Reaction of Antibodies That Cause Paroxysmal Cold Hemoglobinuria (PCH) With Globoside and Forssman Glycosphingolipids

By Gerald A. Schwarting, Samar K. Kundu, and Donald M. Marcus

Many antibodies that cause paroxysmal cold hemoglobinuria (Donath-Landsteiner antibodies) appear to be directed against the blood group P antigen. We recently identified this antigen as the glycosphingolipid globoside GalNAc(β,1-3)Gal(α,1-4)Gal(β,1-4)GlcCer, and we examined the reaction of four Donath-Landsteiner antibodies with anti-P specificity, and an anti-P acid autoagglutinin, with globoside and Forssman glycolipids. Forssman glycolipid, GalNAc(α,1-3)GalNAc(β,1-3)Gal(α,1-4)Gal(β,1-4)Glc-Cer, contains the globoside structure plus an additional terminal nonreducing α-GalNAc residue. All five antibodies were inhibited effectively by globoside. Two of the Donath-Landsteiner antibodies were inhibited much more effectively by globoside than Forssman glycolipid, and the other two antibodies were inhibited more effectively by Forssman glycolipid. The two glycolipids were approximately equally effective in inhibiting the acid anti-P agglutinin. We suggest that the populations of antibodies that react with both glycolipids are directed against an aspect of the globoside structure that is accessible in the Forssman compound, whereas the antibodies that react best with globoside are probably directed against the terminal β-GalNAc residue of this glycolipid. Some human "anti-P" antibodies are probably elicited by immunization against Forssman antigens that are widespread in animal tissues and in microorganisms.

PAROXYSMAL COLD HEMOGLOBINURIA (PCH) is an autoimmune hemolytic anemia in which episodes of intravascular hemolysis, hemoglobinemia, and hemoglobinuria occur when patients are exposed to a cold environment (reviewed in refs. 1 and 2). As demonstrated by Donath and Landsteiner, the sera of these individuals contain antibodies (biphasic hemolysins) that attach to erythrocytes most effectively at 4°C and lyse the cells in the presence of complement when the temperature is raised to 20°-37°C. The syndrome of PCH is heterogeneous with regard to the immunoglobulin class and serologic properties of the antibodies, the nature of associated diseases, and the duration of the hemolytic anemia. The hemolysis may be produced by IgG antibodies that are strongly lytic and have little or no agglutinating activity or by IgM antibodies that are potent agglutinins as well as lysins. Many IgG antibodies appear to have anti-P specificity, i.e., they lyse all erythrocytes except those rare phenotypes P, P, and p, that lack the blood group P antigen. The IgM antibodies exhibit mostly anti-I or anti-i specificity.

Earlier in the century PCH was observed most frequently in patients with syphilis. In the last several decades it has occurred most frequently in children following viral infections or immunization with viral vaccines. The hemolysis...
specificity of pch antibodies

Table 1. Structures of Glycosphingolipids

<table>
<thead>
<tr>
<th>Glycosphingolipid</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactosylceramide</td>
<td>Gal(β1,4)Glc-Cer</td>
</tr>
<tr>
<td>Trihexosylceramide</td>
<td>Gal(α1,4)Gal(β1,4)Glc-Cer</td>
</tr>
<tr>
<td>Globoside</td>
<td>GalNAc(β1,3)Gal(α1,4)Gal(β1,4)Glc-Cer</td>
</tr>
<tr>
<td>Forssman</td>
<td>GalNAc(α1,3)GalNAc(β1,4)Gal(β1,4)Glc-Cer</td>
</tr>
<tr>
<td>Paragloboside</td>
<td>Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc-Cer</td>
</tr>
</tbody>
</table>

Gal, D-galactose; Glc, D-glucose; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; Cer (ceramide), N-acylsphingosine.

associated with viral syndromes is transient, but patients with idiopathic chronic PCH have been described.

We recently identified the blood group P antigen as the glycosphingolipid (GSL) globoside (Table 1), and this identification was based, in part, on the inhibition of anti-P alloantibodies by this compound. We now report the inhibition of anti-P autoantibodies, mostly from patients with PCH, by globoside and the Forssman GSL.

MATERIALS AND METHODS

Antibodies. Sera 1, 2, and 5 were provided by Dr. C. F. Hinz (ref. 6, case 6), G. Garratty, and W. J. Judd, respectively. Case 3, whose serum was provided by Dr. J. H. Crookston, was a 45-year-old man with chronic idiopathic PCH. Serum 4, provided by Dr. C. Grumet, was from a child with transient PCH. Rabbit antisera to globoside were raised as described previously, and purified by affinity chromatography on a globoside column, and the IgG and IgM fractions were separated by chromatography on a column of Sephadex G-200.

GSL. Glycolipids were purified and analyzed as described previously. A total erythrocyte ganglioside fraction was prepared by chromatography of erythrocyte lipids on DEAE-Sephadex, and after alkaline hydrolysis to remove acidic phospholipids the gangliosides were separated into mono-, di-, and trisialoganglioside fractions by the method of Momoi et al. The monosialo fraction was further fractionated by chromatography on latex beads, a porous silica gel.

Streptococcal group C carbohydrate was obtained from Dr. J. Coligan, and α-methyl-N-acetylgalactosamine and β-ethyl-N-acetylgalactosamine were provided by Dr. R. Jeanloz.

Immunologic methods. The quantitative Donath-Landsteiner (DL) hemolytic test was performed by a modification of the method of Hinz. In the cold phase 0.1 ml of a dilution of PCH serum and 0.15 ml of an 8% suspension of human erythrocytes were incubated at 4°C for 30 min with mixing. In our initial studies, complement, 0.1 ml of human serum, was included in this phase. This produced a decrease in hemolysis with some sera, an observation made originally by S. Haddad (Toronto General Hospital) and confirmed by us, and most of our experiments were performed without complement present during the cold phase. After the incubation at 4°C the cells were washed twice with 3 ml of cold barbital buffer and resuspended in 0.1 ml of human serum and 0.2 ml of buffer. After incubation for 30 min at 37°C the cells were sedimented at 4°C, the supernatant was diluted 1:10, and its optical density was determined at 413 nm. When inhibition experiments were performed with GSL, the antibody and inhibitor were incubated at 4°C overnight rather than for 30 min. The GSL preparations contained equal parts by weight of the GSL and egg lecithin (Sigma Chemical).

Hemagglutination inhibition experiments were performed in a microtitraton system at 4°C as described previously.

RESULTS

PCH. The hemolytic activities of all four sera were inhibited by globoside (Table 2), but their reactions with Forssman GSL varied considerably. Serum 1 was not inhibited by Forssman GSL within the range tested, and globoside was five to six times as active as Forssman GSL in inhibiting serum 3. In contrast, sera 2 and 4 were inhibited more effectively by Forssman GSL. These sera were not inhibited by 250 μg/ml of lactosyl ceramide or CTH, by 50 μg/ml of paraglobo-
side, or by 1 mg/ml of streptococcal group C polysaccharide, which has the same terminal disaccharide as Forssman GSL, GalNAc(α,1-3)GalNAc, but differs in its internal sequence.23 Sera 1 and 4 were not inhibited by 0.002 M α-methyl-N-acetylgalactosaminide or by 0.012 M β-ethyl-N-acetylgalactosaminide; sera 2 and 3 were not tested with these glycosides.

The specificity of an anti-P acid autoagglutinin4 (serum 5) and the agglutinating activity of serum 3 were also examined (Table 3). Globoside was twice as active as Forssman GSL in inhibiting serum 5, and Forssman GSL did not inhibit serum 3.

In Table 4 we have summarized data from our previous reports on the inhibition of anti-P alloantibodies by the two GSL. Forssman GSL inhibited all of the sera except one, but it was not more effective than globoside in any instance.

Purified rabbit antigloboside antibodies that had a complement fixation titer of 1:320 with globoside and 1:20 with Forssman GSL had a titer of 1:8 in the hemolysis test. Purified rabbit anti-Forssman IgG antibodies that had a titer of 1:5160 by complement fixation and a titer of 1:20 with globoside did not produce any lysis of human erythrocytes in this assay.

**DISCUSSION**

Anti-P autoantibodies, like the alloantibodies in the sera of P⁰ and p individuals whose cells lack this antigen, are all inhibited by globoside. Forssman glycolipid is a relatively effective inhibitor of most human anti-P sera and inhibits some antibodies (sera 2 and 4) more effectively than globoside. Since essentially complete inhibition of lysis of these sera is obtained with either GSL, these compounds must be reacting with the same population(s) of crossreactive antibodies and not with two different populations. Although most published data on rabbit antibodies raised against these two GSL indicate little or no crossreactivity,24,25 some of our purified rabbit antigloboside antibodies display appreciable crossreactivity with Forssman GSL.

What is the basis of the crossreaction between these two GSL? Immunochemical analyses of carbohydrate antigenic determinants, particularly those without...

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**Table 2. Inhibition of Anti-P Donath-Landsteiner Antibodies**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Concentration (μg/ml) for 50% Inhibition</th>
<th>Ratio of Inhibitory Activity Glob/Forssman</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>2.4</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>16</td>
</tr>
</tbody>
</table>

**Table 3. Inhibition of Anti-P Autoagglutinins**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Concentration (μg/ml) for 50% Inhibition</th>
<th>Ratio, Glob/Forssman</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>3</td>
</tr>
</tbody>
</table>

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repeating sugar sequences, have established the immunodominant role of the terminal nonreducing sugar residue (reviewed in refs. 26 and 27). Antibodies to internal sugar sequences of the O antigen of gram-negative bacteria have been described,28 however, and some antibodies to dextran also appear to be directed against internal sequences.29 The lectin concanavalin A can also react with nonterminal α-mannosyl or α-glucosyl residues if the C-3 and C-4 hydroxyl groups are unsubstituted.30 One explanation of our data is that some anti-P antibodies can react with the inner globoside portion of the Forssman GSL (Fig. 1). Another possibility is that the crossreactive antibodies can react with a terminal GalNAc residue regardless of its anomeric configuration, like the human IgM paraprotein that we described recently.31 The latter explanation is unlikely because the group C streptococcal polysaccharide, which has a terminal disaccharide identical to the Forssman GSL, did not inhibit these sera, nor did the simple glycosides of GalNAc. The more effective inhibition of sera 2 and 4 by Forssman glycolipid suggests that there are conformational differences between globoside and the globoside sequence of Forssman and that some antibodies react preferentially with the latter. Human “anti-P” antibodies of this specificity probably represent an antibody response to Forssman glycolipid rather than to globoside. We are planning to isolate the crossreacting and noncrossreacting fractions of rabbit and human sera in order to perform more detailed immunochemical analyses.

What is the nature of the immunogen(s) that elicit anti-P antibodies? Pk and p

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### Table 4. Specificity of Anti-P Alloantibodies

<table>
<thead>
<tr>
<th>Ratio of Inhibitory Activity</th>
<th>Globoside/Forssman</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pk donors</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>&gt;8</td>
</tr>
<tr>
<td>p donors</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Compiled from ref. 10 and 11.

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**Fig. 1.** Molecular model of the carbohydrate portion of Forssman glycolipid in an extended conformation; a portion of sphingosine is shown on the extreme right-hand side of the picture. Inner four sugar residues, which correspond to the glycolipid globoside, are indicated within a bracket.
individuals are probably immunized by Forssman-like antigens that are found in many bacteria\textsuperscript{32,33} and animal products that contain both GSL. Humans have been considered to be a Forssman-negative species, but recent data indicate that this GSL is present in gastric and colonic epithelia of about 20\% of Chinese people and that it appears in carcinomas of these organs in most individuals who lack Forssman GSL in their normal mucosa.\textsuperscript{34} Three mechanisms have been proposed to explain the production of the antibodies that produce PCH: immunization by a crossreacting antigen present in an infectious agent, an “altered” autoantigen, or an abnormality of the immune system.\textsuperscript{2,35} There are no data that document the existence of crossreacting antigens in viruses associated with either the PCH syndrome or \textit{Treponema pallidum}. The infectious agents may induce an alteration in the surface of erythrocytes, or other cells containing globoside, that increases the immunogenicity of this compound. We would like to propose another version of the altered antigen hypothesis. Enveloped viruses bud through the plasma membrane and contain host cell phospholipids and GSL on their surface.\textsuperscript{36,37} The GSL may become immunogenic when they are on the surface of the virus in association with immunogenic viral antigens.

Another hypothesis is that the primary abnormality resides in the immune system, which produces autoantibodies in increased quantities as a consequence of a disturbance in immune regulation or lymphocyte activation produced by the infectious agent. The best example of such a state is infectious mononucleosis, which is caused by the Epstein-Barr virus.\textsuperscript{38,39} Patients with infectious mononucleosis have elevated serum immunoglobulin levels, particularly IgM, and they make IgM antibodies to many antigens,\textsuperscript{40,41} including the Forssman antigen. PCH has also been observed as a complication of infection with \textit{Mycoplasma pneumoniae};\textsuperscript{42} another infectious agent that may elicit the production of autoantibodies.\textsuperscript{43} Antibodies to common carbohydrate antigens, such as I, i, and Forssman, may be part of the polyclonal antibody response exhibited by “activated” lymphocytes. In view of the heterogeneous nature of the PCH syndrome it is likely that any of these three mechanisms may be operative in individual cases. Patients with chronic PCH (cases 2 and 3) may have an underlying lymphoproliferative disorder.

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GA Schwarting, SK Kundu and DM Marcus