PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

A Specific Test for the Disease Based on the Ability of Thrombin to Activate the Hemolytic Factor

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Paroxysmal nocturnal hemoglobinuria (Marchiafava-Micheli syndrome) is a rare form of chronic hemolytic anemia characterized by the presence of abnormal erythrocytes which are susceptible to lysis by a factor normally present in plasma or serum. It has recently been suggested that this factor resembles closely or may even be identical with the coagulation accelerator globulin. This globulin exists in blood as an inert proenzyme which is activated by thrombin.

Numerous tests have been proposed for the laboratory diagnosis of paroxysmal nocturnal hemoglobinuria (PNH).

1. **Hemosiderinuria** was noted by Marchiafava as being constantly present in the 2 cases of PNH which he studied. The importance and ease of this test has recently been emphasized by Stats, Wasserman and Rosenthal. They point out, however, that hemosiderin is occasionally lacking in the urine of patients with PNH and is occasionally found in other types of hemolytic anemia. We have also found that hemosiderinuria is present in every patient with hemolytic anemia associated with hemoglobinemia. Conversely, in a patient with PNH of six years’ duration the usual hemoglobinemia, hemoglobinuria and hemosiderinuria all disappeared completely following a severe transfusion reaction which occurred ten months previously. The test is therefore not specific.

2. **"Heat sensitivity."** Micheli first observed intensification of the PNH hemolytic reaction in vitro as the temperature was increased. Heggelin and Maier suggested that this phenomenon might be adapted to a specific test for PNH. However, the test is by no means specific. The blood from cases of hemolytic anemia associated with a hemolysin antibody shows marked hemolysis in vitro at 37 C. at 6 hours, provided that the antibody is sufficiently potent.

3. **Acid hemolysis.** Hijmans van den Bergh first noted intensification of hemolysis of PNH cells in acidified serum. This phenomenon has been intensively studied, and although no explanation has been proposed it is a very characteristic feature of the hemolytic mechanism. T. H. Ham has standardized a diagnostic test involving this reaction. The acid-hemolysis test when done according to his detailed instructions is pathognomonic of PNH. Ham lists the following specific points for diagnosis:

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* It has somehow become customary in the literature to cite a paper by George C. Ham et al. as the original description of this test. Although G. C. Ham used the test, it had previously been evolved, standardized and published by Thomas Hale Ham.
PAROXYSMAL NOCTURNAL HEMOGLOBINURIA: A TEST

1. The patient's red cells are hemolyzed only by fresh human serum containing a thermolabile component of human serum.
2. At the normal pH of blood, hemolysis occurs frequently during incubation for one hour at 37.5 °C, but not always.
3. Hemolysis is always produced by increasing the acidity of serum within or beyond the physiological range of variation of pH.
4. Hemolysis is decreased or eliminated at an alkaline pH of 7.6 to 8.0 or above.
5. Destruction of the hemolytic factor by heat or other procedures eliminates the hemolysis in acidified serum.
6. The addition of fresh guinea-pig serum, as a source of complement, does not re-establish the hemolysis for serum inactivated by heat.
7. Any compatible fresh, normal, human serum when acidified will hemolyze the patient's washed red blood cells.
8. The patient's serum will not hemolyze compatible red blood cells from a normal subject, i.e., there is no hemolysin in the patient's serum.

We have used the Ham test as a basic method of investigation in 7 cases of PNH and are convinced of its specificity. Nevertheless, the test must be done carefully.

In reviewing hospital records of several cases of PNH we have encountered laboratory reports which stated that the test was negative. There is in the literature at least one case of unmistakable PNH in which the Ham test was performed and reported as negative. Such "false negatives" may occur in several ways:

1. Loss of CO₂. If the serum, after acidification, is allowed to stand in an unstoppered tube, carbon dioxide diffuses into the air, the pH of the serum rises, and the hemolytic activity of the serum decreases in proportion.
2. Anticoagulants. Since the hemolytic factor in the plasma of a patient with PNH is one of the proteins of the coagulation system, all anticoagulants are capable of blocking the hemolytic mechanism. Oxalate and citrate in concentrations capable of preventing coagulation completely prevent hemolysis. Heparin in concentrations above 1:3000 also blocks hemolysis, although in concentrations of less than 1:15,000 it facilitates activation of the hemolytic enzyme (though preventing coagulation). The Ham test may be performed successfully with lightly heparinized plasma, but it is negative if an excess amount of heparin is used.
3. Transfusion. If a patient with severe anemia is transfused extensively the proportion of his own red cells may become quite small. This is especially true of PNH, in which transfusions are not infrequently accompanied by hemolytic reactions which destroy only the patient's cells. The transfused red cells are not susceptible to the PNH hemolytic mechanism. If the Ham test is performed after a series of transfusions it may be only slightly positive due to the large proportion of normal cells.
4. Inactive serum. We have occasionally encountered sera from normal individuals which were completely inert in the Ham test. These sera showed a very low value for accelerator globulin activity in the coagulation system. Such a serum may be artificially prepared by gently drawing blood through a large gage needle into an oiled syringe, placing it carefully in a clean tube and allowing it to coagulate undisturbed. When this serum is then acidified and PNH cells are added, hemolysis
does not occur. This type of serum can be made hemolytically active by the test described below:

**The Test**

This test depends upon the activation by thrombin of an enzyme which is normally present in plasma. This enzyme when thus activated is specifically hemolytic against PNH red blood cells.

**Method**

1. Blood compatible with that of the patient is drawn from a normal person and allowed to stand in ice water in a stoppered tube until chilled. The tube is centrifuged, and the serum is withdrawn and kept cold and stoppered until ready for use.

2. Red cells from the patient are washed three times in normal saline. After the last centrifugation the saline is withdrawn and no more is added.

3. Two clean small test tubes are set up. Into Tube 1 is measured 2 cc. of the normal serum; 0.1 cc. of N/3 HCl is added and mixed. Then 0.2 cc. of the patient’s packed red cells are added. Into Tube 2 are placed approximately 50 units of thrombin. The cells and serum in Tube 1 are mixed and 1.0 cc. of the cell suspension is transferred to Tube 2.

4. Both tubes are corked and incubated at 37 C. for fifteen minutes. The tubes are then centrifuged. If the cells are those of PNH, the serum in both tubes is stained red, but the serum in Tube 2 is stained more intensely red than in Tube 1. In a hemolytic reaction due to an antibody, hemolysis is less in Tube 2 probably because thrombin is anticomplementary. The test may be controlled by treating a suspension of normal red cells with acidified serum and thrombin. With normal cells there is no hemolysis in either tube.

**Results**

The test has been performed well over a hundred times in patients with PNH and was invariably positive. Red cells from normal subjects and from a variety of cases of hemolytic anemia of types other than PNH always gave negative results.

**Discussion**

This test depends upon the activation by thrombin of an inert plasma factor which when active becomes hemolytic against PNH red cells. This precursor may be identical with the coagulation accelerator (Seegers’ Ac-globulin, Quick’s labile factor, Owren’s Factor V). It is partially activated during coagulation. Addition of thrombin to serum activates the residual. This explains the intense hemolysis of PNH red cells in the test tube which contains thrombin. We believe that hemolysis

* Bovine thrombin suitable for this test is commercially available as a hemostatic. Even in an opened vial this thrombin will remain active for months if stored in the freezing compartment of a laboratory refrigerator. Thrombin is quickly inactivated at room temperature and should be refrozen immediately after use. In this test the thrombin may be added as a powder by estimating 50 units, or the contents of the vial may be dissolved in 1 cc. of cold saline and 50 units measured with a pipet. The amount need be only approximate, but thrombin in excess of 100-150 units per cc. destroys some of the hemolytic activity of the serum.

If thrombin is not available, thromboplastin may be substituted. The thromboplastin should be prepared by incubation in saline as for the determination of prothrombin time. Add 0.05 cc. to Tube 2 in place of thrombin. Since the blood has clotted in the cold, there is much residual prothrombin in the serum. Addition of thromboplastin reacts to form thrombin, which in turn activates the hemolytic factor.
by this hemolytic factor is specific for PNH and, therefore, that the test is specific. Experience in using the test in PNH and in a wide variety of other types of hemolytic anemia lends support to this belief.

The test further commends itself for its simplicity and does not require the numerous procedures recommended by Ham for making a specific diagnosis.

**Summary**

1. A simple and rapid test for paroxysmal nocturnal hemoglobinuria is described. The test is based upon the activation by thrombin of the hemolytic factor in serum which destroys the red cells of this disease.

2. It is believed that the test is specific for paroxysmal nocturnal hemoglobinuria.

**References**

2. ———, and ———: The significance of hemoglobinemia and associated hemosiderinuria with particular reference to various types of hemolytic anemia. (In preparation.)
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