Serum Anti-γG Globulin Factors in Paroxysmal Nocturnal Hemoglobinuria

By Manuel E. Kaplan, Shaul Kochwa, Louis R. Wasserman and Richard E. Rosenfield

In paroxysmal nocturnal hemoglobinuria (PNH) accelerated destruction of abnormal red cells occurs within an apparently benign extracorpulcular environment. Although the underlying cellular defect has not yet been fully elucidated, it has long been recognized that, in vitro, PNH erythrocytes are extraordinarily susceptible to immune lysis. The possibility exists that, in vivo, destruction of the PNH red cell may be provoked by immunologic mechanisms. However, evidence for such a process is usually lacking, and to the present time there has been no consistently recognized abnormality in the serum globulins or in serum complement activity. It was, therefore, of considerable interest that serum from a patient with PNH was found to agglutinate compatible red cells that had been sensitized with some incomplete Rh antisera. This finding suggested that the patient's serum contained rheumatoid factor (RF). However, neither the patient's history nor physical findings suggested the diagnosis of rheumatoid arthritis. Since a search of the literature revealed no previously noted association between PNH and anti-γG globulin factors, additional sera from PNH patients were sought; a total of 25 such patients were finally collected for study. The results of this investigation, previously reported in preliminary form, indicate that anti-γG globulin factors are commonly present in the sera of patients with PNH.

Materials and Methods

1. PNH Sera

Serum specimens were for the most part, collected within a week prior to testing and kept frozen at −20 to −60 C. When available, older sera from individual patients were also obtained in order to compare their serologic activity with that of more recently collected samples. These older sera had been stored at −20 C. for as long as 10 years. In five cases...
death of the patient precluded collection of fresh sera and only aged sera (2 to 5 years) were available for study.

2. Tests for Anti-γG Globulin Activity

A. Sensitized Human Red Cell Agglutination Test (SHC). To one drop of washed, packed red cells of type Rh:1, −2, 3, 4, −5 (cDE/cDE; Rh, Rh,) were added one drop of anti-Rh,2 (CD; Rh,) serum Ripley* (a serum which is known to provide a red cell coat reactive with most RF's) , and 8 drops of saline. After incubation for 2 hours at 37°C, the red cells were washed three times and diluted in saline to yield a 2 percent cell suspension. Serial dilution of the PNH sera in saline was carried out in 8 × 75 mm. tubes. To 2 drops of each serum dilution 2 drops of the sensitized red cell suspension were added. Appropriate controls, consisting of sensitized red cells in saline and nonsensitized red cells in PNH serum, were always included. The contents of the tubes were mixed and allowed to stand for 1 1/2 to 2 hours at room temperature. The degree of red cell agglutination was then observed using a hand lens (6 × magnification). Thereafter, the test tubes were centrifuged briefly at 1000 g and the strength of agglutination again estimated after gentle dislodgement of the cell button. Agglutination observed after centrifugation was graded from trace (questionable agglutination) to 4. Inhibition studies were performed by adding equal volumes of inhibitor to 10 agglutinating units of serum, mixing thoroughly, and adding 2 drops of sensitized red cell suspension.

B. Gm Typing. This was performed by the slide method of Steinberg.† In addition, the technic described by Waller and Lawler was employed when Ripley-coated red cells were utilized for Gm typing.

C. Sheep Cell Agglutination Tests. Prior to testing, PNH sera were diluted 1:3.5 in cold saline and absorbed twice, at 0°C, with one-fourth volume of washed, packed sheep cells.

1. Tanned sheep cell agglutination test (FRI SC). Sheep cells were tanned and coated with human Cohn Fraction II (Squibb. Lot 1812) and the titrations performed as described by Heller et al.9

2. Sensitized sheep cell agglutination test (SSC). This test was performed according to the Heller modification of the Waaler-Rose test utilizing one-half the minimal agglutinating dose of rabbit antisheep hemolysin.11

D. FIT-Latex Agglutination Test. The Hyland R.A. Test Kit was employed as directed.

3. Immunochemical Studies

A. Reduction with 2-Mercaptoethanol (2-ME). Sera were diluted 1:6 in saline and sufficient 2-ME was added to give a final concentration of 0.1 M. After incubation for 2 hours at room temperature, the sera were dialyzed overnight against cold 0.15 M NaCl.

B. Chromatography on DEAE cellulose was performed according to the methods of Kochwa et al.12 and Sober et al.14 Chromatographic fractions were dialyzed against saline before being tested for serologic activity.

C. Immunoelectrophoresis was performed by the method of Grabar and Williams.13

D. Analytical ultracentrifugation was carried out in a Spinco Model E ultracentrifuge at 59,600 r.p.m. at 18°C.

RESULTS

Table 1 summarizes the results of the various hemagglutination studies.

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*We are grateful to Dr. Henry Kunkel of the Rockefeller Institute for supplying this serum.
†We are indebted to Dr. A. Steinberg for his generous gift of reagent sera and for his guidance in the performance of these studies.
‡Rabbit amboceptor was the gift of Dr. Irwin Oreskes of the Mount Sinai Hospital.
§Hyland Laboratories, Los Angeles, California.
*Schleicher and Shuell, Keene, New Hampshire.
Table 1.—Agglutination Titers of PNH Sera

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<th>Patient</th>
<th>SHC*</th>
<th>FII SC†</th>
<th>FII Latex‡</th>
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* Sensitized human red cell agglutination test.
† Tanned sheep cell agglutination test.
‡ FII Latex agglutination test.
§ Sensitized sheep cell agglutination test.
†† tr. = trace.
** mod. = moderate.
††† Sera from non-transfused patients.
‡‡ n.d. = not done.
‡‡‡ st. = strong.
|| These sera when tested with sensitized red cells from another donor were negative on one occasion.

**SHC Agglutination Test**

Sera from 14 of the 25 patients with PNH were found to agglutinate Ripley-sensitized red cells in titer from 1:8 to 1:2048. Essentially identical results were obtained utilizing cells sensitized with anti-Rh serum Heyman (anti-Rh1,2) which closely resembles Ripley in its serologic and immunochemical properties. If a serum supported agglutination only at dilutions of 1:8 or less, it was regarded as nonspecific and the serum recorded as negative. Slight prozones were noted among most of the active sera, hemagglutination being somewhat weaker in undiluted serum than in dilution of 1:4 to 1:8. It was found that if a given serum was unable to agglutinate Ripley- or Heyman-sensitized red cells, it would not agglutinate cells sensitized with any other Rh antiserum tested. Furthermore, most of the PNH sera that agglutinated Ripley-sensitized cells in high titer reacted weakly, if at all, with red cells coated with Rh antiserum routinely used for Gm typing.
In the case of four patients (Va, Sv, Fo and Ka), specimens of sera were obtained before the patients had received even a single transfusion; three of the four sera were found to agglutinate Ripley-sensitized red cells. Later sera obtained from patients Va, Sv and Fo, after each had been transfused repeatedly, revealed that no additional serologic activity had developed. (Patient Ka has not required transfusion to date.)

Gm Typing Studies

When standard Gm typing reagents were employed, serum from only one patient (Ka) could be shown to exhibit anti-Gm specificity (anti-Gm⁺). Efforts to demonstrate anti-Gm specificity among the PNH agglutinators using red cells more weakly sensitized with antisera Ripley were uniformly unsuccessful, whereas a non-rheumatoid agglutinator or known anti-Gm⁺ specificity could easily be typed under similar conditions. The agglutination of Ripley-coated cells by various PNH sera was inhibitable by relatively high concentrations of normal human serum (i.e., dilutions of 1:4 or less), regardless of Gm type and by Cohn FII (2 mg./ml.). The inhibitory activity of FII was only slightly enhanced by heating at 63 C. for 15 minutes.

Sheep Cell Agglutination Tests

Of the 14 sera that agglutinated Ripley-sensitized cells, 9 agglutinated FII-coated tanned sheep cells and 5 agglutinated FII-coated latex particles in significant titer. Serum from only one patient (Ra) agglutinated sensitized sheep cells. This unusual patient exhibited the clinical picture of rheumatoid arthritis as well as PNH.

It was observed that heating of serologically active sera to 56 C. for 30 minutes significantly reduced their ability to agglutinate FII-coated sheep cells and latex particles. A similar phenomenon was noted in the case of serum Co when it was tested in the sensitized human red cell system.

Exposure of PNH sera to 2-mercaptoethanol invariably resulted in total loss of their agglutinating activity.

Figure 1 illustrates the fractionation of serum Kun by DEAE-cellulose chromatography. Serologically active fractions were eluted at relatively high salt concentrations and contained, on immunoelectrophoresis, primarily γM globulin and albumin. Other sera fractionated in the same manner yielded identical results, and in two-stage separation the serologically active fraction was eluted with 1 M NaCl. These findings strongly suggest that these anti-globulin factors are γ macroglobulins.

Analytic ultracentrifugation of selected sera revealed no abnormalities. Serum components sedimenting more rapidly than the normal 18S peak were not observed, no intermediate (7S-18S) peaks were seen, and the 7S peaks were normal in size.

Discussion

A variety of anti-γG globulin factors have, to the present time, been detected in human sera. (1) Rheumatoid agglutinators (RAggs) are 19S γM globulins which combine, in whole serum, with 7S γG globulin to form 22S complexes. They are heat stable and react with heterologous γG
globulins. Some rheumatoid factors, by virtue of their serologic specificity, are capable of differentiating various genetically determined factors (Gm) present in human γG globulin.

(2) Rheumatoid-like factors have been found in a group of apparently unrelated disorders accompanied by marked hypergammaglobulinemia—e.g., liver disease, syphilis, leprosy, tuberculosis, leishmaniasis, sarcoidosis, and subacute bacterial endocarditis. These antiglobulins, too, are typically 19S γM globulins, but as a group their serologic properties differ somewhat from those of classical rheumatoid factors. They are usually nonreactive with heterologous γG globulins and are generally useless for Gm typing.

(3) Nonrheumatoid agglutinating factors
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(SNaggs) are found occasionally in normal individuals but occur much more commonly in the sera of repeatedly transfused patients. These agglutinators are, for the most part, 19S γM globulins, but occasional 7S γG agglutinators of this type occur. They frequently make excellent Gm- or Inv-typing reagents, exhibiting serologic specificity directed against Gm or Inv groups not present in the donors γG globulin. Characteristically they are nonreactive with heterologous γG globulins. (4) Milgrom factors (“anti-antibodies”) are thermostable, antiglobulin factors capable of agglutinating red cells coated with essentially all incomplete Rh antibodies. This agglutination is not inhibitible by undiluted human serum or by high concentrations of pooled human Fraction II. Milgrom factors appear to react specifically with human 7S γG globulin only after the latter has assumed the structural configuration of cell-bound antibody.

The macroglobulin agglutinators found in the sera of many patients with PNH closely resemble, physicochemically and serologically, the previously described anti-γG globulin factors. However, in some respects they differ. In the PNH sera studied, hypergammaglobulinemia was not encountered and interaction between the 19S antiglobulin factors and 7S γG globulin could not be demonstrated. The PNH antiglobulins reacted with relatively few coating Rh antisera and not at all with heterologous γG globulin. With the exception of only one serum, they did not exhibit serologic specificity for any of the known Gm or Inv groups on human γ globulin. (It is, of course, possible that they are capable of defining yet unrecognized genetically determined structures within the γ globulin molecule.) The agglutination of Rh sensitized red cells that they induced was inhibitable by a high concentration of human serum or Cohn Fraction II. And, as a group, the PNH agglutinators appeared to be somewhat more heat-labile than the other classes of antiglobulins.

What stimulates the appearance of these macroglobulins in PNH and what role, if any, do they play in the pathogenesis of this disorder? At present insufficient information is available to answer either of these crucial questions. It is generally believed that the various anti-γG globulin factors heretofore described are antibodies synthesized in response either to genetically foreign γG globulin or to autologous antibody γG globulin which has been engendered and structurally altered by a protracted antigenic stimulus.

In PNH, a common source of foreign γG globulin is blood transfusion. Indeed, this may account for many of the antiglobulin factors found in this disorder. However, in at least three patients these factors were present prior to transfusion. The propositus (patient Kun) had received only 2 units of blood 3 months before a potent antiglobulin was discovered. Thus, it is unlikely that isoimmunization can explain all the anti-γG globulin factors observed in this group of patients. As for the possibility that a persistant antigenic stimulus incites the production of the PNH antiglobulin factors, no evidence for such a process has yet been developed.

In an effort to demonstrate possible involvement of these factors in the pathogenesis of PNH the following studies were performed:

1. Type O PNH red cells from patient Kun were heavily sensitized with
antiserum Ripley and, thereafter, incubated at 37 C. in fresh, undiluted, complement-containing autologous serum. Agglutination, but no hemolysis, ensued. It was concluded, therefore, that the Kun agglutinator lacked hemolytic activity in vitro and probably in vivo.

2. Packed, Ripley-sensitized PNH erythrocytes, 0.1 ml., were injected intradermally into patient Kun (the red cell donor), and the site of injection carefully inspected over the next 4 days. No reaction, immediate or delayed, was observed.

Since the survival of PNH red cells in normal recipients is shortened and since the sera of normal subjects usually do not exhibit antiglobulin activity, one might conclude that these factors are entirely irrelevant to the hemolytic process. However, the failure of normal sera to react in the various tests employed to detect antiglobulin factors may simply reflect a paucity rather than the complete absence of such factors.

Although the results of these studies do not implicate anti-γG globulin factors in the hemolysis of PNH red cells in vitro or in vivo, recent observations by von Felten and Frick suggest that serum macroglobulins are required for complement-dependent, acid hemolysis of PNH erythrocytes (Ham test). They report that treatment of fresh human serum with penicillamine (dimethylcysteine) renders them hemolytically inactive in the Ham test while their serum complement levels (measured by lysis of sensitized sheep erythrocytes) remain essentially unchanged. Although these investigators attribute this effect of penicillamine on properdin, it is as likely that the primary effect of this sulfhydryl compound is to dissociate and to serologically inactivate serum γM globulin.

Isliker and Dacie have postulated that because of surface imperfections, PNH erythrocytes may adsorb immunologically active substances present in serum. The anti-γG globulin factors herein described are potentially able to react in vivo with γG globulin, and perhaps with components of the complement system. The resulting macromolecular complexes may then be absorbed onto, or otherwise interact with, the PNH red cell membrane, thereby initiating red cell lysis. Indeed, Yachnin and Ruthenberg have recently reported that fluid-phase (i.e., occurring in plasma) immunologic events, not necessarily involving fixation of antibody to red cell, may be involved in PNH lysis.

Although highly conjectural, such a series of events may explain the frequent hemolytic reactions observed in patients with PNH following transfusion with whole blood. It has long been recognized that such reactions may be avoided by the administration of washed, packed red cells. It is conceivable that the interaction of PNH antiglobulins with “incompatible” transfused γG globulin within the circulation of the PNH patient may trigger the destruction of the immunologically fragile PNH red cell.

**SUMMARY**

Sera from 16 of the 25 patients with PNH were found to exhibit anti-γG globulin activity as demonstrated by their ability to agglutinate human red
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cells sensitized with certain incomplete Rh antibodies. Of the 16 active sera, 9 agglutinated human FII-coated tanned sheep cells and 5 FII-coated latex particles in significant titer. Only one serum exhibited anti-Gm specificity. In 3 cases serum anti-γG globulin activity was found prior to transfusion of the patient, suggesting that isoimmunization is not in all cases responsible for this phenomenon. The chromatographic behavior of the serologically active substances and their inactivation by treatment with 2 ME suggest that they are γM globulins related to the rheumatoid factors. The possible role of these antiglobulin factors in the lysis of the PNH erythrocyte is discussed.

REFERENCES


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