A STUDY OF SICKLING OF YOUNG ERYTHROCYTES IN SICKLE CELL ANEMIA

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THE COMPARATIVE scarcity of sickled reticulocytes and normoblasts in patients with active sickle cell disease has been sufficiently striking to arouse comment by several students of that disease.[1, 1a, 2, 3] This observation led Murphy and Shapiro[3] to postulate that the occurrence of hemolytic crises might be due in part to the increasing tendency of the red cells to sickle on aging, in which case the reticulocytosis of a crisis might of itself be beneficial. The fact that their patient showed a higher percentage of sickle cells in the plain smear before crises than afterwards seemed to lend further support to this theory. In reviewing many blood smears of sickle cell patients, I was able to find only two normoblasts and one reticulocyte in the sickled form. A quantitative study of the sickling of reticulocytes was therefore undertaken.

MATERIAL

Three patients with active sickle cell anemia from the Hematology Clinic of Kings County Hospital were chosen for study because they consistently showed a high percentage of reticulocytes and sickle cells in their blood. The Wintrobe oxalate mixture was used as an anticoagulant for the venous blood obtained.

METHODS AND RESULTS

1. *Sealed Preparation*

(a) *Blood.* Oxalated blood was mixed with equal parts of 0.5 per cent brilliant cresyl blue in 0.85 per cent NaCl solution. One drop of the mixture was used to make the standard slide-cover slip preparation. This was sealed with paraffin, incubated at 37C., and examined for sickling at frequent intervals. In all cases sickling was complete within two to four hours. This included the reticulocytes as well as the occasional normoblasts present. It was the impression that most of the reticulocytes sickled as soon as the mature red cells, with the exception of some of the most immature reticulocytes which were packed full of reticulum. The normoblasts appeared to be the last to sickle. The progressive sickling of the reticulocytes could not be counted accurately by this method, however, because of the irregularity of sickling in different parts of the same preparation. This irregularity has been mentioned by others, and is probably largely due to uneven distribution of the leukocytes, which have an accelerating effect on the sickling of the red blood cells,[4, 5] presumably through lowering oxygen tension by metabolism. Platelet distribution may also be a factor.[6]

(b) *Bone Marrow.* One cc. of sternal marrow was obtained from one patient (W. B.). The buffy layer was used in a sealed preparation, made as described above, in order to study the sickling of normoblasts. Cresyl blue stains the cytoplasm of the basophilic and polychromatophilic normoblasts, but not that of the orthochromatic normoblasts unless it happens to be reticulated. The reticular network is easily distinguished from the diffuse blue staining of the basophilic normoblasts. It was found that none of the basophilic and polychromatophilic normoblasts were sickled after 24 hours incubation at 37C., whereas most of the orthochromatic normoblasts were in the sickled state. Two of the latter type containing Howell Jolly bodies, however, failed to sickle. Since the orthochromatic normoblasts have a full quota of hemoglobin in their cytoplasm, their ability to sickle is not surprising. Further evidence for the primary role of hemoglobin in sickling has been presented recently by Ponder,[7] who showed that when menisocytes were made into ghosts by lysis of their hemoglobin, they lost their ability to sickle.

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2. *Gas Chamber Method*

The apparatus used was essentially that described by Hahn and Gillespie[8] except that the chamber was made of paraffin instead of glass. A water sealed outlet was found necessary.[4] Both carbon dioxide and nitrogen were used for sickling. The same saline cresyl blue blood mixture was used. Since the red cells in the hanging drop can be studied directly with the oil immersion lens, this method has the advantage that the active dynamic process of sickling can be watched easily. Here again most of the reticulocytes seemed to sickle as fast as the other red cells, but the great rapidity of the sickling—about two minutes for complete sickling—made impossible the quantitative timing of the transformation of the two types of cells. Rarely would the rearrangement of the hemoglobin in the process of sickling result in the reticulum being lost from view. Occasionally the formation of the Sherman "holly wreath" forms of sickle cells[4] made this method, as well as that of the sealed preparation, unsatisfactory. Because of these disadvantages a chamber method which would permit slower sickling and the periodic removal of cells for counting purposes was devised as follows.

3. *Gas Test Tube Method*

A test tube, 15 x 40 mm., with a capacity of 5 cc. was set up with a gas inflow and outflow via 24-gage needles through a rubber stopper. The outflow was equipped with a water seal. Carbon dioxide was used for sickling. One cc. of the saline cresyl blue blood mixture was introduced into the inverted test tube. Small samples of blood were removed at appropriate intervals under oil with an oiled tuberculin syringe and a 22-gage needle through the rubber stopper. The blood was immediately injected into formalin for fixation. Reticulocyte and sickle cell counts had to be done immediately in order to avoid inaccuracy due to slow fading of the reticulum in formalin. A 2 per cent formalin solution in normal saline was found to be as good a fixative as the standard 10 per cent solution and had less of a fading effect. A drop of the red cell suspension was placed on a slide under a cover slip, and a count was made under oil immersion of the reticulocytes and sickle cells. Only 200 cells were counted because of the time factor of fading.

TABLE 1.—*Progressive Sickling of Reticulocytes upon Aeration with Carbon Dioxide*

Patient	*Time in* CO_2	*Reticulocytes (per 100 RBC)*	*Sickle Cells (per 100 RBC)*	*Sickled Reticulocytes (per 100 RBC)*	*Sickled Reticulocytes (per 100 retics.)*
	min.				
L. J.	0	10.5	9.5	0.0	0.0
	2	10.0	45.0	4.5	45.0
	5	9.0	81.0	7.0	77.7
	10	9.0	92.5	8.5	94.4
W. B.	0	20.5	15.0	0.0	0.0
	2	19.5	28.0	4.0	20.5
	5	21.0	69.5	13.5	64.3
	10	20.0	90.0	17.5	87.5
J. W.	0	15.0	11.5	0.0	0.0
	2	16.5	33.5	4.5	27.3
	5	14.0	76.0	9.5	67.8
	10	14.5	93.5	13.0	89.6

The data obtained are shown in table 1. The number of sickled reticulocytes found per 100 RBC was divided by the number of reticulocytes per 100 RBC in order to calculate the percentage of sickled reticulocytes in terms of total reticulo-

cytes. By comparing these figures with those for the percentage of total sickled cells, it is evident that the rate of sickling of the reticulocytes is quite similar to the rate of sickling of the whole red cell population. A reticulocyte in the sickled form is shown in figure 1.

A possible theoretic objection to the fact that sickle cells are not reticulated in an ordinary smear is that the abnormal shape interferes with the supravital staining. In answer to this objection, blood was completely sickled in the test tube chamber, after which an equal amount of saline cresyl blue, previously aerated with carbon dioxide, was introduced into the test tube under oil. The blood was examined after two minutes, and the sickled reticulocytes were found to be well stained.

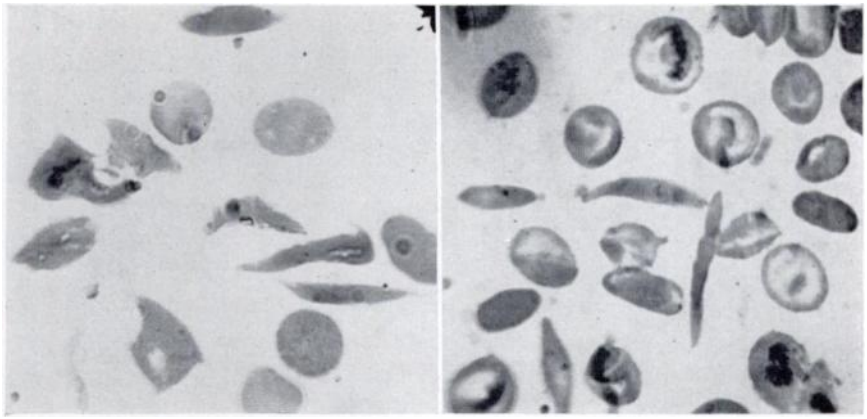

FIG. 1 FIG. 2

FIGURE 1. Meniscocytes sickled by carbon dioxide, stained with cresyl blue and immediately counterstained with Wright's stain. Cells are shown in the process of unsickling with transition forms from the sickled shape to the biconcave disk. Note the sickled reticulocyte at the left. Two crescent forms have developed elongated processes at the ends. The black spots with refractile contours are artefacts in photography.

FIGURE 2. Peripheral blood smear stained with cresyl blue and Wright's stain. Three reticulocytes and seven crescent, elliptical and oval shaped sickle cells can be seen. None of the sickle cells are reticulated. These sickled cells lack the bizarre shapes seen in Fig. 1.

In all three methods used, sickling of all erythrocytes and normoblasts was quickly reversible within a few seconds upon admission of oxygen to the system.

DISCUSSION

Direct counting of the progressive sickling of reticulocytes on aeration with carbon dioxide (table 1) by means of a gas test tube chamber has shown that most reticulocytes sickle as well as the more mature cells. However, it was noted in this and in the other methods used that the reticulocytes with the largest amount of reticulum were usually late in sickling. The few normoblasts observed were also somewhat slow. Although this may be due to the possibility that the most immature cells have a lower oxygen tension threshold for sickling, there is an al-

ternative explanation that the presence of a nucleus or of a large quantity of stained reticulum may mechanically interfere with the sickling process.

The virtual absence of reticulated sickle cells in the ordinary smear (fig. 2) seems paradoxic at first glance. The sickle cells seen in the fixed blood smear appear different from the ones produced by in vitro sickling.[2, 6, 9] The former are crescent shaped, elliptic, or oval and do not have the long processes seen in sickled preparations. When viewed directly in a hanging drop, these forms are seen to send out one or more elongated processes at the pointed ends on aeration with carbon dioxide or nitrogen, and to revert to their original shape on aeration with oxygen without going on to the normal discoid forms. For this reason these abortive sickle cells should perhaps have a distinctive terminology. It may be that they are old cells that have lost their "elasticity" from being kept in the sickled shape for long periods of time in stagnant blood vessels. Good evidence for the role of stagnation is presented by Diggs and Bibb[2] who had three patients with sicklemia who had irreversible sickle cells in their pleural or ascitic fluid, although they had none in their stained blood smears. It should be possible to produce these crescent forms in vitro by keeping cells in their sickled shape for long periods of time, but attempts so far have been unsuccessful.* It is a well known fact that sickle cells are not seen in the blood smears of persons with the sickle cell trait. This could be predicted since the oxygen tension necessary for in vitro sickling (18 mm. Hg)[4] would never occur in vivo.

Reticulocytes, being young cells, may have more "elasticity," so that they are not fixed in the sickled shape in vivo. Or it may be that the length of time for stagnation of erythrocytes to produce these abnormal forms may surpass the estimated five to six day life span of the reticulocyte.[10] If we accept Tomlinson's finding[11] that sickled cells are actually stuck in the interstices of the spleen pulp and cannot be perfused out, it would seem that the spleen could be an important dragnet of sickled erythrocytes in those patients in whom that organ had not become entirely fibrotic.

There is disagreement as to whether the number of circulating sickle cells differs from time to time in the same patient. Diggs and Bibb[2] and Smith[12] found little variation, while Sydenstricker[13] and Murphy and Shapiro[3] found that the sickle cells increased before the crisis and fell after the onset. Emmel[14] also noted significant variation in his patient. The high viscosity of sickled cells[15] and their abnormal shape tend toward their sequestration in organs, so that there could be an increasing accumulation of sickled forms without this increase necessarily being reflected in the peripheral blood. If this is so, the diverse reports among various investigators is not surprising. The increased mechanical fragility of the sickled cells[2, 16] must be an important factor in their final demolition. Thus, a crisis results in the destruction of the old sickled cells and in the outpouring of new young red cells. Although the reticulocytes appear to sickle as well as other cells, they are

* A personal communication from Dr. Shu Chu Shen indicates that sterile incubation in vitro of erythrocytes maintained in the sickled form renders them unable to reassume the discoid form upon exposure to oxygen. The technic employed was the sterile incubation of defibrinated blood samples from either anemic patients or those showing the trait for only twenty-four hours. This incubation was carried out after preliminary equilibration with and during continuous exposure to a gas mixture composed of 90 per cent nitrogen and 10 per cent carbon dioxide.

not found in the irreversibly sickled shape. As the maturing red cell stagnates in anoxic organs, irreversible sickle forms appear, and augment the vicious cycle of stagnation, anoxemia, increasing sickling, thrombosis, and hemolysis.

Summary

1. Data have been presented to show that most reticulocytes from patients with the sickle cell trait or sickle cell anemia sickle as readily as do more mature red blood cells. The most immature reticulocytes and normoblasts tend to sickle more slowly.

2. Orthochromatic normoblasts were the only type of normoblasts which sickled; the basophilic and polychromatophilic types could not be sickled.

3. It is suggested that the sickle cell forms seen in ordinary stained smears represent old cells which have lost their "elasticity" while stagnating in the sickle shape, and are unable to revert to a biconcave disk. This would explain the fact that these forms are so rarely found to be reticulated when stained with brilliant cresyl blue.

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