Humoral Regulation of Erythropoiesis. XI. The Pattern of Response to Specific Therapy in Iron Deficiency Anemia

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Initially, macrocytic red cells with a shortened life span are produced in response to intense erythropoietic stimulation, e.g., in phenylhydrazine-induced anemia, severe blood loss, exposure to hypoxia or the administration of erythropoietin. We have postulated that these macrocytes result from a shortening of the interval between differentiation of stem cells and emergence of reticulocytes together with skipping of divisions in the erythroid compartment. In severe iron deficiency anemia, an intense erythroid stimulation may be present but microcytes are produced. Correction of the iron deficiency in the presence of this intense erythroid stimulation might, therefore, result in the production of macrocytes. This thesis was tested in human beings and rats with iron deficiency anemia.

Materials and Methods

Weanling female Sprague-Dawley rats were given a powdered milk diet supplemented with essential vitamins and minerals except iron. The control rats received a similar diet to which 200 mg. of FeCl₃ was added per Kg. of diet. Two experiments were done. In one, 110 rats were given the milk diet and 50 received the milk diet with iron supplement. In the second experiment, 70 animals were given the milk diet and 50 received the milk diet with supplemental iron. Red cells were counted electronically; hemoglobin, hematocrit, and reticulocytes were measured by standard methods; cell size distribution curves were made electronically with a Coulter particle size distribution plotter. Red cells were separated by centrifugation in a Wintrobe tube at 3000 rpm for 30 minutes. The blood column was then divided into either three equal fractions or four unequal portions (starting from the top: 10 per cent, 20 per cent, 30 per cent, and 40 per cent) and resuspended in 90 per cent Eagles Solution for measurement of cell size distribution and cell constants.

The survival of newly formed cells was measured with radioiron as previously described.

Ten patients were studied during recovery from iron deficiency anemia. These patients could only be seen at irregular intervals and most of them had started iron therapy before they were seen by us. The observations, therefore, served primarily to indicate the relationship of experimental and clinical findings.

Results

The results of the two separate experiments were indistinguishable and were, therefore, pooled for purposes of the present report. The gradual development of an iron deficiency anemia was followed by spot checks on randomly selected animals. Samplings indicative of the general trend were as follows:

The decrease in mean corpuscular volume (MCV) preceded the fall in mean
corpuscular hemoglobin concentration (MCHC); for example, after 6 weeks two animals had a hemoglobin of 8.0 and 8.2 Gm., respectively; the MCHC were 36 and 35 (normal 34-37); MCV on both animals was 30 μℓ (normal 52-58). After 10-12 weeks the iron-deficient animals weighed 159 ± 6.4 Gm. (10 animals) as compared with 188 ± 3.4 Gm. in the controls. The mean hemoglobin value for five animals was 7.5 ± 1.2. The MCHC was 27.4 ± 1.3; the MCV was 31 ± 1.5 μℓ. The distribution of cell size is shown in figure 1. At this point the rats were treated with daily intramuscular injections of 5 mg. of Imferon.* By the second day there was a demonstrable increase in the size of emerging cells (fig. 2). The most macrocytic cells were in the top fraction, which has been previously shown to contain those cells with the lowest MCHC.2 Macrocytes, however, were seen in all fractions after separation by centrifugation (fig. 2). Reticulocytes were also scattered throughout the separated fractions, a finding compatible with the notion that the macrocytes distributed throughout the centrifuged column were newly emerged cells. By the 5th day the macrocytes were found primarily in the bottom fraction of the centrifuged column (fig. 3), presumably because the hemoglobin content of the early macrocytic cohort of cells had increased. In the top fraction there were only the iron-deficient pretreatment cells which had a lower MCHC. The cells produced thereafter, though still macrocytic, were more nearly normal.

*Imferon—a dextran-iron complex. We are grateful to Drs. H. M. Leyland and Howard Albright of the Lakeside Laboratories, Milwaukee, Wis., who generously supplied us with the Imferon used in this study.
Fig. 2.—Red cell size distribution in whole blood and in the fraction of a centrifuged column of blood on the 2nd day of iron therapy. Note presence of macrocytes in all fractions, most prominently in top layer.

Fig. 3.—Red cell size distribution in whole blood and in the fractions of a centrifuged column of blood on the 5th day of iron therapy, showing a predominance of macrocytes in the bottom layers of the column.
Fig. 4.—Red cell survival (using Fe$^{59}$) of iron-deficient animals during iron therapy, and of controls. The radioactive iron was administered on the day therapy was initiated.

in size; on day 15 the modal value was $64 \mu^3$, compared with $90 \mu^3$ on day 2 and the control of $52 \mu^3$ (fig. 1).

As recovery progressed, the upper fraction of the separated blood continued to contain predominantly microcytic cells; e.g., on day 20 the top 10 per cent of the centrifuged column contained over 80 per cent microcytic cells (MCHC $< 38 \mu^3$). At this time the bottom fraction contained 23 per cent microcytes (MCV $< 38 \mu^3$) and 40 per cent macrocytes (MCV $> 64 \mu^3$).

The macrocytes were short-lived as evidenced by the shift in the cell size distribution curve between day 15 and day 31 after the start of treatment (fig. 1). Further support for shortening the life span of these cells was provided by direct measurement of survival with Fe$^{59}$. Radioiron was given on the day on which treatment was instituted. The red cell survival curve from one experiment is shown in figure 4. In a second experiment, comparable results were obtained.

In the patients with iron deficiency, the degree of anemia and the magnitude of the macrocytic response to iron was variable. In one patient, who had 4 Gm. of hemoglobin at the start of treatment, the response to iron was comparable to that seen in the severely iron-deficient rats. By day 4 a slight macrocytosis was evident which was quite striking by day 14 (fig. 5). The size distribution curves of fractions of blood separated at that time (14 days after start of therapy) are shown in figure 6. At that time the hemoglobin had increased to 6.5 Gm. As in the rats, the early cohorts of cells produced in response to iron therapy were the most macrocytic. Thereafter, as recovery progressed, more
Fig. 5.—Red cell size distributions obtained during treatment of a severely iron-deficient patient (K. B.) with oral iron.

Fig. 6.—Red cell size distributions in whole blood and in the fractions of the centrifuged column in patient (K. B.) on the 14th day of iron therapy.
nearly normal-sized cells were produced (fig. 5). Although isotope administration to measure cell survival was not thought warranted in this patient, inspection of the cell size distribution curve suggested a shortening of life span of macrocytes produced during the early part of recovery. It should be noted that the change in the shape of the curve is accounted for, in part, by a continuous increase in the number of normal-sized cells produced as the severity of the anemia, and thereby the stimulus for a macrocytic response decreased. In fact, the total RBC increased from $2.89 \times 10^6$ on day 14 to $4.39 \times 10^6$ on day 35. The number of cells with a MCV of greater than 113 $\mu^3$, however, decreased from $6.25 \times 10^6$ to $5.5 \times 10^6$ during this 20-day period, indicating that there was some shortening of red cell life span in this cohort of newly formed cells.

In a second patient (L. L.), the hemoglobin on admission was 7.2, the MCV 66 and the MCHC 24. The MCV increased to 74 and the MCHC to 26 during the first 2 weeks of treatment. During that entire period normocytes and macrocytes were seen predominantly in the bottom layer of centrifuged blood. The size distribution, illustrated in figure 7, indicates the presence of significant numbers of macrocytic cells in all post-treatment samples. However, on successive dates, cells of normal size became relatively more frequent as indicated by the progressively steeper slope of the right-hand side of the distribution curve. Reticulocytes, which originally concentrated in the top layer, later
gravitated to the middle and bottom layers (table 1). This is in keeping with the observations on iron-deficient rats.

In a third patient (L. I.), the hemoglobin was 7.8, the MCV 62, and the MCHC 27. This patient did not respond as promptly to iron therapy, and the reticulocyte count exceeded 2 per cent only on a single occasion. The shift of reticulocytes from the top to the middle and bottom layers was barely discernible, presumably because the total reticulocyte count had fallen to less than 1 per cent by the time the shift occurred. The initial macrocytosis was also minimal.

In seven additional patients the hemoglobin at the start of treatment was greater than 9 Gm. and the modal value for cell size was of the order of 60 μm, except one patient in whom the initial hemoglobin was 7.6, the MCV 82 and the MCHC 28. In all of these patients macrocytes were produced, but the degree of macrocytosis was not comparable to that seen in the rat. Moreover, a clear-cut separation of macrocytic reticulocytes in the centrifuged column of blood was not achieved.

**Discussion**

When iron deficiency was produced by restriction of dietary intake in rats, the first red cell abnormality was a reduction in the size of the cells, a finding similar to that reported by Crosby in patients made iron deficient by phlebotomy. Thereafter, the MCHC diminished. After 2 to 3 months the classical picture of iron deficiency anemia evolved and a single population of hypochromic microcytic cells was present.

After treatment with intramuscular iron, macrocytes were produced. The modal value for cell size of this initial cohort of cells was 90 μm with some cells in excess of 120 μm.

If macrocytes result from skipped divisions and early release, the largest cells would be those with the shortest emergence interval. Because of the decreased maturation interval, it might be expected that hemoglobin synthesis would not have progressed to the level normally seen in reticulocytes. This has now been demonstrated by direct measurement by Borsook et al. These cells would then have a low MCHC and appear in the top fraction of a centrifuged column of blood. A tendency toward segregation of the largest cells in the top layer was indeed seen (fig. 2). However, macrocytic reticulocytes were present throughout the column, presumably reflecting varying
degrees of hemoglobin concentration of the emerging cells. These reticulocytes continue to synthesize hemoglobin. Thus, the MCHC increases to normal values, which exceed that of the majority of pretreatment iron deficient cells, causing the newly formed macrocytes to gravitate to the bottom of the column. The pretreatment microcytes with a low hemoglobin content were found in the upper fractions of blood. It should be noted that microcytes were present in significant numbers throughout the centrifuged column (figs. 3 and 6), indicating that many microcytes are well hemoglobinized.

Continued observation of the cell size distribution curve pointed to the fact that the initial cohort of cells had a shortened cell life span. Further support for this was gained from the measurement of red cell life span with the radiiron technic. The finding of the production of defective macrocytes which are short-lived supports the hypothesis that under intense erythroid stimulation and in the presence of adequate nutrients, there is shortening of the maturation interval with skipping of divisions. As recovery from the anemia progressed, the intensity of the erythroid stimulation decreased and more nearly normal cells were produced.

In one patient with a hemoglobin of 4 Gm., a clear-cut macrocytic response was seen after treatment with iron as in the experimental animals. Although survival was not measured directly, data derived from the cell size distribution curve pointed to the fact that the initial cohort of macrocytes produced in this patient did, in fact, have a shortened life span. In another patient, the characteristic shift of reticulocytes through a column of centrifuged blood was observed. In the other patients studied, the anemia was not severe enough to evoke a clear-cut macrocytic response seen in the more severely iron-deficient rats and man.

The mechanism of macrocytic response may be mediated by erythropoietin. Erythropoietin was demonstrated in cases of iron deficiency anemia with hemoglobin levels below 5 Gm., but was not found at higher hemoglobin levels. This inability to demonstrate erythropoietin at higher levels of hemoglobin is thought to be due to utilization of erythropoietin by a functionally competent stem cell compartment.

**SUMMARY**

Short-lived macrocytes were produced in response to specific therapy in severely iron-deficient rats and in two patients. These findings add weight to our hypothesis that the production of defective, short-lived macrocytes is a phenomenon common to states in which there is marked erythroid hyperactivity in the presence of adequate nutrients.

**Summario in Interlingua**

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ACKNOWLEDGMENTS

The authors gratefully acknowledge the able technical assistance of Mrs. Helen Grimes, Mr. Donald Howard, and Miss Antoinette Beland.

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