Inverse expression of P^k and Luke blood group antigens on human RBCs

Laura L. Cooling and Kathleen Kelly

BACKGROUND: Luke (LKE) is a high-frequency RBC antigen, related to the P blood group system. A LKE-negative phenotype is found in 1 to 2 percent of donors and may be associated with increased P^k. Because P^k and similar glycolipids are receptors for shiga toxin on cell membranes, a LKE-negative phenotype could have implications for infections by *Shigella dysenteriae* and enterohemorrhagic *Escherichia coli*.
STUDY DESIGN AND METHODS: Volunteer donors (n = 257) were serologically typed for LKE with a LKE MoAb, MC813-70. LKE-strong-positive, LKE-weak-positive and LKE-negative RBCs were analyzed for P^k, P, LKE, and shiga toxin binding by immunofluorescence flow cytometry, high-performance thin-layer chromatography, scanning densitometry, and high-performance thin-layer chromatography immunostaining.
RESULTS: Among Iowa donors, 78.6 percent were LKE-strong-positive, 20.2 percent were LKE-weak-positive, and 1.2 percent were LKE-negative. There was an inverse expression of P^k and LKE on RBCs. P^k expression was increased on LKE-negative RBCs and was associated with increased shiga toxin binding. A LKE-active glycolipid was identified in the ganglioside fraction of LKE-strong-positive RBCs.
CONCLUSION: A LKE-negative phenotype is associated with increased expression of P^k on RBCs. Differences in P^k and LKE expression may play a role in host susceptibility to infection with *S. dysenteriae* and *E. coli*.

The Luke (LKE) blood group antigen is a high-incidence RBC antigen expressed by the majority (99%) of blood donors.[1-4] Like many blood group antigens, LKE can vary in strength, with approximately 78 to 89 percent of donors typing as LKE-strong-positive (LKE-str+) and 10 to 20 percent typing as LKE-weak-positive (LKE-wk+). A LKE-negative (LKE-neg) phenotype has also been described in 0.07 to 2 percent of donors, probably as an autosomal recessive phenotype.[1,5] It is interesting that p and P^k RBCs also type as LKE-neg, which suggests a possible relationship between LKE and the P blood group family.[1-3,5] Even among P_1 and P_2 donors, there is evidence of an interaction between LKE and P blood group family antigens.[3,5] In a family study including three LKE-neg persons, Bruce et al.[3] reported elevated P^k expression on LKE-neg RBCs. To date, alloantibodies against LKE have been rare and clinically insignificant.[1,3-6]

ABBREVIATIONS: C-M = chloroform-methanol; C-M-W = C-M-water; DPA = diphenylamine; GSL(s) = glycosphingolipid(s); HPTLC = high-performance thin-layer chromatography; HUS = hemolytic uremic syndrome; LKE-neg = Luke-negative; LKE-str+ = Luke-strong-positive; LKE-wk+ = Luke-weak-positive; MCFI = MCF intensity; MSGG = monosialo-galactosyl-globoside; SSEA = stage-specific embryonic antigen; ST = shiga toxin; STEC = ST-producing *E. coli*.

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan; and the Department of Pathology, The University of Iowa College of Medicine, Iowa City, Iowa.

Address reprint requests to: Laura L. Cooling, MD, MS, Clinical Assistant Professor, Department of Pathology, Room 2F226 Blood Bank, University Hospital, Box 0054, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0054; e-mail: lcooling@med.umich.edu.

Supported in part by the National Blood Foundation and the College of American Pathologists Foundation.

Received for publication October 13, 2000; revision received January 20, 2001, and accepted January 30, 2001.

TRANSFUSION 2001;41:898-907.

LKE has not yet been isolated from RBCs; however, there is evidence indicating that LKE is a monosialo-galactosyl-globoside (MSGG). Originally isolated from a teratocarcinoma cell line,[7] MSGG is a sialic acid-containing glycosphingolipid (GSL) or ganglioside, biosynthetically related to the P blood group system.[8] MSGG expression is dependent on prior synthesis and subsequent elongation of P^k and P antigens.[9] Like LKE, MSGG is not expressed on the RBCs of p and P^k persons, which are unable to synthesize P^k and P, respectively.[6,8] Furthermore, MoAbs to MSGG have anti-LKE activity in hemagglutination studies.[2] In addition to teratocarcinoma cells, MSGG has been identified on human endothelium, smooth muscle,[10] dorsal horn,[11] and kidney cells, and platelets.[12-14]

Clinically, LKE may play a role in embryonic development and infection. In mice, LKE is a stage-specific embryonic antigen (SSEA) on oocytes, early-cleavage-stage embryos, and midgestation embryos, where LKE is a marker of extraembryonic visceral endoderm and visceral yolk sac cells.[15,16] LKE is also a receptor for P-fimbria, a bacterial adhesin expressed by uropathogenic *Escherichia coli* strains, which facilitates *E. coli* colonization and infection of vaginal and uroepithelium.[12] LKE could also play an indirect role in infectious diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), because of infections with *Shigella dysenteriae* and shiga toxin-producing *E. coli* (STEC).[17] A ricin-like cytotoxin, shiga toxin (ST) binds to renal endothelium and tubular epithelium via recognition of P^k on cell membranes.[17-19] Increases in P^k expression, by either enzymatic modification of cell membranes or stimulation of P^k synthesis by inflammatory cytokines, are accompanied by parallel increases in ST binding and cytotoxicity.[18,20,21] Likewise, elevated P^k expression associated with an LKE-neg phenotype could potentially increase host susceptibility to ST through increased binding of toxin to target cell membranes.[22]

In this study, we initially examined the prevalence of LKE phenotypes in a Midwest donor population. The relationship between LKE and P blood group system antigens and the biochemical basis of the relationship were examined by flow cytometry and high-performance thin-layer chromatography (HPTLC) of isolated RBC GSLs. In addition, we examined the influence of the LKE phenotype on binding of ST to human RBCs.

MATERIALS AND METHODS

Cells

RBC samples were collected in EDTA from volunteer blood donors in accordance with the human investigation review committee at one of our institutions (DeGowin Blood Center, University of Iowa). For most experiments, RBCs were enzymatically modified as described.[1,2] Briefly, 1 drop of packed, washed RBCs was incubated with 4 drops of diluted papain (EC 3.4.22.2, Sigma Chemical Co., St. Louis, MO; 3.3 units/mL final in 0.067 *M* phosphate buffer, pH 7.7) at 37°C for 15 minutes. In selected donors, separate aliquots of washed RBCs were digested with neuraminidase in parallel (EC 3.2.1.18, from *Clostridium perfringens*, 0.2 units/mL in PBS-1% BSA; Sigma) as described.[2] After incubation, cells were washed three times in normal saline and then resuspended as a 1-percent RBC suspension in cold Alsever's solution with 12-percent BSA for serologic typing or as a 3-percent RBC suspension in PBS with 1-percent BSA for flow cytometry.

Immunologic reagents

MoAbs against P antigen (MoAb MC631, mouse IgM), LKE (MoAb MC813-70, mouse IgG), and Lewis^x antigen (MoAb MC480, anti-CD15; mouse IgM) were purchased as hybridoma supernatants (MoAbs MC631 and MC480) or purified mouse ascites (MoAb MC813-70) from the Developmental Studies Hybridoma Bank maintained by the Department of Biology (contract NO1-HD-2-3144, University of Iowa, Iowa City, IA).[7,23,24] MoAbs Pk002 (anti-P^k, -P_1) and P2 (anti-CD62, mouse IgG) were purchased from Accurate Chemical (San Diego, CA).[25] ST (holotoxin, B subunits) and rabbit polyclonal anti-ST were a gift of Arthur Donohue-Rolfe, MD (Tufts University, Boston, MA). Anti-A, anti-B, and anti-P_1 were purchased from Gamma Biologicals (Houston, TX). For flow cytometry, isolated ST B subunits were labeled with FITC (isomer I; Sigma) as described.[22,26] Polyclonal anti-mouse IgG and FITC-labeled anti-mouse immunoglobulins (IgG and IgM) were purchased (Sigma). Biotinylated anti-rabbit IgG, anti-mouse IgG, anti-mouse IgM, avidin-linked alkaline phosphatase (ABC kit), and alkaline phosphatase substrate (SK-5200) were also purchased (Vector Laboratories, Burlingame, CA).

Serologic tests

All donors were typed for ABO and Rh at the DeGowin Blood Center. Selected donors were also typed for P_1 with a human polyclonal anti-P_1. ABO, Rh, and P_1 typing were performed as described according to manufacturers' instructions.

All donors were typed for LKE antigen as described, with modification.[2] Briefly, 1 drop of a 1-percent papainized RBC suspension in Alsever's solution with 12-percent BSA and 1 drop of a murine monoclonal anti-LKE (MoAb MC813-70, concentrated mouse ascites diluted 1-in-100 in PBS-6% BSA) were incubated for 20 minutes at 4°C and then centrifuged (3400 rpm, 25 sec). Samples that showed macroscopic agglutination after 20-minute incubation were typed as LKE-str+. Donor samples giving weak or negative reactions were washed and then incubated with a poly-clonal mouse IgG (50 µg/mL in PBS-1% BSA; Sigma) for 10 minutes at 4°C and then centrifuged. Donor samples that agglutinated in the

antiglobulin phase were typed as LKE-wk+. Donor samples that failed to agglutinate were typed as LKE-neg.

To exclude the possibility that weak or absent hemagglutination was the result of insufficient papain digestion, all LKE-wk+ and LKE-neg samples were typed for P, a high-incidence, cryptic RBC GSL antigen.[27] For P typing, 1 drop of a 1-percent papainized RBC suspension and 1 drop of MoAb MC631 supernatant (neat) were incubated for 20 minutes at 4°C.[2,7,23] Cells were then centrifuged and the results read macroscopically (3-4+ agglutination). For both LKE and P typing, all solutions were stored and used at 4°C.

Flow cytometry

Flow cytometry was performed on untreated, neuraminidase-treated, and papainized RBCs from 13 donors. For MoAb staining, 25 µL of a 3-percent RBC suspension was incubated with MoAb diluted in PBS with 1-percent BSA (100 µL final vol) for 30 minutes at 4°C. Cells were washed twice with PBS with 1-percent BSA and then labeled with an FITC-labeled anti-mouse immunoglobulin (IgG or IgM) for 30 minutes at 4°C. To label RBCs with ST, 25 µL of 3-percent RBC suspension, 20 µL of FITC-labeled ST (100 µg/mL stock, 2 µg/sample total), and 55 µL of PBS with 1-percent BSA buffer were incubated for 2 hours at 4°C. After staining, cells were washed again and then resuspended in PBS with 1-percent paraformaldehyde (400 µL final vol). Controls included P_2 MoAb (IgG isotype), MC480 MoAb (IgM isotype), and ST-free, FITC dialysate filtrate.[22]

Samples were analyzed on a flow cytometer (440, Becton Dickinson, Brea, CA) equipped with an argon laser. FACS data were collected on a computer equipped with software (DESK; Wayne Moore, Stanford University, Palo Alto, CA). Cells were gated on forward and orthagonal scatter with a minimum of 10,000 events measured per sample. All samples were performed in duplicate and the results recorded as the percentage of FITC-positive RBCs and log MCF intensity (MCFI). Final graphic output was performed using canvas software (Macintosh, Apple, Cupertino, CA).

GSL isolation

GSLs were isolated from individual RBC samples (approx. 1 mL packed RBCs) as described by Ledeen and Yu[28] with modification.[22] Briefly, RBCs were extracted with 20 mL of chloroform-methanol (C-M) 1:1 (vol/vol) for 72 hours. The total lipid extract was dried, resuspended in 200 mL of C-M-water (C-M-W) 30:60:8 (vol/vol) and applied to an anion exchange column (DEAE, A25, Sigma; 10-mL bed vol). Neutral lipids were isolated by washing the column with 200 mL of C-M-W 30:60:8 (vol/vol). The column was then stripped of acidic lipids with 150 mL of C-M with 0.8*M* sodium acetate 30:60:8 (vol/vol). The neutral and acidic lipid fractions were dried in vacuo, suspended in methanolic NaOH (0.1N-0.3N, 2 hours, 37°C), dried, and then dialyzed against distilled water (MW cutoff, 14000; Spectra-Por, Houston, TX). For neutral lipids, the dried dialysis residue was resuspended in 50 mL of chloroform and applied to a silicic acid column (10-mL bed vol, 40 µm; J.T. Baker, Phillipsburg, NJ). The column was sequentially washed with an additional 100 mL of chloroform and 50 mL of ethyl acetate and then stripped of neutral GSL with 50 mL each of acetone-methanol 9:1 and 7:3 (vol/vol). The last two fractions were pooled as the total neutral GSL fraction and dried. For acidic lipids, the dried dialysis residue was resuspended in 30 mL of C-M 85:15 (vol/vol), applied to a silicic acid column (10 mL, 40 µm), and then washed with 170 mL of C-M 85:15 (vol/vol) to remove sulfatides. Gangliosides were isolated by stripping the column with 130 mL of C-M 1:2 (vol/vol).

HPTLC

HPTLC was performed according to published procedures using glass or aluminum-backed HPTLC plates (Merck, Darmstadt, Germany).[29] GSLs were blotted onto HPTLC plates and then developed in a solvent of C-M-W 65:25:4 (vol/vol). For neutral RBC GSLs, dried samples were resuspended in 100 µL of C-M 1:1 (vol/vol), and 5 µL was applied on each HPTLC lane. Because RBCs express little ganglioside (0.7 µg lipid-bound sialic acid/mL RBC),[28,30] RBC gangliosides were diluted in 25 µL of C-M 1:1 and the entire sample was blotted. GSL controls included isolated glucosylceramide (CMH, Table 1), lactosylceramide (CDH), globotriaosylceramide (Gb_3 or P^k), globoside (Gb_4, P; Sigma; 5 µg/lane), and total kidney ganglioside (40 µg/lane). GSLs were detected by spraying with either diphenylamine (DPA) reagent (Sigma) or by immunostaining as described below. GSL bands were characterized by intensity (% total staining density) and relative mobility (R_f) by scanning densitometry at 370 nm (Shimadzu Instruments, Columbia, MD).[28,29] Error in mobility measurements ($R_f \pm$ SD) was less than 0.01 unless otherwise stated. For relative expression of individual GSL bands, GSL samples were examined in triplicate and reported as the adjusted percentage of total RBC-neutral GSL. The latter was obtained by subtracting the area (mm^2) of minor RBC GSLs (<15%) from the total DPA positivity.

HPTLC immunostaining

HPTLC immunostaining was performed as described by Magnani et al.[31] and modified by Buehler and Macher.[32] Briefly, air-dried, solvent-developed plates were dipped in a hexane solution of 0.4-percent poly(iso-butyl)methacrylate (Polysciences, Warrington, PA) for 60 seconds and then air-dried again. The plates were blocked with Tris buffer (40 m*M* Tris-HCl, 150 m*M* NaCl, 1% BSA, 0.1% sodium azide; pH 7.8) for 45 minutes and then overlaid with primary antibody diluted in Tris buffer for 1 hour. Plates were gently washed with PBS, incubated with a biotinylated anti-mouse Ig antibody (1 hour), washed, and then allowed

to bond with an avidin-linked alkaline phosphatase for 30 minutes. Bound antibody was detected by overlying plates with an alkaline phosphatase substrate in cold (4°C) 100 m*M* Tris-HCl, pH 9.5. Overlays with ST were performed as described, using ST holotoxin (final concentration, 50 ng/mL; 2-hour incubation), rabbit ST polyclonal antibody (final concentration, 1:5000 in PBS-1% BSA), and biotinylated anti-rabbit IgG (Vector). A toxin-free, buffer control was run in parallel.[22]

Statistics

GSL expression was compared by using a two-tailed *t* test and the Mann-Whitney U test. A p value <0.05 was considered significant.[33] Graphics and statistical correlation between flow cytometry and HPTLC data was obtained by using software (Cricketgraph, Apple) for the Macintosh.

RESULTS

Distribution of Luke phenotypes among Iowa blood donors

A total of 257 volunteer whole-blood donors were serologically typed for LKE antigen by use of the LKE MoAb, MC813-70.[2,4] Because the LKE-neg phenotype is reportedly higher among group A and P_1 phenotypes,[1] all LKE-wk+ and LKE-neg samples were serologically typed for ABO and P_1 antigens. As shown in Table 2, 254 (98.8%) of 257 Iowa donors typed as LKE-positive. Among LKE-positive donors, 78.6 percent typed as LKE-str+, 20.2 percent as LKE-wk+, and 1.2 percent as LKE-neg. In contrast to early reports,[1] there was no association between either a group A or P_2 phenotype and a LKE-wk+ and LKE-neg phenotype (data not shown). In general, the distribution of LKE phenotypes among Iowa donors was comparable to that reported among Northern European donors (Table 2).[1-4]

LKE-negative donors express LKE antigen

The presence of LKE was also examined by one-color immunofluorescence flow cytometry. Papainized RBCs from 13 donors (3 LKE-neg, 2 LKE-wk+, and 8 LKE-str+) were incubated with MoAb MC813-70, followed by an FITC-labeled anti-mouse immunoglobulin secondary antibody. As an internal positive control, RBCs were also stained for the presence of P antigen with MoAb MC631. To correct for nonspecific binding, RBCs were incubated with mouse monoclonal IgG (P_2 MoAb, anti-CD62) and IgM (MoAb MC480, anti-Lewis^x) isotype controls. Results were reported as the percentage of FITC-positive cells and MCFI.

As shown in Table 3, nearly 100 percent of LKE-str+ and LKE-wk+ RBCs were MoAb MC813-70-positive. As reported

TABLE 1. GSL structures

Name/symbol*	GSL structure
Glucosylceramide (CMH)	Glcβ1→1´Cer
Lactosylceramide (CDH)	Galβ1→4Glcβ1→1´Cer
Globotriaosylceramide (Gb_3, P^k)	Galα1→4Galβ1→4Glcβ1→1´Cer
Globotetraosylceramide (Gb_4, P, globoside)	GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1´Cer
IV^3-β-Gal-globotetraosylceramide (Gal-Gb_4, SSEA-3)	Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1´Cer
IV^3-β-Galα1-4Gal-globotetraosylceramide (band 0.03)	Galα1→4Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1´Cer
IV^3-β-Fucα1-2Gal-globotetraosylceramide (globo-H)	Fucα1→2Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1´Cer
IV^3-β-NeuAcα2-3Gal-globotetraosylceramide (MSGG, SSEA-4, LKE)	NeuAcα2→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1´Cer
$V^{3,6}$-α-$(NeuAc)_2$-galactosylglobotetraosylceramide (DG-4)	NeuAcα2→3(NeuAcα2→6)Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1´Cer
IV^6-α-NeuAc, IV^3-β-NeuAcα2-3Gal-globotetraosylceramide (DSGG)	NeuAcα2→3Galβ1→3(NeuAcα2→6)GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1´Cer

* GSL structures and trivial names as recommended by the International Union of Pure and Applied Chemistry–International Union of Biology Commission on Biochemical Nomenclature.[9]

TABLE 2. Distribution of LKE phenotypes among volunteer blood donors

LKE phenotype	Iowa*† Number	Iowa*† (%)	London[1]‡ Number	London[1]‡ (%)	London[2]† Number	London[2]† (%)	Glasgow/Western Scotland[3]†‡ Number	Glasgow/Western Scotland[3]†‡ (%)	Netherlands[4]† Number	Netherlands[4]† (%)
Positive	254	(98.8)	445	(98.0)	503	(99.0)	2396	(99.8)	891	(99.3)
LKE-str+	202	(78.6)	380	(83.7)	453	(89.2)	NA	NA	706	(78.7)
LKE-wk+	52	(20.2)	65	(14.3)	50	(9.8)	NA	NA	185	(20.6)
Negative										
LKE-neg	3	(1.2)	9	(2.0)	5	(1.0)	4	(0.17)	6	(0.7)
Total	257	(100)	454	(100)	508	(100)	2400	(100)	897	(100)

* This study.
† Donors typed with MoAb MC813-70.
‡ Donors typed with human polyclonal anti-LKE.

TABLE 3. P^k, P, and LKE expression on RBCs by flow cytometry, LKE phenotype, and enzyme treatment

Donor LKE phenotype	Enzyme treatment*	MoAb MC18-70†		MoAb MC631†		ST†	
		Percentage positive	MCFI	Percentage positive	MCFI	Percentage positive	MCN
LKE-str+	Untreated	1.2 ± 0.1	30.9 ± 1.0	79.2 ± 4.8	54.8 ± 11.6	58.0 ± 14.3	46.5 ± 8.8
	Papain	93.7 ± 2.5	113.8 ± 1.1	73.4 ± 10.6	88.1 ± 9.8	57.3 ± 13.4	46.2 ± 7.2
	Neuraminidase	91.5 ± 2.4	110.3 ± 2.4	79.4 ± 9.9	112.8 ± 10.2	NT	NT
LKE-wk+	Untreated	1.2 ± 0.1	31.4 ± 1.0	70.2 ± 2.8	78.1 ± 1.1	83.6 ± 1.3	62.4 ± 1.0
	Papain	95.5 ± 1.5	93.1 ± 3.6	80.3 ± 7.4	96.4 ± 12.4	83.7 ± 1.0	61.6 ± 1.2
	Neuraminidase	89.9 ± 3.4	87.2 ± 5.0	88.9 ± 3.0	115.7 ± 5.3	NT	NT
LKE-neg	Untreated	1.0 ± 0.1	30.5 ± 1.0	37.0 ± 18.3	54.8 ± 11.6	98.8 ± 1.3	90.2 ± 7.7
	Papain	61.0 ± 18.3	73.9 ± 3.0	70.9 ± 10.2	85.8 ± 9.7	98.9 ± 1.1	86.4 ± 6.7
	Neuraminidase	33.0 ± 14.8	53.7 ± 7.4	77.6 ± 6.4	94.9 ± 10.4	NT	NT
Control‡	Untreated	3.2	35.0	2.6	35	1.7	17

* Untreated, papain-modified, or neuraminidase-modified RBCs.
† Flow cytometry results reported as the mean ± SD of both the percentage of positive cells and the MCFI.
‡ ST, IgM, and IgG isotype controls as described in Materials and Methods (n = 2).

by Tippet et al.,[2] weak MoAb MC813-70 staining was also observed with LKE-neg RBCs. A comparison of LKE phenotype and MCFI showed a good correlation, with a progressive decrease in MoAb MC813-70 MCFI with a LKE-str+, LKE-wk+, and LKE-neg phenotype, respectively (Table 3). In contrast, there was no significant difference in P expression (MoAb MC631) among the three LKE phenotypes.

LKE is sensitive to neuraminidase

Neuraminidase is reported to inhibit MoAb MC813-70 binding to RBCs by removing a terminal sialic acid necessary for MoAb MC813-70 recognition (Table 1).[2,7] To confirm the sensitivity of LKE to neuraminidase, MoAb MC813-70 binding to untreated, papain-treated, and neuraminidase-treated RBCs was compared by flow cytometry. As before, MoAb MC631 was included as a control: Unlike LKE, P is a nonsialylated or "neutral" GSL and, therefore, insensitive to neuraminidase.

As shown in Fig. 1, MoAb MC813-70 binding was observed to papain (heavy solid line) and neuraminidase-treated (hatched line) RBCs; however, the percentage of positive cells and MCFI were relatively decreased in neuraminidase-treated RBCs. This was particularly striking with LKE-neg RBCs, in which neuraminidase treatment resulted in a 44.8-percent decrease in the percentage of FITC-positive cells and a 17.2-percent decrease in MCFI. In contrast, neuraminidase treatment increased MoAb MC631 binding, as determined by the MCFI, in most samples. The observed increase in MoAb MC631 binding is consistent with the conversion of LKE to Gal-Gb_4 (Table 1), which is strongly recognized by MoAb MC631.[7,23] It is notable that no MoAb MC813-70 binding was observed in the absence of enzyme modification (thin line).

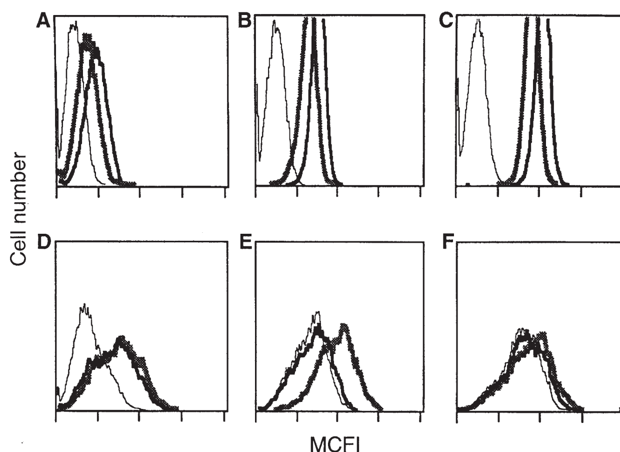

Fig. 1. LKE antigen is sensitive to neuraminidase. Untreated (thin line), papain-modified (bold line), and neuraminidase-modified (hatched line) RBCs were stained with MoAb MC813-70 (anti-LKE, Panels A-C) or MoAb MC631 (anti-P, Panels D-F). Samples shown include LKE-neg (A and D), LKE-wk+ (B and E), and LKE-str+ (C and F) RBCs.

ST binding is related to donor LKE phenotype

In a family study of several LKE-neg donors, Bruce et al.[3] reported an apparent association between LKE phenotype and P^k expression. Specifically, LKE-neg RBCs appeared to express significantly higher P^k, as determined by agglutination with a polyclonal anti-P^k. To determine if elevated P^k is a common finding on LKE-neg RBCs, we compared P^k and LKE expression on RBCs by flow cytometry. To detect P^k, RBCs were stained with FITC-labeled ST B subunits, which bind P^k and P_1 via a terminal Galα1-4Gal-R epitope.[17,22]

In agreement with Bruce et al.,[3] there was a noticeable increase in ST binding to LKE-neg RBCs (Table 3).[5] Furthermore, there was an inverse relationship between LKE and P^k antigen expression. As shown in Fig. 2, a direct comparison of the fluorescence spectra by LKE phenotype shows a progressive decrease in ST MCFI with increasing MoAb MC813-70 fluorescence. We also attempted to examine P^k expression on RBCs with an anti-P^k MoAb, Pk002.[24] Despite multiple attempts, we were unable to demonstrate any binding by MoAb Pk002 to intact RBC membranes.

Analysis of RBC GSLs

Both hemagglutination and immunofluorescence studies suggested an increase in P^k expression on LKE-neg RBCs.

These findings may suggest either an absolute increase in P^k content, decreased crypticity of P^k on RBC membranes, or synthesis of a novel P^k-like antigen on LKE-neg RBCs.[3,5,34] To investigate the basis underlying P^k expression on LKE-neg RBCs, the RBC-neutral GSL fractions from 11 donors, representing all three phenotypes, were isolated and examined by HPTLC. GSLs were visualized and identified by chemical staining with diphenylamine reagent (DPA) and HPTLC immunostaining relative to known standards. Confirmation of P^k antigen (Gb_3, Table 1) was performed by HPTLC immunostaining with ST and MoAb Pk002 as described.[22] Differences in GSL expression were quantitated by scanning densitometry of DPA-stained HPTLC plates and reported as the adjusted percentage of total neutral GSL.

As reported by others (Fig. 3),[35] the major neutral GSLs of human RBCs were monoglycosylceramides to tetraosylceramides, which accounted for >85 percent of the total RBC-neutral GSL. From the literature, mobility, and reactivity with GSL-specific MoAbs, the four major neutral GSLs were identified as glucosylceramide (CMH, relative mobility [R_f] 0.59; Table 1), lactosylceramide (CDH, R_f 0.40), globotriaosylceramide (Gb_3, R_f 0.25), and globoside (Gb_4, R_f 0.16). As shown in Fig. 4, Gb_3 (P^k) was confirmed by HPTLC immunostaining with both ST and MoAb Pk002. ST and MoAb Pk002 also recognized a second, faint GSL band (R_f 0.06), which was identified as P_1 (data not shown).[22] The identification of both Gb_3 and Gb_4 in Samples 1 through 3 (LKE-neg) excluded the possibility of a LKE-neg phenotype arising from a rare p or P^k background (Fig. 4, Table 4).[1,2,6,8]

On scanning densitometry, significant differences were noted in the relative expression of Gb_3 (% total GSL). Specifically, the percentage of Gb_3 in LKE-neg RBCs was nearly twice that in LKE-pos RBCs (Table 4, Fig. 3). Increased Gb_3 was accompanied by significant increases in the Gb_3:CDH and Gb_3:Gb_4 ratios, which suggest an increase in Gb_3 and/or a decrease in Gb_4 synthesis.[35,36] Although statistically insignificant, because of the small number of samples examined, the percentage of Gb_3 and the Gb_3:CDH ratio were also increased in LKE-wk+ RBCs. Overall, there was a good correlation between HPTLC and flow cytometry data, with both methods showing increased P^k on LKE-neg and, possibly, LKE-wk+ RBCs.

Binding of ST to RBCs is related to the percentage of Gb_3

To determine the relationship between the percentage of Gb_3 and the expression of P^k on RBC membranes, ST MCFI was plotted against Gb_3 (% total RBC neutral GSL; Table 4). As expected, the percentage of Gb_3 and the ST MCFI were significantly related where the (log) ST MCFI = 10.2 + %Gb_3, R^2 = 0.93 (Fig. 5).

LKE-positive RBCs express a glycolipid with LKE activity

It has been hypothesized that LKE on RBCs is a ganglioside.[2] To determine whether RBCs express a sialylated glycolipid with LKE activity, the entire ganglioside fraction from 1 mL of packed RBCs was immunostained with MoAb MC813-70. Human kidney ganglioside was included as a positive control. As shown in Fig. 6, a LKE-str+ band (R_f 0.01) was observed in 4 of 4 LKE-str+ RBCs (Lanes 6-11). In LKE-neg and LKE-wk+ samples, the concentration of LKE per mL of RBCs was below the threshold for detection by HPTLC.[31,32]

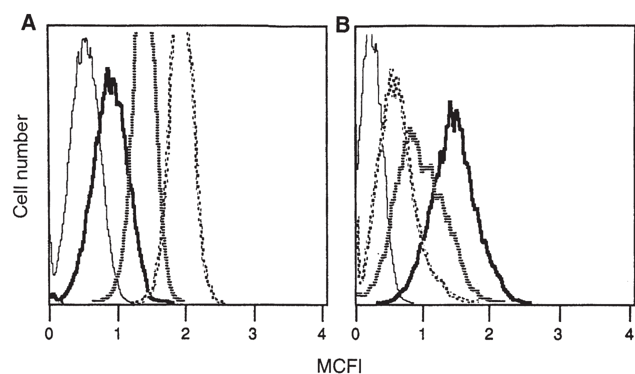

Fig. 2. Inverse expression of P^k and LKE on RBCs. Papain-modified RBCs from a LKE-neg donor (bold line), a LKE-wk+ donor (hatched line), and a LKE-str+ donor (dotted line) stained with MoAb MC813-70 (Panel A) or ST (Panel B). Negative controls (thin line) included P_2 MoAb (IgG, Panel A) and ST-free, dialysate control (Panel B).

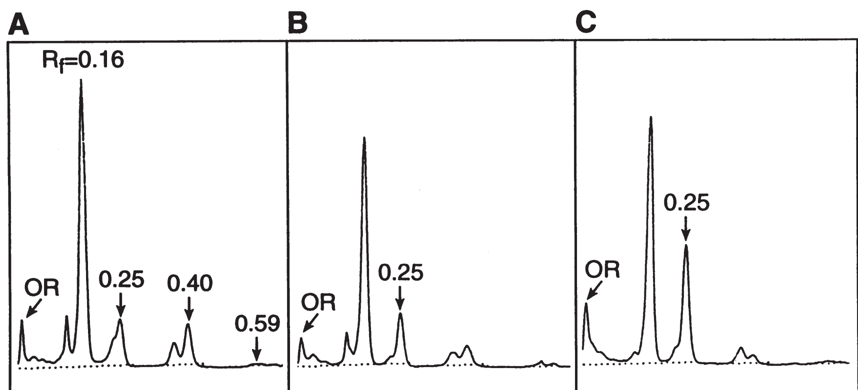

Fig. 3. Neutral GSL expression on RBCs by LKE phenotype. The total RBC-neutral GSL fractions from a LKE-str+ donor (Panel A), a LKE-wk+ donor (Panel B), and a LKE-neg donor (Panel C) were separated by HPTLC, chemically stained with DPA, and then subjected to scanning densitometry: The numbers refer to the relative mobility (R_f) of the major RBC GSL bands (Table 1); glucosylceramide (CMH, R_f 0.59), lactosylceramide (CDH, R_f 0.40), globotriaosylceramide (Gb_3, P^k; R_f 0.25), and globotetraosylceramide (Gb_4, P; R_f 0.16). OR, lane origin. Solvent was C-M-W 65:25:4 (vol/vol).

DISCUSSION

The LKE blood group is a high-frequency RBC antigen among Iowa donors. Overall, nearly 99 percent of Iowa donors were identified as serologically positive for LKE by the LKE MoAb, MC813-70. That is slightly more than the 98 percent reported with human polyclonal anti-LKE, which suggests that MoAb MC813-70 is more sensitive than polyclonal anti-LKE.[1,2] Among Iowa donors, the distribution of LKE-str+ and LKE-wk+ phenotypes is very similar to that reported for donors in the Netherlands (Table 2).[4] In contrast, the incidence of LKE-neg is twice that among Danish donors, but very similar to the rate in British donors.[1-4] Overall, the similarity among Iowa and European blood donors in the distribution of LKE phenotypes is not surprising, given the predominantly white, European ancestry of most Iowa donors.

We also examined the expression of LKE on RBCs by flow cytometry. With the exception of LKE-neg RBCs, there was a good correlation between serologic phenotype and immunofluorescence (MCFI). The weak MoAb MC813-70 positivity observed with LKE-neg RBCs may reflect the greater sensitivity of flow cytometry in comparison to hemagglutination methods.[37] Despite the latter, we observed no MoAb MC813-70 binding to any RBC tested, regardless of LKE phenotype, in the absence of enzyme modification (Fig. 1). The absence of MoAb MC813-70 binding to native, untreated RBCs underscores the cryptic nature of LKE on RBC membranes.[1] A common finding among GSL antigens,[27,38] the crypticity of LKE on RBC membranes may reflect the small numbers of antigens and masking by protein glycoconjugates.

We also screened RBCs for the presence of a LKE-active glycolipid by HPTLC immunostaining. To date, it has been hypothesized that LKE is an RBC ganglioside. This hypothesis is based on the LKE-like activity of MoAb MC813-70, which recognizes the globo-ganglioside MSGG (Table 1).[2,7] Originally isolated from human teratocarcinoma, MSGG has been identified in the ganglioside fraction of several human tissues including human kidney,[13] endothelium,[10] and platelets.[14] In addition, human RBCs reportedly express one and, possibly two disialo-globo-gangliosides related to MSGG (DSGG and DG-4; Table 1).[39,40] Although preliminary, a LKE-active glycolipid was identified in the ganglioside fraction of all four LKE-str+ RBC samples tested. Unfortunately, the amount of RBC ganglioside available from our individual donor samples (1 mL packed RBCs) was insufficient to permit additional studies to elucidate the structure of the LKE-active glycolipid identified. We have subsequently performed large-scale isolation of RBC gangliosides to characterize the structure of this LKE-active glycolipid, as well as two additional globo-gangliosides.[41] The results will be published in a subsequent report.

Clinically, the density or relative expression of LKE on RBCs varies among individuals, and it gives rise to three serologic phenotypes—LKE-str+, LKE-wk+, and LKE-neg. In all populations studied to date, the vast majority of donors (98-99%) are LKE-positive, with only 1 to 2 percent typing as LKE-neg. In fact, a LKE-positive phenotype is more frequent than reported, as

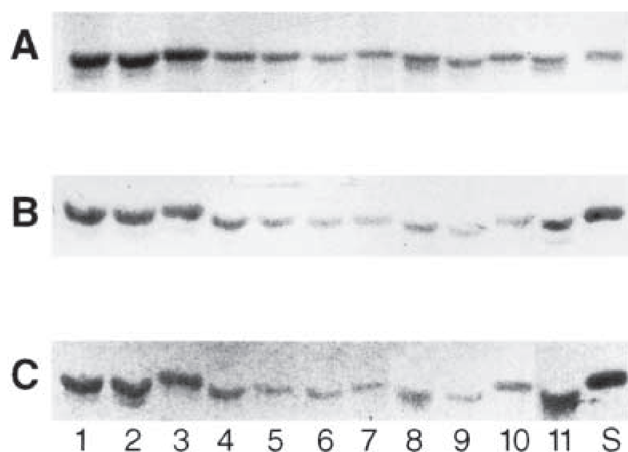

Fig. 4. Confirmation of Gb_3 in RBCs. To confirm that DPA Band 0.25 (Panel A) was Gb_3, the RBC-neutral GSL fraction was immunostained with ST (Panel B) and Pk002 MoAb (Panel C). Lane numbers refer to Donors 1-11 (Table 4). Lane S is a Gb_3 standard.

TABLE 4. RBC GSL distribution by LKE phenotype

RBC phenotype*				Adjusted percentage of total RBC-neutral GSLs†				Ratio area (mm²)‡	
Donor‡	ABO/Rh	P_1	LKE‡	CMH	CDH	Gb_3 (P^k)	Gb_4 (P)	Gb_3/CDH	Gb_3/Gb_4
1	A+	+	neg	3.3	12.0	26.9	57.8	2.2	0.46
2	O+	+	neg	0.8	12.6	37.6	49.0	3.0	0.77
3	B+	+	neg	4.1	11.2	31.9	54.2	3.5	0.56
4	O+	+	wk+	3.3	17.9	17.3	61.5	0.9	0.28
5	A+	+	wk+	1.7	20.1	21.7	56.5	1.1	0.38
6	O+	+	str+	2.8	14.5	16.0	66.7	1.1	0.24
7	A+	+	str+	2.5	11.4	16.1	70.0	1.4	0.23
8	O+	+	str+	1.0	19.4	17.0	62.6	0.90	0.27
9	A+	+	str+	2.2	19.1	17.7	61.0	0.90	0.29
10	O+	+	str+	6.6	19.7	17.7	56.0	0.9	0.35
11	O+	+	str+	1.0	12.6	19.1	67.3	1.5	0.28
Mean ± SD									
LKE-negative (n = 3)				2.9 ± 1.7	11.2 ± 1.9	31.9 ± 5.4	54.2 ± 4.0	2.9 ± 0.65	0.60 ± 0.16
LKE-positive (n = 8)§				2.6 ± 1.8	16.8 ± 3.5	17.8 ± 1.8	62.7 ± 5.0	1.1 ± 0.24	0.29 ± 0.05
p value‖				>0.50	<0.02	<0.001	<0.02	<0.001	<0.001

* RBC phenotype by serologic test.
† Relative distribution of CMH, CDH, Gb_3, and Gb_4 as determined by scanning densitometry of DPA-stained HPTLC plates. The percentages were normalized to equal 100 percent.
‡ Ratio of area (mm²) as determined by densitometry of DPA-stained HPTLC plates.
§ LKE-str+ and LKE-wk+.
|| Two-tailed *t* test.

even LKE-negative donors express small quantities of LKE. It is likely that only rare persons of the p and P^k phenotypes are truly negative for LKE (Table 2).[8] The high incidence of LKE in the population, coupled with its relative crypticity on RBC membranes, may account for the apparent rarity of LKE alloantibodies.[1,3,4,6,34]

We also examined P^k or Gb_3 expression on RBCs relative to LKE phenotype. Bruce et al.[3] previously observed an apparent increase in P^k expression on LKE-neg RBCs. However, the study was confined to a single kindred containing multiple LKE-neg donors and, therefore, may not be applicable to all LKE-neg RBCs. In addition, the results were based on hemagglutination scores using a human polyclonal anti-P^k, which suggests either an increase in total P^k, altered exposure of P^k, or cross-reactivity with a novel, P^k-like GSL related to LKE. The latter was hypothesized to reflect replacement of the terminal sialic acid of MSGG with an αGal, giving rise to a Galα1-4Gal-Gb_4 structure with P^k-like activity (Band 0.03, Table 1).[5,22,34]

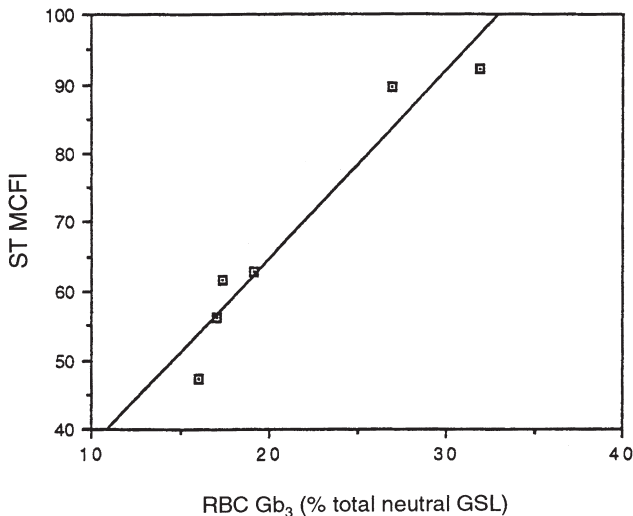

Fig. 5. ST binding to RBCs is directly related to total RBC Gb_3. Gb_3 expression by HPTLC (adjusted % total neutral GSL, Table 4) and flow cytometry (ST MCFI) was compared in six donors. As shown, there was a first-order correlation in which FITC-ST (MCFI) = 10.2 + percentage of Gb_3; R^2 = 0.93.

As a consequence, we compared LKE and P^k antigen expression on RBCs of different LKE phenotypes by both flow cytometry and HPTLC. As reported by Bruce et al.,[3] all three LKE-neg donors showed increased P^k expression by flow cytometry and HPTLC. Because the three donors were unrelated, these results suggest that increased P^k is a common feature of LKE-neg RBCs.[5] Furthermore, there was an inverse relationship between P^k and LKE expression, with the weakest P^k expression observed on LKE-str+ RBCs (Fig. 2, Table 3). Elevated P^k on LKE-neg RBCs was due to increased total P^k, and not to increased membrane exposure, as evidenced by the increased percentage of Gb_3 and its correlation with ST MCFI (Fig. 5). This increase in P^k may reflect either an increase in Gb_3 synthase activity or a decrease in Gb_4 synthase activity, as suggested by the Gb_3:CDH and Gb_3:Gb_4 ratios.[35,36]

Although phenotypic differences in P^k (Gb_3) expression are sufficient to explain increased ST binding to LKE-neg RBCs, they do not exclude the possibility that LKE-neg RBCs may also express a LKE-related GSL with P^k-like activity, similar to that isolated from human platelets (Band 0.03, Table 1).[22] In platelets, "antithetical LKE" may serve as an additional ST receptor in 20 percent of donors and is associated with an increase in both the percentage of Gb_3 and the Gb_3:CDH ratio. It was hypothesized that band 0.03 may reflect elevated Gb_3 synthase activity, which successfully competes with α2-3sialyltransferase for Gal-Gb_4 substrate (SSEA-3, Table 1).[22] Elevated Gb_3 synthase activity, therefore, would present with both increased P^k and decreased LKE expression, analogous to that observed on LKE-neg RBCs. We have subsequently identified an apparently novel, ST-binding GSL in the neutral GSL extracts of LKE-neg RBCs; however, the quantity is small (<0.5% of total RBC neutral GSL) compared to Gb_3. As a consequence, the elevated ST binding to LKE-neg and LKE-wk+ RBC primarily mirrors increased Gb_3 in these cells.

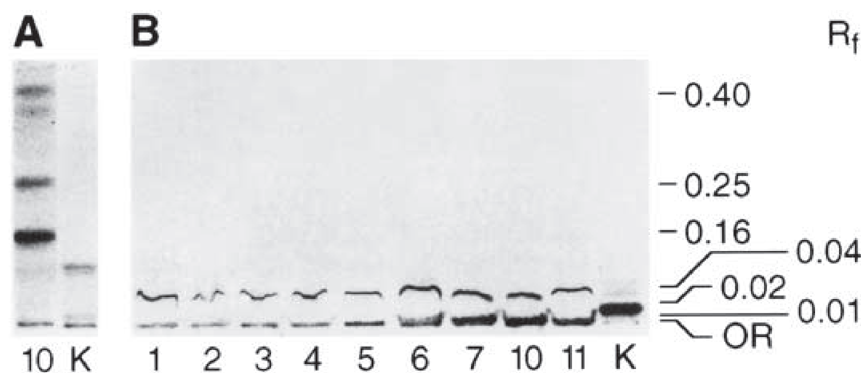

Fig. 6. LKE-active glycolipid is present in human RBCs. A) RBC-neutral GSL and kidney ganglioside stained with DPA. B) The total ganglioside fractions from 1 mL of packed RBCs immunostained with MoAb MC813-70. Lane numbers refer to Donors 1 through 11 (Table 4) and kidney ganglioside control (Lane K). A MoAb MC813-70-positive RBC glycolipid (R_f 0.01) is identified near the origin in Lanes 6, 7, 10, and 11. Band 0.04 in Samples 1 through 11 represents nonspecific binding. Numbers on right refer to mobility R_f of CDH (R_f 0.40), Gb_3 (R_f 0.25), Gb_4 (R_f 0.16), or MoAb MC813-70-positive bands (R_fs 0.02 and 0.01). Solvent was C-M-W 65:25:4 (vol/vol).

The inverse relationship between P^k and LKE may have important clinical implications in the pathophysiology of several infectious diseases. LKE was recently

identified as the preferred physiologic receptor for P-fimbria, a bacterial adhesin expressed by uropathogenic *E. coli* strains, which facilitates bacterial colonization of vaginal epithelium and uroepithelium.[12,13] It was recently noted that the risk of urinary tract infections by P-fimbriated *E. coli* is decreased in persons of the secretor phenotype, Le (a–b+).[42] It is hypothesized that the expression of LKE is decreased in the secretor phenotype, because of preferential synthesis of globo-ABH over LKE on vaginal epithelium and uroepithelium.[12,43] It is possible that LKE may also be depressed on renal epithelial and uroepithelial cells in LKE-neg and LKE-wk+ persons. If true, a LKE-neg and LKE-wk+ phenotype could similarly decrease the risk of infection by uropathogenic *E. coli*, much as occurs with the inheritance of secretor.

In contrast, a LKE-neg and LKE-wk+ phenotype could potentially increase host susceptibility to infections by *S. dysenteriae* and STEC. Both organisms are associated with infectious diarrhea, hemorrhagic colitis, and HUS, as a result of the production of ST.[17] A 72-kDa multimeric protein, ST is composed of a ricin-like A subunit and five B subunits, which mediate toxin binding to P^k (Gb_3) on gut mucosa, endothelium, and other target tissues.[17,22] Increased ST binding and cytotoxicity can be induced in several tissues by exposure to inflammatory cytokines, which increase synthesis and expression of P^k on cell membranes.[18,21] If LKE and P^k expression on renal epithelial and/or endothelial cells mimics that observed on RBCs, LKE-neg and LKE-wk+ persons may have an inherited, increased sensitivity to STEC infections due to constitutively increased P^k expression on cell membranes. Conversely, increased P^k on RBCs of LKE-neg and LKE-wk+ persons could potentially decrease morbidity from STEC infections by acting as a "sink" for circulating ST.

In summary, LKE is a high-incidence (>99%) glycolipid antigen on RBC membranes. The expression of LKE is inversely related to P^k expression, with LKE-neg donors expressing nearly twice the P^k (%Gb_3) of LKE-str+ donors. Increased P^k expression is associated with increased ST binding by flow cytometry and HPTLC. Differences in LKE and P^k expression with LKE phenotype may be associated with differences in host susceptibility to *S. dysenteriae*, STEC, and uropathogenic *E. coli* infections.

ACKNOWLEDGMENT

The authors thank Kathy Walker for technical assistance.

REFERENCES

1. Tippett P, Sanger R, Race RR, et al. An agglutinin associated with the P and the ABO blood group systems. Vox Sang 1965;10:269-80.
2. Tippett P, Andrews PW, Knowles D, et al. Red cell antigens P (globoside) and Luke: identification by monoclonal antibodies defining the murine stage-specific embryonic antigens -3 and -4 (SSEA-3 and SSEA-4). Vox Sang 1986;51:53-6.
3. Bruce M, Watt A, Gabra GS, et al. LKE red cell antigen and its relationship to P_1 and P^k: serological study of a large family. Vox Sang 1988;55:237-40.
4. Moller B, Jorgensen J. Phenotype frequency of LKE in the Danish population. Hum Hered 1988;38:375-7.
5. Tippett P. Contributions of monoclonal antibodies to understanding one new and some old blood group systems: In Garratty G, ed. Red cell antigens and antibodies. Arlington: American Association of Blood Banks, 1986:83-98.
6. Issitt P, Anstee DJ. Applied blood group serology. 4th ed. Durham, NC: Montgomery Scientific Publications, 1998.
7. Kannagi R, Cochran NA, Ishigami F, et al. Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. EMBO J 1983;2:2355-61.
8. Spitalnik PF, Spitalnik SL. The P blood group system: biochemical, serological, and clinical aspects. Transfus Med Rev 1995;9:110-22.
9. The nomenclature of lipids. International Union of Pure and Applied Chemistry-International Union of Biology Commission on Biochemical Nomenclature. J Lipid Res 1978;19:114-28.
10. Gillard BK, Jones MA, Marcus DM. Glycosphingolipids of human umbilical vein endothelial cells and smooth muscle cells. Arch Biochem Biophys 1987;256:435-45.
11. Holford LC, Case P, Lawson SN. Substance P, neurofilament, peripherin and SSEA4 immunocytochemistry of human dorsal root ganglion neurons obtained from post-mortem tissue: a quantitative morphometric analysis. J Neurocytol 1994;23:577-89.
12. Stapleton AE, Stroud MR, Hakomori SI, Stamm WE. The globoseries glycosphingolipid sialosyl galactosyl globoside is found in urinary tract tissues and is a preferred binding receptor in vitro for uropathogenic *Escherichia coli* expressing pap-encoded adhesins. Infect Immun 1998;66:3856-61.
13. Stroud MR, Stapleton AE, Levery SB. The P histo-blood group-related glycosphingolipid sialosyl galactosyl globoside as a preferred binding receptor for uropathogenic *Escherichia coli*: isolation and structural characterization from human kidney. Biochemistry 1998;37:17420-8.
14. Cooling LL, Zhang DS, Koerner TAW. Human platelets express gangliosides with LKE activity and ABH blood group activity. Transfusion 2001;41:504-16.
15. Shevinsky LH, Knowles BB, Damjanov I, Solter D. Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. Cell 1982;30:697-705.
16. Fox NW, Damjanov I, Knowles BB, Solter D. Stage-specific embryonic antigen 3 as a marker of visceral extraembryonic endoderm. Dev Biol 1984;103:263-6.

17. O'Brien AD, Holmes RK. Shiga and Shiga-like toxins. Microbiol Rev 1987;51:206-20.
18. Obrig TG, Louise CB, Lingwood CA, et al. Endothelial heterogeneity in Shiga toxin receptors and responses. J Biol Chem 1993;268:15484-8.
19. Karpman D, Hakansson A, Perez MT, et al. Apoptosis of renal cortical cells in the hemolytic-uremic syndrome: in vivo and in vitro studies. Infect Immun 1998;66:636-44.
20. Sandvig K, Prydz K, Ryd M, van Deurs B. Endocytosis and intracellular transport of the glycolipid-binding ligand Shiga toxin in polarized MDCK cells. J Cell Biol 1991;113:553-62.
21. Van de Kar NA, Monnen LA, Karmali MA, van Hinsbergh VW. Tumor necrosis factor and interleukin-1 induce expression of the verocytotoxin receptor globotriaosylceramide on human endothelial cells: implications for the pathogenesis of the hemolytic uremic syndrome. Blood 1992;80:2755-64.
22. Cooling LL, Walker KE, Gille T, Koerner TA. Shiga toxin binds human platelets via globotriaosylceramide (P^k antigen) and a novel platelet glycosphingolipid. Infect Immun 1998;66:4355-66.
23. Kannagi R, Levery SB, Ishigami F, et al. New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to a developmentally regulated antigen, stage-specific embryonic antigen 3. J Biol Chem 1983;259:8934-42.
24. Kannagi R, Nudelman E, Levery SB, Hakomori S. A series of human erythrocyte glycosphingolipids reacting to the monoclonal antibody directed to a developmentally regulated antigen SSEA-1. J Biol Chem 1982;257:14865-74.
25. Brodin NT, Dahmen J, Nilsson B, et al. Monoclonal antibodies produced by immunization with neoglycoproteins containing Galα1→4Galβ1→4Glcβ1-O and Galα1→4Galβ1→4GlcNAcβ-O residues: useful immunochemical and cytochemical reagents for blood group P antigens and a differentiation marker in Burkitt lymphoma and other B-cell malignancies. Int J Cancer 1988;42:185-94.
26. Bitzan M, Richardson S, Huang C, et al. Evidence that verotoxins (Shiga-like toxins) from *Escherichia coli* bind to P blood group antigens of human erythrocytes in vitro. Infect Immun 1994;62:3337-47.
27. Hakomori SI. Differential reactivities of fetal and adult human erythrocytes to antisera directed against glycolipids of human erythrocytes. Vox Sang 1969;16:478-85.
28. Ledeen RW, Yu RK. Gangliosides: structure, isolation and analysis. In: Ginsberg V, ed. Methods in Enzymology, vol 83. New York: Academic Press, 1982:139-91.
29. Koerner TA, Weinfeld HM, Bullard LS, Williams LC. Antibodies against platelet glycosphingolipids: detection in serum by quantitative HPTLC-autoradiography and association with autoimmune and alloimmune processes. Blood 1989;74:274-84.
30. Schultz H, Mischer O, Schenk H. [Contents and patterns of gangliosides in the membranes of human erythrocytes] (Engl abstract). Biomed Biochim Acta 1984;43:1127-34.
31. Magnani JL, Smith DF, Ginsburg V. Detection of gangliosides that bind cholera toxin: direct binding of ^{125}I-labeled toxin to thin-layer chromatograms. Anal Biochem 1980;109:399-402.
32. Buehler J, Macher BA. Glycosphingolipid immunostaining: detection of antibody binding with an avidin-biotin enzyme system. Anal Biochem 1986;158:283-7.
33. Bruning FL, Kintz BL. Computational handbook of statistics. Chicago: Harper Collins, 1987.
34. Mollison PL, Engelfriet CP, Contreras M. Blood transfusion in clinical medicine. 9th ed. Oxford: Blackwell, 1993:188-94.
35. Fletcher KS, Bremer EG, Schwarting GA. P blood group regulation of glycosphingolipid levels in human erythrocytes. J Biol Chem 1979;254:11196-8.
36. Bieberich E, Yu RK. Multi-enzyme kinetic analysis of glycolipid biosynthesis. Biochim Biophys Acta 1999;1432:113-24.
37. Garratty G, Arndt P. Applications of flow cytofluorometry to transfusion science. Transfusion 1995;35:157-78.
38. Wiels J, Holmes EH, Cochran N, et al. Enzymatic and organizational difference in expression of a Burkitt lymphoma-associated antigen (globotriaosylceramide) in Burkitt lymphoma and lymphoblastoid cell lines. J Biol Chem 1984;259:14783-7.
39. Kundu SK, Samuelsson BE, Pascher I, Marcus DM. New gangliosides from human erythrocytes. J Biol Chem 1983;258:13857-66.
40. Levery SB, Salyan ME, Steele SJ, et al. A revised structure for the disialosyl globo-series gangliosides of human erythrocytes and chicken skeletal muscle. Arch Biochem Biophys 1994;312:125-34.
41. Cooling L, Zhang DS, Koerner T. Isolation of Luke antigen and related, novel gangliosides from human red cells and kidney (abstract). Transfusion 1999;39(Suppl):91S.
42. Sheinfeld J, Schaeffer AJ, Cordon-Cardo C, et al. Association of the Lewis blood-group phenotype with recurrent urinary tract infections in women. N Engl J Med 1989;320:773-7.
43. Stapleton A, Nudelman E, Clausen H, et al. Binding of uropathogenic *Escherichia coli* R45 to glycolipids extracted from vaginal epithelial cells is dependent on histo-blood group secretor status. J Clin Invest 1992;90:965-72.