# A MORPHOLOGIC INVESTIGATION DURING EXPERIMENTAL HETERIMMUNE HEMOLYTIC ANEMIA

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In recent years in connection with observations on anti-erythrocyte antibodies in the blood of many patients with hemolytic anemias significant interest in the subject has arisen.

The relation of hemolysis with the presence of anti-erythrocyte antibodies has been shown in a number of investigations [4, 8, 16, 17, 21] of which the first was reported in 1902 by G. D. Belonovsky [4]. Nonetheless many problems on the pathogenesis of immune hemolytic anemias remain unclarified. Up to the present time the cause and mechanism of the formation of anti-erythrocyte antibodies are unknown. The role of the spleen in the development of the disease cannot be considered as settled nor can the site of erythrocyte destruction. The pathological changes remain poorly studied.

Understanding of the morphologic changes in the hemolytic anemias can be broadened on the basis of experimental investigation which allows the development of these changes to be followed dynamically. With this aim in mind we carried out an investigation on 25 white mice.

## METHODS

The hemolytic anemia in the mice was evoked by the intramuscular administration of rabbit serum from donors immunized with mouse erythrocytes. Two mice were given 1 ml and 0.5 ml, 10 mice 0.25 ml, and 13 mice 0.15 ml of the hemolytic serum. Six mice served as controls, of which three were given 0.5 ml of normal rabbit serum, one was killed by hemorrhage and two died before the beginning of the experiment from pneumonia.

After administration of the serum, changes in the color of the urine were observed. In 18 experimental and 3 control mice, up to and at varying periods after the injection of the antiserum, erythrocyte and leukocyte counts were done on blood from the tail vein.

Eight mice died and the remaining were killed at varying periods (from 6 hours to 1 and ½ months) after the administration of the antiserum. The dead and killed mice were rapidly autopsied at which time pieces of soft tissue from the injection site were taken as well as portions of the liver, kidneys, spleen, lungs, heart, sternum and a portion of the femur, which, after fixation in Ort's solution, were sectioned in paraffin. The sections were stained with hematoxylin-eosin, azure-eosin and for iron by the method of Terman-Shmeltzer, an in some cases in parallel by the method of Perls.

## RESULTS

Soon after the administration of the hemolytic antiserum hemolysis of the mouse's blood began. The number of erythrocytes, which varied from 6,840,000 to 8,850,000/cc in different animals before administration of the antiserum, rapidly fell reaching the lowest level in 48 to 72 hours. The degree of hemolysis depended on the amount of administered antiserum.

After giving the mice 0.25 to 1 ml of hemolytic antiserum, the number of erythrocytes in their blood fell to 1,500,000 to 1,000,000/cc. In 24 hours after the administration of the antiserum, hemoglobinuria appeared and in 32 to 48 hours after the beginning of the experiment the mice died.

After the administration of 0.15 ml of hemolytic antiserum the number of erythrocytes in the blood of the mice fell to below 2,000,000/cc. The urine became yellow but hemoglobinuria usually did not develop. Furthermore

the number of erythrocytes gradually increased reaching the initial level by the 20th day after the beginning of the experiment. Only in 2 of the thirteen mice in this group did a more severe hemolysis occur, accompanied by hemoglobinuria and death on the third to fifth day after the administration of the antiserum.

The greatest changes in the organs and tissues were noted in the eight mice dying from the hemolytic anemia. At autopsy, in all the mice, jaundice and pallor were noted with the exception of the spleen. The spleen, in comparison with the controls, was increased $2\frac{1}{2}$ to 3 fold and on section was deep red and congested. The liver had a brownish color; in two mice, under the capsule and on cut section there were tiny, poorly outlined yellow spots. Dark brown bile was found in the overexpanded gall bladder. The kidneys were of the usual size and on cut section were gray-pink in color with a jaundiced tint. The bone marrow of the thigh and sternum was red. No changes were observed in the heart and lung. The subcutaneous and intermuscular tissue in the region of the injection were edematous.

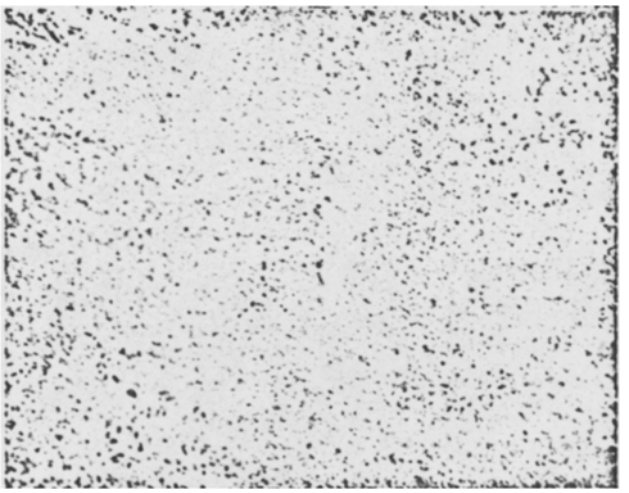

Fig. 1. Congestion of the red pulp of the spleen 34 hours after the administration of the anti-serum. Stained with hematoxylin and eosin. Magnification 100 X.

Microscopically the red pulp of the spleen was engorged (Fig. 1) and the follicles seemed diminished. In the periphery of the lymph follicles, under the capsule and in the red pulp, as is usually observed in mouse spleen, there were magakaryocytes and nucleated formed of erythrocytes and myeloid cells in varying stages of development. The reaction for iron was weakly positive for hemosiderin in isolated reticular cells situated primarily at the periphery of the follicles.

In the livers of eight mice smaller or larger areas of necrosis were seen in the central portion of the hepatic lobule. Small accumulations of leukocytes were found in the capillaries between the necrotic areas. No hemosiderin was found in the liver.

In the tubules, particularly in the collecting tubules, of the kidney there were hemoglobin or slightly granular masses (Fig. 2), staining pink with hematoxylin and eosin and orange red (as erythrocytes) with azure-eosin. In the mice dying of hemolytic anemia at a later stage there was greenish brown material. There was a negative reaction for iron with the material in the tubules. In 5 mice dying after 34, 44, 48 hours and 3 to 5 days after the administration of the hemolytic antiserum the epithelium of the tubules took a deep stain for iron. In three other mice dying in 32, 37 and 41 hours after the administration of the hemolytic antiserum the reaction for iron in the kidneys was negative.

In the subcutaneous and intermuscular tissue from the area of injection of the hemolytic antiserum, edema and a meager leukocytic infiltration were noted. In the marrow of the femur and sternum no changes were noted. There was no hemosiderin deposition.

In order to follow the character of the changes developing in the earliest stages of the fatal form of hemolytic anemia, 2 mice were killed after six hours, two after 12 hours and two after 24 hours following the administration of 0.25 ml of hemolytic antiserum. In the mice killed in 6 or 12 hours only an increase in spleen size and congestion were noted. In those killed after 24 hours, together with the increase in spleen size and congestion, jaundice was observed, and in one case, also observed, small foci of necrosis of the central portion of the hepatic lobule and pink masses in the renal tubule which did not react for iron.

Of 13 mice which were give 0.15 ml of the hemolytic antiserum only two died of hemolytic anemia. Two mice were killed at four, 2 at ten, one at thirteen, one at nineteen and two at forty-five days after administration of the hemolytic antiserum.

In the mice killed on the fourth day jaundice, anemia and increase in spleen size with red pulp congestion were noted. In the mice killed on the tenth day only an insignificant increase in spleen size was noted and in those killed at a later date no significant changes were seen. Slightly positive reactions were found in them as well as in the control mice only in isolated reticular cells of the spleen. No significant hyperplasia of the bone marrow was observed.

Thus the most consistent change in the presence of hemolytic anemia evoked by the injection of hemolytic antiserum appears to be the congestion and increase in size of the spleen. Similar changes are observed also in the majority of cases of acquired and congenital forms of chronic hemolytic anemia in man [6, 10, 13, 15, 19]. The essence and significance of these changes must not be considered as finally explained. Previously they were considered to be the cause of the illness. However the observation that removal of the spleen does not always lead to a cure and that these changes can be experimentally evoked by the administration of hemolytic antiserum bear witness to the fact that this is not the cause but only one of the manifestations of the disease.

In the experiments just cited there was no evidence of erythrophagocytosis. Contradictory data are given in the literature as regards erythrophagocytosis in the spleen in acquired forms of hemolytic anemia in man. While some authors [6, 19] describe erythrophagocytosis in the spleen during these illnesses, others do not observe it or note it only rarely [10, 13, 14]. This gave Eppinger the basis for assuming that erythrocytes in the spleen are subjected to some effect after which they are rapidly destroyed in the blood stream.

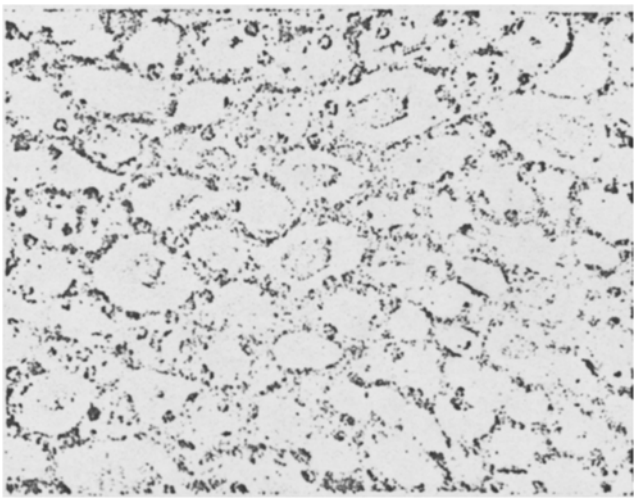

Fig. 2. Hemoglobin casts in the straight tubules of the mouse kidney. Death from hemolytic anemia with the appearance of hemoglobinuria occurred after 37 hours following the administration of the hemolytic antiserum. Staining with hematoxylin and eosin. Magnification 400 ×.

The given facts allow us to consider that erythrophagocytosis in the spleen, if it occurs, is not the principal and natural cause of erythrocyte destruction. The absence of evident hemosiderosis in the spleen in our experiments and also in a number of observations on people suffering from hemolytic anemia [5, 15, 18, 19] point up the fact that the destruction of erythrocytes in this disease can occur outside the spleen.

The appearance of hemoglobin casts in the kidney tubules is a consequence of hemoglobinemia which results from massive destruction of erythrocytes. It should be considered that filtration of hemoglobin through the glomeruli in the presence of an insignificant plasma concentration does not occur and begins only when the concentration reaches 100 to 150 % [7, 9]. Proceeding through the glomeruli, the hemoglobin is partially excreted in the urine which causes the appearance of hemoglobinuria and is partially converted to hemosiderin which gives the histochemical reaction.

Hemoglobin casts were observed in 8 mice dying as a result of hemolytic anemia. In 5 of them dying at a later period, hemosiderosis of the epithelial cells of the renal tubules was also noted. Upon administration of 0.15 ml of hemolytic antiserum hemolysis of the blood usually occurred to a lesser degree. The concentration of hemoglobin in the plasma apparently did not reach such magnitude that glomerular filtration might result in hemoglobinuria, and hemoglobin casts and hemosiderosis were not observed.

The observation of hemoglobin casts in the tubules and hemosiderosis in the renal epithelium can serve as indicators of the degree of hemolysis of the blood. Experiments to determine the site of erythrocyte destruction on the basis of the presence or absence of renal hemosiderosis [1, 2] should be recognized as inadequately established. If the presence of renal hemosiderosis allows one to think of intravascular hemolysis then its absence does not in the same measure contradict this possibility.

Somewhat unanticipated was the absence in our experiments of hepatic hemosiderosis despite the fact that there was considerable hemolysis of blood. Hemosiderin was not only absent from the mice dying from hemolytic anemia in two or three days, but was also absent from the mice killed at later periods after the administration of the hemolytic antiserum. The absence of hemosiderosis of the liver during hemolysis of the blood might be explained by the fact that the breakdown products of the hemoglobin are utilized at once in the organism by new blood formation or that it is stored in the liver in the form of ferritin [3, 11, 12] which is not detected by the ordinary histochemical techniques.

The existence of a definite relationship between the degree of anemia and the development of necrosis in the liver permits us to consider this as anemic necrosis.

## SUMMARY

Hemolytic anemia was induced in white mice by intramuscular administration of rabbit's serum immunized with mice erythrocytes. Anemia and jaundice developed in mice soon after the serum administration by the degree of manifestation that they were in direct relationship to the amount of the serum administered. Enlargement of the spleen was constantly observed in connection with a marked congestion of its pulp. With a reduction of the blood erythrocyte count down to 2,000,000 per cubic mm the mice survived. The number of erythrocytes in their blood gradually reverted to normal and the changes in the spleen disappeared. In decrease of the erythrocyte count below 2,000,000 per cubic mm hemoglobinuria developed and the mice perished. Apart from anemia, jaundice and congestion of the splenic pulp, hemoglobin casts were found in the tubuli and hemosiderosis of the renal epithelium, as well as anemic necroses in the liver.

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