THE INVOLVEMENT OF THE NUCLEAR SUBSTANCE OF ERYTHROCYTES IN EXPERIMENTAL HEMOLYTIC ANEMIAS

A. Ya. Kreimer

Institute of Pathological Physiology (Head – Prof. E. I. Goldberg)
Tomsk Medical Institute
(Presented by Active Member of the AMN SSSR V. N. Chernigovskii)
Translated from Byulleten' éksperimental'noi biologii i meditsiny Vol. 49
No. 3, pp. 35-38, March, 1960
Original article submitted February 18, 1959

There is no agreement concerning the nature of the degenerate remains found in erythrocytes lysed by various hemolytic agents. The fact that only mature erythrocytes are damaged, no degenerate structures are found in the young red cells, and the Feulgen reaction is negative have led Ya. Ya. Sokolovskaya [7, 8] to think that they result from injury and coagulation of the hemoglobin. E. I. Freifel'd [9] maintains that these structures are of nuclear origin. The fact that the hemolytic poisons do not affect the reticulocytes [9] is explained as being due to the presence of a basophil substance which prevents the degenerative structures from forming. There are other views, intermediate between those expressed above, concerning the origin of these structures.

Nevertheless, this problem is of considerable importance because an explanation of the nature of the degenerate structures may give information concerning their relation to certain preformed substances or to substances developing under the influence of the poison, and, in this way, may lead to a deeper understanding of the process whereby the erythroblasts lose their nuclei to form normal erythrocytes.

To study the nature of the degenerate structures, we investigated whether the hemolytic poisons affect the erythroblast nucleus and nuclear remains of young erythrocytes, which are stained by the hemolytic stain Azur II-eosin of D. I. Goldberg's method [1] and give a positive Feulgen reaction.

METHOD

We used our own method [4, 5] of a differential supravital stain for the internal structures of the erythrocyte. It differentiates the degenerative structures, which are stained green by brilliant green, from all the remaining regenerating structures which are stained red by neutral red. It was therefore possible to distinguish the numerous granular degenerate remains obtained from erythrocytes under the action of phenylhydrazine from the granular and thread-like substance of the reticulocytes, and also the Heinz bodies from the remains of the basophil cytoplasm and Howell bodies. By this method we have been able to reveal degenerate structures in cells where control smears treated with a single stain would have shown only the granular and fibrillar substance of the reticulocytes (Figs. 1 and 2).

To obtain degenerate structures in red cells, hemolysins (phenylhydrazine, aniline, benzidine, methylene blue) were injected subcutaneously. In all 33 mice, 14 rabbits, 5 guinea pigs, 6 frogs, and 3 pigeons were investigated. According to the particular poison used and the particular study being made, blood for a smear was taken every few minutes, or hours, or daily, or every other day, or sometimes less frequently. The smears were stained supravitally both by the differential method and by Nile blue (as a control) at intervals during the whole of the time from the beginning of the experiment until all red cells containing any remains had disappeared from the circulating blood. At the same time, smears were also prepared fixed in methyl alcohol and stained in Azur II-eosin.

To show the genetic relationship between the degenerate structures in the reticulocytes and the nuclear structures, fresh and supravitally stained smears were counterstained with the hemolytic stain Azur II-eosin after first fixing by the "time" method of D.I. Goldberg [1]. The Feulgen reaction was also performed for the same purpose. In rabbits, the same methods were used to study smears made from bone marrow punctures of the tibia and calcaneum.

Most of the experiments were carried out using phenylhydrazine, the strongest and most rapidly acting hemolytic agent whose action on the red cell has been thoroughly studied [2, 3, 6 - 8].

RESULTS

A few minutes after injecting the rabbits or mice with 50 mg/kg of aqueous phenylhydrazine, a fine granularity differentially stained green appeared in the erythrocytes. In the mature cells, most of the grains lay in groups, and sometimes in more or less massive conglomerates which sometimes formed a reticular structure. The resemblance of these erythrocytes to reticulocytes was so great that they could be distinguished from each other on the smear only by differential supravital staining. We were able to show that, contrary to the usual opinion, the hemolytic poison damages the reticulo-

cytes of the circulating blood. When it is injected into an animal with a normal number of reticulocytes the granularity representing degeneration which was found in them resembled that found in mature erythrocytes, but was coarser and stained an intense green color.

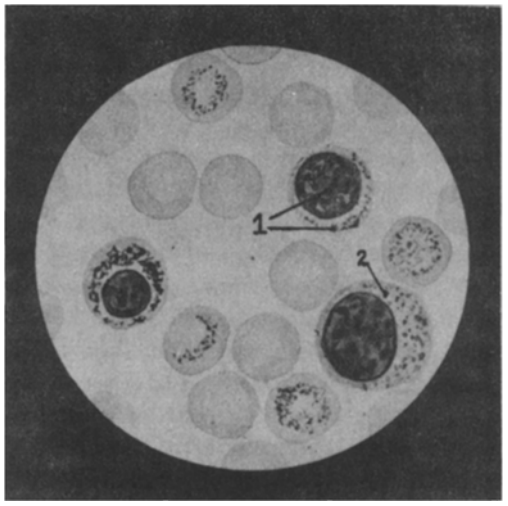

Fig. 1. Picture of bone marrow of rabbit before injecting poison. Differential supravital stain neutral red – brilliant green. Nuclei of all cellular elements and the granular and fibrillar substance are stained by neutral red. N.N.V. Objective.

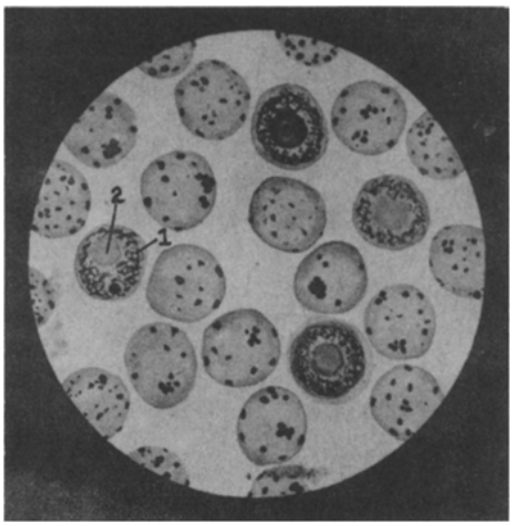

Fig. 2. Blood stained differentially with the supravital stain neutral red – brilliant green two days after poisoning the mouse with phenylhydrazine. The granulofibrillar substance continues to be stained by neutral red, while the necrotic nuclei and the granules which have degenerated are stained by brilliant green. N. N. V. Objective.

Original color plates were not reproduced due to technical difficulties. 1) Stains red; 2) Stains green – Publisher's note.

By frequent bleeding or repeated injection of the hemolytic agent, we stimulated the bone marrow strongly as was shown by the appearance of a large number of young forms of reticulocytes having an abundance of the granulofibrillar substance. When these cells are acted on by the hemolytic agent, reticulocytes with single large or numerous small homogeneous green bodies appear. The number of these cells is greater the stronger the stimulus to the erythropoietic system. As regeneration proceeds more rapidly, the degenerative structures found in the reticulocytes become larger. Then quite often reticulocytes are found with large degenerate structures in the center of the cell, where their roundness and the sharpness of their outline make them resemble an erythroblast nucleus (Fig. 2). Five to eight such cells per field of view were found in circulating blood, chiefly on the second or third day after injecting the hemolytic agent. The nuclei of the leucocytes did not change their color after the injection, and as before were stained red with the differential stain. There was no change in the morphological features of the degenerate granules which was related to the amount of the injected hemolytic agent. Repeated injections caused a change only in the number of unaffected reticulocytes.

Wheras mature erythrocytes with degenerate granules were found in circulating blood over a period of 8 – 10 days or more, reticulocytes with such granules survived for a time which was shorter the younger the cell; when only reticulocytes were present and hemolytic agent was injected, the damaged cells were replaced by new reticulocytes from the bone marrow in 2-3 days.

During the development of phenylhydrazine anemia, when there are a large number of reticulocytes, erythroblasts are almost absent from the circulating blood. Occasional cells were found which were stained by Azur II-eosin and which had homogeneous disk-shaped nuclei which did not stain cherry red, but blue or pale blue.

These results led us to study hemopoiesis in phenylhydrazine anemia. Bone-marrow punctures were examined at different time intervals after injecting a hemolytic agent, but no degenerate structures could be observed in the protoplasm of erythroblasts, though there were changes in their nuclei. Some of the nuclei, instead of staining bright red began to show a green shade and the result of the mixture of the two colors was to produce a diffuse brown spot. In some nuclei which were stained red, there were isolated bright green areas. After some hours, these cells lost their resemblance to erythroblasts, because their nuclei took up the green stain more strongly, as though changing into the usual disk-shaped reticulocyte bodies.

A careful study of the damaged cells showed that the rate at which the degenerate structures appeared in the erythrocytes after the injection depended on their age; the first to be affected were the erythrocytes and the more mature reticulocytes, and then the more mature erythroblasts. This feature is shown more clearly when a

more slowly acting agent is used (aniline, methylene blue, nitrobenzene).

In spite of repeated injections of large does of phenylhydrazine, the nucleated red cells of frogs and pigeons showed no degenerate structures in the protoplasm. However, after the injection green granules could be revealed by differential staining. Sometimes all the nuclei stained brown. In some erythrocytes, the nucleus was not clearly outlined.

The resemblance between the degenerate and the Azurophil structures in the reticulocytes, and particularly the relationship between their appearances and the degree of maturity of the cell, led us to investigate the relationship between the structures. The nuclei of the erythroblasts and the Azurophil structures gave a positive Feulgen reaction, but the later the time after the injection the less clearly did these structures appear, and after 2–3 hours from the time of the injection they could not be made out, even when before the injection they had appeared in each cell.

We have concluded that normally the nucleus of an erythroblast becomes lost by becoming gradually more dense, and at the same time passing into solution. The Azurophil nuclear remnants are detected at first by using an hemolytic stain and Feulgen's reaction, when they appear in the young erythrocyte as coarse threads, clumps, and granules, whereas in the more mature cells the granularity no longer occurs. Hemolytic agents cause many intracellular changes and bring about the swelling and the change in adsorptive properties of the nuclear fragments, so that they are no longer shown up by Feulgen's test for nucleic acid, though they do appear as degenerate structures when smears are stained supravitally. Degeneration of the substance of the nucleus takes place more rapidly in mature cells than in young reticulocytes or in erythroblasts. The fact that after the degenerate structures have been formed the cell dies more quickly the younger it is suggests that the irreversible process by which the nuclear substance forms degenerate products leads to the loss of some substrate, whose function is of greater importance the younger the cell.

Our experiments have shown that the hemolytic agents, and particularly phenylhydrazine, have a more widespread action than was previously supposed. Besides destroying hemoglobin they also induce necrotic changes in erythroblast nuclei and in Azurophil nuclear remnants, and in this way cause the formation of the degenerate structures of nuclear origin.

SUMMARY

A study was made of the effect of hemolytic agents (phenylhydrazine and aniline) on the nuclei of erythroblasts and the nuclear remnants of young erythrocytes in mice, rabbits, guinea pigs, frogs, and pigeons. The author's own method of supravital staining with neutral red – brilliant green was used, as well as D. I. Goldberg's method for smears, and Feulgen's reaction. It was shown that hemolytic poisons act on erythroblast nuclei and erythrocytic nuclear remnants and assist the formation of degenerate nuclear structures.

LITERATURE CITED

[1] D. I. Goldberg, Byull. Éksptl. Biol. i Med. 5, 3, 292 (1938).

[2] D. I. Goldberg, Byull. Éksptl. Biol. i Med. 6, 1, 126 (1938).

[3] D. I. Goldberg, Byull. Éksptl. Biol. i Med. 24, 4, 256 (1947).

[4] A. Ya. Kreimer, Problems of Experimental Biology and Medicine [in Russian] (Moscow 1951) (1st Ed.) p. 243.

[5] A. Ya. Kreimer, Labor. Delo 1, 28 (1955).

[6] K. I. Polkovnikova, Transactions of the Hospital Surgery Clinic and Blood Transfusion Station of the Tomsk Med. Inst. 2, 146, (1949).

[7] Ya. Ya. Sokolovskaya, The Morphology, Dynamics, and Genesis of Degenerative Structures in Erythrocytes in Phenylhydrazine Anemia (Dissertation) [in Russian] (Tomsk, 1946).

[8] Ya. Ya. Sokolovskaya, Arkh. Pat. 4, 9 (1948).

[9] E. I. Freifel'd, Hematology [in Russian] (Moscow, Leningrad, 1947).