A hemolytic anti-LKE associated with a rare LKE-negative, "weak P" red blood cell phenotype: alloanti-LKE and alloanti-P recognize galactosylgloboside and monosialogalactosylgloboside (LKE) antigens

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BACKGROUND: "Weak P" is a rare red blood cell (RBC) phenotype, characterized by a global decrease in P^k and P antigens. We now describe a second weak P individual who also typed LKE-negative (LKE-N) and possessed a clinically significant anti-LKE.

STUDY DESIGN AND METHODS: Patient RBCs and plasma were examined by standard serology and flow cytometry. Glycosphingolipids (GSLs) from patient, P^k, and LKE-strong (LKE-S) RBCs were isolated and analyzed by high-performance thin-layer chromatography (HPTLC). To confirm antibody specificity, patient serum and 30 human polyclonal controls, including alloanti-P and anti-PP_1P^k, were tested against a panel of GSLs by HPTLC immunostaining.

RESULTS: The patient typed P_1+, P+, and LKE-N and possessed a "P-like" panagglutinin. In a two-stage indirect antiglobulin test, the patient's plasma caused hemolysis of LKE-S cells but not p, P^k, or LKE-N cells. Clinically, transfusion of P+ RBCs compatible by a prewarmed technique had shortened RBC survival with laboratory evidence of hemolysis. Analysis of the patient's isolated RBC GSLs showed a 30% relative decrease in Gb3 (P^k) and Gb4 (P) and a 90% decrease in monosialogalactosylgloboside (MSGG, LKE), accompanied by increased lactosylceramide (CDH), paragloboside, and GM3. On HPTLC immunostaining, the patient's plasma strongly bound MSSG with weak binding to galactosylgloboside (Gb5). Binding to MSGG, Gb5, and Gb4 was also observed with some examples of alloanti-P from P^k individuals, but not anti-PP_1P^k, autoanti-P, or normal controls.

CONCLUSIONS: We describe the first example of a clinically significant anti-LKE in the setting of a rare weak P background. Human alloanti-LKE and some alloanti-P recognized Gb5 and MSGG.

LKE is a sialylated, globo-family glycosphingolipid (GSL)[1,2] that is biosynthetically related to the P^k (Gb3) and P (Gb4) antigens. Like P^k and P antigens, LKE is a high-frequency antigen on human red blood cells (RBCs), with 98% to 99% of donors typing LKE-positive.[2-7] LKE varies in strength between individuals, with 80% to 90% typing LKE-strong (LKE-S) and 10% to 20% individuals as LKE-weak (LKE-W). LKE-negative (LKE-N) is reported in 1% to 2% of P+ donors, as well as rare p and P^k individuals who are deficient in complex globo-GSLs. Interestingly, there is an apparent inverse relationship between LKE and P^k, with many LKE-N donors showing elevated P^k expression reminiscent of a "P^k variant" phenotype.[4,7,8] LKE may also have interactions with other carbohydrate blood groups. In uroepithelial

ABBREVIATIONS: DSGG = disialogalactosylgloboside; GSL = glycosphingolipid; HMW = high-molecular-weight; HPTLC = high-performance thin-layer chromatography; IS = immediate spin; LKE-N = LKE-negative; LKE-S = LKE-strong; LKE-W = LKE-weak; MSGG = monosialogalactosylgloboside; PCH = paroxysmal cold hemoglobinuria; RT = room temperature; Stx = Shiga toxin.

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TABLE 1. Examples of anti-LKE reported in the literature

Case	Age (years)/sex	Ethnicity	Diagnosis	Prior sensitization	Antibody titer: Unmodified RBCs	Antibody titer: Enzyme RBCs	IAT*	Significant	Reference
1	32/male	Black	Hodgkin's lymphoma	No	256	512	C3	NR	Tippett, 1965[5]
2	NA/female	NA	Prenatal	Yes, G_2P_1†	2	4	C3 only	No	Bruce, 1988[7]
3	32/female	Danish	Prenatal	Yes, G_2P_1‡	4	16, IAT	IgG‡	No	Moller, 1988[6]
4	NA/male	Algerian	Cardiac	No	NR	NR	NR	NR	Race, 1965[10]
5	NA/female	Turkish	NR	NR	"Weak"	NR	NR	NR	Race, 1965[10]
6	58/male	White	Large B-cell lymphoma	Unknown§	ND	256	C3	Yes	This study

* Reactivity in the IAT (anti-C3, anti-IgG).
† Weak panagglutinin recognized at 4 months during first pregnancy. Antibody specificity not identified until her second pregnancy.
‡ Antibody reportedly sensitive to dithiothreitol, suggesting an IgM antibody. Patient also possessed an anti-P_1 reactive at 37°C and anti-IgG.
§ Patient received a 2-unit RBC transfusion 1 week before the discovery of a panagglutinin. The pretransfusion results are not available.
ND = not done; NR = not reported.

tissues, LKE can be weakened in the presence of secretor.[9] Serologic studies with rare human anti-LKE also suggest an association between LKE-W, A_1, and P_2 phenotypes.[5,6]

Although 1% to 2% of the population types as LKE-N, alloantibodies to LKE are actually quite rare, with only five cases mentioned in the literature.[5-7,10] All acted as direct panagglutinins, with most capable of fixing complement in vitro (Table 1). The first example was found in an untransfused man with Hodgkin's disease.[5] Two examples were identified in prenatal samples[6,7] and an additional two were identified in routine testing.[10] Based on available literature, anti-LKE appears to be clinically insignificant.

We now report a sixth example of an alloanti-LKE. Unlike prior cases, this patient had clinical evidence of shortened RBC survival and hemolysis after repeated transfusions with LKE+ blood that was compatible in prewarmed testing. In addition, we show evidence that the LKE-N phenotype arose from a "weak P" RBC phenotype,[11] with weakened expression of all globo-GSLs. This is the second case of a weak P and the *first* associated with a LKE-N phenotype. Finally, we provide the first direct evidence of human alloanti-LKE binding to monosialogalactosylgloboside (MSGG), the putative LKE antigen.

CASE REPORT

The patient was a 58-year-old white man with a history of gastroesophageal reflux disease, hypertension, Type II diabetes, atrial fibrillation, congestive heart failure with an ejection fraction of 25% to 30%, chronic microcytic anemia, and a 1-year history of a nonpainful right groin mass. He was initially seen in a local primary care facility, where he was transfused with 2 RBC units for a hemoglobin (Hb) of 9.7 g/dL. He was referred to a local hospital 1 week later for further evaluation of his anemia. At that time, his Hb was 7.0 g/dL with a weakly positive direct antiglobulin test (DAT; 1+, anti-C3 only). In addition, a cold agglutinin with "P-like" specificity was identified in his plasma. He received no additional transfusions due to difficulty finding compatible blood. He was subsequently transferred to a large regional hospital for management of his anemia and investigation of his left groin mass.

Upon admission to Hospital 2, the patient was afebrile, tachycardic (pulse 104) with a 5×3-cm firm, nontender, left groin mass. A complete blood count was significant for a hypochromic, microcytic anemia (Hb 7.1 g/dL, MCV 79, MCH 25.9), mild leukocytosis (17.6×10^9/L), and thrombocytosis (614×10^{11}/L). Chemistry studies showed a normal serum iron, normal bilirubin, mildly depressed transferrin, and elevated ferritin (11,600 ng/mL; normal range, 12-300 ng/mL) and haptoglobin (375 mg/dL; normal range, 23-200 mg/dL). There was no evidence of gastrointestinal bleeding by endoscopy and stool guaiac tests. A CT scan revealed splenomegaly with periaortic, iliac, and inguinal lymphadenopathy: There was no evidence of retroperitoneal or intrabdominal hemorrhage.

On Hospital Day 2, the patient became hypotensive and was transfused with 2 units of group O RBCs. Both units were strongly incompatible (3+) by immediate-spin (IS) cross-match and weakly (±) reactive in polyethylene glycol (PEG) indirect antiglobulin tests (IATs), but were compatible using a prewarmed technique.[12,13] Both units were transfused without incident but failed to elicit the expected increase in Hb, with a posttransfusion Hb of only 7.7 g/dL (Fig. 1).

On Day 5, the patient received an additional 2 RBC units before a scheduled lymph node biopsy. As before, units were cross-matched by a prewarmed technique. The patient was medicated before transfusion with acetaminophen, diphenhydramine, and dexamethasone. The patient tolerated transfusion with an appropriate 2 g/dL increase in Hb; however, his Hb quickly decreased to pretransfusion levels within 24 hours. Over the next 6 days, the patient was transfused an additional 5 units with no sustained increase in Hb (Fig. 1A). Transfusions were accompanied by increased lactate dehydrogenase (LDH), total bilirubin, and decreasing haptoglobin (Fig. 1B). By Day 11, the patient was noted to be jaundiced with a total

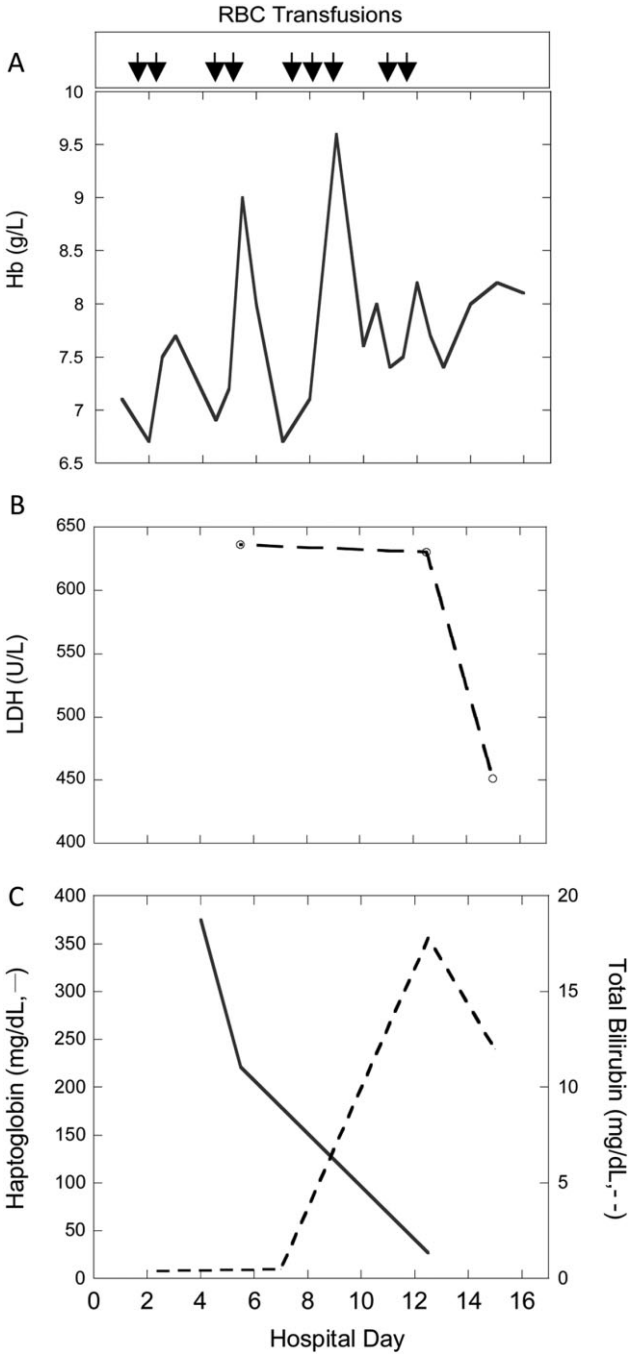

Fig. 1. Patient clinical course following transfusion of P+ RBC units compatible by a prewarmed cross-match technique over the course of hospitalization at Hospital 2. (A) Patient's Hb, (B) LDH, and (C) haptoglobin (–) and total bilirubin (- -). Arrows indicate transfusion of group O+, leukoreduced irradiated RBC units.

bilirubin of 17.9 mg/dL (64% direct bilirubin), elevated reticulocytes (4.9%), and low haptoglobin. In addition, his DAT was 2+ (anti-C3) with spherocytes and rouleaux noted on a peripheral blood smear.

Given laboratory evidence of a delayed hemolytic transfusion reaction, it was decided to forego any further transfusions unless the patient became symptomatic. A histologic examination of his left inguinal lymph node revealed a large B-cell lymphoma. On Day 15, the patient was treated with one course of rituximab, cyclophosphamide, and vincristine. He was discharged on Day 17 to the care of a local oncologist. The patient died 3 months after discharge of unknown causes.

MATERIALS AND METHODS

Immunologic reagents

Monoclonal antibodies (MoAbs) against P/Gb4 (MoAb MC631),[14] LKE/MSGG (MoAb MC18-70),[1] and lactosamine (MoAb FeA5)[15] were obtained from the Developmental Studies Hybridoma Bank held at the University of Iowa (Iowa City, IA). Shiga toxin (Stx) and anti-Stx IgG were purchased from Toxin Technology (Sarasota, FL). Stx was labeled with fluorescein isothiocyanate (FITC) as described.[4] The resulting Stx-free dialysate was retained as a negative control. SC802, a recombinant P-fimbriated *Escherichia coli* strain, and HB101, a nonfimbriated *E. coli* control, were a gift from S. Clegg (Department of Microbiology, University of Iowa).[16] Biotinylated anti-mouse (IgG, IgM) and anti-human (IgG, IgM) antibodies, streptavidin-alkaline phosphatase, and alkaline phosphatase substrate were purchased from Vector Laboratories (Burlingame, CA). FITC-labeled anti-mouse antibodies (IgG, IgM) and *Clostridium perfringens* Type IV neuraminidase were purchased from Sigma (St Louis, MO). The immunologic reactivity and structure of various GSL antigens is shown in Table 2.

Rare human RBCs and antibodies were obtained via the SCARF International Exchange Program[17] and the University of Michigan Immunohematology Reference Laboratory. P_1^k cells for GSL extraction were a gift from R. Thompson (University of Minnesota). Anti-mouse IgG gel cards were a gift from H. Malyska (Micro Typing Systems, Pompano Beach, FL). All other serologic reagents were from Ortho Clinical Diagnostics (Raritan, NJ) or ImmucorGamma (Norcross, GA) and used according the manufacturers' instructions.

RBC studies

LKE typing was performed with MoAb MC813-70 as described.[4,18] Samples agglutinating at IS were typed as LKE-S. Negative samples were tested by the IAT using anti-mouse IgG. 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 MoAb MC631.[4,14]

Other serologic tests were performed as described previously.[12,13] Reactions were graded and scored

TABLE 2. GSL structures

Name/family	Specificity	Structure	Reagent*
CMH	Glucosylceramide	Glcβ1-1Cer	
CDH	Lactosylceramide	Galβ1-4Glcβ1-1Cer	
GM3	Gd†	NeuAcα2-3Galβ1-4Glcβ1-1Cer	
Globo-series			
Gb3	P^k, CD77	Galα1-4Galβ1-4Glcβ1-1Cer	Stx
Gb4	P	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	MC631
Gb5	SSEA-3	Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	MC631
Forssman		GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	
Globo-H	Type 4 H	Fucα1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	MBr1
MSGG	LKE, SSEA-4	NeuAcα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	MC813-70
DSGG		NeuAcα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	nm/MC631
		NeuAcα2-6/	5F3
Neolacto-series			
nLc4	Paragloboside	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	FeA5
αGal-nLc4	P_1 antigen	Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	Stx
βGalNAc-nLc4	PX2, P-like	GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	
snLc4	Sialylparagloboside	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	nm/FeA5‡
snLc6		NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer	nm/FeA5‡
Ganglio-series			
GM1		Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer	Cholera toxin
GD1a		NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer	
GD1b		Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1Cer	

* MoAbs and lectins against GSL antigens.
† GM3 is recognized by Gd (glycolipid-dependent) cold agglutinins.
‡ Nm/FeA5, reactive after in situ digestion of gangliosides with neuraminidase, followed by staining with MoAb FeA5.
Cer = ceramide; Fuc = fucose; Gal = galactose; GalNAc = *N*-acetylgalactosamine; Glc = glucose; GlcNAc = *N*-acetylglucosamine; Gd = glycolipid-dependent cold agglutinin; NeuAc = *N*-acetylneuraminic acid; nm = in situ neuraminidase digestion; SSEA = stage-specific embryonic antigen.

according to the method described by Marsh.[19] Bacterial hemagglutination assays using P-fimbriated *E. coli* were performed on buffered gel cards (Ortho) as previously described.[20]

Flow cytometry

Washed, ficin-treated RBCs were stained with MoAbs or FITC-labeled Stx and analyzed by one-color flow cytometry (FACScan, Becton Dickinson, Hialeah, FL) as described.[4] All samples were stained in duplicate and recorded as the mean percent positive cells and mean channel fluorescence intensity (MFI).[4]

High-performance thin-layer chromatography

RBC GSLs were isolated by a modified Leeden and Yu method as previously described.[4,18,21] High-performance thin-layer chromatography (HPTLC) was performed using either glass or aluminum-backed HPTLC plates (E. Merck, Darmstadt, Germany).[22,23] For neutral GSL analysis, HPTLC plates were developed in standard neutral solvent (C-M-W 65:25:4, vol/vol). Gangliosides were developed in C-M-0.02% aqueous $CaCl_2$. GSLs were visualized by chemical staining (diphenylamine, charring) or HPTLC immunostaining.[22,23] GSLs were characterized by relative mobility (R_f) and relative intensity by scanning densitometry (Quantiscan, Biosoft, Ferguson, MO). GSL standards were purchased from Sigma or Matreya (Pleasant Gap, PA). Paragloboside (nLc4), Gb5, MSGG, neutrophil, and ACHN GSLs were isolated previously.[23]

HPTLC immunostaining

For immunostaining, HPTLC plates were coated in 0.2% poly(iso)butyl-methacrylate in hexane for 1 minute and dried for at least 1 hour at room temperature (RT).[18,23] For staining with human antisera, plates were initially blocked for 1.5 hours with phosphate-buffered saline (PBS)-3% bovine serum albumin (BSA) and then overlaid with antisera diluted 1:20 in PBS-5% BSA for 2 hours. After washing, bound antibody was detected by sequential incubation with biotinylated anti-human immunoglobulin (IgM, IgG), streptavidin-linked alkaline phosphatase, and black alkaline phosphatase substrate.[18] To estimate relative antibody binding, the amount of bound antibody was divided by total GSL as determined by scanning densitometry (antibody staining [area]/GSL quantity [area by charring]). Immunostaining with murine MoAbs and Stx were performed as previously described.[4,23,24]

Statistical analysis

Quantitative data were expressed as the mean and standard deviation (SD). Differences in relative GSL expression were compared by t test. Graphics and statistical analysis

were performed with commercial software (Kaleidograph, Synergy Software, Reading, PA).

RESULTS

Serology

RBC typing

The patient's RBCs typed as group O, D+, I+, Le(a–b+), P_1+. RBCs were reactive with several anti-globo reagents by direct agglutination, including anti-PP_1P^k, alloanti-P, MoAb MC631, and P-fimbriated *E. coli* (Table 3). The patient typed LKE-N with MoAb MC813-70: No agglutination was observed with ficin-treated patient RBCs after 4°C incubation or in the IAT with anti-mouse IgG (tube and gel method).[2,4,18]

The patient's DAT was weakly reactive (1+) due to complement after transfusion of 2 RBC units at a local facility. A DAT drawn after his last transfusion was 2+ (anti-C3, 11 RBCs total). An acid eluate of both samples was negative by tube method at IS and after incubation at RT (15 min) and 4°C (30 min, Table 3).

Serum findings

The patient's plasma contained a weak RT panagglutinin reactive with 14 of 16 P+ RBCs: No agglutination was observed against p (n = 4), P_1^k, P_2^k, LKE-N (n = 2), and autologous RBCs (Table 3). Reactivity could be enhanced by 15-minute RT incubation, 30-minute incubation at 4°C (4+), and use of ficin-treated RBCs. The direct agglutination titer against LKE-S RBC was 256 at 4°C.

The plasma was nonreactive at 37°C with low-ionic-strength saline (30- or 60-min incubation) and in the IAT by tube and gel method with anti-IgG. The serum was weakly reactive in PEG-IAT cross-matches with some random-donor RBCs, but nonreactive with the same cells by a prewarmed technique.[12,13] The serum caused hemolysis of ficin-treated, LKE-S cells in a two-stage IAT by tube method, using fresh normal serum as a source of complement and polyspecific anti-human globulin (Table 3).[13]

Flow cytometry

Patient cells were initially screened for P^k, P, and LKE expression by flow cytometry.[4] RBCs from LKE-S (n = 3), LKE-N, P_1^k, and p individuals were included as controls. Consistent with serologic typing, little or no detectable LKE was identified on patient RBCs with MoAb MC813-70 (Fig. 2). Unlike the LKE-N and P_1^k controls, the patient had no evidence of increased P^k expression.[4,7] There was no significant difference in P^k and P expression between the patient and the LKE-S controls.

Neutral RBC GSLs

To confirm the results of flow cytometry, GSLs from the patient's cells were isolated and analyzed by HPTLC. GSLs from human granulocytes and P_1^k and LKE-S RBCs were included as controls. Neutrophils, like p RBCs, are devoid of globo-GSLs and express predominantly neolacto-family GSLs (Table 4).[23,25]

TABLE 3. RBC and serum reactivity by LKE phenotype*

	RBC typing*†								Serum testing‡				
				Anti-P		*E. coli*§			Direct Agglutination‖			Two-stage IAT¶	
Cell	ABO	Anti-P_1	Anti-PP_1P^k	MC631	P-titer**	SC802	HB101	LKE††	IS	15 min, RT	60 min, 37°C	AHG	Comments
p	O	0	0	0	0	0	0	N	0	0	–	–	
P_1^K	O	+	3+	0	0	0	0	N	0	0	–	–	
1	O	+	–	4+	32	512	0	N	0	0	0	0	
2	O	+	–	3+	16	256	0	N	0	+/–	0	0	
3	O	+	–	–	–	1048	0	W	–	$1+^w$	0	0	
4	O	+	–	–	32	256	0	W	–	$1+^w$	0	0	
5	O	+	3+	4+	32	512	0	S	2+	2+	0	$1+^w$	Hemolysis
6	O	+	–	4+	64	1048	0	S	2+	3+	0	$1+^m$	Hemolysis
7	O	+	–	–	–	–	–	S	+/–	$1+^s$	0	$2+^s$	
8	O	+	–	–	–	–	–	S	–	–	0	2+	Hemolysis
Pt.	O	3+	3+	2+	8	512	0	N	0	0	0	$1+^w$	2+ DAT‡‡

* A total of 32 RBC samples were tested against patient serum/plasma. Shown are the reactivity of cells used in the two-stage IAT and flow cytometry (Fig. 2).
† RBC typing with commercial ABO, anti-P_1, human anti-PP_1P^k, MoAb MC631, human alloanti-P, and MoAb MC813-70.
‡ Performed with the "posttransfusion" sample obtained by Hospital 2. Patient had been transfused with 11 units of RBCs to date.
§ Gel titer with *E. coli* SC802 (P-fimbriated) and HB101, a nonfimbriated control against unmodified RBC in buffer-only gel cards.[16,20]
‖ Direct agglutination (tube) at IS, 15-minute RT incubation, and 60-minute incubation at 37°C.
¶ Two-stage IAT performed with ficin-treated RBC, polyspecific AHG, and fresh plasma (serum) as a source of complement.[13] Cells were chosen based on their LKE-phenotype as noted.
** P-titer using human polyclonal alloanti-P with strict Gb4 specificity by HPTLC immunostaining. Testing performed with unmodified RBC (buffer gel, 1 hour, 4°C). Titer reported as the inverse dilution giving 1+ agglutination.
†† LKE phenotype as determined with MoAb MC81-70.[2,4]
‡‡ Patient had 2+ DAT after transfusion with 11 units of RBCs.
– = not done; 0 = no agglutination; + = positive. Hemagglutination scoring per routine.[13]

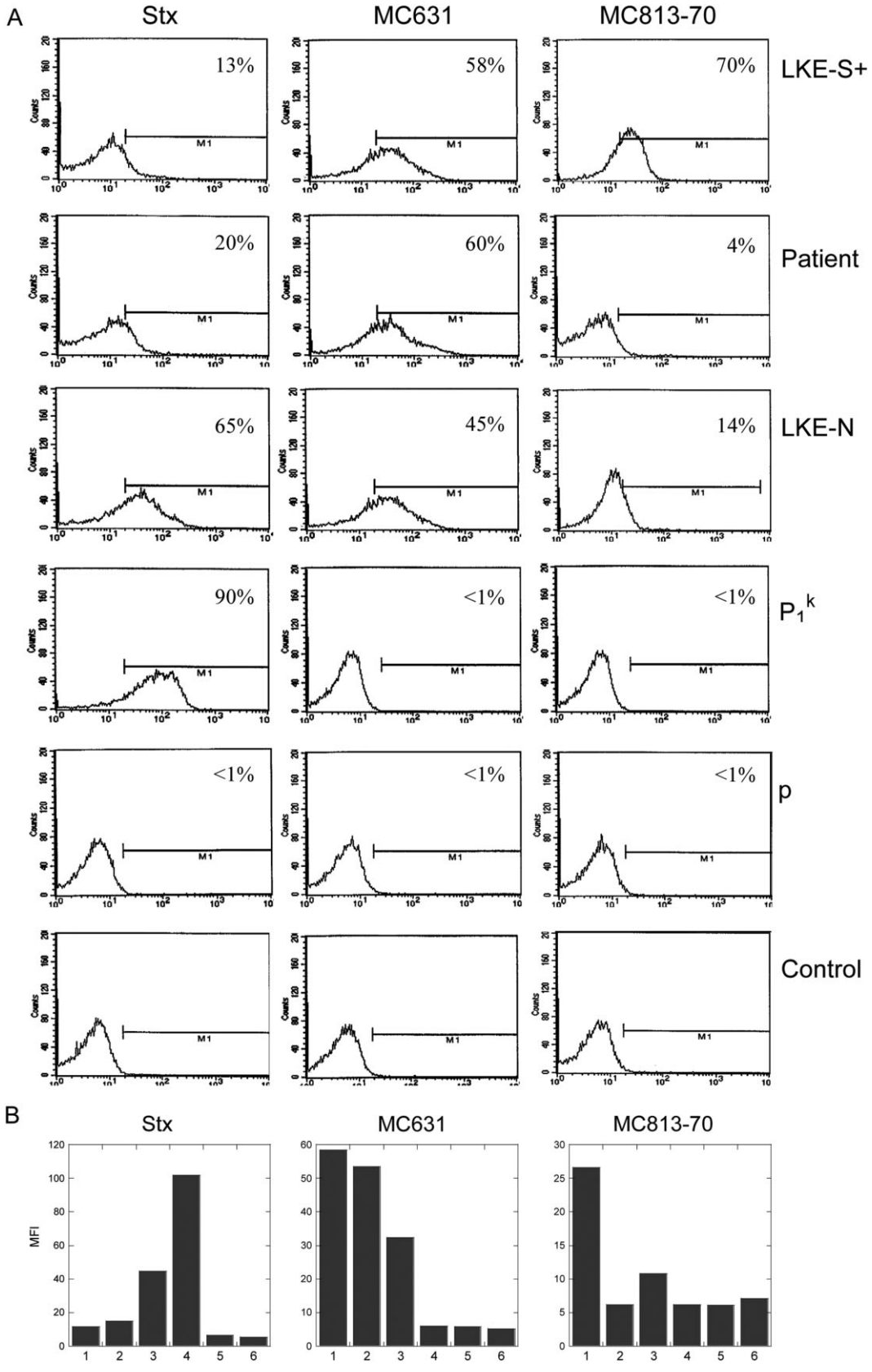
A
Stx
MC631
MC813-70
13%
58%
70%
LKE-S+
20%
60%
4%
Patient
65%
45%
14%
LKE-N
90%
<1%
<1%
P1k
<1%
<1%
<1%
p
Control
M1
Counts
B
Stx
MC631
MC813-70
MFI

Fig. 2. Globo-GSL analysis by flow cytometry. (A) Flow cytometric histograms of patient, LKE-S, LKE-N, P_1^k, and p ficin-treated RBCs stained with FITC-Stx (anti-Gb3), MoAb MC631 (anti-Gb4), and MoAb MC813-70 (anti- LKE, Table 2). Patient sample is posttransfusion of 2 units of group O RBCs. Also included are negative isotype controls for murine IgM (anti-CD15), IgG (anti-CD31), and Stx (FITC dialysate[4]). Line demarcates positively stained cells. Percentages indicate the percent positive cells for each marker. (B) The MFI of RBCs stained with Stx, MoAb MC631, and MoAb MC813-70. Lane 1 = LKE-S; Lane 2 = patient; Lane 3 = LKE-N; Lane 4 = P_1^k; Lane 5 = p; Lane 6 = negative control.

TABLE 4. Neutral GSL expression by P and LKE type

			Percent neutral GSL (normalized, mean ± SD)*					Ratio (area)†	
RBCs	ABO	No.‡	CMH	CDH	Gb3	Gb4	nLc4	Gb3/CDH	Gb4/Gb3
LKE-S	O, A	8	1.3 ± 0.7	11.3 ± 3.7	23.5 ± 3.0	58.4 ± 3.6	4.4 ± 0.6	2.38 ± 0.80	2.45 ± 0.44
	O	4	1.3 ± 0.7	10.3 ± 1.9	24.4 ± 2.5	57.7 ± 3.2	4.4 ± 0.6	2.56 ± 0.45	2.22 ± 0.16
	A	4	1.3 ± 0.8	11.1 ± 1.9	21.6 ± 5.6	54.2 ± 4.6	2.3 ± 0.5	2.20 ± 1.08	2.67 ± 0.54
LKE-N§	A,B,O	3	2.3 ± 1.3	11.1 ± 1.9	31.6 ± 5.6	54.2 ± 4.6	4.3 ± 0.7	2.90 ± 0.65	1.75 ± 0.43
P^k variant‖	O	1	NR	11.6	63.8	24.6	NR	5.52	0.38
P_1^k	A	1	1.5 ± 0.4	33.9 ± 3.7	52.0 ± 3.2	0	11.5 ± 1.6	1.65 ± 0.02	0
Weak P¶	A	1	NR	22.5	16.7	40.2	20.6	0.73	2.41
Patient	O	1	0.7 ± 0.1	30.6 ± 0.3	13.5 ± 1.8	42.7 ± 4.0	12.3 ± 4.6	0.47 ± 0.06	3.15 ± 0.44
p (PP_1P^k-)**	NR	4	7.2 ± 0.6	67.4 ± 2.9	0	0	13 ± 2.1	0	0
Granulocytes	B	1	1.9 ± 0.6	75.8 ± 2.1	0	0	18.2 ± 2.9	0	0
Patient vs. LKE-S††			NS	<0.0001	0.009	0.002	0.0005	0.03	0.046
Patient vs. LKE-N††			NS	<0.0001	0.006	0.031	0.019	0.003	0.017

* Relative distribution of CMH, CDH, Gb3, Gb4, and nLc4 as determined by scanning densitometry. The percentages were normalized to equal 100%.
† Substrate/product ratios for Gb3 (Gb3/CDH) and Gb4 (Gb4/Gb3) synthesis, as determined by scanning densitometry.
‡ Number of RBC GSLs tested or published in the literature.
§ Data taken from Cooling and Kelley.[4]
‖ Data taken from Kundu et al.[8]
¶ Data from Kundu et al.[11]
** Data taken from Kundu et al.[25] Lactotriaosylceramide (Lc3) content of p RBC averaged 12.2 ± 1.4%.[26]
†† t-test.
NA = not applicable; NR = not reported; NS = not significant ($p > 0.05$).

Normal RBCs express four major neutral GSLs (Fig. 3A): glucosylceramide (CMH, $R_f = 0.59$), lactosylceramide (CDH, $R_f = 0.40$), globotriaosylceramide (Gb3, $R_f = 0.25$), and globoside (Gb4, $R_f = 0.16$). Patient RBCs (Fig. 3A, Lane 7) displayed a 40% decrease in Gb3 and a 25% decrease in Gb4, for an overall 30% decrease in globo-GSLs relative to LKE-S controls (Table 4). Decreased globo-GSLs were accompanied by a threefold increase in CDH and increased expression of a paragloboside-like GSL (nLc4, $R_f = 0.13$). An increase in CDH and nLc4 were also observed in a P_1^k control.

Neutral GSLs were subsequently immunostained with reagents against Gb3 (Stx, Fig. 3B),[4] Gb4 (MoAb MC631, Fig. 3C),[14] and nLc4 (MoAb FeA5, Fig. 3D; Table 2)[15] to verify the results of chemical staining. The patient had weak Stx staining to Gb3 and P_1 (Fig. 3D, Lane 7). Likewise, MC631 staining to Gb4 was reduced 50% relative to LKE-S RBCs. Strong FeA5 staining to nLc4 was observed in patient, P_1^k, and a neutrophil control.

As an indirect measure of relative glycosyltransferase activity, the ratio of product to substrate was determined by scanning densitometry (Table 4).[28,29] Unlike LKE-N RBCs,[4] patient cells had a relatively normal Gb4/Gb3 ratio. In contrast, there was a fivefold decrease in the Gb3/CDH ratio, consistent with decreased conversion of CDH→Gb3. A decrease in Gb3/CDH ratio, accompanied by increases in CDH and nLc4, is also reported for the weak P phenotype.[11] As shown, relative neutral GSL expression in our patient and a known weak P sample was virtually identical (Table 4).

RBC gangliosides

HPTLC analysis of the total RBC ganglioside fraction showed subtle but significant differences in ganglioside expression between patient (Fig. 3E, Lane 3), P_1^k (Fig. 3E, Lane 2), and LKE-S RBCs (Fig. 3E, Lanes 4-6). All RBCs expressed ganglioside GM3 ($R_f = 0.48$) and sialoparagloboside (snLc4, $R_f = 0.38$), which together comprised 60% to 70% of the total RBC ganglioside by scanning densitometry (Figs. 3H-3J). In LKE-S cells, the ratio of snLc4:GM3 was approximately 2:1, with snLc4 representing nearly 50% of the total RBC ganglioside. In patient and P_1^k RBCs, the snLc4:GM3 ratio was reduced (1:1) due to a 20% relative increase in GM3 expression (Figs. 3H and 3I). All RBC samples, including the patient's, had evidence of snLc6 and long-chain, high-molecular-weight (HMW) Type 2 sialylated gangliosides[30-32] upon immunostaining with MoAb FeA5 (Fig. 3F).[15,24]

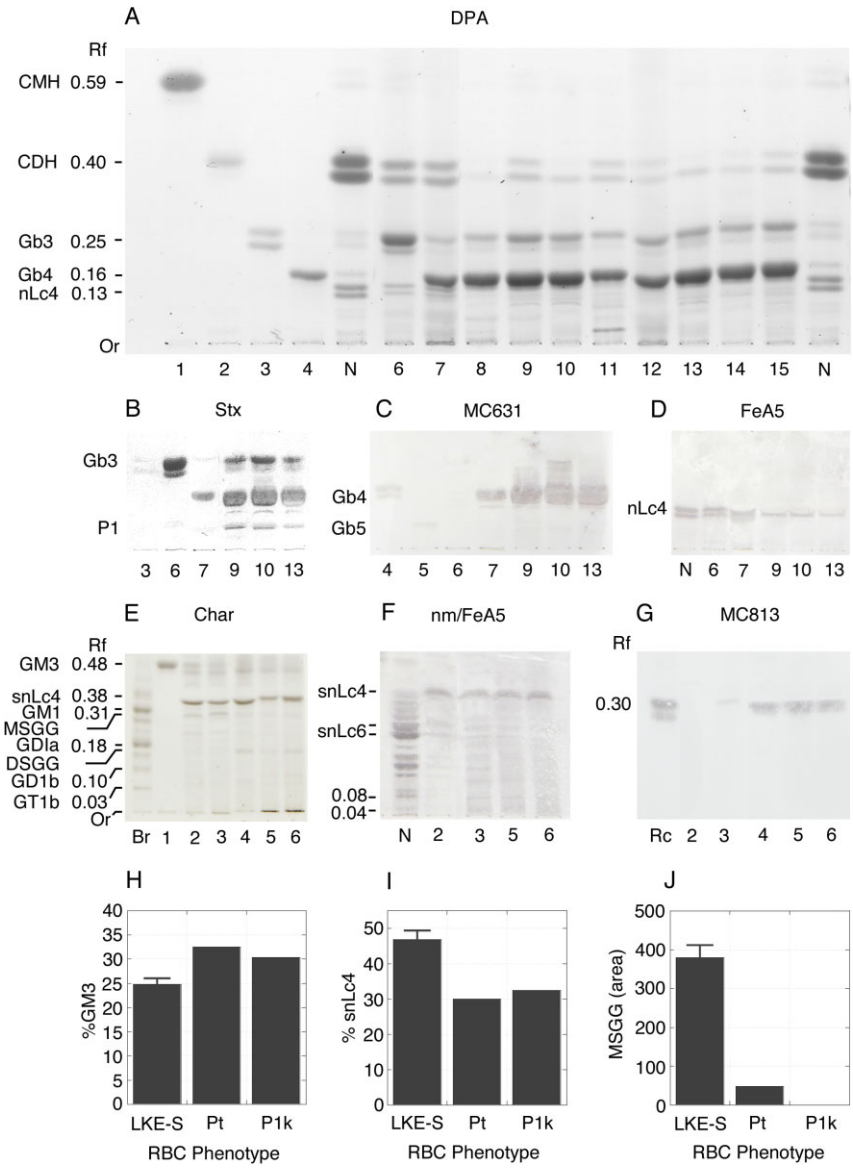

Fig. 3. HPTLC analysis of isolated RBC GSLs. (A-D) Neutral RBC GSLs chemically stained with (A) diphenylamine reagent, (B) Stx, (C) MoAb MC631, and (D) MoAb FeA5. Note faint Stx binding due to high Gb4 concentrations.[26,27] Lane 1 = CMH; Lane 2 = CDH; Lane 3 = Gb3 (P^k antigen); Lane 4 = Gb4 (P antigen); N = neutrophils; Lane 6 = P_1^k RBCs; Lane 7 = patient; Lanes 8 through 11, group A LKE-S RBCs; Lanes 12 through 16 = group O, LKE-S RBCs. Each RBC lane contains total neutral GSL from approximately 250 µL of RBC. Neutral GSLs were separated in neutral solvent C-M-W, 65:25:4 vol/vol. (E-G) RBC gangliosides stained by (E) charring reagent, (F) neuraminidase/FeA5,[24] and (G) MoAb MC813-70. MSGG identified in ACHN control (Rc, R_f = 0.30) and LKE-S RBCs, with only faint band in patient sample. Lane 1 = GM3 standard; Lane 2 = P_1^k RBCs; Lane 3 = patient; Lanes 4 through 6 = LKE-S RBC; Br = human brain; Rc = ACHN renal carcinoma cell (MSGG+, DSGG+); N = neutrophils. Gangliosides were separated in C-M-0.2% aqueous $CaCl_2$ 50:40:10 vol/vol. Each RBC lane contains the total ganglioside fraction from approximately 500 µL of RBCs. (H-J) Scanning densitometry of RBC gangliosides showing (H) percent GM3, (I) percent snLc4, and (J) relative concentration of MSGG based on MoAb MC813-70 staining.

To identify MSGG/LKE antigen, RBC gangliosides were immunostained with MoAb MC813-70 (Fig. 3G).[1,2] ACHN cells were included as an MSGG-positive control.[33,34] As shown, a strong MC813-70 band was identified in the ACHN control (Rc, R_f = 0.30) and all three LKE-S RBCs. A faint, but detectable MC813-70 band was identified in the patient sample (Fig. 3G, Lane 3). Based on MC813-70 staining intensity, MSGG in patient samples was 10% that observed in LKE-S RBCs (p = 0.001, Fig. 3J). As expected, no staining was observed in P_1^k cells, which lack Gb4 and longer chain globo-GSLs.

Titration of globo-antigens on patient RBCs

The weak P phenotype is reported to show weakened P expression in titration studies with some examples of anti-PP_1P^k.[11] In titration studies, the patient had a significantly decreased titer with one alloanti-P (titer, 8) relative to LKE-N (n = 3; median titer, 16), LKE-W (n = 4; titer, 16-32), and LKE-S cells (n = 4; titer, 32-128).[18] No difference was observed with a second alloanti-P sample (data not shown).

We also performed titration studies with SC802, a recombinant P-fimbriated *E. coli* strain (Table 3).[16,20] The P-fimbria adhesion recognizes all globo-GSLs but is reported to preferentially recognize MSGG.[35,36] There was no difference in agglutination titer or Marsh score (not shown) between patient and LKE-S RBCs. As expected, no agglutination was observed with p RBCs, which are devoid of globo-GSLs, or with HB101, a nonfimbriated *E. coli* control.

Patient's antibody recognizes MSGG and Gb5

To confirm that the patient's anti-LKE recognized MSGG, we performed HPTLC-immunostaining with patient serum against isolated RBC gangliosides from LKE-S, P_1^k, and the patient (Fig. 4). GSLs from ACHN cells were also included as a control.[33] Patient

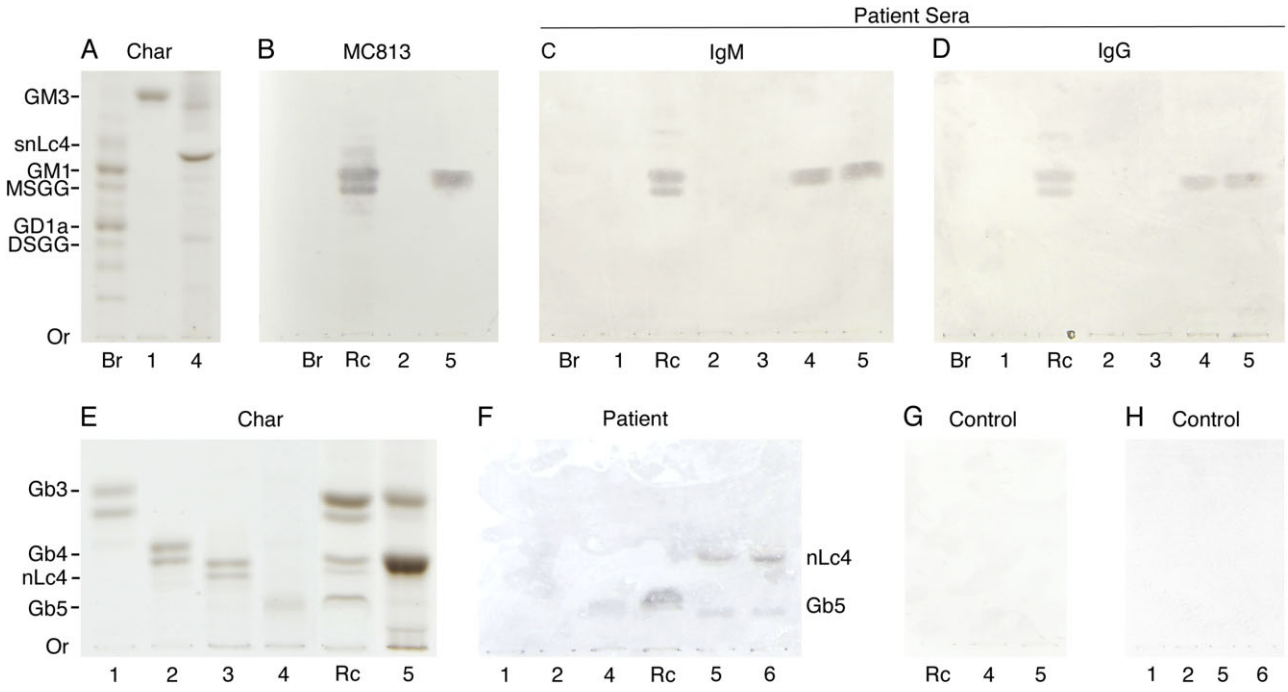

Fig. 4. Patient sera binds MSGG and Gb5 by HPTLC immunostaining. (A-D, G) Gangliosides stained with (A) charring reagent, (B) MoAb MC813-70, (C) patient serum and anti-human IgM, (D) patient serum and anti-IgG, and (G) control serum, anti-IgM. Patient serum recognized MSGG in ACHN and LKE-S RBCs. Patient serum did not recognize DSGG present in ACHN cells. Very faint IgG binding to HMW Ii-active gangliosides (R_f = 0.08, 0.04) was also observed. Lane 1 = GM3 standard; Lane 2 = P_1^k RBCs; Lane 3 = patient RBCs; Lanes 4 and 5 = group A LKE-S RBCs; Br = human brain; Rc = ACHN. Solvent C-M-0.2% aqueous $CaCl_2$ 50:40:10 vol/vol. (E, F, and H) Neutral GSLs stained with (E) charring reagent, (F) patient serum, or (H) normal control. Lane 1 = Gb3; Lane 2 = Gb4 standard; Lane 3 = nLc4 standard; Lane 4 = Gb5 standard; Rc = ACHN; Lanes 5 and 6 = RBC neutrals. Secondary antibody (F, H) was anti-human IgM. Solvent C-M-W 65:25:4 vol/vol.

serum strongly bound MSGG in ACHN and LKE-S RBCs: no staining was observed to patient and P_1^k RBCs. The patient's antibody did not recognize GM3, ganglio-series gangliosides (Br, Table 2), or disialogalactosylgloboside (DSGG, Table 2; ACHN cells[37]). The alloanti-LKE present in patient serum included both anti-human IgM (Fig. 4C) and anti-IgG (Fig. 4D). Very faint binding to two HMW RBC gangliosides (R_f = 0.08, 0.04), consistent with complex sialyl-Ii gangliosides (Fig. 3F), was observed with anti-IgG only.

We also tested patient serum against neutral GSLs. Faint binding was observed to galactosylgloboside (Gb5, Fig. 4F), the immediate biosynthetic precursor to MSGG. On scanning densitometry, the patient's sera preferentially recognized MSGG over Gb5 when normalized for the amount of GSL present (p = 0.0001; Fig. 5C). Antibody binding was also observed to a GSL band near Gb4 in RBCs, but not in ACHN cells or the Gb4 control. Subsequent studies confirmed that patient sera contained antibodies recognizing nLc4 (data not shown). Antibody binding to nLc4 was also observed in one normal control (Table 5).

Reactivity of other globo antibodies to MSGG

We subsequently screened a series of human polyclonal globo antibodies including alloanti-PP_1P^k, alloanti-P, and an autoanti-P from a patient with paroxysmal cold hemoglobinuria (PCH). Results of human polyclonal reagents were compared to known anti-globo MoAbs and lectins (Table 5). Relative antibody binding to individual GSLs was determined by scanning densitometry (Fig. 5C).

To confirm antibody activity and specificity, antisera was initially tested against neutral GSLs (Fig. 5A). As expected, sera from p individuals reacted with Gb_3, Gb_4, and P_1 antigen with a preference for Gb3 (Fig. 5C). Heterogeneous activity was observed with alloanti-P from P^k individuals, with some examples showing nearly pure anti-P activity and other examples displaying broader reactivity (Table 5, Fig. 5C). Four examples of alloanti-P showed activity against Gb5. PCH serum was specific for Gb4, as was a historical low-titer "anti-LKE" (UM1314, Table 5).[6,18]

When tested against gangliosides (Fig. 5B), two alloanti-P recognized MSGG, with one example showing

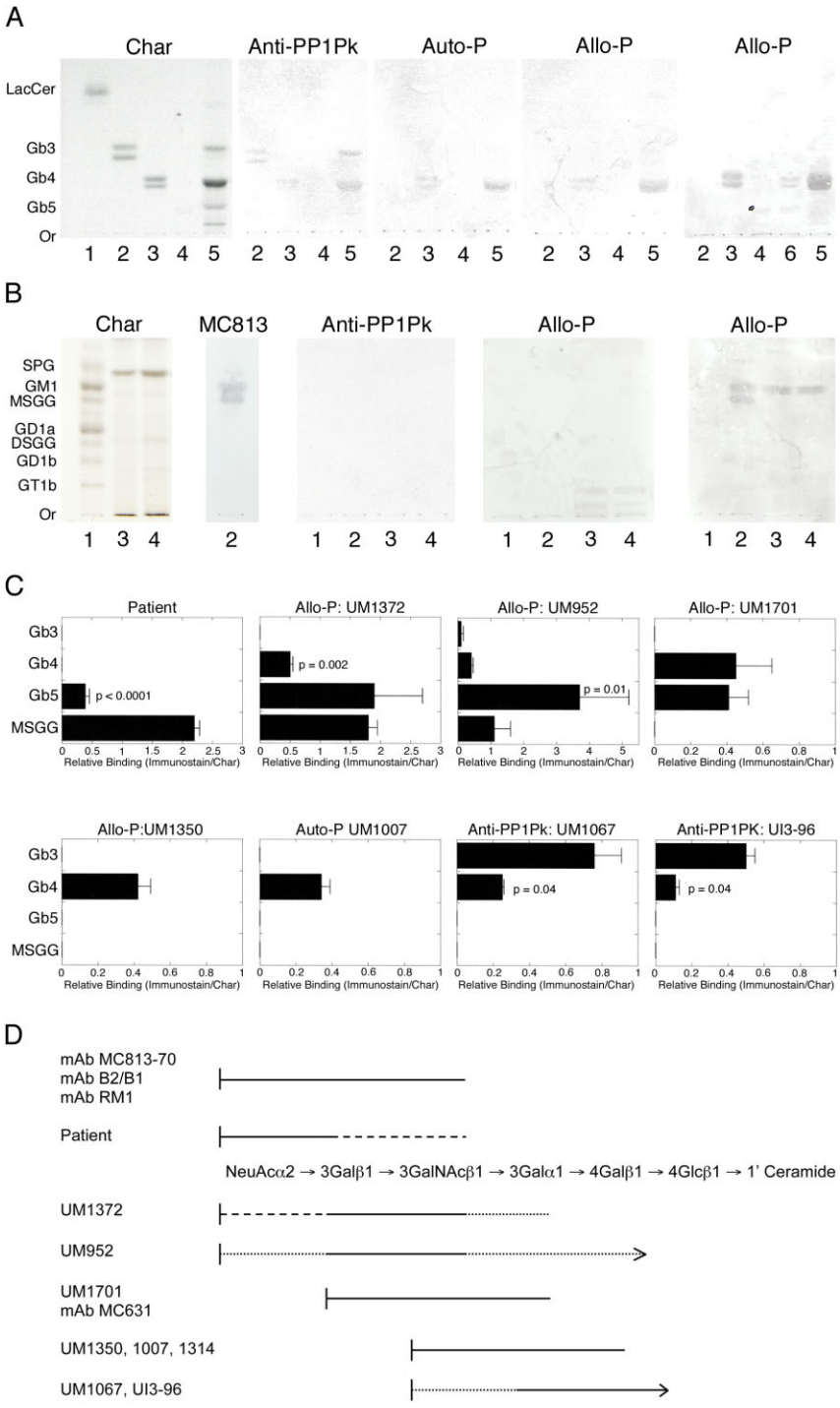

Fig. 5. Relative binding and epitope mapping of globo antibodies. (A) Neutral GSLs stained by charring or with human anti-PP1Pk, auto-P (PCH), and alloanti-P serum. Antibody staining detected with human anti-IgM. Lane 1 = CDH; Lane 2 = Gb3; Lane 3 = Gb4; Lane 4 = Gb5; Lane 5 = RBCs; Lane 6 = ACHN. Solvent C-M-W 65:25:4 vol/vol. (B) Gangliosides stained by charring, MoAb MC813-70, anti-PP_1P^k, and alloanti-P. Lane 1 = Brain; Lane 2 = ACHN; Lanes 3 and 4 = LKE-S RBCs. Solvent C-M-0.2% aqueous $CaCl_2$ vol/vol. (C) Relative antibody binding to Gb3, Gb4, Gb5, and MSGG. Relative binding determined by scanning densitometry (area [immunostain]/area [charring]). (D) Epitope mapping of anti-globo antibodies. Both patient's anti-LKE and many alloanti-P recognize the Galβ1→3GalNAc motif present in Gb5 and MSGG. The patient's anti-LKE was significantly enhanced by the presence of a terminal α2→3 sialic acid. Relative binding (— = strong; - - - = moderate; · · ·, weak).

TABLE 5. Reactivity of human polyclonal and monoclonal globo antibodies and lectins by HPTLC

	Neutral GSLs						Gangliosides				
	CDH	Gb3	Gb4	Gb5	P1	nLc4	GM3	snLc4	MSGG	DSGG	Sialyl-Ii
Human											
Anti-PP_1P^k*	0/5	5/5	5/5	0/5	5/5	0/5	0/2	0/2	0/2	0/2	0/2
Allo-P*	0/8	3/8	8/8	4/8	0/8	0/8	0/4	0/4	2/4	0/4	1/4
PCH	0	0	++	0	0	0	0	0	0	0	0
UM1314[6,18]	0	0	+	0	0	0	0	+	0	0	w+
Patient	0	0	0	+	0	+	0	0	+++	0	w+
Normal*	0/8	0/8	0/8	0/8	0/8	1/8	0/6	0/6	0/6	0/6	0/6
Murine											
MC631[14]	0	0	++	++	0	0	0	0	+/–	0	0
Nm/MC631[1]†	0	0	++	++	0	0	0	0	+++	++	0
MC813-70[1]	0	0	0	0	0	0	0	0	+++	0	0
B2/B1[34]	0	0	0	0	0	0	0	0	+	0	0
RM1[34,37,38]	0	0	0	0	0	0	0	0	++	0	0
5F3[37]	0	0	0	0	0	0	0	0	0	++	0
Lectins											
Stx	0	+++	+/–	0	+	0	0	0	0	0	0
E. coli SC802	0	+	+	+	+	0	0	0	+	+	0
E. coli HB101	0	0	0	0	0	0	0	0	0	0	0

* Results expressed as number positive/total number of samples tested.
† In situ digestion of gangliosides to expose cryptic Gb5 epitopes, followed by immunostaining with MoAb MC631.

stronger reactivity to MSGG and Gb5 than Gb4 (UM1372; Figs. 5B and 5C). No binding to MSGG was noted by PCH, anti-PP_1P^k, UM1314, or normal control sera (Table 5). No sample tested recognized human brain gangliosides.

DISCUSSION

The first example of alloanti-LKE was identified in "Mr. Luke P.," a 32-year-old, group B, P+, untransfused, African American man with diffuse Hodgkin's lymphoma.[5] Serologic studies showed that this "anti-Luke" recognized a high-incidence RBC antigen that was expressed by P_1 and P_2 donors, but not on rare p and P^k RBCs, suggesting that the Luke antigen was related to the globo-GSL family. This was strengthened by family and population studies showing increased P^k expression on LKE-N RBCs.[4,7] Over the next decade, four additional anti-Luke were identified in P+ individuals.[6,7,10] Unlike the original anti-Luke, these later examples were low-titer panagglutinins (Table 1). Despite a 1% incidence of LKE-N in the general population, anti-LKE remains a rarely encountered antibody. The latter may reflect the fact that LKE is also expressed on many nonerythroid tissues.[4,39-43]

The formal identification of "Luke" as the globoganglioside MSGG occurred in 1988 with the demonstration that MoAb MC813-70, a MoAb developed against murine embryonic stem cells, had "Luke-like" activity in serologic testing.[1,2] This was also consistent with the known RBC serology since MSGG would be absent on p and P^k RBCs due to the absence of required upstream GSL precursors necessary for MSGG synthesis (Fig. 6). With the presumed identification of MSGG as "Luke," the antigen was officially renamed LKE for "Luke antigen on erythrocytes."[3] Direct evidence of human anti-LKE binding to MSGG, or any other RBC GSL, has been lacking due to the scarcity of anti-LKE antisera.

We now present a sixth case of alloanti-LKE. Like the original Mr. Luke P., the anti-LKE in our patient was a high-titer, complement-fixing, direct agglutinin capable of in vitro hemolysis in a two-stage IAT with enzyme-treated RBCs. Unlike previous anti-LKE described,[5-7,10] our patient's antibody was also clinically significant with evidence of shortened RBC survival, a positive DAT, and laboratory evidence of hemolysis in vivo. It is interesting that the only two known high-titer anti-LKE both occurred in male patients with newly diagnosed lymphoma, raising the possibility that anti-LKE arose in response to malignancy. Carbohydrate antibodies are not uncommon in patients with advanced lymphoma, which commonly express Gb3, Gb4, and GM1 (Galβ1-3GalNAc, Table 2).[44-47] Globo-specific antibodies have been reported in other globo-rich cancers.[48-52]

We were also able to demonstrate, for the first time, direct binding of a human anti-LKE to MSGG, thus confirming MSGG as the LKE antigen. In addition to MSGG, the patient's antibody showed weak reactivity to Gb5, the biosynthetic precursor to MSGG. Gb5 is also recognized by MoAb MC631 and many alloanti-P. Furthermore, alloanti-P demonstrating strong Gb5 binding also recognized MSGG, suggesting that the Galβ1→3GalNAcβ1-R may be a common epitope among alloanti-P (Fig. 5D). Our findings complement an earlier study by Hansson and colleagues,[53] who demonstrated broad reactivity by a high-titer alloanti-P, which recognized Gb4, Gb5, Forssman, and a placental ganglioside running near snLc4. The demonstration of alloanti-P with anti-LKE

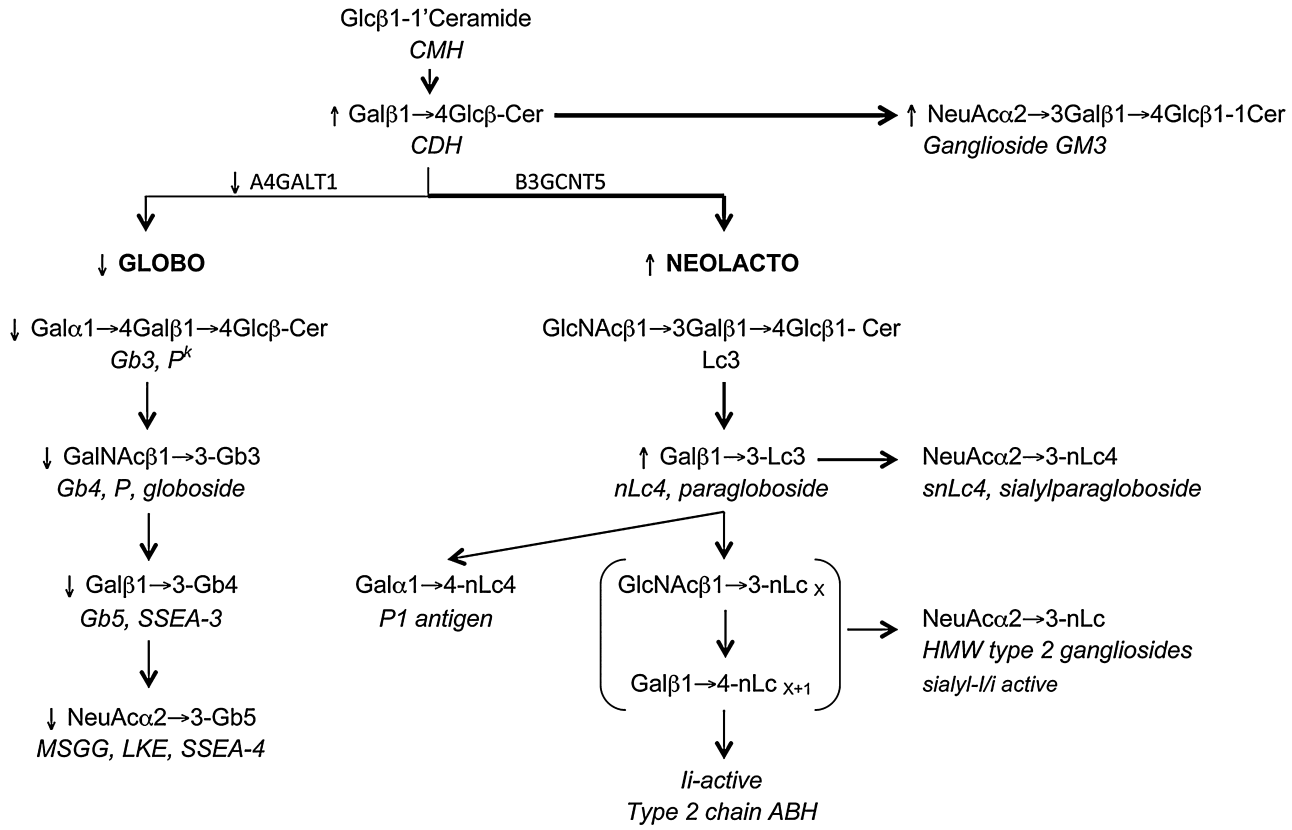

Fig. 6. Schematic of GSL synthesis in patient and weak P individuals. Unlike normal individuals, there is an apparent decrease in α4GalT1 activity, the first committed enzyme for globo-GSL synthesis. As a result, there is a global decrease in all globo-GSLs including P^k, P, and LKE and a compensatory increase in neolacto-series GSLs. There is also an increase in CDH and GM3 in these individuals.

activity suggests that some examples of alloanti-P could behave as an anti-P/LKE with stronger reactivity against LKE-S RBCs due to higher expression of both P and LKE antigens (Table 3).

Most examples of LKE-N described to date share features of the P^k variant phenotype, with elevated Gb3/P^k expression.[4,8] Our patient is the first known example of LKE-N arising from a weak P phenotype and is only the second example of weak P characterized in the literature. The first weak P was identified serendipitously in a normal donor after routine phenotyping showed weaker agglutination with some anti-PP_1P^k.[11] Analysis of the donor's RBC GSLs showed a 30% decrease in globo-GSL expression, accompanied by a two- to fourfold increase in CDH, nLc4, GM3, and total RBC ganglioside. As we have shown, our patient also displayed significant decreases in Gb3 and Gb4 with compensatory increases in CDH, nLc4, and GM3. In fact, a comparison of relative neutral RBC GSL expression between our patient and the original weak P are virtually identical (Table 4). Unlike our patient, the original weak P donor was of Chinese ancestry and did not possess any atypical RBC antibodies in his plasma.

The etiology of the weak P phenotype is unknown. Kundu and coworkers[11] hypothesized a partial block in α4GalT1, the α1,4-galactosyltransferase responsible for Gb3 synthesis. As a result, weak P RBCs have a decrease in all globo-GSLs with a compensatory increase in CDH, GM3, and neolacto-GSLs (nLc4, snLc4) similar to that observed in the P^k and p phenotype (Fig. 6).[11,25] The basis for decreased α4GALT1 activity is unknown although one obvious possibility is heterozygosity for a null *A4GALT1* allele. Family studies in p kindreds, however, have shown normal RBC GSL expression in heterozygous family members.[11,25] Alternatively, Kundu and colleagues[11] proposed inheritance of a mutant *A4GALT1* allele with functionally decreased enzyme activity. Unfortunately, we were unable to sequence the patient's *A4GALT1* gene.

In summary, we report the first case of a clinically significant anti-LKE, capable of binding MSGG, in a male patient with lymphoma. This is the second case of weak P described and is the first associated with an anti-LKE.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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