The Effect of Bleeding on the Proportion of Red Fluorescing Forms among the Total Normoblasts of Bovine Porphyric Bone Marrow

By Walter Runge and C. J. Watson

IN CONNECTION with the designation of porphyria congenita (Günther) as p. erythropoietica it was recognized that while many of the bone marrow normoblasts fluoresce and, in particular, exhibit strong nuclear fluorescence, many others, indistinguishable by ordinary morphologic criteria, did not exhibit porphyrin fluorescence. Many of the fluorescing forms exhibited heme containing nuclear inclusions but these were not invariably correlated, either positively or negatively, with excessive porphyrin content as indicated by fluorescence. At the time of these early papers the possibility of a bimodality of the normoblasts was suggested: 1) An abnormal type characterized by the genetic disturbance in porphyrin metabolism and the presence of large amounts of uroporphyrin; 2) a normal non-fluorescing type. If such a bimodality existed it might be explained as a form of mosaicism.

Others have described morphologic evidence favoring a bimodality in the human disease, and have referred to the fluorescing normoblasts as "porphyroblasts" in contrast to the normal type. It is possible, however, that the changes described might in some way be related simply to the abnormal metabolism evidenced by the great excess of uroporphyrin. It was conceivable that the normoblasts might be unimodal, the metabolic disturbance and hence the fluorescence, depending on dynamic factors such as the stimulus to erythropoiesis and consequent rate of normoblast maturation or the extent to which developing red cells, even though possessing the genetic trait, might be in a "resting" state in terms of excessive porphyrin formation at the time of examination.

The first of our human cases of erythropoietic porphyria had a marked hemolytic anemia and was splenectomized after which the porphyria decreased greatly in terms of the erythrocyte, urinary and fecal porphyrin concentrations. This was construed as the sequel of a reduced erythropoiesis in response to removal of the stimulus of excessive hemolysis. In retrospect it would have been of much interest to determine the percentage of fluorescing vs non-fluorescing normoblasts during the post-splenectomy state but this was not...
done or even considered at that time and subsequently an opportunity has not presented to obtain bone marrow under these conditions.

More recently, in studies of the bovine form of erythropoietic porphyria, it became evident that bleeding was followed promptly by a marked increase in concentration of circulating erythrocyte porphyrins, especially Uro-7. This was in accord with the belief that increased erythropoiesis augmented the abnormal formation of porphyrins. It was therefore of interest to determine whether this was associated with a proportional increase of two types of normoblasts, fluorescing and non-fluorescing, in accord with the concept of bimodality, or whether the percentage of fluorescing normoblasts might increase to a degree more suggestive of a single mode. It was considered that if the great majority of nucleated red cells exhibited red fluorescence as a result of bleeding, this would be difficult to reconcile with a bimodal theory as it would be highly unlikely that but one of two types would respond to the stimulus, especially in view of the well known response under normal conditions.

With these considerations in mind counts of fluorescing vs non-fluorescing normoblasts have been made on bone marrow of homozygous bovine porphyrics, and on that of normal controls, before and after bleeding. In most cases the identity of the animal and its status was not known to the individual making the counts.

**Materials and Methods**

Bleeding of various amounts over varying short periods was carried out in nine animals, five of which were homozygous for the disease, with pink teeth and red urine. Four normal animals were studied similarly, as controls (Table 1).

Bone marrow was aspirated from the sternum into heparinized syringes. Care was taken to avoid dilution with peripheral blood. In one porphyric and one normal calf, bone marrow was obtained by squeezing a piece of resected rib in a vise. Bone marrows were obtained before and shortly after bleeding.

As many direct bone marrow smears as possible were made, at least 25 per bone marrow sample. A great number of smears had to be available in order to avoid photo fading of the porphyrin fluorescence in the course of cell counts made with the fluorescence microscope.

The dry smears were mounted with pyridine-glycerine (1:3) and were immediately placed under a suitable fluorescence microscope. A Reichert Biozet microscope equipped with phase contrast objectives and Polyphos condensor was used on a previously modified Reichert Fluorescence Base. An aspherical relay lens had been installed into this base. The Polyphos condensor provided bright field, phase contrast or dark field illumination, optionally and sequentially, without disturbing the field of view in focus.

For the recording of fluorescence spectra of individual normoblast nuclei, the micro-fluorospectrophotometer (MFSP), as previously described was used.

In the mounted bone marrow smears, the red fluorescing normoblasts were counted upon excitation under dark field illumination, and the total number of normoblasts in the same field of view was recorded after adjustment of the Polyphos condensor to phase contrast illumination with blue light.

Excitation filter for the mercury light was a Corning glass filter #5113 CS5-58, and the barrier filter was composed as follows: 1) FG10, 0.2 mm.; 2) Wratten #4; 3) Harrison and Harrison #C4; 4) Corning #7380, 0.1 mm. These were cemented with Gurr's Fluormount in the order given.

At least 200 normoblasts were counted in a maximum of two widely separated fields of view per slide. Great care was taken not to expose any slide for more than two minutes.
Table 1.—Percentage of Fluorescing Normoblasts in the Bone Marrow of Porphyric and Normal Bovine Subjects Before and After Bleeding

<table>
<thead>
<tr>
<th>No.</th>
<th>State</th>
<th>Sex</th>
<th>Age, months</th>
<th>Wt., kilo.</th>
<th>Incl. dates of bleeding</th>
<th>Total amount of blood removed, liters</th>
<th>Per cent fluorescing normoblasts Before</th>
<th>Source of bone marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>2318</td>
<td>Porph.</td>
<td>F</td>
<td>64</td>
<td>156</td>
<td>6-5 to 6-8</td>
<td>2.5</td>
<td>32 91</td>
<td>Sternum</td>
</tr>
<tr>
<td>2966</td>
<td>&quot;</td>
<td>M</td>
<td>10-21 to 10-30</td>
<td>2.0</td>
<td>26 78</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2252</td>
<td>&quot;</td>
<td>M</td>
<td>7-17 to 7-21</td>
<td>4.0</td>
<td>50 81</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26XM</td>
<td>&quot;</td>
<td>M</td>
<td>10</td>
<td>220</td>
<td>4-0</td>
<td>50 81</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>3033</td>
<td>&quot;</td>
<td>M</td>
<td>6-26 to 7-16</td>
<td>11.2</td>
<td>46 80</td>
<td>Rib</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2327</td>
<td>Normal</td>
<td>F</td>
<td>43</td>
<td>100</td>
<td>8-21 to 8-25</td>
<td>1.5</td>
<td>2 2</td>
<td>Sternum</td>
</tr>
<tr>
<td>2326</td>
<td>&quot;</td>
<td>F</td>
<td>43</td>
<td>100</td>
<td>8-21 to 8-25</td>
<td>1.5</td>
<td>1 2</td>
<td>&quot;</td>
</tr>
<tr>
<td>26WM</td>
<td>&quot;</td>
<td>M</td>
<td>10</td>
<td>300</td>
<td>6-26 to 7-16</td>
<td>12.7</td>
<td>0.3</td>
<td>Rib</td>
</tr>
</tbody>
</table>

* Data unrecorded.
† The difference before and after is not believed significant, each value, however, significantly below 1 per cent.

to the illuminating beam of light. The number of red fluorescing normoblasts was noted as a percentage of the total number of normoblasts. The values are believed reproducible at the 1 per cent level, and are so indicated in the following, with one exception (Table 1).

### RESULTS

The percentages of fluorescing normoblasts, before and after bleeding, are given in Table 1, together with the amounts of blood removed over given periods of time. It is apparent that a striking increase in proportion of the fluorescing cells occurred in each of the five porphyric animals, the final value ranging from 66 per cent to 91 per cent in contrast to the initial range of 26 per cent to 50 per cent.

The intensity of the red fluorescence emitted by the normoblasts varied considerably among individual cells. In confirmation of earlier observations, the red fluorescence of the porphyric normoblasts was mainly confined to the nuclei whereas the fluorescence of the cytoplasm was much less noticeable. In the small percentage of fluorescing normoblasts from the normal animals, the fluorescence was mainly cytoplasmic, relatively weak and disappeared rapidly under illumination, these characteristics suggesting that it was mainly protoporphyrin. The fluorescence intensity was too weak to permit recording in the MFSP. The intensity of the more pronounced nuclear fluorescence of the porphyric normoblasts ranged from barely visible red to bright orange-red. After about three minutes of fluorescence excitation, the red fluorescence in the majority of cells changed to a muddy yellow green. In a significant number, however, red fluorescence was still superimposed on the blue phase contrast image. It was noticeable that this photo fading and change in color of the porphyrin related fluorescence occurred first in the cells which initially had lesser fluorescence intensity. Because of the time factor the counting of the fluorescing normoblasts was accomplished during the first 1.5 minutes of exposure of any given field to the exciting light. Studied promptly in this way it was determined that mounting of the dry bone marrow smear in
pyridine-glycerine did not adversely affect the quality of the fluorescence image, nor was the phase contrast image impaired. In about six minutes after mounting of the smear the phase image of all cell types becomes washed out in appearance.

Inclusion bodies in the nuclei of fluorescing normoblasts were only observed rarely in the present study, in contrast with previous observations in the human disease. It was determined with the MFSP that the nuclear fluorescence of bovine normoblast nuclei originates from a mixture of uro- and coproporphyrin and derived oxyporphyrin. In glycerol-pyridine, 100:1, the λ max. of the fluorescence spectral bands of these porphyrins were as follows: Uroporphyrin 6260A, coproporphyrin 6230A, oxy-uroporphyrin 6590A, oxy-coproporphyrin 6540A. The fading and color shift properties of such cells are sufficiently characteristic that a muddy yellow-green fluorescence after considerable exposure to the exciting light indicates that the cell in the fresh state would have exhibited red fluorescence.

It seems logical to anticipate that those fluorescing normoblasts, in which the fluorescence intensity is close to or is below the threshold of color vision will escape counting. The magnitude of this error is unknown but it is safe to assume that the actual number of fluorescing normoblasts is somewhat greater than expressed in the percentages given in Table 1.

**Comment**

The increase in percentage of fluorescing cells after bleeding is of a magnitude scarcely in accord with a bimodal concept (Table 1). While it is conceivable that some imbalance might exist in rate of response if there were two basically different forms of normoblasts in terms of porphyrin metabolism, it is highly unlikely that the present disproportions could be on this basis. It is evident from Table 1 that very few fluorescing normoblasts are observed in normal bovine, as in normal human bone marrow, and that the increase after bleeding is slight, yet the increase of normoblasts in the normal bovine marrows, after bleeding, appeared to correspond roughly to that of the porphyric marrow.

The relatively large amount of oxyporphyrin in the bovine as contrasted with the human normoblasts may be related to the longer periods that the bovine bone marrow was exposed to air, in transit from farm to laboratory. The red fluorescing oxyporphyrin is thus regarded as a chemical artifact, having the same basic significance as the uro- or coproporphyrin from which it was derived. The above-mentioned muddy yellow-green fluorescence is believed due to further oxidation to compounds as yet unidentified.

If, as the present findings indicate, the normoblasts of the bovine disease are unimodal in respect to the genetic abnormality, it will remain to be determined whether the human disease is fundamentally similar. It is true that the bovine porphyric normoblast nuclei exhibit inclusions with less frequency than is true in the human disease; also, it appears that there is considerable variation from time to time in the same animal. Furthermore, it was pointed out in an earlier paper that the erythrocytes of the bovine porphyric contain much larger amounts of protoporphyrin than those of the human disease.
SUMMARY AND CONCLUSION

The percentage of fluorescing, porphyrin rich normoblasts in the bone marrow of five bovine porphyrics increased after bleeding to a range of 66–91 per cent, as contrasted with 26–50 per cent in the same animals before bleeding. This is believed to be more in accord with a unimodal distribution of normoblasts in respect to the genetic abnormality and the resultant overproduction of porphyrin.

REFERENCES

The Effect of Bleeding on the Proportion of Red Fluorescing Forms among the Total Normoblasts of Bovine Porphyric Bone Marrow

WALTER RUNGE and C. J. WATSON