.
Fig. 4. Relationship between radiation dose and the iron uptake by erythrocytes at 18 hours after irradiation of animals. Each circle indicates the mean of duplicate determinations on 5 individual samples from C57 BL mice. Vertical bars represent the standard error of the mean.

Fig. 5. Changes in 5 hours iron uptake \textit{in vivo} by erythropoietic tissues and the iron uptake \textit{in vitro} by erythrocytes on CF\#1 mice irradiated with 200 R. The results are expressed as the percentage of the values obtained on unirradiated control mice. Closed circle; the 5 hours iron uptake \textit{in vivo} by erythropoietic tissues (total bone marrow plus spleen), open circle; the iron uptake \textit{in vitro} by erythrocytes.

with 400 R was compared with those in reticulocyte counts by the ordinary smear method. The result were shown in Fig. 6. The iron uptake decreased steeply by 3 days and then gradually increased. At 7 days there was an overshooting. The decrease in reticulocyte counts by 3 days after irradiation was less steeper than in the iron uptake. The rise at 7 days was found but did not exceed the control level. The pattern of changes in the iron uptake was similar to that seen in the case of 200 R irradiation (Fig. 5). However, the time when the abortive rise reached the maximum delayed about one day. This time-delay seems to correspond with the difference of the time of abortive rise in the iron uptake by erythropoietic tissues between 200 R and 400 R irradiation\textsuperscript{1).}
DISCUSSION

Since Walsh, et al. have reported that intact immature red cells (human reticulocytes) are capable of taking up iron bound to plasma or serum \textit{in vitro} \cite{5}, it is generally accepted that the radioiron from iron binding serum protein (transferrin) is taken up only by reticulocytes but not by mature erythrocytes. In contrast, radioiron from $^{59}$FeCl$_3$ in saline is taken up or adsorbed by mature erythrocytes as well as immature erythrocytes \cite{5}.

In this study, erythrocytes were incubated with $^{59}$Fe previously bound to serum protein. Thus, non-specific adsorption onto mature erythrocytes could be eliminated. Under these condition, it has been reported that the entire process from the initial binding of iron to cell surfaces until the incorporation of iron into hemoglobin required only 6 to 9 minutes at 37°C \cite{2}, and that 70 per cent of the iron taken up by
reticulocytes was found in heme\textsuperscript{4,9}. Our preliminary results indicated that the heme extracted with acid methylethylketone by the method of Teale\textsuperscript{6} contained about 70 per cent of total activity remaining the hemolysate. This implies that the iron uptake by erythrocytes in the present study is most likely to be by reticulocytes in erythrocyte suspension. However, the circulating reticulocytes vary in age from those just released into circulation to those about to become mature erythrocytes. In rat, young reticulocytes appearing in blood during induced erythropoiesis appear to take up more iron than older reticulocytes\textsuperscript{4,9}, and in the normal duck the younger reticulated cells apparently take up more iron than older forms\textsuperscript{9}. Therefore, the amount of iron uptake of reticulocytes in a given erythrocyte population depends not only on their number, but on their age distribution. When the release of cells by erythropoietic tissues is suppressed, the proportion of aged reticulocytes in the circulating blood increases progressively. As the result, the iron uptake in vitro decreases. Conversely, when the rate of release of cells by erythropoietic tissues increases, the proportion of young reticulocytes in the blood increases, and this may lead to an increased iron uptake. This view is supported by the finding that the iron uptake by erythrocytes after irradiation of mice decreased more rapidly by 3 days than the reticulocyte counts, and that the abortive rise in the reticulocyte counts did not exceed control level, whereas that in the iron uptake overshooted beyond control level (Fig. 6). Thus, it is conceivable that the observed changes in the iron uptake by erythrocytes after irradiation of animals reflects the changes in the rate of release of cells from erythropoietic tissues into the blood of irradiated animals.

In the present study, it is noteworthy that the biphasic changes were found within 48 hours after irradiation with doses as low as 25 R and 50 R. Odartchenko, et al. reported that the mitotic delay of about 2 hours was induced in most mature erythroblasts in dog by irradiation with 50 R\textsuperscript{9}. The first decrease in the iron uptake after low dose irradiation might be responsible for the delay of release of reticulocytes as the result of such a mitotic delay in mature erythroblasts.

The recovery of the in vitro iron uptake by erythrocytes was found to delay about 2 days from the recovery of the in vivo iron uptake by erythropoietic tissues. This delay of 2 days can be attributed to the time taken for the erythroblast to mature and be released as the circulating blood reticulocyte. Hence, it appears that the abortive rise found during the recovery of erythropoietic tissues is due to an increase of erythroblasts, which are capable of taking up iron.

From the results presented, it is concluded that the changes in the iron uptake by mouse erythrocytes in vitro is a manifestation of the changes in the rate of release of reticulocyte into blood and that it provides one of sensitive indicators of erythropoietic depression after irradiation.

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REFERENCES


