BRIEF REPORT

Decreased Hemolytic Capacity of Sera From Patients With Paroxysmal Nocturnal Hemoglobinuria

By STUART F. BLUM AND FRANK H. GARDNER

The erythrocytes of patients with Paroxysmal Nocturnal Hemoglobinuria (PNH) hemolyse at 37 C. in normal compatible human sera in the absence of demonstrable antibody. The reaction is complement dependent and in vivo causes a hemolytic anemia of varying severity.

The diagnostic acid hemolysis test for this disorder is performed by demonstrating hemolysis of suspect erythrocytes in the patient's serum or in serum from a serologically compatible normal subject. It is generally stated that normal serum is preferable for this test because the patient's serum may be less hemolytic than normal. Moreover it has been noted that the hemolytic process in vivo is markedly accelerated by the transfusion of fresh whole blood to these patients, but the transfusion of washed normal erythrocytes ameliorates the hemolytic process. Although the washing process will remove leukocytes thereby reducing the possibility of leukoagglutinins triggering a hemolytic reaction, these data suggest that the patient's plasma may be deficient in its hemolytic capacity, and the transfusion of fresh plasma restores the concentration of a low titer hemolytic component. Since variation in the hemolytic capacity of the plasma may be a critical factor in controlling the in vivo PNH hemolytic rate, studies were performed to determine if there is a diminution in the hemolytic capacity of the patient's plasma.

MATERIALS AND METHODS

Whole blood from patients with PNH was collected in ACD. The erythrocytes were washed three times in saline prior to use. Sera, both normal and patient, were prepared by collecting whole blood into cold plastic tubes without anticoagulant, centrifuging at 4 C., separating the plasma and allowing it to clot 30 minutes at 37 C. Compatibility of the normal sera and the PNH cells was determined by ABO group and by Coombs testing.

Acid hemolysis tests were performed as described previously. Incubations of PNH cells

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Table 1.—Hemolysis of PNH erythrocytes in the patient’s serum and in various normal sera at hematocrit 40 per cent pH 7.5 and in the routine acid hemolysis test, hematocrit 1 per cent pH 6.5. The normal sera are more potent than the patient’s serum. The difference is greater at a normal hematocrit.

<table>
<thead>
<tr>
<th>Source of Serum</th>
<th>% Hemolysis Hematocrit 40%</th>
<th>% Hemolysis in Normal Serum</th>
<th>% Hemolysis Acid Hemolysis Test</th>
<th>% Hemolysis in Normal Serum</th>
<th>% Hemolysis in Patient’s Serum</th>
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</thead>
<tbody>
<tr>
<td>Patient M.S.</td>
<td>0.8</td>
<td>11.8</td>
<td>19.5</td>
<td>2.0</td>
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</tr>
<tr>
<td>Normal 1</td>
<td>9.4</td>
<td>11.8</td>
<td>38.4</td>
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<tr>
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<td>7.5</td>
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<tr>
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<td>3.2</td>
<td>4.1</td>
<td>30.0</td>
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<td>11.1</td>
<td>13.9</td>
<td>46.2</td>
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<td>11.4</td>
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<td>2.9</td>
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<tr>
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<td>35.7</td>
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<tr>
<td>Mean</td>
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</table>
HEMOLYTIC CAPACITY

Fig. 1.—Autologous and isologous survival of Cr51 labeled PNH erythrocytes. The survival in the normal recipient is biphasic with a rapid destruction of short lived red cells while the residual red cells have a normal Cr51 survival.15 The autologous T1/2 survival is longer and the curve is monophasic. The initial 100 per cent value for comparison is the mean of values in the 90 minute interval after infusion.

in sera at a hematocrit of 40 per cent were performed by suspending packed red cells in appropriate volumes of sera. These high hematocrit incubations, designed to simulate in vivo conditions, were performed in a shaking incubator at 37 C. with the pH maintained at 7.45 ± 0.05. Plasma hemoglobin was measured by the benzidine method at the beginning and at the conclusion of a 60 minute incubation and per cent hemolysis calculated using the hemoglobin concentration of the whole blood sample as 100 per cent.

To define any in vivo diminution in PNH lytic capacity of the patient's sera, the erythrocytes in 20 cc of blood from a patient with PNH were labeled with 100 microcuries Na Cr51 O4. Aliquots were infused into the patient and into a serologically compatible normal volunteer. Venous blood samples were obtained 30, 60 and 90 minutes post-injection and at daily intervals thereafter for determination of red cell survival.4 The initial 100 per cent values represent the mean of the 30, 60 and 90 minute samples.

RESULTS

In Vitro Studies

Results of a typical study comparing the hemolytic potency of sera from one patient with PNH to that of six normal controls are shown in Table 1. Not shown are the similar results obtained using 8 additional normal sera. Studies comparing the sera of 4 other PNH patients with the same 14 normal sera gave similar results. Half the normal subjects were male and half female. PNH sera are not as hemolytic as normal sera either at pH 6.5 or at physiologic pH. It is of interest that the defect in hemolytic capacity of the patient's sera is much more easily demonstrable in a 40 per cent hematocrit incubation than it is in the routine acid hemolysis test. This may result from greater stress being placed on the serum factors by the low ratio of serum to cells at this high hematocrit as compared with the 99:1 ratio of serum to cells in the acid hemolysis test.

In Vitro Studies

The survival curves, Figure 1, of the labeled red cells clearly demonstrate a shorter overall survival in the normal subject. The T1/2 lifespan in the pa-
tient was 15 days. The curve in the normal could be resolved into 2 distinct components, representing a short lived population with a $T_{1/2}$ of 1 day and a normally lived population with a $T_{1/2}$ of 26 days. This biphasic curve is in keeping with the biclonal theory proposed for the pathogenesis of PNH.

**DISCUSSION**

These observations regarding the hemolytic capacity of PNH sera are an attempt to define more clearly previous observations relative to this measurement. Widespread variation exists in the hemolytic capacity of sera. In addition to the comments of Dacie, Hinz cites a four-fold variation in the hemolytic capacity of normal sera from different subjects. The possibility exists that the hemolytic capacity may vary with time in the patient with PNH, and such patients may have hemolytic capabilities approaching that of normal sera. In our experience, however, all five patients tested had hemolytic capacity less than the 14 normal subjects. These five patients, at the time of study, were in their usual "steady" clinical state without evidence of any recent exacerbations of their persistent hemolysis. The spectrum of clinical disease represented by these patients ranged from one subject with severe hemolytic disease requiring approximately 10 transfusions yearly to maintain a hematocrit of 25 per cent to another patient with compensated hemolytic disease maintaining a hematocrit of 35 per cent with 4 per cent reticulocytes.

In vivo survival curves previously reported in PNH from many laboratories have established the variable nature of the shape of the curve. Most authors describe two-component curves in autologous survivals, suggesting two populations of cells although this concept has been challenged. There is no report in the literature of simultaneous autologous and isologous survivals as we performed to detect any in vivo difference in PNH hemolytic capacity. Our data demonstrate a clearly shorter survival in the isologous study and support the in vitro observations.

The specific defect responsible for the decreased hemolytic capacity of the patient's sera is not known. Measurements of whole complement titers ($C'$ $H_{50}$) in patients with PNH have been normal. Measurements of the titer of the second component, $C'2$, have been low but adequate to promote a normal degree of hemolysis. $C'3$ levels also are greater than are required for lysis.

The observation that the sera of patients with parenchymal liver disease hemolyse PNH cells minimally may be an important clue for the understanding of our observations. Liver pathology has been noted repeatedly in PNH although the etiology is not clear. The requirement for transfusion in many patients with PNH may result in unrecognized episodes of serum hepatitis with resultant impairment of liver function. In addition, the tendency to portal thrombosis and hepatic infarction may likewise compromise hepatic function. A review of the liver function of the patients herein studied revealed biopsy documented hepatic infarction in one, liver disfunction as a residual of serum hepatitis in another, a history of portal vein thrombosis with portal hypertension and a portacaval shunt in the third, abnormal liver func-
tion tests of uncertain etiology in the fourth and abnormal function tests in the fifth patient who had suffered from recurrent cholecystitis. All patients had diffuse hypergammaglobulinemia in addition to other abnormalities of liver function but this particular abnormality probably is of little significance in affecting PNH lysis. Studies performed with sera from patients who had diffuse hypergammaglobulinemia with high titer rheumatoid factor, Waldenstrom’s Hypergammaglobulinemia and Systemic Lupus Erythematosus revealed normal hemolytic capability. Previous measurements of whole complement titers of liver disease have revealed marked changes only with severe disease\textsuperscript{14} and the changes do not correlate with the PNH hemolytic capacity of the sera\textsuperscript{12}. The whole complement titers have been normal in liver disease sera studied in this laboratory although these sera barely supported PNH hemolysis. It is possible however that a small depression in the titer of one complement component is critical for PNH lytic capacity and may occur with moderate liver dysfunction to explain the decreased hemolytic capacity observed in these five patients.

In addition to the implications of these findings for understanding the PNH hemolytic mechanism and the variation in the in vivo hemolytic rate, these data reemphasize the necessity for using normal sera rather than patient’s sera when performing the acid hemolysis test as a diagnostic tool.

**SUMMARY**

The sera of patients with Paroxysmal Nocturnal Hemoglobinuria have been shown, in vitro and in vivo, to be less hemolytic for PNH erythrocytes than are normal sera. This defect may result from the liver dysfunction encountered in many of the patients although the exact defect has not been defined.

The necessity for using normal sera when performing the acid hemolysis test is emphasized.

**SUMMARIO IN INTERLINGUA**

Le seros de patientes con paroxysmic hemoglobinuria nocturne (PHN) se ha monstrate in vitro e in vivo minus hemolytic relative a erythrocytos ab subjectos con PHN que seros normal. Iste defecto resulta possibilemente ab le dysfunction hepatic incontrate in multes del patientes ben que le precise identitate del defecto ha non ancora essite establite.

Es sublineate le necissitate de usar seros normal in effectuar le acide test hemolytic.

**REFERENCES**


Brief Report: Decreased Hemolytic Capacity of Sera From Patients With Paroxysmal Nocturnal Hemoglobinuria

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