DUAL-LABELING OF ERYTHROCYTES: PROPERTIES AND SPLENIC HANDLING OF ^{75}Se- AND ^{59}Fe-LABELED CELLS

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Radioactive chromium (^{51}Cr) has been used as an erythrocyte label to follow splenic sequestration of cells. However, radiochromium does not provide information about the subsequent handling of erythrocyte components by splenic tissue, such as the possible differential release of heme and globin breakdown products. For this reason we have investigated the properties of dual gamma-labeled erythrocytes. We used ^{75}Se-selenomethionine to label red-cell globin and ^{59}Fe to label the iron in heme. The preparation, properties and initial uses of the dual-labeled cells are described in this paper.

MATERIALS AND METHODS

Dual counting of ^{75}Se and ^{59}Fe in the same *in vitro* sample was carried out using two gamma-ray spectrometer readings. At a 1.05–1.15-MeV setting, a Picker Spectroscaler III with a 3-in. NaI(Tl) crystal recorded ^{59}Fe activity but none from ^{75}Se. At a setting of 0.23–0.33 MeV, ^{75}Se was recorded, as well as 51% of the ^{59}Fe counts at the higher spectrometer setting. ^{75}Se counts were corrected for ^{59}Fe content and compared with standards to correct for physical decay.

Initial studies were performed with rabbits. However, in the rabbit the spleen is only 1/5,000 of the body weight and the erythrocytes are relatively fragile. Therefore we performed later studies with rats in which the spleen weighs approximately 1/300 of the body weight. Radioactivity of the whole blood, plasma and saline-washed cells (four washes) was determined.

In rats the time course of erythrocyte labeling was followed after a single intravenous injection of 5 μCi of ^{59}Fe-ferric chloride and 50 μCi of ^{75}Se-selenomethionine. When rats were used to prepare labeled blood for donation to other rats, 50 μCi of ^{75}Se-selenomethionine and 5 μCi of ^{59}Fe were given intravenously twice weekly for 3 weeks. At 4–5 weeks after the initial injection, blood was drawn from the aorta in a heparinized syringe or in a syringe containing acid-citrate-dextrose (2 parts blood to 1 of ACD).

Saline-washed, dual-labeled cells made up to the original volume with saline were denatured by heating at 49°C for 30 min in a water bath (temperatures 50°C or above were not satisfactory). At 5-min intervals the blood was shaken slowly for uniform distribution. Denatured cells were washed six times with saline before being injected into recipient animals. For reproducible results the volume of injection had to be kept at 0.4 cc or below. After the erythrocytes were injected into the tail vein, blood samples were withdrawn from the abdominal aorta and compared with an aliquot of a standard. The blood was separated into plasma and cells, and each portion was recounted separately. Animals were sacrificed periodically, and activity in the spleen was determined by counting at two gamma-ray spectrometer settings.

Preliminary studies revealed that similar results were obtained with the dual-labeled erythrocytes from a single animal or with those from the pooled blood of several animals. Hence, when the precise time course of the splenic discharge of ^{59}Fe and ^{75}Se was followed, the blood of three donor rats (injected at identical times) was pooled before heat denaturation and injection into 51 recipient rats.

In studies of the fate of hemolyzed cell components, hemolysis was achieved by shaking erythrocytes with half their volume of distilled water; when

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stroma-free hemoglobin was desired, centrifugation (30,000 g) was used to bring down the red-cell ghosts.

To compare dual-labeled cells with those tagged with radiochromium, 8 ml of freshly drawn rat blood was incubated with one-half its volume of ACD and 40 μCi of ^{51}Cr-sodium chromate for 30 min. The reaction was stopped with 40 mg of ascorbic acid, and the cells were washed three times with saline. A sample of the ^{51}Cr-labeled erythrocytes was also heat denatured. When the comparison was made with dual-labeled erythrocytes, they were also prepared in the ACD solution with added ascorbic acid followed by saline washing.

Dialysis of cells was carried out with 2 ml samples (in saline, and also in pH 7.4 phosphate buffer) contained in Visking dialysis tubing. The outside volume was 50 ml of saline (or buffer). Incubation periods of up to 24 hr were used at 37°C in a water bath. Small aliquots of the outside solution were periodically removed for counting. At the end of the dialysis period, both the volume and radioactivity of the solution within the bag were determined.

To study the effects of tryptic digestion (*1*), hemoglobin was prepared by freezing and thawing erythrocytes and discarding the sediment after centrifugation. To render hemoglobin susceptible to trypsin attack, it was denatured by heating a 5% solution (in 0.05 *M*, pH 8.0 phosphate buffer) at 90°C for 4 min in a water bath. The sample was quickly cooled and 4 ml mixed with 0.1 ml of 2% trypsin (CalBiochem). The admixture was placed in Visking dialysis tubing and kept in 100 ml of 0.5 *M* sodium phosphate buffer (pH 8.0, 37°C) for 48 hr in an oscillating water bath. Two drops of toluene were added to reduce bacterial growth. At intervals aliquots of the outer solution were removed for counting, and at the end of the dialysis the outer and inner fluids were both counted. As a control, inactivated trypsin was added to the hemoglobin solution (a trypsin solution heated at 100°C for 12 min).

Heme and globin were separated from dual-labeled cells as follows (*2*). Washed red cells from 4 ml blood were lysed with 10 ml distilled water. Two milliliters of toluene were added and vigorously shaken to form an emulsion. After centrifugation for 20 min at 1,500 g, the clear hemoglobin layer was removed and filtered through Whatman No. 1 paper. The hemoglobin solution was added dropwise to 10 volumes of acetone containing 1.2% of concentrated HCl. The supernatant was saved for heme isolation. The precipitated globin was washed twice with the acid-acetone.The globin was redissolved in water and precipitated again with acid-acetone. The precipitate was dissolved in 30 ml water, precipitated with 10 ml

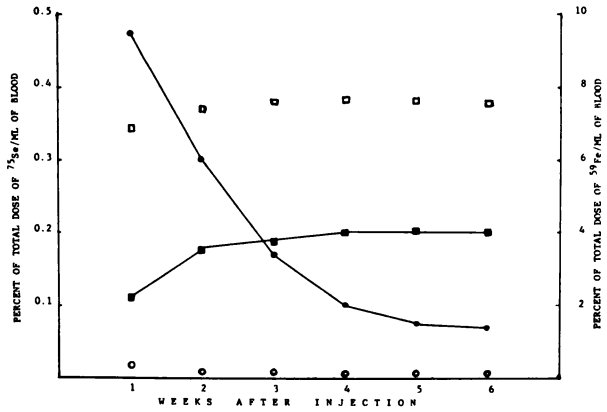

FIG. 1. Erythrocyte and plasma content of ^{75}Se and ^{59}Fe following single intravenous injection in rats. Hollow squares represent erythrocyte iron while plasma iron is shown as hollow circles. Solid squares indicate erythrocyte selenium; plasma selenium is depicted by solid circles.

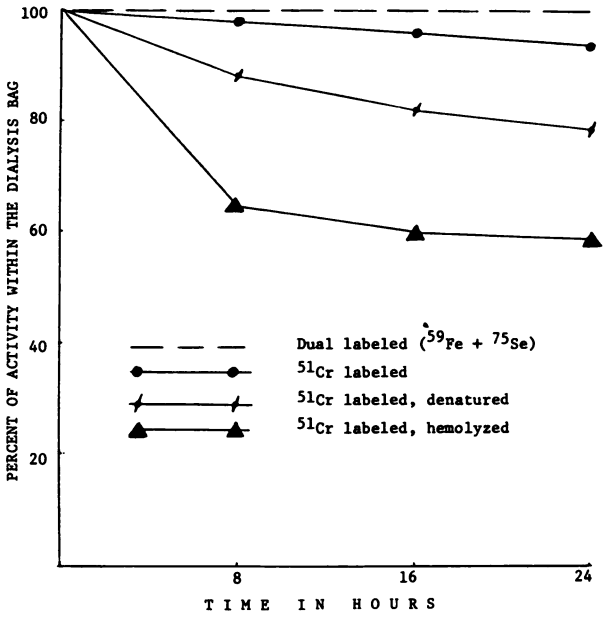

FIG. 2. Comparison of behavior of rat dual-labeled (^{75}Se plus ^{59}Fe) erythrocytes as well as ^{51}Cr-labeled erythrocytes during dialysis.

of 14% trichloracetic acid and washed with 30 ml of 7% trichloracetic acid followed by distilled water. The globin was counted for ^{59}Fe and ^{75}Se. The supernatant that had been saved for heme isolation was filtered, and 1 volume of distilled water was added. The precipitated heme was washed with water and samples checked for both radioisotopes.

To follow the splenic content of intravenously administered iron, 5 μCi of ^{59}Fe-ferric chloride were given to rats, and the spleen was removed at various time intervals for comparison with standards.

RESULTS

In rats following a single intravenous dose of ^{75}Se-selenomethionine and ^{59}Fe-ferric chloride, the eryth-

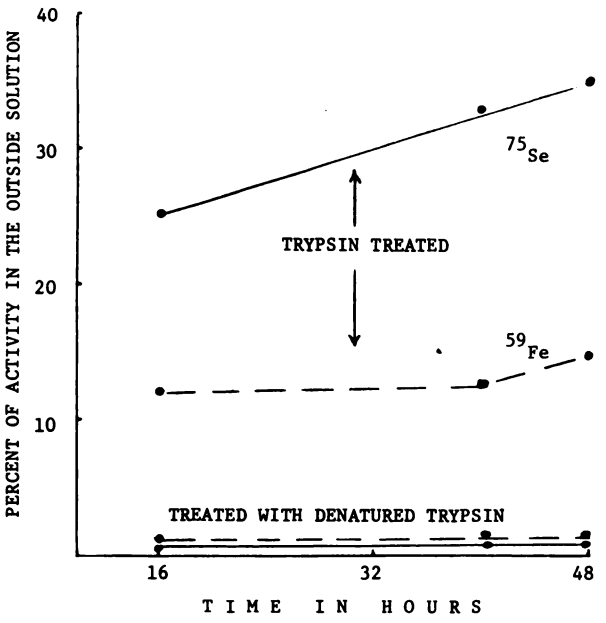

FIG. 3. Release of radioactivity upon dialysis from dual-labeled hemoglobin prepared from rat erythrocytes and studied before and after trypsin treatment.

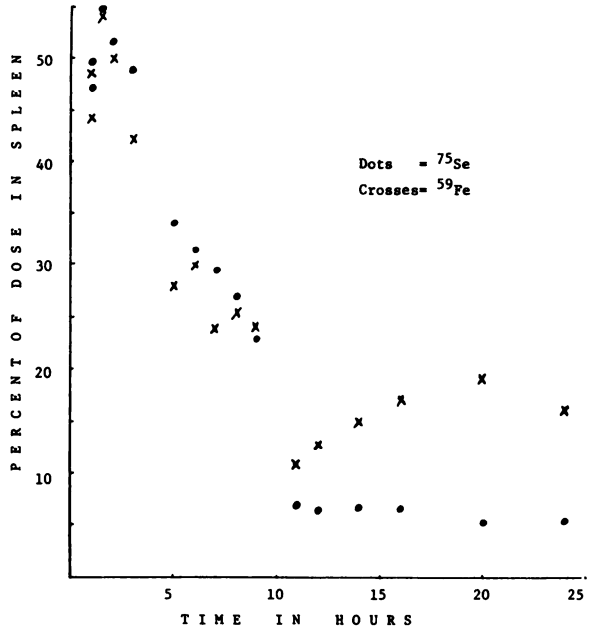

FIG. 5. Splenic content of ^{75}Se and ^{59}Fe in rat given as function of time following injection of dual-labeled heat-denatured erythrocytes. Each point is mean of three rats.

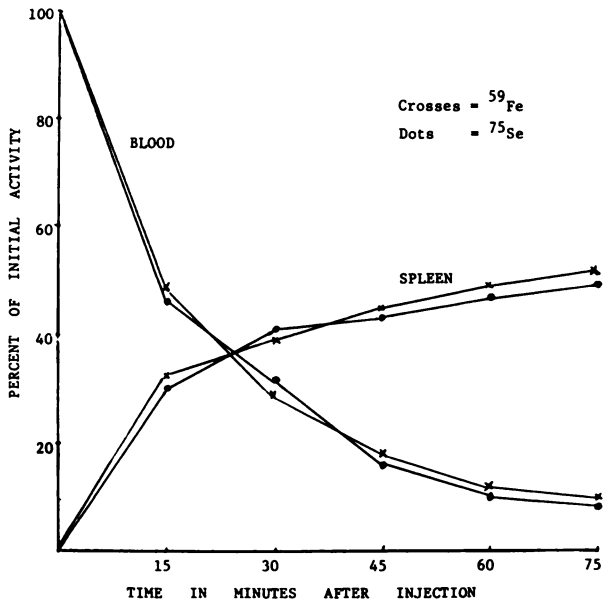

FIG. 4. Disappearance of two radiolabels from circulation and their splenic accumulation after injection of heat-denatured, dual-labeled erythrocytes in rat. Each point is mean of two rats.

rocyte content of radioiron peaked by the second week and the ^{75}Se content reached a maximum at about the same time (Fig. 1).

Dual-labeled erythrocytes lost none of their radiolabel upon dialysis, even after denaturation or hemolysis (Fig. 2). By contrast, significant ^{51}Cr was lost from chromium-labeled cells, and this loss was increased by denaturation or by hemolysis.

Treatment of dual-labeled hemoglobin with trypsin resulted in loss of both ^{75}Se and ^{59}Fe from the material placed in a dialysis bag (Fig. 3). There was greater percent loss of ^{75}Se than of ^{59}Fe.

Following intravenous injection of dual-labeled heat-denatured erythrocytes in the rat, the cells disappeared from the bloodstream with a half-time of about 15 min (Fig. 4). Within the limits of error of the experiment, the ^{59}Fe and ^{75}Se disappeared from the bloodstream simultaneously. The splenic uptake of the two radiolabels proceeded simultaneously, reaching a peak of about 55% of the injected radiolabel. Isolated and washed heme from rat erythrocytes contained ^{59}Fe with less than 3% contamination of ^{75}Se. Isolated, washed globin contained ^{75}Se with less than 5% contamination from ^{59}Fe.

After the initial peaking of ^{59}Fe and ^{75}Se in the spleen following injection of the heat-denatured dual-labeled erythrocytes, the splenic content of the radiolabels began decreasing (Fig. 5). The halftime for the decrease was approximately 7 hr (after the first 60–90 min required for maximum uptake). The splenic content of ^{75}Se fell toward a low value while that of ^{59}Fe began increasing again at about 10 hr postinjection. When ^{59}Fe-ferric chloride was given intravenously to rats, a maximum of about 10% of the dose entered the spleen at 10 hr (Fig. 6).

DISCUSSION

The ability of erythrocyte precursors to incorporate ^{75}Se-selenomethionine as well as ^{59}Fe provides

a technique for doubly labeling erythrocytes. Hence, a method is available for following the heme and globin portions of hemoglobin (or their breakdown products). The ability of both organic and inorganic selenium compounds to transfer their label to developing erythrocytes has been noted (*3–5*). Apparently, both ^{75}Se and ^{59}Fe enter erythrocytes early in their life cycle, and we would expect cohort labeling after a single intravenous injection of both radionuclides (Fig. 1). In the case of radioiron there is reuse of the radiolabel; such reuse is minimal for ^{75}Se. To provide labeling of a larger portion of the erythrocyte population, repeated intravenous injections of the radiolabels can be used (as was done here with the rats).

Unlike ^{51}Cr labeling, the ^{59}Fe and ^{75}Se in hemoglobin are an essential part of the molecule. The ^{59}Fe and ^{75}Se cannot be dialyzed away from intact hemoglobin (Fig. 2). Although the exact amino acid sequence of rat hemoglobin is not known, the size of the human hemoglobin molecule has been found to be approximately 50 A across its smallest dimension (*6*). This is slightly larger than the pore size of the Visking dialysis tubing. Addition of heat-denatured trypsin to the rat hemoglobin did not allow any increased escape of radiolabel out of the dialysis sack. However, when native trypsin was added to the contents of the dialysis bag, there was increased escape of both ^{75}Se and ^{59}Fe. A possible explanation for this is that in human hemoglobin methionine (and presumably selenomethionine) occupies the 32nd and 76th positions of the 141 amino acid α chain of hemoglobin, as well as the 55th position of the 146 amino acid β chain (*6*). Thus, a human hemoglobin A molecule of composition $\alpha_2\beta_2$ might contain six selenomethionine residues. Trypsin can hydrolyze linkages in the α and β chains of hemoglobin. The preferred site of attack of trypsin is the peptide bond between the carboxyl group of arginine or lysine and the amino group of another amino acid. There are 13 such sites in each hemoglobin chain. It is therefore to be expected that multiple fragments will be formed by tryptic digestion of hemoglobin and that the radiolabeled peptides might escape through the Visking tubing, and this apparently occurs with rat hemoglobin (Fig. 3). Heme itself is a small molecule (approximately 14 $\times$ 17 A) which could also potentially escape through the pores of the dialysis tubing (*6*). The observation that less ^{59}Fe than ^{75}Se escaped from the dialysis sack is probably due to the fact that heme chloride precipitated within the dialysis bag. Experiments with purified heme and globin from the dual-labeled cells indicate that heme can be obtained with a ^{59}Fe tag relatively free of ^{75}Se; similarly, globin can be purified so that ^{75}Se is present relatively free of ^{59}Fe.

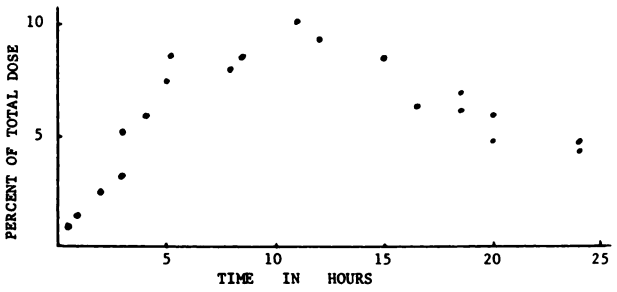

FIG. 6. Splenic content of ^{59}Fe after intravenous injection of ^{59}Fe-ferric chloride. Each point represents one rat.

Following intravenous injection of heat-denatured, dual-labeled erythrocytes, ^{59}Fe and ^{75}Se disappeared simultaneously from the blood stream. Further, the labels had the same time-course of entry into the spleen (Fig. 4). After radioactivity has peaked in the spleen, a discharge begins to take place. The half-time for this event is about 7 hr (Fig. 5). At about the tenth hour following injection of the heat-denatured dual-labeled erythrocytes, a disassociation appeared to occur between the two radiolabels. Disappearance of the ^{75}Se continued while the splenic content of ^{59}Fe began to increase. This increase might be due to uptake of iron freed from previously trapped cells. The latter part of the iron curve grossly resembled the splenic handling of intravenously injected ^{59}Fe (Fig. 6). This point could be further investigated by means of iron loading before or during the experiment.

There is an aspect of these studies that is of interest in the comparative evaluation of reticuloendothelial function. Bilirubin appears within a few hours after erythrocyte sequestration (*7*). This is consistent with the present results of discharge of ^{75}Se and ^{59}Fe from heat-denatured red cells. It might be possible to quantitate the kinetics of uptake and release of the labels from other sites such as the bone marrow and liver. The exact kinetics of the multiple events occurring will have to be determined. However, dual labeling of erythrocytes has possible applications in following splenic function. A useful technique may thus be present for following the differential splenic handling of erythrocyte protein (globin) and the iron in heme.

SUMMARY

Erythrocytes can be dual-labeled with ^{59}Fe and ^{75}Se by injecting ferric chloride and selenomethionine in rats. The two radiolabels are an integral part of the erythrocyte, and cannot be removed by dialysis. Trypsin treatment, however, apparently cleaves dual-labeled hemoglobin into smaller fragments, which

permits some radiolabel to pass out of the dialysis sacks.

Heat denatured dual-labeled erythrocytes are rapidly cleared from the blood stream of rats after intravenous injection ($T_{1/2}$ = about 15 min). The ^{59}Fe and ^{75}Se radiolabels disappear simultaneously and enter the spleen together. About 55% of the injected radiolabels can be found in the spleen.

After splenic accumulation, a discharge of ^{59}Fe and ^{75}Se occurs. The disappearance half-time is about 7 hr. As the ^{75}Se label approaches a low level, an increase in the ^{59}Fe content of the spleen occurs. This might represent uptake of previously freed iron since the curve approximates that of intravenously injected ^{59}Fe.

ACKNOWLEDGMENT

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