A new look at an old case: An auto-anti-P with pseudo-LKE activity

Laura Cooling

ABSTRACT

Aims: LKE is a high-incidence, minor RBC glycosphingolipid, related to both Pk and P antigens. Approximately 1% individuals are LKE-negative. However, antibodies against LKE are rare, with only six cases mentioned in literature. Past examples of anti-LKE have relied on serologic testing, with no direct testing against RBC glycosphingolipid (GSL). To test a historical ‘anti-LKE’ against a panel of RBC and glycosphingolipid standards by high performance thin layer chromatography and standard serology. Methods: Serum samples included human polyclonal anti-LKE, alloanti-P, alloanti-P1Pk and untransfused controls. Hemagglutination was performed by gel method with ficin-treated RBC of known LKE, P and P1 phenotype. P antigen expression was determined by titration with a well characterized alloanti-P. Antibody specificity was determined by incubating serum against glycosphingolipids on high performance thin layer chromatography plates. Results: The patient’s serum reacted with most LKE+ RBC but not ficin-treated p, Pk, or LKE-negative donors, consistent with an anti-LKE. However, on direct testing, the patient’s antibody failed to recognize monosialogalactosylgloboside, the LKE antigen. The patient’s serum did recognize globoside (P) antigen. This was confirmed by hemagglutination, which showed a correlation between LKE phenotype, P antigen expression and serum reactivity. The patient’s weak auto-anti-P was not inhibited by solubilized globoside. Conclusion: This historical anti-LKE is an auto-anti-P with ‘pseudo-LKE’ activity due to differences in P antigen expression between LKE+ and LKE—donors.

Keywords: LKE, Glycosphingolipid, P blood group

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Article ID: 100010IJBTILC2013

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doi:10.5348/ijbti-2013-10-OA-1

INTRODUCTION

The LKE antigen or ‘Luke antigen on erythrocytes’ is a high-incidence red cell (RBC) antigen, present on 99% of donors (Table 1). Like P1 antigen, LKE has variable expression, with 80–90% typing LKE-strong (LKE-S), 10–20% typing LKE-weak (LKE-W) and 1–2% typing LKE-negative (LKE-N) depending on the population and reagent used for typing [1–3]. The LKE antigen has been identified as monosialogalactosylgloboside (MSGG) or stage-specific embryonic antigen-4, a globofamily ganglioside [2]. As a globo-GSL, LKE/MSGG is biosynthetically and serologically related to Pk and P
antigens, which are upstream substrates necessary for MSGG synthesis (Figure 1). As a consequence, p and Pk RBC are always LKE-negative (LKE-N) due to the complete absence of P/Gb4, Gb5 and MSGG (Table 1). In contrast, normal P+, LKE-N donors still have low levels of MSGG when examined by sensitive methods [3, 4].

Despite the incidence of LKE-N in the general population, examples of anti-LKE are quite rare, with only six examples mentioned in the literature [1, 5-9]. We had the opportunity to reexamine a historical ‘anti-LKE’ from an untransfused, group A, P2, 32 years old woman in her second pregnancy [5, 6]. At 16 weeks, a low titer, IgM panagglutinin (titer 4), reactive with P+, but not p, Pk or autologous RBC, was identified in her serum. Furthermore, her antibody showed evidence of ABO-specificity in population studies, reacting stronger with group O, LKE+ donors [5]. Because the patient typed LKE-N, it was presumed that her alloantibody was an anti-LKE.

We retested this sample against a panel of well-characterized LKE-typed RBC by standard serology, as well as direct testing against a series of RBC and GSt standards. In addition, we attempted to inhibit antibody reactivity using a modified hemagglutination inhibition assay for use by gel method.

**MATERIALS AND METHODS**

**Reagents:** Monoclonal antibodies (mAb) MC813-70 (NeuAcα2-3Galβ1-3GalNAcβ1-R, IgG), MC631 (Galβ1-3GalNAcβ1-3Galα1-4Gal-R, IgM) and Fe-A5 (Galβ1-4GlcNAcβ1-R, IgM) were purchased as either ascites or hybridoma supernatant from the Developmental Studies Hybridoma Bank held at the University of Iowa, Iowa City, IA, USA [2, 10, 11]. Biotinylated secondary antibody (anti-mouse, human), streptavidin-linked alkaline phosphatase and substrate were purchased from Vector Laboratories, Burlingame, CA, USA. Lactosylceramide (CMH, LacCer), globotriaosylceramide (Gb3, Pk antigen), globoside (Gb4, P antigen), gangliotriaosylceramide (Gg3, αGM2), gangliotetraosylceramide (Gg4, αGM4), and ganglioside GM3 were purchased from Matreya Inc. (Pleasant Gap, PA, USA). The total neutral and ganglioside fraction from human RBC (group A, P1, LKE-S), ACHN renal carcinoma (Gb5+, MSGG+, DSGG+) [12] and human brain were isolated using a modified Ledeon and Yu method. [3, 13, 14] Paragloboside (nLc4, PG) and galactosylgloboside (Gb5, SSEA3) were previously isolated by high performance liquid chromatography from chronic myelogenous leukemia cells and human kidney, respectively [14]. All solvents were HPLC-grade (Sigma, St. Louis, MO USA).

**Samples:** Patient RBC and serum were previously obtained from the University of Arhus, Denmark via the SCARF (Serum, Cells And Rare Fluids) network and stored frozen at -80°C [5, 6]. Per original typing, the patient was group A, D+C+E-c-e-, M+N+S+s+, K-, Fy(a+b+), Jk(a+b+), Le(a-b+), Vel+, P1-, P+ and LKE-N with mAb MC813-70. A direct antiglobulin test (DAT) was negative. Her serum was reported to contain anti-B, anti-P1 and anti-LKE. Her anti-LKE was weak (titer 4), sensitive to dithiotreitol (DTT), and enhanced by low ionic saline (LISS) and predigestion of RBC with enzymes [6]. The serum sample used for testing had remained frozen, without thawing, since receipt in 1989.

Other RBC and serum used in the study were obtained from SCARF or the University of Michigan Immunohematology Reference Laboratory (Tables 1 and 2). LKE type was reconfirmed on all RBC samples as described below. Typing for ABO, A1, P1 (Ortho Clinical Diagnostics, Raritan, NJ, USA), and Lewis (Immucor, Norcross, GA, USA) were performed by direct hemagglutination using commercially available reagents in accordance with manufacturer’s instructions.

**LKE Typing:** For LKE phenotyping, washed red cells were digested with 0.1% ficin, washed and resuspended in saline (3% final) as described [15]. One drop of RBC, two drops mAb MC813-70 (ascites 1:100 in phosphate buffered saline with 1% bovine serum albumin [PBS-1% BSA]) and one drop 22% commercial BSA (Ortho Clinical Diagnostics) were incubated by tube method for 2 hours at 4°C, followed by centrifugation (30 s, 3400 rpm) and immediate macroscopic reading [2, 3]. Samples agglutinating at immediate spin (IS) were typed as LKE-S. Negative samples were tested by the indirect antiglobulin test (IAT) using anti-mouse IgG (50 μg/mL in PBS-1%BSA, Sigma). Samples negative at IS, but positive by the IAT, were typed as LKE-W. Samples failing to agglutinate by IS and IAT were typed as LKE-N. LKE-N samples were tested for P antigen with mAb MC631 [2, 3].

**P antigen titer:** P antigen expression on RBC was determined by direct hemagglutination with a well-characterized human polyclonal alloanti-P from a group A, Pk individual. A series of two-fold dilutions of alloanti-P serum were prepared in normal saline using calibrated pipets. Titration were performed in Buffered Gel™ cards (Micro Typing Systems, Pompano Beach, FL, USA) against washed, 0.8% unmodified RBC resuspended in a commercial low ionic saline suspension (MTS Diluent 2, Micro Typing Systems). Gel cards were incubated for 1 hour at 4°C and then centrifuged at 895 rpm for 10 minutes per manufacturer’s instructions. The P-titer was the last dilution resulting in 1+ agglutination [14].

**Hemagglutination Inhibition:** Inhibition studies were performed as described by Naiki et al. with modification to gel method [16, 17]. GSlS for inhibition studies were initially suspended at a concentration of 1 μg/mL in chloroform-methanol (C-M) 1:1 v/v with sonication. Aliquots were dried in glass microvials under nitrogen, resuspended in taurodeoxycholate buffer (0.5 mg/mL taurodeoxycholate in PBS) and sonicated for 1 to 2 minutes in a bath sonicator. For most experiments, GSlS were diluted to a working concentration of 0.1-0.2 μg/mL (10-20 μg GSlS/50 μL buffer) [16, 17]. All GSlS solutions were prepared fresh, immediately prior to each experiment. Pilot studies indicated a progressive loss of inhibitory activity with solutions stored greater than 24 hours at 4°C.
For antibody inhibitions, serum (or plasma) was diluted in normal saline to a final working titer of 4 against LKE-S RBC [16, 17]. For weak antibodies, serum was used neat. To minimize the serum dilution, the amount of inhibitor was limited to 20% of the final serum volume (5 μL GSL and 20 μL plasma). At 0.2 μg/μL working stock, this resulted in a final inhibitor concentration of 40 μg/mL serum. GSL and serum were incubated in microtubes for 3 hours at 4°C. Treated serum was subsequently incubated with 0.8% RBC in buffer gel cards for 1 hour at 4°C, followed by centrifugation as before. Alloanti-P and patient sera were tested against ficin-treated P2, LKE-S RBC. Group O plasma was tested against group A1, unmodified RBC. Negative controls included serum incubated with PBS and taurodeoxycholate–PBS buffer, respectively.

High Performance Thin Layer Chromatography: High performance thin layer chromatography (HPTLC) was performed as described [3, 13, 18]. Gb4 and other neutral GSLs were separated in standard neutral solvent (C-M-water; 65:25:4 v/v). Gangliosides were separated in C-M-0.2% aqueous CaCl2 (50:40:10, v/v). GSLs were chemically visualized with charring reagent (3% cupric sulfate) on glass HPTLC plates [18]. GSLs were characterized based on their intensity and relative mobility (Rf) by scanning densitometry (QuantiscanTM, BioSoft, Cambridge, UK).

HPTLC-immunostaining was performed on aluminum-backed HPTLC plates (E. Merck, Darmstadt, Germany) as described with modification [14, 19, 20]. HPTLC plates were developed in the appropriate solvent, air-dried and then coated with a 0.2% poly(isobutyl)methacrylate solution (Polysciences, Warrington, PA) for 60 s and air dried overnight. Immunostaining with mAbs was performed as described using PBS-1% BSA as buffer [14, 19]. To detect sialylated type 2 chain gangliosides, plates were first digested with neuraminidase in situ (0.2 units/mL in PBS-1% BSA, 2 hours at 37°C), washed and then incubated with mAb Fe-A5 [11, 19].

For immunostaining with human sera, HPTLC plates were blocked with PBS-5% BSA for 1 hour, room temperature. HPTLC plates were subsequently overlaid with human plasma diluted 1:15-1:20 in PBS-5% BSA for 2 hours, washed thrice with PBS on an orbital rotator [19] followed by incubation with a biotinylated anti-human antibody in PBS-3% BSA. To detect bound antibody, plates were washed and sequentially incubated with avidin-linked alkaline phosphatase and alkaline substrate as described [12, 19, 20]. Controls included plasma or serum from p (anti-PP1pK; Table 1), P1k (alloanti-P) and 5 normal group A adults with a negative antibody screen, and no history of ABO discrepancies or RBC alloantibodies.

Table 1: P and LKE RBC Phenotypes.

<table>
<thead>
<tr>
<th>Type</th>
<th>Incidence</th>
<th>CDH</th>
<th>Pk</th>
<th>P</th>
<th>Gb5</th>
<th>LKE</th>
<th>P1</th>
<th>Antibody</th>
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<tbody>
<tr>
<td>p</td>
<td>rare</td>
<td>+++</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>PP1Pk</td>
</tr>
<tr>
<td>Pk</td>
<td>rare</td>
<td>++</td>
<td>+++</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>P</td>
</tr>
<tr>
<td>P1c</td>
<td>&gt;80%</td>
<td>+</td>
<td>+</td>
<td>+++</td>
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<td>tr</td>
<td>+</td>
<td>o</td>
<td>P1</td>
</tr>
<tr>
<td>LKE-S</td>
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<td>+</td>
<td>+</td>
<td>+++</td>
<td>tr</td>
<td>++</td>
<td>+/o</td>
<td>none</td>
</tr>
<tr>
<td>LKE-W</td>
<td>10-20%</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>tr</td>
<td>+</td>
<td>+/o</td>
<td>none</td>
</tr>
<tr>
<td>LKE-N</td>
<td>1%</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>tr</td>
<td>tr</td>
<td>+/o</td>
<td>‘LKE’</td>
</tr>
</tbody>
</table>

a. GSL antigen expression on RBC based on P and LKE phenotype. Tr, trace expression on high performance thin layer chromatography (HPTLC).
b. Alloantibodies against globo-antigens.
c. P+ RBC are subclassified as P1 or P2, based on the expression of P1 antigen, a neolacto-series GSL (see Fig. 1).
d. P antigen (globoside) expression is required for synthesis of LKE (Fig. 1). P1 and P2 RBC can be further classified as either LKE- strong (S), LKE-weak (W) and LKE-negative (N) based on their reactivity at immediate spin (IS, LKE-S) and indirect antiglobulin test (IAT, LKE-W). Unlike p and Pk RBC, LKE-N RBC have trace amounts of LKE by HPTLC-immunostaining.
RESULTS

Confirmation of Patient Red Cell Phenotype: The patient’s phenotype was reported as P2, Tj(a+) and LKE-negative [5, 6]. Given the age of the sample, we repeated P1, P and LKE typing on the patient and an unrelated frozen sample of equivalent age. The patient again typed P+, P1- with mAb MC631, commercial anti-P1 and one example each of anti-PP1Pk and alloanti-P. Likewise, the patient typed LKE-N with mAb MC813-70, which recognizes the globo-ganglioside MSGG or LKE antigen (Figure 1) [2, 10]. To assure that the LKE-N phenotype was not an artifact of inadequate ficin-digestion [2, 3] patient’s RBC were typed for Jk(a) before and after ficin digestion [15].

Serum Testing: Given the age of the sample, patient sera was tested against a panel of RBC of known p, Pk, P1 and LKE phenotype to verify serologic activity (Table 2). In the original report [5, 6], the patient plasma contained both anti-LKE and anti-P1. A weak anti-P1 was identified in the IAT by tube: No anti-P1 was observed by gel method using buffered gel cards. To avoid anti-P1 activity, subsequent studies were performed with 0.8% ficin-treated RBC in buffered gel cards (1 hour, 4°C). A gel titer of the patient’s antibody against LKE-S, P2 ficin-treated red cells was 1, indicating some loss in antibody activity over prolonged storage [6].

Consistent with the original report, patient sera did not agglutinate p or Pk RBC (Table 2). The autocontrol was negative. Among confirmed LKE-N RBC, most samples were negative or only trace positive. In LKE-W samples, agglutination was observed in 4/7 (57%) samples. Variable reactivity (1+ to 4+) was noted in LKE-S samples and was independent of donor ABO type. Interestingly, the strongest reactivity to patient sera was observed in a group A, P2 donor with relatively weak LKE expression (+, IS) when compared to other LKE-S donors (+ to 4+, IS).

Reactivity to Neutral RBC GSLs: To determine whether the patient’s antibody recognized a RBC GSL (Table 3), patient serum was initially tested against CDH, Gb3 (Pk), Gb4 (P), Gb5, as well as the total neutral GSL fraction from ACHN cells and P1, LKE-S RBC by HPTLC-immunostaining (Figures 2A and C). As controls, serum from p (anti-PP1Pk, Table 1), P1-type RBC, Gb1+ and Gb2+ RBCs were used. Neutral GSLs were also stained with mAb MC631, which recognizes Gb4 and Gb5 (Figure 2B).

Patient sera showed weak reactivity against Gb4 in RBC, ACHN cells and the Gb4 control: No reactivity was observed to CDH, Gb3, Gb5 or P1 (Figure 2C). As expected, antibody binding to Gb4 was also observed with alloanti-P (Figure 2D, Table 3). Binding to Gb3, Gb4 and P1 antigen were noted with anti-PP1Pk from p individual (Figure 2E). No binding was noted with normal sera from P+ individuals (data not shown).

No Binding to MSGG or LKE Antigen: Patient sera was also tested against isolated RBC and ACHN

Figure 1: Schematic of major glycosphingolipids in human RBC. GSL synthesis proceeds by the stepwise addition of sugars (underlined) from glucosylceramide (CMH, ceramide monohexose). Lactosylceramide (CDH, ceramide dihexose) is the first major branch point, giving rise to GM3, Gb3 and Lc3. Gb3 (Pk antigen) is the first committed globo-series GSL (red) and is a biosynthetic precursor to Gb4 (globose, P antigen) and MSGG (LKE antigen). Lc3 is rapidly galactosylated to form paragloboside (nLc4), the first neolacto-series or type 2 chain GSL (teal blue) and immediate precursor of sialylparagloboside (snLc4) and P1 antigen. GM3, sLc4 and MSGG are gangliosides. Nonsialylated GSLs are considered neutral GSLs.
Table 2: Serum Reactivity by LKE Type and P-titer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ABO</th>
<th>P</th>
<th>LKE</th>
<th>Serum(^a)</th>
<th>Titer(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>O</td>
<td>p</td>
<td>N</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>A(_1)</td>
<td>P(_1)(^k)</td>
<td>N</td>
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<td>0</td>
</tr>
<tr>
<td>3.(^c)</td>
<td>A(_1)</td>
<td>P(_2)</td>
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<td>0</td>
<td>8</td>
</tr>
<tr>
<td>4.(^d)</td>
<td>O</td>
<td>P(_1)</td>
<td>N</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>5.</td>
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<td>N</td>
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<td>16</td>
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<tr>
<td>6.</td>
<td>O</td>
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<td>32</td>
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<td>N</td>
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<td>8.</td>
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<td>P(_1)</td>
<td>W</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
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<td>P(_1)</td>
<td>W</td>
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<td>16</td>
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<tr>
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<td>W</td>
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<td>32</td>
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<tr>
<td>11.</td>
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<td>W</td>
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<tr>
<td>12.</td>
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<tr>
<td>13.</td>
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<td>W</td>
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<tr>
<td>14.</td>
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<td>S</td>
<td>2+</td>
<td>32</td>
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<tr>
<td>15.</td>
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<td>P(_1)</td>
<td>S</td>
<td>2+(^8)</td>
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<tr>
<td>16.</td>
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<td>P(_1)</td>
<td>S</td>
<td>3+</td>
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<tr>
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<td>O</td>
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<td>S</td>
<td>3+</td>
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<td>S</td>
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<tr>
<td>19.</td>
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<tr>
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<td>21.</td>
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<td>22.</td>
<td>A(_2)</td>
<td>P(_2)</td>
<td>S</td>
<td>3+</td>
<td>NT</td>
</tr>
</tbody>
</table>

\(^a\) Reactivity of patient serum against ficin-treated RBC by gel method.
\(^b\) P-antigen titer against washed, unmodified RBC. NT, not tested.
\(^c\) Autocontrol.
\(^d\) Weak P sample.

The data in Table 2 illustrate the reactivity of patient sera against different RBC phenotypes. The sera were tested against RBCs with various ABO, P, and LKE types. The table shows that sera exhibit specificity towards certain antigens, as indicated by the serum titer and reactivity. For example, Serum 6 reacts with P\(_1\) and LKE-N, indicating the presence of a specific antigen that mediates hemagglutination.

Gangliosides, which are rich in MSGG and disialogalactosylgloboside (DSGG) [12], play a significant role in RBC function and immune response. The data in Table 2 suggest that these gangliosides are important targets for the immune system, as indicated by the reactive sera. The presence of these antigens can influence the specificity of the immune response and the development of antibodies against RBCs.

**Hemagglutination is Related to P Antigen Expression**: Although the patient's sera did not react with MSGG/LKE by HPTLC-immunostaining, her
serum did recognize Gb4. Serologic and biochemical studies have indicated subtle changes in Gb4 expression by LKE type, characterized by increased Gb3/Pk and decreased Gb4 on LKE-N RBC [3, 7]. It was possible, therefore, that the apparent ‘anti-LKE’ activity reported, and observed in our testing, was actually an auto-anti-P with pseudo-LKE activity due to decreased P antigen expression on LKE-N RBC. To assess the latter, we performed P-antigen titers on 15 RBC samples used in patient testing. All P-titers were performed on the same day with a well-characterized anti-P with strict Gb4 specificity on HPTLC-immunostaining [21].

As shown in Table 2, the median P-titer of LKE-N, LKE-W and LKE-S cells was 16, 24 and 48, respectively. The two lowest P-titers were observed in the patient and a rare LKE-N, “weak P” red cell sample [22, 23]. There was a good correlation between P-titer and reactivity with patient serum. The patient’s sera failed to recognize donors with P-titers < 1:32 but displayed progressively stronger reactivity at higher P-titers.

**GSL Inhibition Studies:** Based on HPTLC-immunostaining and hemaggulutination studies, the patient’s sera actually contained a weak, clinically-insignificant auto-anti-P. Although auto-anti-P is a common specificity in cold paroxysmal hemoglobinuria [15] weak IgM panagglutinins with auto-anti-P activity are known [24, 25]. In at least one example, auto-anti-P was partially inhibited by solubilized Gb4 [24]. To test the latter, we developed a modified GSL hemagglutination inhibition assay for use in gel cards. As a positive control, testing was performed in parallel with an alloanti-P from a group A, P⁺ donor. Group O serum, which contains anti-A (αGalNAc-R, Table 3), was included as a negative control.

As expected, alloanti-P showed marked inhibition with Gb4 (40 μg/mL plasma, Figure 4A, lane 4) [15, 16].

Table 3: Serum Reactivity Against Specific Glycosphingolipids

<table>
<thead>
<tr>
<th>Name</th>
<th>Alias</th>
<th>Structure</th>
<th>HPTLCb</th>
<th>HAIc</th>
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<td></td>
<td></td>
<td>PP1Pk</td>
<td>P</td>
</tr>
<tr>
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<td></td>
<td>Pt</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pt</td>
<td></td>
</tr>
</tbody>
</table>

**Neutral GSLs**
- LacC CDH Galβ1→4Glcβ1-1’Ceramide - - - -
- Gb3 Pk, CTH Galα1→4Galβ1→4Glcβ1-1’Cer + + + +
- Gb4 P GalNAcβ1→3Galα1→4Galβ1-1’Cer - N NT - T
- Gb5 SSEA3 Galβ1→3GalNAcβ1→3Galα1→4Galβ1-1’Cer - - NT NT
- Gg3 aGM2 GalNAcβ1→3Galβ1→4Glcβ1-1’Cer - N NT - T
- Gg4 aGM1 Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1-1’Cer NT N NT - T
- nLe4 PG Galβ1→4GlcNAcβ1→3Galα1→4Glcβ1-1’Cer - - - -
- P1 Galα1→4Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1-1’Cer - - - -
- A GalNAcα1→3(Fucα1→2)Galβ1-(R) - - - -

**Gangliosides**
- GM3 NeuAcα2→3Galβ1→4Glcβ1-1’Cer - - - -
- smLe4 SPG NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1-1’Cer - - + - -
- MSGG LKE, SSEA4 NeuAcα2→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1-1’Cer - - - -

a. Abbreviations: Cer, ceramide; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; NeuAc, N-acetyleuraminic acid; NT, not tested; PG, paragloboside; Pt, patient sample; (R), core oligosaccharide; SPG, sialylparagloboside.

b. Binding by HPTLC-immunostaining with anti-PP1Pk, allo-P and patient (Pt) sera.

c. Hemagglutination inhibition with allo-P and patient (Pt) sera.
Inhibition was specific: alloanti-P was not inhibited by taurodeoxycholate, CDH, Gb3, nLc4, GM3, RBC gangliosides or gangliotriaosyleramid (Gg3), which also terminates in a βGalNac-R (Table 3). Gb4 inhibition was also dose-dependent, with a progressive decrease in agglutination strength with increasing Gb4 concentrations (Figure 4B). As expected, Gb4 did not inhibit anti-A in group O serum (Figure 4C; Table 3, αGalNac-R).

Unlike alloanti-P, Gb4 failed to inhibit patient sera (Figure 4D, lane 4), even at concentrations of 100 μg Gb4/mL plasma (not shown). There was no demonstrable inhibition of patient plasma by any GSL tested, including Gg3, Gg4 (Galβ1-3GalNAc-R, T-like terminus), nLc4 (II-like), and GM3 (Gd sialoagglutinin) [15, 26]. Patient plasma was also not inhibited by RBC gangliosides (100 μL packed RBC/mL serum, lane 11) despite evidence of antibody binding to snLc4 by HPTLC-immunostaining (Figure 2B).

**DISCUSSION**

LKE is a high-incidence, sialylated globo-family glycosphingolipid (GSL) or ganglioside, on RBC and other tissues (Figure 1) [1-3]. Like antibodies to P and Pk antigens, alloantibodies to LKE antigen are rare (Table 1), with only 6 cases mentioned in the literature.
[5-9]. A well-known example of anti-LKE was described by Moller et al., [5, 6] who reported a low-titer, clinically-insignificant LISS-dependent panagglutinin in a prenatal sample of a group A, P+, LKE-N woman. We recently had the opportunity to reexamine this sample and show that the antibody actually recognizes Gb4 or P antigen. The absence of reactivity with autologous and LKE-N RBC appears to reflect weakened P/Gb4 expression on patient and other LKE-N RBC. This case suggests that some examples of anti-LKE might actually represent weak auto-anti-P with ‘pseudo-LKE’ activity in serologic testing. In addition, we describe a modified GSL inhibition assay for use by gel method.

The original identification of this patient’s antibody as an alloanti-LKE was consistent with both her RBC phenotype and the results of serologic testing. This patient typed as P+, LKE-N and contained an antibody reactive with all cells except rare p, Pk cells and autologous RBCs. Furthermore, antibody reactivity was enhanced by enzyme-digestion and sensitive to DTT, consistent with an IgM antibody against a GSL antigen, including all prior cases of anti-LKE [5-9, 15]. In addition, the antibody was clinically insignificant, consistent with most (5/6) examples of anti-LKE [6-8].

At delivery, there was no clinical or laboratory evidence of HDN: the infant’s DAT was negative and no antibody was identified in the baby’s serum [6].

We also observed an agglutination pattern consistent with anti-LKE after testing patient serum against a panel of well-characterized LKE-N, LKE-W and LKE-S RBC. However, direct testing against a panel of GSLs by HPTLC-immunostaining revealed a weak anti-P with no antibody binding to MSGG/LKE. On further study, the presence of anti-P was also consistent with the ‘LKE-like’ pattern we observed by serology (Table 2). There was a clear correlation between relative P expression, LKE phenotype and reactivity with patient serum. Specifically, the patient’s serum agglutinated RBC with P-titers ≥ 32, whereas LKE-N and many LKE-W RBC had P-titers ≤ 16 (Table 2). It is interesting that the lowest P-antigen expression were observed with a rare ‘weak P’ sample and patient RBC (titer 8), which may account for the lack of reactivity with autologous RBC.

These findings are consistent with other studies examining the LKE-N phenotype. In many LKE-N donors examined to date, decreased MSGG is often
accompanied by relative decreases in P/Gb4 and increased Pk/Gb3, reminiscent of a variant-Pk phenotype [3, 7, 27]. At least one case of LKE-N has arisen on a "weak P" background, with marked depression of all globo-GSLs [22, 23]. It is not unusual, therefore, for LKE-N RBC to have weakened P/Gb4 expression.

Although we were able to demonstrate Gb4/P specificity by HPTLC-immunostaining and hemagglutination, we were unable to inhibit the patient’s anti-P with purified Gb4 in a modified GSL hemagglutination inhibition assay. We also found no inhibition with a broad panel of GSLs representing globo-, ganglio- and neolacto-families (Table 3, Figure 3). In contrast, we were able to demonstrate specific, dose-dependent inhibition of alloanti-P with solubilized Gb4, with nearly complete inhibition at 20 μg Gb4/mL serum.

Our inability to inhibit the patient’s auto-anti-P may reflect either weak antibody avidity and/or specificity. Judd et al. reported only partial inhibition of a LISS-dependent auto-anti-P (titer 16) by Gb4 [24]. Likewise, anti-PP1Pk sera is inhibited by Gb3, but not Gb4, in hemagglutination inhibition assays [16] despite discernable antibody binding to Gb3 and Gb4 by HPTLC-immunostaining (Figure 2E, Table 3) [21]. Alternately, the patient’s antibody could be a nonspecific anti-βGalNAC, with crossreactivity to Gb4/P [28]. Gb4 accounts for over 35% of all the terminal GalNAC epitopes on RBC membranes [29] and typically exists in large membrane microdomains, [30] presenting a high density antigen favoring antibody binding. Anti-βGalNAC can have apparent P-specificity, agglutinating P+ RBC, but not p or Pk RBC [28].

Finally, we cannot exclude the possibility that our modification of the GSL inhibition method may have hampered our ability to detect weak or partial inhibition. In the original method, assays were performed by passive hemagglutination in microtiter plates [16, 17]. Unfortunately, the microtiter method requires a strong direct agglutinating antibody, a working titer of 4 to compensate for dilution, and sufficient material to perform the assay. Given the limited material available and weak antibody reactivity (titer = 1), we adapted the assay to gel method with minimal serum dilution (20%). Unlike the microtiter method, however, the gel method requires centrifugation, which may have obscured any subtle inhibition by Gb4 or other GSL.

Auto-anti-P is a common specificity in PCH patients, a clinically-significant autoimmune anemia due to a bispicic IgG hemolysin [31]. Unlike the patient, PCH patients typically have a positive DAT (C3 only) and a negative antibody screen in routine testing. In contrast, panagglutinins with auto-anti-P specificity are rarely reported in the literature. Judd previously described three auto-anti-P, all in female patients [24, 25]. Two auto-anti-P were identified in LISS whereas a third auto-P was pH-dependent. Like the index patient, these auto-anti-P were relatively low-titer, enhanced by ficin, nonreactive with p and Pk RBC, and predominantly of IgM isotype based on studies with DTT and antihuman globulin (Table 4). Unlike the patient, the autocontrol was positive when autologous RBC were suspended in LISS (cases 2, 3) or acidic saline (case 4). Neither P-antigen expression nor LKE type were performed; however, given the incidence of LKE in the population, it is likely that all three cases were LKE+ with normal P-antigen expression.

It is tempting to speculate that other examples of anti-LKE may also represent auto-P with ‘pseudo-LKE’

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**Table 4: Low-Titer Globo Panagglutinins.**

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Age</th>
<th>ABO</th>
<th>P</th>
<th>p</th>
<th>Pk</th>
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<th>37°C</th>
<th>IAT</th>
<th>DTT&lt;sup&gt;c&lt;/sup&gt;</th>
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<th>Antibody&lt;sup&gt;e&lt;/sup&gt;</th>
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<td>0</td>
<td>4</td>
<td>+</td>
<td>0</td>
<td>0</td>
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<td>8</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>C3</td>
<td>S</td>
<td>↑</td>
<td>Auto-P</td>
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<td>4</td>
<td>+</td>
<td>16</td>
<td>+</td>
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<td>C3</td>
<td>S</td>
<td>↑</td>
<td>'Anti-LKE'</td>
</tr>
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</table>

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a. ABO and P1 RBC phenotype. DAT, direct antiglobulin test (DAT). NR, not reported.

b. Reported serologic testing results against p, Pk and P+ RBC. Also shown are the titer (Tit) results and IS reactivity against group O, P+ RBC at different temperatures and in the IAT. For IAT results, samples reactive were reactive with anti-C3 but not anti-IgG.

c. Tit, antibody titer against LKE+ RBC.

d. Reactivity of serum after treatment with either dithiothriitol (DTT) or 2-mercaptoethanol (2ME).

e. Reactivity of serum/plasma after incubation with enzyme-modified RBC.

f. Reported antibody specificity.

g. ESRD, end-stage renal disease.

h. Case 3 showed reactivity with ficin-treated p RBC [23]. Case 5 reacted with 1/5 p RBC samples [7].

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activity. Of the 6 known anti-LKE mentioned in the literature, four were apparently low titer, clinically-
insignificant panagglutinins [4–7]. Very little information is available on 2 examples [8], however, a third ‘anti-LKE’ is highly suspicious for an auto-anti-P (Table 4, case 5) [7]. Like the patient sample, this ‘anti-LKE’ was a low-titer, IgM antibody identified in a pregnant woman. Interestingly, this ‘anti-LKE’ was weakly reactive with an occasional p RBC sample, suggesting possible anti-P-like activity. A similar phenomena can be observed with some alloanti-P due to crossreactivity to PX2, a ‘P-like’ GSL on p RBC that also possesses a terminal βGalNAc (PX2, GalNAcβ1-3Galβ1-
4GlcNAcβ1-3Galα1-4Galβ1-4Cer) [32].

In addition to an auto-anti-P, we also observed antibody binding to sialylparagloboside (snLC4, SPG), the predominant neolacto-series GSL and ganglioside on human RBC [22, 27]. SnLC4 is recognized by many sialoagglutinins and is an epitope for Gd or ‘glycolipid-
dependent’ cold agglutinins [26]. Although no I/1-like cold agglutinins were reported in the sample, [5, 6] the patient did possess an apparent anti-P1, a related type 2 chain antigen (Table 3), in routine serologic testing. Some examples of anti-P1 can display both P and II specificity (iP1, iP1) due to crossreactive epitopes shared by type 2 chain antigens [32, 33]. These antibodies behave as normal anti-P1 in routine serologic testing unless carefully titrated against adult and cord cells [32]. The activity we observed, therefore, could suggest a ‘Gd-type’ agglutinin with both snLC4 and P1 activity.

CONCLUSION

In summary, we have reexamined a historical anti-
LKE and have reclassified this antibody as a weak anti-auto-P with ‘pseudo-LKE’ activity in serologic testing. It is possible that other previously identified weak ‘anti-
LKE’ are low-titer autoantibodies with anti-P or anti-
βGalNAc-activity. Like other weak auto-anti-P described in the literature, antibody activity was enhanced by LISS and ficin-treatment. Our results indicate that serology is not sufficient to identify anti-LKE, but requires direct serum testing against MSGG and related globo-GSLs. We also describe a modification of the GSL inhibition assay for use by gel method.

ABBREVIATIONS

paragloboside or SPG.

ACKNOWLEDGEMENTS

I wish to thank Louann Dake for helpful discussions and access to rare sera. I also thank Dr. De-Sheng Zhang and Dr. Theodore Koerner for their past help in isolating GSL standards used in this study.

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Author Contributions

Laura Cooling – Substantial contributions to conception and design. Acquisition of data, Analysis and interpretation of data. Drafting the article. Revising it critically for important intellectual content, Final approval of the version to be published

Guarantor

The corresponding author is the guarantor of submission.

Conflict of Interest

Authors declare no conflict of interest.

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