PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 78-83 (1976)

The Utilization of Senescent Red Cell and Hemolysate Iron for Erythropoiesis[1] (39147)

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Iron from senescent red cells provides the primary source for synthesis of hemoglobin by maturing red cells. This process involves removal of senescent red cells by the reticulo-endothelial system, separation of the iron from the heme, and release of the iron to transferrin for transport to erythropoietic sites. The rate at which iron is processed by the reticulo-endothelial cells has been implicated as the site of the defect contributing to the anemia associated with chronic infections and malignant disease (1). It also has been shown that iron deposited in the storage sites is eventually converted to a form which is relatively stable and consequently relatively unavailable for transport to erythropoietic sites (2).

Another variable that influences the manner in which iron is stored is the condition in which it is presented for storage. Iron injected into experimental animals in the form of hemolysates (simulating intravascular hemolysis) is preferentially taken up by the hepatocytes, whereas that from heated red blood cells (simulating extravascular hemolysis) is taken up by the reticulo-endothelial cells (Kupffer cells of the liver) (3, 4). Transferrin bound iron, not taken up by maturing erythroid cells, is also taken up preferentially by hepatocytes (5). These cell lines (hepatocytes and Kupffer cells) may process iron differently and this might affect the availability of iron for subsequent hemoglobin synthesis.

Heated red blood cells (RBC's) have been shown to be rapidly sequestered in the reticulo-endothelial system and consequently serve as a model for extravascular hemolysis. Since erythropoiesis is almost totally abolished in plethoric mice and can be restimulated in controlled amounts by either injection of erythropoietin or reexposure to hypoxia, this experimental model seems suitable for studying the availability of different forms of iron stores at various times after deposition of the iron.

This paper describes the results of studies on the availability of storage iron at various times after deposition and the relative availability of radioiron derived from heated RBC's and from hemolysates for hemoglobin synthesis.

Methods and Materials. CF_1 mice weighing 20 to 25 g were used. Mice were exposed to 0.5 atm by placing them in a steel chamber attached to a vacuum pump and fitted with a spring valve set to open when the pressure in the chamber reached the desired amount. Mice were made polycythemic by exposure to 0.5 atm for 3 weeks (in the chamber Monday through Friday from 5 PM to 9 AM, and all weekend). Mice prepared in this manner will be referred to as polycythemic mice. Red cells labeled with ^{59}Fe were obtained from the retro-orbital plexus 36 hr after injecting mice with 20 μCi of ^{59}Fe intravenously. Heat-damaged ^{59}Fe-labeled RBC's were prepared by incubating 1 ml of these cells in 9 ml of saline in a water bath at 50° for 30 min except in Experiment I, in which the effect of heating for different times was studied. The cells were then centrifuged and suspended in sufficient saline to provide the desired volume of RBC's in 0.5 ml. Hemolysate was prepared by diluting the ^{59}Fe-labeled red cells in sufficient distilled water to provide the indicated quantity of RBC's in a volume of 0.5 ml. Sodium chloride was then added to render the solutions isotonic. Cell stroma were not removed so as to simulate the conditions of intravascular hemolysis more accurately. Red cell suspensions and hemolysate solutions were administered intravenously by tail vein in a volume of 0.5 ml.

[1] Supported by the Veterans Adminstration Research Funds and the National Institutes of Health Grant No. AM-12936.

Experiment I. Distribution of iron from RBC's heated for various lengths of time. ^{59}Fe-labeled RBC's were heated at 50° for 10, 30, or 60 min. They were then centrifuged, suspended in saline, and 0.03 ml of RBC's were injected into groups of 6 *N* recipients. Six mice received ^{59}Fe-labeled hemolysate containing the equivalent of 0.03 ml of RBC's. Three hours later, the mice were sacrificed and the radioactvity in their RBC's, spleen, livers, femurs, and kidneys was measured and recorded as the percentage of ^{59}Fe administered.

Experiment II. Availability of iron for erythropoiesis at various times after deposition into stores. This experiment was performed to determine the availability of iron deposited in the reticulo-endothelial system of polycythemic mice at various times in relation to an erythropoietic stimulus and subsequent increase in hemoglobin synthesis. Previous studies have shown that the wave of erythropoiesis stimulated by reexposure of polycythemic mice to hypoxia or the injection of erythropoietin reaches the stage of maximal hemoglobin synthesis 2 days later (6). Polycythemic mice received 0.01 ml of ^{59}Fe-labeled heat-damaged RBC's 7 days after termination of their exposure to hypoxia. At various times ranging from 2 days before to 2 days after the injection of the cells groups of mice were reexposed to hypoxia at 0.5 atm. Controls received heated and labeled RBC's but were not reexposed to hypoxia. Five days after continuous exposure to hypoxia all mice were sacrificed, and 0.5 ml of blood was obtained by intracardiac puncture and counted in a Picker automatic well counter. A standard containing the dose given the animals was also counted and the red cell iron incorporation determined by multiplying the calculated blood volume of the animal by the counts per milliliter of whole blood divided by the standard. Blood volume was calculated by multiplying the weight of the animal by 0.06. The control group, which was not reexposed to hypoxia, was sacrificed 5 days after injection of heated, labeled RBC's.

Experiment III. Comparison of utilization of RBC iron and hemolysate iron. Polycythemic mice were kept at atmospheric pressure for 5 days and then were reexposed to hypoxia; 48 hr later groups of five animals received various amounts of heat damaged RBC's or hemolysate labeled with ^{59}Fe. Control groups of polycythemic animals, not reexposed to hypoxia, were included. Seventy-two hours after administration of the labeled RBC's or hemolysate, the whole animals were counted in a Packard small animal counter. The animals were then bled and the kidneys and 0.5 ml of whole blood were counted in a Picker automatic gamma counter. A standard containing the amount of ^{59}Fe injected into each group of animals was also counted. The data were recorded as the percentage of the counts per minute in each animal at the time of sacrifice compared with the standard, and the percentage of the counts per minute in the two kidneys compared with the whole animal. Since [^{59}Fe]hemoglobin removed by the kidney is relatively unavailable for new red cell production, and since this fraction is greater in mice given hemolysate iron as compared to red cell iron, it was not considered appropriate to determine the percentage of the ^{59}Fe utilized by reference to the number of counts injected. Therefore, to determine the percentage of the red cell and hemoglobin ^{59}Fe utilized, the counts in the red cell mass were divided by the counts in the whole animal at the time of sacrifice subtracted by the counts in the kidneys. The results were recorded as the mean ±1 SE.

Experiment IV. Effect of progressively increasing amounts of erythropoietin on utilization of storage iron. Groups of 30 polycythemic mice received injections of either 10, 1.0, 0.1, or 0 units of erythropoietin[2] 7 days after discontinuing hypoxia. Five members of each group then received either 0.5 μCi of [^{59}Fe]ferric chloride (S.A. $\simeq$ 20), 0.01 ml of ^{59}Fe-labeled hemolysate or 0.01 ml of ^{59}Fe-labeled RBC's (2 days prior to

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[2] Erythropoietin was procured by the Department of Physiology, University of the Northeast, Corrientes, Argentina, and processed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, for distribution by the National Heart and Lung Institute under Research Grant HE-100880. It was authorized for distribution by the National Heart and Lung Institute Committee on Erythropoietin.

the stage of maximal hemoglobin synthesis). The others received one of these forms of iron 2 days later (at the time of maximal hemoglobin synthesis). Five days after injection of erythropoietin, the amount of iron incorporated into the RBC's expressed as a percentage of the iron in the total body minus that in the kidneys was determined on all mice. This experiment was repeated one time using injections of 25, 10, 5, and 0 units of erythropoietin as stimuli. The results were recorded as the mean ±1SE.

Results. Experiment I. Distribution of radioiron derived from hemolysates or from RBC's heated for various lengths of time. Results in Fig. 1 are 3 hr after injection of the hemolysates or heated RBC's. Radioiron derived from hemolysates or RBC's heated for 60 min is deposited in lesser amounts in the spleen and liver and in greater amounts in the kidneys than is that derived from RBC's heated for either 10 or 30 min. A significantly smaller amount of radioiron derived from RBC's heated for 10 min is deposited in the spleen and a larger amount remains in the circulation than is the case for that derived from RBC's heated for 30 min. Almost all of the RBC's heated for 30 min are destroyed within 3 hr, and a large proportion of these are processed by the liver and spleen. Most of the RBC's heated for 10 min are also destroyed in the spleen and liver within the first 3 hr. However, significantly more of these remain in the circulation suggesting that this amount of heating was not sufficient. RBC's heated for 30 min were accordingly used in subsequent studies to prepare cells which are representative of those handled by the host similarly to senescent RBC's.

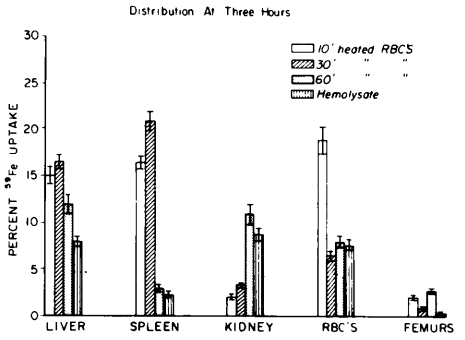

FIG. 1. Distribution of iron from RBC's heated for various lengths of time.

Experiment II (Fig. 2). Availability of RBC radioiron for erythropoiesis at various times after deposition into stores. The utilization of RBC radioiron declines from 25% of the administered dose when maximal hemoglobin synthesis (Day −2 on the abscissa of Fig. 2) corresponds with the entry of iron into the reticulo-endothelial system to 11% when maximal hemoglobin synthesis occurs 24 hr to 4 days after injection of labeled, heated RBC's (Days −1 to +2 on the abscissa of Fig. 2). Animals that recieved no erythropoietic stimulation incorporated less than 1% of the administered red blood cell ^{59}Fe into new red cells.

Experiment III (Table I). Comparison of utilization of RBC radioiron and hemolysate radioiron. A larger percentage of the injected RBC ^{59}Fe remained in the mice after 72 hr than was the case for hemolysate ^{59}Fe, reflecting the greater urinary excretion of iron injected in the form of hemolysate ^{59}Fe. Also, a greater proportion of the hemolysate ^{59}Fe than of the RBC ^{59}Fe was deposited in the kidneys. This iron deposited in the kidneys is for the most part unavailable for delivery to the erythron. For the purposes of this study it was assumed to be totally unavailable.

Available radioiron from heated RBC's (that which remained in the mouse after 72 hr in sites other than the kidney) was better utilized for new hemoglobin production

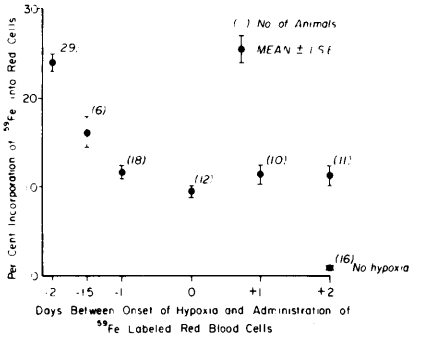

FIG. 2. The utilization of ^{59}Fe from heat-damaged RBC's for hemoglobin synthesis as related to the time during which the ^{59}Fe is in the reticulo-endothelial system before maximal hemoglobin synthesis is produced by hypoxia.

than was available radioiron from hemolysates. As the amount of administered RBC radioiron was increased, the percentage utilization of the radioiron for new hemoglobin synthesis declined from 30% to 17%. On the other hand, increasing the amount of administered hemolysate radioiron from 0.01 to 0.15 ml did not affect the percentage of hemolysate radioiron utilized (11–16%). The utilization of RBC and hemolysate radioiron in polycythemic animals which were not reexposed to hypoxia was less than 1.2% at all the dose levels.

Experiment IV (Table II). Effect of progressively increasing amounts of erythropoietin on utilization of storage iron. When iron was given at the time of maximal hemoglobin synthesis (48 hr after injection of erythropoietin), the iron given in the form of ferric chloride was most efficiently utilized and that given in the form of hemolysate was least well used. In all forms, the

TABLE I. COMPARISON OF UTILIZATION OF HEMOLYSATE AND RBC IRON.

Stimulus	Amount injected (ml)	% Animal/std		% Kidney/animal		% RBC/animal-kidney[a]	
		RBC ^{59}Fe	Hemolysate ^{59}Fe	RBC ^{59}Fe	Hemolysate ^{59}Fe	RBC ^{59}Fe	Hemolysate ^{59}Fe
Hypoxia	0.01	84.6	65.0	6.5	46.4	30.2 ± 2.4	16.2 ± 1.0
	0.03	90.4	56.7	8.7	40.1	23.5 ± 1.3	10.7 ± 1.3
	0.07	92.3	63.2	11.0	28.6	19.0 ± 2.7	11.2 ± 0.8
	0.15	82.9	67.9	14.9	22.9	17.0 ± 1.4	12.2 ± 0.9
No hypoxia	0.01	83.3	69.0	5.7	50.4	0.4 ± 0.04	0.7 ± 0.04
	0.03	90.0	59.4	7.5	43.7	0.5 ± 0.06	0.5 ± 0.06
	0.07	89.5	61.7	11.1	30.7	0.5 ± 0.05	0.4 ± 0.17
	0.15	83.2	63.3	16.6	23.6	1.08 ± 0.5	1.2 ± 0.14

[a] Results represent the mean ±1 SE of values obtained from five mice.

TABLE II. EFFECT OF ERYTHROPOIETIN ON UTILIZATION OF STORAGE IRON.

Expt	Form of ^{59}Fe injected	Dose of erythropoietin	%RBC ^{59}Fe uptake[a]	
			^{59}Fe given at time of erythropoietin	^{59}Fe given 2 days after erythropoietin
1	RBC ^{59}Fe	10	3.7 ± 0.8	12.2 ± 0.9
		1.0	3.0 ± 0.3	5.9 ± 0.5
		0.1	3.0 ± 0.2	2.3 ± 0.2
		0.0	2.4 ± 0.2	1.9 ± 0.1
	Hemolysate ^{59}Fe	10	1.3 ± 0.2	2.5 ± 0.3
		1.0	0.8 ± 0.3	0.9 ± 0.2
		0.1	0.1 ± 0.1	0.7 ± 0.2
		0.0	0.1 ± 0.0	0.0 ± 0.0
	$^{59}FeCl_3$	10	3.5 ± 0.8	25.0 ± 2.0
		1.0	3.4 ± 1.0	16.2 ± 1.2
		0.1	0.0 ± 0.0	0.8 ± 0.2
		0.0	0.3 ± 0.2	0.6 ± 0.3
2	RBC ^{59}Fe	25.0	14.4 ± 3.7	30.0 ± 5.1
		10.0	7.4 ± 1.2	13.6 ± 1.7
		5.0	7.1 ± 0.4	14.8 ± 1.5
		0.0	0.6 ± 0.1	2.1 ± 0.5
	Hemolysate ^{59}Fe	25.0	4.3 ± 0.9	28.7 ± 4.3
		10.0	3.5 ± 0.4	7.3 ± 0.8
		5.0	2.5 ± 0.3	4.6 ± 0.8
		0.0	1.1 ± 0.4	0.4 ± 0.1
	$^{59}FeCl_3$	25.0	4.8 ± 0.2	50.7 ± 1.9
		10.0	4.2 ± 0.5	40.7 ± 4.2
		5.0	5.4 ± 0.6	36.0 ± 4.7
		0.0	1.1 ± 0.4	0.8 ± 0.3

[a] Results represent the mean ±1 SE of values obtained from five mice.

amount incorporated increased with increase in the dose of erythropoietin injected. When the iron was given simultaneously with the erythropoietin (2 days prior to the time of maximal hemoglobin synthesis), then the amount utilized was decreased in the case of all three forms of iron. However, the mice injected with heat damaged RBC's utilized a larger percentage of the injected iron than did those injected with either ferric chloride or hemolysate. Although the amount of iron utilized was less at each dose of erythropoietin than that utilized by comparably stimulated mice given iron 2 days later, the response was still roughly dose related.

Discussion. These experiments indicate that a greater proportion of the radioiron of heated RBC's is incorporated into newly formed RBC's if hemoglobin synthesis is in progress at the time of heated RBC injection than is the case if hemoglobin synthesis is initiated one or more days afterwards. The data also show that a constant proportion of the radioiron from heated RBC's is utilized whether hemoglobin synthesis is initiated 1 or 4 days after injection of the heated RBC's. This suggests that there are at least two pools of iron within the cells that process red cell iron. The first pool consists of readily available radioiron. The second pool contains less available iron and it is the pool which the radioiron enters when it is administered to animals with depressed erythropoiesis. An alternative possibility is that the first pool into which red cell iron enters is small so that radioiron entering it is not significantly diluted. If hemoglobin synthesis is taking place at the time of injection of heated RBC's, the radioiron enters the first pool and is then delivered to the marrow for new hemoglobin synthesis. If, however, hemoglobin synthesis is suppressed at the time of injection, the radioiron leaves the first pool and enters a second larger pool where the radioactivity becomes diluted with nonradioactive iron. If hemoglobin synthesis is then stimulated, iron from the second pool will be utilized but less radioactivity will enter newly formed cells because of its dilution.

A larger percentage of the heated RBC radioiron is immediately available for utilization in developing erythroid cells than is the case for radioiron derived from hemolysates. This is so even when allowance is made for the increased loss of hemolysate iron in the urine and kidney. This difference is most apparent when small amounts of these substances are given. Bissel, Hammaker, and Schmid (5) and Cook, Herschko, and Finch (4) have shown in rats that whereas radioiron derived from heated RBC's is deposited preferentially in the Kupffer cells of the liver, that derived from hemoglobin is deposited in the hepatocytes. This difference in the anatomic distribution of the radioiron may account for the difference in its availability for delivery to erythropoietic sites. With adminstration of larger amounts of heated RBC's there is perhaps a shift of radioiron into hepatocytes or a saturation of the processing ability of the Kupffer cells.

Further evidence for a difference in the body's processing of radioiron derived from heated RBC's and from hemolysate is the observation that 2 days after deposition a larger percentage of the radioiron derived from heated RBC's is available for hemoglobin synthesis than is that from hemolysate. This also suggests that radioiron from these two sources is deposited into different storage pools. These may differ in size and consequently in the amount of dilution of the label or in their chemical form and consequently in their availability. If the observation in rats that hemolysate iron is deposited in hepatocytes and heated RBC's in Kupffer cells applies for mice, this might explain the two different pools.

Radioiron administered as $FeCl_3$ is initially more available for use by developing red cells than either hemolysate or heat-damaged RBC iron because it is attached directly to unsaturated transferrin in the plasma. However, once deposited in a storage form it becomes available in an amount comparable to that derived from hemolysate, and significantly less available than that derived from heated RBC's. This can be explained by the concept that transferrin bound iron which exceeds receptor sites on erythroid cells is deposited in hepatocytes rather than in Kupffer cells, and accordingly enters a pool which is either larger

than or less accessible than is that of the Kupffer cells.

In conclusion, radioiron derived from heated RBC's is deposited in a relatively available form and within 24 hr either mixes with a larger pool, or one which is less readily available. Radioiron derived from hemolysate is deposited into a different pool than is that from heated RBC's. This pool is either less accessible or larger than is that into which heated RBC iron is deposited. Finally, injected $FeCl_3$ is immediately most available for hemoglobin synthesis; however, when such iron is deposited in a storage pool its availability for hemoglobin synthesis is comparable to that of hemolysate iron and it is less available than heated RBC iron.

Summary. We report experiments to determine the availability for new hemoglobin production of radioiron from nonviable red cells at various times after deposition in the reticulo-endothelial system and to determine the relative availability of radioiron derived from hemolysates versus that derived from nonviable red cells. When heated nonviable red cells labeled with ^{59}Fe are injected into polycythemic mice the iron is deposited in the reticulo-endothelial system, and less than 1% of it is reutilized for hemoglobin synthesis. If the polycythemic mice are given nonviable red cells 48 hours after exposure to hypoxia, when hemoglobin synthesis is maximal, 25% of the iron is reutilized. When the cells are given 36 hr after exposure to hypoxia, iron reutilization declines to 16%, and when exposure to hypoxia is further delayed, reutilization of the iron falls to a plateau level of 11%. Radioiron from hemolysates, primarily deposited in parenchymal cells of the liver, is less available for new hemoglobin synthesis than is radioiron from nonviable red cells, which is primarily deposited in Kupffer cells of the liver. When transferrin-bound iron is given to polycythemic mice, this iron is also deposited in parenchymal cells of the liver and is also less available for new hemoglobin synthesis. Thus, in relation to an erythropoietic stimulus, the site and time of deposition of iron influence its accessibility for erythropoiesis.

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Received July 25, 1975. P.S.E.B.M., 1976, Vol. 151.