Preferential Hemolysis of Immature Erythrocytes in Experimental Iron Deficiency Anemia: Source of Erythropoietic Bilirubin Formation

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ABSTRACT Bilirubin-\(^{14}\)C production was measured in rats transfused with labeled erythrocytes from animals with iron deficiency anemia, a condition associated with ineffective erythropoiesis. With labeled reticulocytes harvested 1 day after the administration of glycine-\(^{2}\)\(^{14}\)C, conversion of hemoglobin-\(^{14}\)C to bilirubin averaged 47.3\% over the 3 days of observation; the corresponding value for reticulocytes from normal rats was only 1.7\%. Findings were not altered by splenectomy. Bilirubin-\(^{14}\)C production fell to 35.8\% with iron-deficient cells harvested 3 days after glycine-\(^{14}\)C administration, and declined further to a plateau averaging 25\% with cells labeled 5, 7, 10, or 15 days earlier. The latter values still far exceed those for mature erythrocytes from normal animals.

The findings indicate that experimental iron deficiency anemia is associated with hemolysis of red cells of various ages, but with preferential destruction of the youngest cells. Degradation of hemoglobin from reticulocytes is sufficient to account for a major fraction of the increase in erythropoietic bilirubin production found in this disorder, as has also been shown for physiologically regulated erythroid hyperplasia. However, the defect is quantitatively much more striking in experimental iron deficiency, and this and perhaps a similar defect in bone marrow cells appear to explain the decrease in net hemoglobin production that is characteristic of pathologic ineffective erythropoiesis.

INTRODUCTION

As adduced from isotopic studies, approximately 15\% of normal bile pigment production originates from sources other than the hemoglobin of senescent erythrocytes (3-5). In rats under normal conditions, most of this "early-labeled" bilirubin fraction is derived from extra-erythroid sources, primarily in the liver (5-10). In addition, an erythropoietic component is contained within the later phases of the early-labeled fraction (5, 10). This component is small in rats under physiological conditions (5), but appears to have a larger role in species such as man and the dog (10, 11).

Enlargement of the erythropoietic phase of bile pigment production is found with both accelerated red cell formation in normal animals and man (5, 10-13) and disordered erythropoiesis in subjects with certain hematologic diseases (14-17). It has generally assumed that this is due to ineffective erythropoiesis (18), i.e., hemolysis of immature erythroid cells. However, this concept has been difficult to substantiate directly. Moreover, it has not been ascertained whether the erythropoietic bilirubin fraction has similar origins in subjects with physiologically regulated as compared with pathologically disordered red cell production (19).

Recent studies in this laboratory have shown that degradation of hemoglobin from immature erythroid cells in the peripheral blood, i.e., reticulocytes, accounts for a major portion of the enlarged erythropoietic fraction in rats undergoing a physiologic response to blood loss (21). A rise in erythropoietic bilirubin production has also been found in rats with iron deficiency anemia (22); however, this is associated with a decrease rather than an increase in the net production of red cell hemoglobin heme, a pattern characteristic of pathologic ineffective erythropoiesis (5, 14-17). Investigative

1 For convenience, the term "reticulocyte" is used for circulating red cells that are labeled 24 hr after the administration of glycine-\(^{2}\)\(^{14}\)C. Only immature erythroid cells have the capacity to synthesize hemoglobin heme from this physiologic precursor (20). However, some such young cells might not actually stain as reticulocytes.
TABLE I

Conversion of Reticulocyte Hemoglobin Heme-14C to Bilirubin-14C under Different Conditions

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Condition</th>
<th>No. of recipients</th>
<th>72 hr conversion of Hb heme-14C to bilirubin-14C* %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron-deficient reticulocytes</td>
<td>Splenectomy (donors/recipients)†</td>
<td>5</td>
<td>49.5 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>Sham/sham</td>
<td>4</td>
<td>46.6 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>Sham/splenex</td>
<td>4</td>
<td>49.8 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>Splenx/sham</td>
<td>5</td>
<td>45.9 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td>13</td>
<td>47.3 ± 8.3</td>
</tr>
<tr>
<td>Normal reticulocytes</td>
<td>Cells from normal rats</td>
<td>6</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>Normal reticulocytes</td>
<td>Cells from iron-treated controls§</td>
<td>2</td>
<td>1.0, 2.7</td>
</tr>
<tr>
<td>Normal reticulocytes</td>
<td>Incubated with iron-deficient plasma</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>Normal reticulocytes</td>
<td>Iron-deficient recipients</td>
<td>2</td>
<td>4.1, 4.9</td>
</tr>
</tbody>
</table>

Values are means ±SD (26).
*Transfusions contained 1.46 to 4.51 × 10⁴ dpm in hemoglobin heme-14C.
†Sham donors include both nonoperated and sham-operated rats because of lack of difference between the two.
§Previously iron-deficient rats treated with iron dextran (see Methods).

Experiments blood from three donors was combined for transfusion into two recipient rats. Each transfusion contained 0.1-0.3 g hemoglobin in a volume of 2-3 ml. Disintegrations per minute in transfused heme were calculated from the hemoglobin content (23) and heme specific activity (21) of the transfused blood.

Incorporation of glycine-14C into hemoglobin heme at the different intervals studied was calculated from the specific activity of hemin and the specific activity of the donor blood volumes. Blood volumes in the controls were estimated on the basis of body weight according to a formula (24) which has been corroborated in this laboratory. Blood volume was measured directly in five iron-deficient rats with 51Cr-labeled red cells (21); donor cells were obtained from other anemic animals. Values ranged from 6.6 to 7.3% of body weight, and the mean of 6.8% was applied to all other iron-deficient animals.

**Bilirubin-14C excretion in recipient rats.** Except as noted, recipients were normal rats in which the common bile duct had been cannulated 18 hr before transfusion. Donor cells were injected into a tail vein over 2-3 min, and bile samples were then collected in the dark and on ice at 3.5, 24, 48, and 72 hr, and in a few experiments also at 96 and 120 hr. The excretion of bilirubin-14C during each collection period was determined from the specific activity of bilirubin and the rate of total bilirubin excretion during that interval (21). Results were expressed as per cent conversion of erythrocyte hemoglobin heme-14C to bilirubin-14C during the first 72 hr, with correction for the loss of one-eighth of the glycine-14C label in heme during degradation to bile pigment (25).

**Conditions affecting bilirubin-14C production.** The role of the spleen was evaluated in experiments in which donor and/or recipient rats had been splenectomized. In the donor rats splenectomy or sham-splenectomy was performed 9-11 days before the administration of glycine-14C. Recipients underwent splenectomy at the time of bile duct cannulation.

To determine whether iron deficiency anemia might lead to extracorpuscular changes conducive to reticulocyte hemolysis, bilirubin excretion was measured in two iron-deficient rats given transfusions of labeled reticulocytes from normal donor rats. In an additional experiment, labeled normal reticulocytes were incubated with radioactive plasma from iron-deficient rats, 4 volumes of red cells to 7 of plasma, at 37°C in air for 45 min with mild agitation in a metabolic incubator; the cells were then prepared for transfusion into a normal recipient rat with external bile drainage.

**Reticulocytes labeled with 57Fe**

Labeled reticulocytes were obtained from iron-deficient rats given 15 µCi citrate-57Fe4 1 day earlier (21). Studies with 57Fe were not performed with older cells. Recipients were normal rats that had been prepared with injections of iron-dextran (21) to minimize 57Fe reutilization. Transfusion volumes were small, equivalent to 0.13 g of hemoglobin, and hematocrits remained stable during the subsequent 3 days of observation. 0.4-ml samples of blood were collected from a tail vein at 15 min, and then at 4, 24, 48, and 72 hr for measurement of the specific activity of packed red cells (21). The animal was then sacrificed, and the spleen, liver, and kidneys were removed for radioassay. The per cent of the blood volume normally present in these organs had been measured earlier (21), and the contribution of circulating 57Fe-14C was negligible.

**Preparation of labeled cells for transfusion.** Donor rats were given 50 µCi glycine-2-14C intravenously, and were exsanguinated 1, 3, 5, 7, 10, or 15 days later to obtain cohorts of labeled erythrocytes of different ages. Cells were prepared for transfusion as described previously (21). In most

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4Ferrous citrate-57Fe, 16.5 mCi/mg; Abbott Laboratories, North Chicago, Ill.

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labeled red cells to total organ radioactivity was calculated from these values by correcting for the decline in red cell-
"Fe specific activity during the 72 hr since transfusion; this
assumes that intact iron-deficient cells have a distribution similar to that of normal cells. Data for normal reticulo-
cytes are from recent investigations from this laboratory
(21).

Incorporation of "Fe into red cells was measured in two
normal and three iron-deficient rats. Citrate-"Fe 4 was slowly
added to fresh heparinized plasma (5 µCi/ml) from normal
or iron-deficient rats, according to the type of animal
studied; the mixture was incubated for 30 min at 37°C with
occasional agitation. Approximately 2.5 µCi of transferrin-
bound "Fe (0.14 µg iron) was injected into a lateral tail
vein, and 0.05-ml samples of blood were obtained by tail
puncture in heparinized capillary tubes at 1, 3, 5, 7, 10, and
15 days. The tube was centrifuged, the hematocrit measured,
and the column of packed red cells counted (21) after
discarding the plasma layer. "Fe incorporation was calcu-
lated from counts per minute per milliliter of erythrocytes,
hematocrit, and blood volume.

RESULTS
Hematologic values in 20 groups of iron-deficient donors
were as follows: hemoglobin 2.9 g/100 ml (± 0.1),
mean corpuscular hemoglobin concentration 19.1% (± 0.4),
and reticulocytes 12.4% (± 3.4). Corresponding
values for 10 groups of control rats were 13.6 (± 0.8),
31.8 (± 1.3), and 1.7 (± 0.8), respectively.

Erythrocytes with "C-labeled hemoglobin heme

Incorporation of glycine-2-14C into hemoglobin heme
(Fig. 1). Initially, heme radioactivity in circulating
red cells increased at similar rates in both normal and
iron-deficient rats. Between 1 and 3 days, however,
labeling continued to rise substantially in the anemic
rats, but increased only slightly in the anemic animals;
thus, maximum incorporation of glycine-14C in the
iron-deficient animals was approximately half normal. From
days 3 to 15, the level of radioactivity remained more
or less constant in the control rats, but fell by a factor
of 3-4 in the anemic animals.

Conversion of reticulocyte hemoglobin heme-14C to
bilirubin-14C. In rats transfused with labeled reticulo-
cytes from normal donors given glycine-14C 1 day earlier,
a mean of 1.7% of the injected heme-14C was excreted
as bilirubin-14C in 3 days (Table I). In marked contrast,
with transfusion of labeled reticulocytes from
iron-deficient donors, conversion to bilirubin-14C aver-
aged 47.3% in 3 days. The rate of labeled bilirubin excre-
tion was greatest during the first 24 hr after trans-
fusion, and then leveled off gradually over the ensuing
2-4 days.

Alterations in the rate of labeled bilirubin production
as a function of the time of labeling of the donor cells
are shown in Fig. 2. The mean 72 hr conversion of
hemoglobin heme-14C to bilirubin-14C fell from 47.3%
with iron-deficient reticulocytes labeled 1 day earlier to
35.8% with cells labeled 3 days previously. With cells
from iron-deficient donors given glycine-14C 5, 7, 10, or
15 days before transfusion, bilirubin production declined
further to a plateau averaging 25%.

Conditions affecting bilirubin-14C production (Table
I). The findings with iron-deficient reticulocytes were
not influenced by splenectomy of either donor or re-
cipient rats, and the results of all of these experiments
have therefore been combined elsewhere in the text.
Transfusion of normal reticulocytes into iron-deficient
recipients led to a small increase in bilirubin-14C produc-
tion, suggesting that extracorpuscular factors might
contribute to reticulocyte hemolysis in intact iron-def-
icient animals. It seemed possible that this might be
related to plasma constituents, since the plasma of these
rats is grossly lipemic as the result of an increase in
triglycerides (27); however, preincubation of labeled
normal reticulocytes with plasma from iron-deficient
animals had little or no effect on the subsequent rate
of labeled bilirubin production.

Reticulocytes labeled with "Fe

Incorporation of "Fe into erythrocytes. Red cell in-
corporation of "Fe in the two normal rats reached maxi-
mal values of 92 and 97% at 5-7 days. By contrast, only
53-61% incorporation was observed in the three iron-
deficient rats; these values were reached at 3-5 days.
Iron labeling then remained more or less constant
through the final measurement at day 15 in both types
of animal, in contrast with the decline observed with
iron-deficient rats when glycine-14C was used as the pre-
cursor (Fig. 1).
Disappearance of transfused reticulocytes. The disappearance of "Fe-labeled iron-deficient reticulocytes transfused into normal recipients (Fig. 3) was similar to the production of bilirubin-"C in the corresponding experiments with glycine-labeled cells (Table I). At 72 hr, specific activities had fallen an average of 38% below the initial values measured at 15 min as compared with only 3.5% with cells from normal donors.

Both the spleens and livers of animals given transfusions of "Fe-labeled iron-deficient reticulocytes contained substantially larger amounts of radioactivity than did those of animals given normal reticulocytes (Table II). These differences are more striking after total organ radioactivity is corrected for the content of "Fe in organ blood (values in parentheses), since the latter accounted for most of the radioactivity in the control experiments. Expressed in either manner, the radioactive content of the spleen exceeded that of the liver in recipients of iron-deficient cells. Only low levels of radioactivity were found in the kidneys in both groups of animals.

**FIGURE 2.** Conversion of transfused hemoglobin heme-"C to bilirubin-"C as a function of the age of the labeled donor cells. Height of each bar represents the mean (±SE) per cent conversion in 72 hr. Transfusions contained 0.82±4.58×10^6 dpm in red cell hemoglobin heme-"C. Values for 1 day old cells are from experiments with both intact and splenectomized animals (see Table I).

**FIGURE 3.** Disappearance of "Fe-labeled reticulocytes in normal recipient rats. Initial specific activities measured at 15 min ranged from 3.47 to 4.08×10^6 cpm/ml in the experiments with iron-deficient reticulocytes, and from 4.78 to 11.85×10^6 cpm/ml in those with normal reticulocytes. Means ±SE are plotted for normal cells.

**TABLE II**
Recovery of Transfused Reticulocyte-"Fe in Organs of Recipients

<table>
<thead>
<tr>
<th>Cell type</th>
<th>% Recovery of &quot;Fe*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>Normal reticulocytes</td>
<td>1.7±0.1 (0.7)</td>
</tr>
<tr>
<td>Iron-deficient reticulocytes</td>
<td>16.5±0.4 (15.8)</td>
</tr>
</tbody>
</table>

Values are means ±SE(26). Numbers in parentheses refer to "Fe recovery after correction for organ blood-"Fe.

* Rats received 3.17×10^6 cpm in "Fe-labeled cells in the experiments with iron-deficient reticulocytes, and 6.42–7.33×10^6 cpm in those with normal cells.
DISCUSSION

These experiments provide direct confirmation both of the concept of ineffective erythropoiesis and of the relationship of this pathophysiologic process to the erythropoietic fraction of bile pigment formation. Together with previous studies, they indicate that degradation of hemoglobin from immature erythroid cells accounts in large measure for the increased production of early-labeled bilirubin both in rats with abnormal erythropoiesis due to severe iron deficiency (22) and in animals with physiologically regulated erythroid hyperplasia (21). This confirms the suggestion of earlier investigators that reticulocyte hemolysis might be a source of early bilirubin formation (3, 12, 28).

Not surprisingly, there was also increased destruction of mature red cells in the iron-deficient rats; the mean value for bilirubin-\textsuperscript{14}C production with transfusion of 10 day old iron-deficient cells was 27.9\% in 3 days (Fig. 2), as compared with only 2.5\% for normal cells of this age (21). Since both the amount of hemoglobin heme-\textsuperscript{14}C present in the donor blood and its rate of conversion to bilirubin-\textsuperscript{14}C diminished with increasing red cell age (Figs. 1 and 2), the absolute magnitude of labeled pigment formation was greatest with transfusion of 1 day old iron-deficient cells, declined moderately with 3 day old cells, and then fell off more steeply, as would be expected from the pattern of early-labeled bilirubin production in intact iron-deficient rats (22).

On the basis of the data in Figs. 1 and 2, hemolysis of labeled reticulocytes could account for 0.11\% glycine-\textsuperscript{14}C incorporation into bilirubin-\textsuperscript{14}C in 3 days. In studies of early-labeled pigment formation from all potential sources, intact iron-deficient rats incorporated 0.13\% of the injected glycine-\textsuperscript{14}C into labeled bilirubin during the first 60 hr (22); some of this is nonerythroid in origin (5-11, 22). Comparison of labeled bilirubin production in rats given transfusions of labeled cells and intact animals given glycine-\textsuperscript{14}C directly serves only as an approximation. Nevertheless, it is apparent that degradation of reticulocyte hemoglobin is of sufficient magnitude to account for a major portion of the enlarged erythropoietic bilirubin component in experimental iron deficiency. Since the earliest transfusions were performed 1 day after the administration of glycine-\textsuperscript{14}C to the donor rats, it is possible that other mechanisms, perhaps including hemolysis of less mature cells in the bone marrow, played a significant role during the first 24 hr.

Study of labeled bilirubin production permits precise evaluation of the rate of degradation of erythrocyte hemoglobin and circumvents a number of problems inherent in other techniques. Measurement of red cell incorporation of \textsuperscript{55}Fe failed to show hemolysis, presumably because of avid \textsuperscript{55}Fe reutilization in the iron-deficient animals. McKee, Wasson, and Heyssel did demonstrate reticulocyte hemolysis in iron-deficient rats with \textsuperscript{55}Fe, by iron loading the animals after the isotope was given (29); however, they found only mild hemolysis of older cells. Reutilization of glycine-\textsuperscript{14}C also occurs to a small extent (30); this probably explains why direct labeling of hemoglobin heme with glycine-\textsuperscript{14}C did not demonstrate preferential hemolysis of the youngest cells and fell off at a rate of only 5-6\% per day (Fig. 1), whereas the bilirubin studies reflected a decreasing rate of hemolysis over a range of 16-7\% per day (Fig. 2). On the other hand, the findings with transfusion of \textsuperscript{55}Fe-labeled reticulocytes (Fig. 3) did correspond to those based on bilirubin-\textsuperscript{14}C production; however, in studies of reticulocytes produced in response to hemorrhage, these two techniques led to somewhat different results that were attributed tentatively to loss of nonheme iron or to some cell sequestration without hemolysis (21). Moreover, measurement of red cell specific activity, unlike measurement of labeled bilirubin production, requires maintenance of the erythropoietic steady state.

Potential liabilities of the bilirubin technique have been considered elsewhere (21), but issues pertinent to the present investigation merit brief comment. It is possible that reutilization of glycine-\textsuperscript{14}C in the donor rats may have "contaminated" the cohorts of older labeled cells with labeled reticulocytes, thus exaggerating the apparent hemolytic susceptibility of these older cells. It is also conceivable that the large amounts of labeled bilirubin produced by the recipients of iron-deficient erythrocytes originated not from hemoglobin heme, but from the excess free protoporphyrin present in such cells (31, 32); however, the almost parallel findings with \textsuperscript{55}Fe- and glycine-labeled reticulocytes implicate the equivalent loss of both the iron and porphyrin moieties of entire heme molecules. Finally, it is perhaps surprising that splenectomy did not influence the rate of bilirubin-\textsuperscript{14}C production (Table I), in view of the high levels of splenic radioactivity observed in the experiments with \textsuperscript{55}Fe-labeled cells (Table II). The liver may have compensated for the absence of the spleen (21). Alternatively, it is possible that the spleen had offsetting effects, both protective (33) and destructive, on these immature cells.

Iron deficiency anemia differs in certain respects in experimental animals and in man. Reticulocytosis is not typical of iron-deficient patients, although a moderate increase in the reticulocyte count is probably not uncommon (29, 34, 35); reticulocytosis is characteristically found in animals with iron deficiency anemia (29, 36, 37). Studies differ with regard to the presence of hemolysis in iron-deficient humans (38-42); hemolysis is readily confirmed in the experimental disorder (29, 1851).
37, 43) (Figs. 1 and 2). Finally, \(^{57}Fe\) incorporation into red cells is rapid and high in patients with iron deficiency (44, 45), but was below normal in this investigation of iron-deficient rats. The present findings may therefore not be entirely applicable to man. However, analyses of \(^{57}Fe\) turnover in human subjects are consistent with ineffective erythropoiesis (44, 45), as are studies of chelatable iron before and after iron therapy (46). It is therefore likely that the differences between the findings in rats and man are largely a matter of degree, with a more marked impairment in survival of both young and old cells in the experimental disorder.

If the increase in erythropoietic bilirubin production in experimental iron deficiency anemia is of similar origin to that in the physiologic response to blood loss (21), what accounts for the decrease in net hemoglobin production in the former and the increase in the latter condition? Since the rate of hemoglobin degradation is as high as 16% per day with iron-deficient reticulocytes (Table 1), as compared with only 2.2% per day with “stress reticulocytes” from rats after blood loss (21), this appears to be largely a quantitative phenomenon. However, recent observations are causing us to reevaluate this attractive and unifying concept. Stress reticulocytes appear to undergo heme loss primarily by a process of fragmentation, with loss of only portions of hemoglobinized cytoplasm (47-49). On the other hand, the magnitude of bilirubin-\(^{14}C\) production with iron-deficient reticulocytes suggests that hemolysis of whole cells must be involved to a large extent. Although this is a subject for further investigation, it is tempting to speculate that fragmentation and hemolysis are parts of a spectrum that underlies much of the increase in erythropoietic bilirubin formation under both physiologic and pathologic conditions.

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