A Simple In Vitro Method for the Assessment of Ineffective Erythropoiesis

By Diana Samson, J. Tikerpae, and Helen Crowne

A simple in vitro method has been developed for the assessment of ineffective erythropoiesis by measuring the release of heme from a labeled cohort of erythroblasts in short-term suspension culture. The release of labeled heme was shown to correlate with the death of erythroblasts in culture determined by cell counting. Heme release was markedly increased in conditions where there is known to be excessive ineffective erythropoiesis, while in hematologic disorders where ineffective erythropoiesis is thought to be normal, heme release was within the normal range.

INEFFECTIVE ERYTHROPOIESIS has long been recognized as a major factor in the pathogenesis of some types of anemia, notably pernicious anemia and thalassemia, but more recent evidence suggests that it may play a role in a wide variety of hematologic disorders. The techniques used to quantitate ineffective erythropoiesis either measure the incorporation of labeled precursors of heme synthesis into early labeled bilirubin or carbon monoxide and depend on computer analysis of the disappearance curve of plasma$^{59}$Fe. These methods are relatively laborious and cannot be repeated until the isotope has been cleared from the body. There is therefore a need for a test that is suitable for large numbers of patients and which can be repeated at frequent intervals, for example to follow the effect of treatment. These criteria would be fulfilled by an in vitro test using aspirated bone marrow.

Myrhe, using short-term suspension cultures of human marrow, measured the incorporation of labeled iron into the erythroblasts as an index of cell proliferation and the release of labeled iron into the supernatant as an index of cell destruction. In pernicious anemia, the death rate of the erythroblasts determined by cell counting and the release of labeled iron into the supernatant were both increased compared with normal marrow and reverted to normal following$\text{B}_{12}$ therapy. In the present work, this technique has been modified in an attempt to provide a simple method of assessing ineffective erythropoiesis.

MATERIALS AND METHODS

Subjects

Three groups of subjects were studied: 28 hematologically normal control subjects, 11 patients with normoblastic erythroid hyperplasia due to conditions in which the amount of ineffective erythropoiesis is thought to be normal, and 27 patients with diseases where ineffective erythropoiesis is known to be increased.

The 28 control subjects included 3 normal volunteers, 8 patients undergoing minor surgery in whom marrow was obtained after induction but before administration of nitrous oxide, 3 patients with fully treated iron or$\text{B}_{12}$ deficiency, 3 patients with raised ESR, 3 patients with localized malignancy, and 8 patients with miscellaneous nonhematologic diseases. All had normal blood counts and marrow morphology. The patients with normoblastic hyperplasia included 5 patients with hemolytic anemia (one case each of warm autoimmune hemolytic anemia, cold agglutinin disease, paroxysmal nocturnal hemoglobinuria, hereditary spherocytosis, and microangiopathic hemolytic anemia), 4 patients with polycythemia (2 with polycythemia rubra vera and 2 with secondary polycythemia), and 2 patients with erythroid hyperplasia following acute hemorrhage. There is no morphological or ferrokinetic evidence for dyserythropoiesis in any of these conditions and evidence from bilirubin studies suggests that ineffective erythropoiesis is normal in hyperplasia following hemorrhage, polycythemia rubra vera, paroxysmal nocturnal hemoglobinuria, microangiopathic hemolytic anemia, and warm autoimmune hemolytic anemia.

The patients with diseases known to involve increased ineffective erythropoiesis included 23 patients with megaloblastic anemia, 3 with idiopathic sideroblastic anemia, and 1 with congenital dyserythropoietic anemia associated with a defect of DNA repair. The patients with megaloblastic anemia were divided into two groups: "severe," with RBC less than $3 \times 10^{12}$/liter, and "mild," with RBC greater than $3 \times 10^{12}$/liter. All had low serum$\text{B}_{12}$ and/or red cell folate except one patient with di Guglielmo's disease. Relevant hematologic data are shown in Table 1. Comparison of the patients with severe and mild megaloblastic anemia shows little difference in mean cell volume (MCV), which was surprising and for which there was no obvious explanation, or in reticulocyte count, serum iron and iron-binding capacity, though the percentage saturation was higher in the severe group. However, there was a marked difference in serum bilirubin level, many of the mild group having a normal bilirubin. The sex age distribution in the two groups was similar; the severe group was comprised of 6 males and 7 females with a mean age of 63 (range 36–82) and the mild group of 4 males and 7 females with a mean age of 67 (range 19–87).

In four patients with severe megaloblastic anemia due to pernicious anemia, measurement of heme release was repeated after$\text{B}_{12}$ therapy. The interval between$\text{B}_{12}$ administration and subsequent study ranged from 24 hr to 13 days.

Principle

A labeled cohort of erythroblasts is produced by incubating aspirated bone marrow in the presence of$^{59}$Fe. After removal of free iron by washing, the cells are reincubated for varying periods, giving total incubation times of up to 144 hr. Death of erythroblasts is

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accompanied by release of $^{59}$Fe heme into the supernatant. The proportion of total $^{59}$Fe appearing in the supernatant at any given time is an index of cell death. It was decided to extract $^{59}$Fe heme rather than count $^{59}$Fe directly in order to exclude any effect of the uptake and release of free iron by the cell membrane.

**Materials**

Folate-free Eagle's minimum essential medium containing 0.11% sodium bicarbonate was used with the addition of 1 ml penicillin/streptomycin 10,000 U/ml, 1 ml 200 mM l-glutamine, and 2500 U preservative-free heparin/100 ml medium. $^{59}$Fe as ferric citrate, 100 μCi/μg, was obtained from the Radiochemical Centre, Amersham, England. It was prepared for use by adding 0.1 ml (10 μCi) to 1.9 ml newly thawed fresh frozen AB plasma and leaving for 30 min at room temperature.

**Method**

Five milliliters of bone marrow was obtained by aspiration and delivered into 10 ml incubation medium, aspirated twice through a 25G × 16mm needle, and centrifuged for 10 min at 900 g. The cells were washed once with medium and resuspended in 50 ml medium. Fifty percent sodium acetate, 0.1 ml, was added to prevent cell division that would interfere with determination of cell loss by heme extraction. Heme was isolated from both supernatant and cell fraction and from the 24-hr supernatant using a modification of the method of Falk. $^{59}$Fe-heme extraction. Heme was isolated from both supernatant and cell fraction and from the 24-hr supernatant using a modification of the method of Falk. $^{12}$Fifteen milliliters cold freshly prepared 5% HCl in acetone was added, and after mixing well, the samples were left at 4°C for 30–60 min and then centrifuged for 10 min at 900 g. The heme was extracted from the supernatant into 10 ml ethyl ether after neutralization with 10 ml saturated sodium acetate. A drop of ferric citrate, 500 μg/ml, was added to aid extraction of any free $^{59}$Fe in the aqueous phase. The ether layer was washed once with 20 ml 5% HCl in saturated sodium acetate and once with 20 ml 5% HCl. A 0.5-ml quantity of NCS solubilizer was added and the ether evaporated under nitrogen to a volume of 2 ml. The efficiency of extraction of heme by this method was shown to be 85% for both supernatant and cell fractions, using $^{59}$Fe labeled red cells prepared in vivo. The samples were counted on a Wallac counter, and the radioactivity in the supernatant and cell layers was corrected by subtraction of the value for the appropriate layer of the blank sample. All counts were at least 10 times the blank value. The supernatant value was further corrected for cell death occurring within the first 24 hr by addition of the $^{59}$Fe heme counts in the 24-hr supernatant. The percentage $^{59}$Fe-heme release was then calculated as the percentage of total $^{59}$Fe-heme counts appearing in the supernatant. The method was shown to have a coefficient of variation of 8%.

In a series of experiments to determine the relationship of heme release to death of erythroblasts, duplicate pairs of 96-hr cultures of random routine bone marrow samples were set up with colcemid, 0.9 μg/ml, in the incubation medium, colcemid being added to prevent cell division that would interfere with determination of cell loss by counting. One pair of cultures was used for the measurement of heme release as described above. The other pair of cultures was used for counting the erythroblast numbers by performing total viable cell counts and differential counts at 0 and 96 hr.

**RESULTS**

**Preliminary Experiments**

Preliminary experiments were undertaken to determine the period of optimum exposure to $^{59}$Fe and the optimum time for incubation of the cultures. Initially, the cells were exposed to $^{59}$Fe for 4 hr, as in Myhre's original work. However, the levels of radioactivity in the supernatant were frequently too low to be measured accurately, especially when ineffective erythropoiesis was normal. For this reason, further studies were carried out exposing the cells to $^{59}$Fe for
Correlation of Heme Release With Loss of Erythroblasts Determined by Counting

There was good correlation between $^{59}$Fe heme release and loss of erythroblasts determined by counting ($r = 0.85$, $p < 0.001$; see Fig. 2), but the percentage loss of erythroblasts always exceeded the percentage release of labeled heme. This may reflect some difference between the stages of maturation at which $^{59}$Fe is taken up compared to the stages most subject to cell death.

Heme Release in Various Types of Anemia

The results are shown in Figs. 3–6 and Table 2. In the conditions with normoblastic erythroid hyperplasia, heme release was within the normal range in all except one of the 11 patients (see Fig. 3), showing that...
hyperplasia per se does not result in increased heme release (although the total amount of labeled heme synthesized is usually greater). In contrast, the release of $^{59}$Fe heme was markedly increased in all patients with severe megaloblastic anemia and sideroblastic anemia and in the patient with congenital dyserythropoietic anemia (see Fig. 4). In a few patients with mild megaloblastic anemia, there was a modest increase in heme release, but in the majority it was within the normal range. When all the cases of megaloblastic anemia was considered, there was a significant correlation between the degree of ineffective erythropoiesis and the severity of the anemia ($r = 0.88$, $p < 0.001$; see Fig. 5).

All the patients with pernicious anemia in whom studies were repeated shortly after $B_{12}$ showed a return of heme release to normal values (see Fig. 6).

**DISCUSSION**

This paper describes a simple in vitro method for the assessment of ineffective erythropoiesis based on the release of $^{59}$Fe heme from a labeled cohort of erythroblasts. The validity of the method depends on the assumption that $^{59}$Fe heme release is an index of cell death, and this has been tested in two ways, first...
by a comparison of $^{59}$Fe heme release with cell death in vitro as determined by counting, and secondly by a study of $^{59}$Fe heme release in various hematologic disorders that are either known to be associated with increased ineffective erythropoiesis or known not to be so associated.

It is difficult to devise a satisfactory in vitro test to check the validity of the method. It would be possible to compare release of heme with that of other cellular constituents, but hemoglobin is virtually the only measurable cellular constituent that is confined to erythroblasts and not present in other cell lines in the marrow. Comparison with death of erythroblasts determined by counting suffers from the disadvantage that one cannot morphologically identify the labeled cohort of cells and determine their survival, but this was the only practical method available. The results of this comparison confirmed that heme release is closely correlated, with death of erythroblasts. The two figures were not identical—heme release always being exceeded by the percentage loss of erythroblasts. This suggests that labeled heme is not lost in significant amounts from viable cells.

The validity of the method is further supported by the data obtained in the various disorders studied. Thus, heme release is invariably increased in conditions where there is known to be excessive ineffective erythropoiesis, i.e., severe megaloblastic anemia, sideroblastic anemia, and congenital dyserythropoietic anemia. In patients with megaloblastic anemia, heme release was correlated with the severity of the anemia. The absence of any increase in heme release in the mildest cases deserves comment. There are two possible explanations, either that the technique is not sufficiently sensitive to detect minor increases in ineffective erythropoiesis, or that the amount of ineffective erythropoiesis is indeed normal, which would be consistent with the normal bilirubin levels observed in these patients. However, as no direct measurements of ineffective erythropoiesis in mild megaloblastic anemia have been reported, it is necessary to perform in vivo studies to confirm this hypothesis. The rapid return to normal of heme release after treatment of pernicious anemia with $B_{12}$ is consistent with in vivo bilirubin studies, showing that reversal of ineffective erythropoiesis occurs within 24 hr of giving $B_{12}$.

### Table 2. $^{59}$Fe Heme Release in Control Subjects and Various Types of Anemia

<table>
<thead>
<tr>
<th>Group</th>
<th>72-hr Incubation</th>
<th>96-hr Incubation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$^{59}$Fe Heme Release (%)</td>
<td>t Value; p Value</td>
</tr>
<tr>
<td>Control</td>
<td>13 9.1 ± 1.8</td>
<td>12 13.7 ± 4.1</td>
</tr>
<tr>
<td>Normoblastic hyperplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A) Hemolytic anemia</td>
<td>1 4.6</td>
<td>4 19.2 ± 3.9</td>
</tr>
<tr>
<td>(B) Acute hemorrhage</td>
<td>1 9.9</td>
<td>1 8.3</td>
</tr>
<tr>
<td>(C) Polycythemia</td>
<td>1 8.0</td>
<td>3 17.2 ± 4.7</td>
</tr>
<tr>
<td>Dyserythropoietic anemias</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A) Congenital dyserythropoietic anemia</td>
<td>— — — 1 34.7</td>
<td>— — — 1 34.7</td>
</tr>
<tr>
<td>(B) Sideroblastic anemia</td>
<td>2 28.8 ± 3.5</td>
<td>3 35.9 ± 1.70</td>
</tr>
<tr>
<td>(C) &quot;Severe&quot; megaloblastic anemia</td>
<td>5 24.0 ± 16.0</td>
<td>6 50.0 ± 10.5</td>
</tr>
<tr>
<td>(D) &quot;Mild&quot; megaloblastic anemia</td>
<td>5 14.9 ± 5.8</td>
<td>8 17.1 ± 6.8</td>
</tr>
</tbody>
</table>

*Student's t test, $(n_1 + n_2) - 2 df$.

NS, not significant at $p$<0.01 level.
Confirmation that the increased heme release observed in the above conditions was due to ineffective erythropoiesis and not to marrow hyperplasia per se is given by the studies in patients with normoblastic hyperplasia of varying etiology. Only one of these patients showed an increase in heme release, the remainder being normal in spite of considerable hyperplasia in some cases. Furthermore, in the patients studied after treatment of pernicious anemia, heme release returned to normal in the presence of continued narrow hyperplasia.

While these observations are consistent with the validity of the present method, they do not substitute for a direct comparison with a quantitative in vivo measurement of ineffective erythropoiesis. Although the ferrokinetic method is the easiest of the in vivo methods to perform, because of the doubts that have been raised as to its accuracy, \(^1,15,16\) as is also the case with “classical” early-labeled peak methodology employing labeled glycine, \(^10\) it would be of interest to use a newer early-labeled bilirubin method, \(^5\) which would also provide data more closely analogous with heme release. However, because of the complexity of this method, it would not be possible to carry out a comparison in a large series of patients.

Although measurement of heme release does not provide an absolute figure for ineffective erythropoiesis, and as yet the precise relation between the values for heme release and values for ineffective erythropoiesis measured in vivo have not been determined, the technique does enable quantitative evaluation of different disorders and of the progress of individual patients. By using marrow obtained at routine diagnostic aspiration, it overcomes the disadvantages of in vivo methods described in the introduction. It is applicable to the majority of patients and can be repeated as often as marrow can be sampled. It therefore provides a valuable means of following the progress of a disease or monitoring the effect of treatment. This method should also prove useful in the study of the effect of drugs, toxins, and abnormal sera on normal marrow.

REFERENCES

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