Glycosphingolipid Expression in Acute Nonlymphocytic Leukemia: Common Expression of Shiga Toxin and Parvovirus B19 Receptors on Early Myeloblasts*

Running Title: Neutral GSL expression in ANLL

Scientific Section Heading: Phagocytes

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Supported by grants from the College of American Pathology, National Blood Foundation, and National Institutes of Health (Grants R01 HL55447 and R29 HL42395)

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Abstract Word Count: 241
Total Word Count (Text): 5088

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ABSTRACT

Glycosphingolipids (GSL) are complex macromolecules on cell membranes, which have been shown to play a role in neutrophil differentiation, activation, phagocytosis and adhesion to both microorganisms and vascular endothelium. Because GSLs are often cryptic antigens on cell membranes, little is known regarding GSL expression in early myelopoiesis. To study the latter, myeloblasts were collected from patients with acute nonlymphocytic leukemia (ANLL) who required therapeutic leukocytopheresis for hyperleukocytosis. The neutral GSLs were isolated and identified by high performance thin layer chromatography (HPTLC), HPTLC-immunostaining, gas chromatography, nuclear magnetic resonance and fast atom bombardment-mass spectrometry. Like mature peripheral blood neutrophils, myeloblasts expressed glucosylceramide, lactosylceramide and the neolacto-family GSLs, lactotriaosylceramide and neolactotetraosylceramide. Unlike neutrophils and chronic myeloid leukemia, most ANLL samples also expressed the globo-series GSLs, globotriaosylceramide and globotetraosylceramide. Globo-GSL expression was strongly associated with a myeloblastic (ANLL M0-M2) and monoblastic phenotype (M5). A weak association was also noted with expression of either lymphoid (P<0.10) or early hematopoietic markers (TdT, CD34; P<0.10). Globo-positive ANLL samples bound both shiga toxin and parvovirus B19 on HPTLC-immunostaining. Based on these findings, we propose that neolacto- and globo-GSLs are expressed during early myeloid differentiation. Globotriaosylceramide expression on myeloblasts, and possibly myeloid stem cells, may have important implications for the use of shiga toxin as an ex vivo purging agent in autologous stem cell transplantation. Expression of globotetraosylceramide, the parvovirus B19 receptor, on myeloblasts may also explain the association between B19 infection, aplastic anemia and chronic neutropenia of childhood. (lcooling@med.umich.edu)

Key Words: Leukemia, Glycosphinoglipids, Shiga Toxin, Parvovirus, Transplantation
INTRODUCTION

Glycosphingolipids (GSLs) are information-rich biomolecules present in the cell membrane of all animal cells. Structurally, GSLs are composed of an antigenically-active carbohydrate head group covalently linked to a ceramide (N-acyl sphingosine) lipid tail, which anchors the molecule within the cell membrane.\(^1\) Over three hundred GSL species have been identified to date, reflecting differences in either the carbohydrate or lipid moiety.\(^{1,2}\) GSLs can be classified by their charge (neutral, acidic) and the presence of chemical modifications, such as sialylation (gangliosides) or sulfation (sulfatides). GSLs are also classified by family, based on the sequence and anomeric linkage of the first three to four carbohydrates. Of twelve GSL families identified, only five are typically expressed in human tissues (Gala, Globo, Lacto, Neolacto, and Ganglio; Table 1).\(^{1,3}\)
Table 1. Glycosphingolipid (GSL) Structures and Families

<table>
<thead>
<tr>
<th>Name</th>
<th>Symbol</th>
<th>Family</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosylceramide</td>
<td>GlcCer</td>
<td>Glc</td>
<td>Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Lactosylceramide</td>
<td>LacCer</td>
<td>Gal</td>
<td>Galβ1→4Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Galactosylceramide</td>
<td>GalCer</td>
<td>Gala</td>
<td>Galβ1→1′Cer</td>
</tr>
<tr>
<td>Galabiosylceramide</td>
<td>Gal₂Cer</td>
<td>Gala</td>
<td>Galα1→4Galβ1→1′Cer</td>
</tr>
<tr>
<td>Globotriosylceramide</td>
<td>Gb₃</td>
<td>Globo</td>
<td>Galα1→4Galβ1→4Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Globotetraosylceramide</td>
<td>Gb₄</td>
<td>Globo</td>
<td>GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Gangliotriaoysylceramide</td>
<td>Gg₃</td>
<td>Ganglio</td>
<td>GalNAcβ1→4Galβ1→4Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Gangliotetraosylceramide</td>
<td>Gg₄</td>
<td>Ganglio</td>
<td>Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Lactotriaoysylceramide</td>
<td>Lc₃</td>
<td>Lacto</td>
<td>GlcNAcβ1→3Galβ1→4Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Lactotetraosylceramide</td>
<td>Lc₄</td>
<td>Lacto</td>
<td>Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Neolactotetraosylceramide</td>
<td>nLc₄</td>
<td>Neolacto</td>
<td>Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Neolactopentaosylceramide</td>
<td>nLc₅</td>
<td>Neolacto</td>
<td>GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Neolactohexaosylceramide</td>
<td>nLc₆</td>
<td>Neolacto</td>
<td>Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1′Cer</td>
</tr>
<tr>
<td>Neolactooctaosylceramide</td>
<td>nLc₈</td>
<td>Neolacto</td>
<td>Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1′Cer</td>
</tr>
</tbody>
</table>
Biologically, GSLs and their derivatives play critical roles in receptor modulation, cell signaling, apoptosis, adhesion, growth and differentiation. Cellular differentiation and malignant transformation are often accompanied by dramatic changes in GSL expression, with many GSLs capable of inducing differentiation, bone marrow suppression, platelet activation and metastasis. GSLs also play a role in the pathophysiology of many infectious diseases through modulation of cytokine receptors, phagocytosis, T cell and neutrophil activation. GSLs also serve as membrane receptors for several viruses, toxins and bacterial adhesins including P-fimbria, HIV, parvovirus B19, and shiga toxin (Stx).

Recently, LaCasse et al. proposed using Stx1 to purge residual tumor cells present in autologous stem cell collections. Stxs are a family of ricin-like bacterial cytotoxins which recognize specific GSLs of the gala, globo and neolacto-families. Once bound, Stx is endocytosed, followed by enzymatic cleavage of rRNA, inhibition of protein synthesis and cell death. GSL receptors for Stx and Shiga-like toxins (Stx1 and Stx2) are galabiosylceramide (Gal$_2$Cer, Table 1), globotriaosylceramide (Gb$_3$, P$_k$ antigen), and P$_1$ antigen, which all terminate in a Gal$_\alpha$1-4Gal or galabiosyl epitope. Stx2e, the causative agent of pig edema disease, preferentially binds globoside (Gb$_4$) or P blood group antigen. In mice, Stx1 successfully purged Burkitt’s lymphoma, which is rich in Gb$_3$, from murine bone marrow grafts prior to transplantation. Preliminary studies in humans indicate Stx1 may be a useful purging agents in breast cancer, non-Hodgins lymphoma, and multiple myeloma. Stx1 does not appear to be toxic to human hematopoietic progenitor cells (HPC) based on flow cytometry and in vitro proliferation assays.

Although evidence suggests early HPC do not bind Stx1, Stx1 receptors might be expressed on committed myeloid progenitors. Gb$_3$ and other globo-family GSLs are richly expressed on early myeloid cells of mice. They are also major neutral GSLs on human peripheral blood monocytes, erythrocytes, and platelets, which ultimately arise from the same pluripotent progenitor cell--the granulocyte-erythroid-monocyte-megakaryocyte colony forming unit (CFU-GEMM). Gb$_3$ and/or Gb$_4$ have also been identified in three immortalized human myeloid leukemia cell lines,
KG1, K526 and THP-1, as well as a small number of clinical acute nonlymphocytic leukemia (ANLL) samples.\textsuperscript{27-32} In addition, there is evidence that parvovirus B19, which recognizes Gb\textsubscript{4}, can infect early myeloid cells, decrease myeloid progenitors and inhibit granulocyte-monocyte colony (CFU-GM) formation in vivo.\textsuperscript{18,33-36} In contrast, globo-GSLs are not expressed by either mature neutrophils or chronic myeloid leukemia (CML) cells.\textsuperscript{24,25,37,38} The presence of globo-GSLs on mature monocytes and possibly myeloblasts, but not neutrophils, suggests that globo-GSLs might be developmentally regulated antigens during myelomonocytic differentiation. The latter has been demonstrated during human lymphopoiesis and murine myelopoiesis.\textsuperscript{23,39-41}

At present, very little is known regarding neutral GSL expression and differentiation during human myelopoiesis. Such studies have been hampered by the cryptic nature of neutral GSLs on cell membranes,\textsuperscript{42-45} coupled with the difficulty in obtaining sufficient numbers of well-characterized myeloid precursors from human marrow for biochemical analysis. To combat these difficulties, we examined myeloid cells collected from thirteen patients with ANLL and CML, who required therapeutic leukocytophresis for severe leukocytosis. This permitted collection of large numbers of well defined, immature myeloid cells for GSL isolation and characterization. GSL expression was examined by high performance thin layer chromatography (HPTLC), HPTLC-immunostaining, compositional analysis, nuclear magnetic resonance spectroscopy (NMR) and fast atom bombardment-mass spectroscopy (FAB-MS). GSL samples were also tested for their ability to bind Stx and parvovirus B19. Unlike earlier studies,\textsuperscript{27,29-31} we were able to examine GSL expression relative to myeloid differentiation, as defined by histologic and immunophenotypic markers. Based on our data and those of earlier investigators,\textsuperscript{27,29} we present a modified model of neutral GSL differentiation during human myelopoiesis and discuss the implications for mature granulocyte function, parvovirus B19 infection, and transplantation.
MATERIALS AND METHODS

Leukemic and Normal Peripheral Blood Cells

Therapeutic leukocytopheresis was performed on 13 patients for treatment of hyperleukocytosis and clinical evidence of leukostasis. Eleven patients were diagnosed with ANLL and two patients with CML and CML in blast crisis with transformation to ANLL M2 (CML-B/M2). Except for patient 6, all patients were newly diagnosed with leukemia at the time of leukocytopheresis and had not received prior chemotherapy. Patient 6 was previously diagnosed with chronic myelomonocytic leukemia three years earlier and was subsequently diagnosed with ANLL M2, ten months prior to leukopheresis. The patient’s chemotherapy in the three years prior to leukopheresis included daunorubicin, cytosine arabinoside, and hydroxyurea. All patients had bone marrow biopsies at the time of admission and were diagnosed according to French-American-British (F.A.B.) criteria.46 In most patients, cytogenetic and immunophenotyping were performed as part of their diagnostic evaluation (Table 2).

Leukemic cells were collected on a Cobe Spectra 2997 (Cobe Laboratories, Inc., Lakewood, CO.). A total of $10^{11}$ to $10^{12}$ leukocytes were collected from each patient with minimal lymphocyte and platelet contamination (average PLT/WBC < 0.23), when compared to a normal granulocyte control (PLT/WBC = 22.6, Table 3). A white blood cell differential of the collected leukocytes showed predominantly blasts (>80%) in 8/11 ANLLs: Exceptions were samples 7 (M3; blasts + promyelocytes=82%), 10 and 11 (M5; monocytes + monoblasts≈85%). Normal peripheral blood lymphocytes, granulocytes and RBC (AB+, P1+) were obtained from the DeGowin Blood Center (Dept. of Pathology, University of Iowa, Iowa City, IA). Outdated peripheral blood lymphocytes and granulocyte concentrates were collected on a Fenwal CS3000 (Fenwal Laboratories, Deerfield, IL) as described.47,48 Outdated platelet concentrates (5-7 days) were purchased from Siouxland Red Cross (Sioux City, IA). Human kidney was obtained from the hospital autopsy service. All tissue procurement was carried out with the supervision and approval of the institutional human investigation committee.
Table 2. Cytogenetic, Cytochemical and Immunophenotypic Characteristics of Myeloid Leukemias

<table>
<thead>
<tr>
<th>Sample</th>
<th>F.A.B. Subtype</th>
<th>Immunophenotype*</th>
<th>Cytogenetic Phenotype†</th>
<th>Cytochemistry ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M0</td>
<td>CD13, CD34, CD45, HLA-DR,</td>
<td>NL</td>
<td>&lt;3%</td>
<td>- -</td>
</tr>
<tr>
<td>2 M1</td>
<td>CD33, CD45, HLA-DR, TdT</td>
<td>NL</td>
<td>&lt;10%</td>
<td>- -</td>
</tr>
<tr>
<td>3 M1</td>
<td>CD5, CD7, CD11b, CD13, HLA-DR, CD45</td>
<td>NL</td>
<td>20%</td>
<td>- -</td>
</tr>
<tr>
<td>4 M1</td>
<td>CD13, CD33, CD45</td>
<td>NL</td>
<td>+</td>
<td>- -</td>
</tr>
<tr>
<td>5 M1</td>
<td>ND</td>
<td>ND</td>
<td>&lt;10%</td>
<td>- -</td>
</tr>
<tr>
<td>6 M2</td>
<td>CD13, CD15, CD34, CD45, TdT</td>
<td>NL</td>
<td>+</td>
<td>- -</td>
</tr>
<tr>
<td>7 M3</td>
<td>CD2, CD13, CD33, CD45</td>
<td>t(15:17)</td>
<td>+</td>
<td>+ -</td>
</tr>
<tr>
<td>8 M4</td>
<td>CD33, CD45</td>
<td>inv(16)(p32q32)</td>
<td>+</td>
<td>- -</td>
</tr>
<tr>
<td>9 M5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND ND ND</td>
</tr>
<tr>
<td>10 M5a</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+ -</td>
</tr>
<tr>
<td>11 M5b</td>
<td>CD5, CD11b, CD13, CD14, CD15, CD24, CD45</td>
<td>ND</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>12 CML-B/M2</td>
<td>CD2, CD13, CD33</td>
<td>47XY t(9:22)</td>
<td>+</td>
<td>- -</td>
</tr>
<tr>
<td>13 CML</td>
<td>ND</td>
<td>NL</td>
<td>+</td>
<td>+ +</td>
</tr>
</tbody>
</table>

* Positive Markers listed only. All patients were negative for cytoplasmic μ, CD3, CD19, glycophorin A and CD41. ND=Not done,

† NL=normal (45 XX or XY).

‡ Per=peroxidase, NSE=nonspecific esterase, PAS=periodate-acid-shiff.
### Table 3. Quantitative Cytometric Analysis of Leukemic and Normal Granulocyte Collections by Cytopheresis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total WBC* (x $10^{11}$)</th>
<th>WBC Differential (% Total WBC)†</th>
<th>Ratio‡</th>
<th>PLT/WBC</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blast</td>
<td>Pro</td>
<td>Myelo</td>
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<tr>
<td>1</td>
<td>2.6</td>
<td>96</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3.5</td>
<td>95</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>6.0</td>
<td>91</td>
<td>1</td>
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<tr>
<td>4</td>
<td>2.6</td>
<td>94</td>
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<td>-</td>
</tr>
<tr>
<td>5</td>
<td>14.0</td>
<td>&gt;99</td>
<td>-</td>
<td>-</td>
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<tr>
<td>6</td>
<td>9.7</td>
<td>85</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>2.9</td>
<td>45</td>
<td>37</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>3.8</td>
<td>97</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>3.1</td>
<td>80</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>1.9</td>
<td>73</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>4.2</td>
<td>96</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>10.0</td>
<td>47</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>13</td>
<td>4.1</td>
<td>0</td>
<td>1</td>
<td>18</td>
</tr>
</tbody>
</table>

Granulocyte Control 0.27 0 92 22.6

* Total collected WBC by leukocytopheresis

† WBC differential of the collected product. Abbreviations: Lymph, lymphocyte; Gran, granulocyte; Meta, metamyelocyte; Mono, monocyte; Myelo, myelocyte; Pro, promyelocyte.

‡ Ratio of platelets (PLT) to WBC in the collected product prior to centrifugation and lipid extraction.

¶ Includes monoblasts and promonocytes.
Glycosphingolipid Isolation

Prior to lipid extraction, all leukocyte concentrates were initially diluted with isotonic ammonium bicarbonate (NH₄HCO₃, 154 mM) to osmotically lyze contaminating erythrocytes and then centrifuged (350g, 15 min). After carefully decanting the supernatant containing platelets and RBC ghosts, leukocytes were washed thrice more with NH₄CO₃, centrifuged (4500g, 30 min), frozen and lyophilized dry. Platelets and RBC were isolated as described. The total GSL fraction of each tissue was isolated using the procedure of Ledeen and Yu with modification. Briefly, lyophilized cell powders were extracted with chloroform-methanol (C-M 1:1 [v/v]; 100 mL/gm dry weight) to obtain a total lipid extract. Neutral lipids were isolated by anion exchange chromatography (A-25 DEAE-Sephadex, Sigma; St. Louis, MI), saponified with 0.3N methanolic NaOH to remove phospholipids, followed by dialysis against distilled water. The dialysis retentate was lyophilized dry, dissolved in chloroform and applied to a silicic acid column (40 mesh, Mallinckrodt-Baker, Phillipsburg, NJ). After washing with chloroform and ethyl acetate, the column was stripped of neutral GSLs with acetone-methanol 9:1 and 7:3 (v/v). To remove alkali-resistant phospholipids, the latter two fractions were pooled and then acetylated as described. The deacetylated, purified neutral GSL was resuspended in C-M 1:1 (v/v) to a final concentration of 10 mg/mL. The total neutral GSL/leukocyte sample was determined by direct measurement of dry weight GSL (in triplicate).

Immunologic Reagents.

Monoclonal antibody (MoAb) Pk002 was purchased from Accurate (San Diego, CA). MoAb MC631 was purchased as a hybridoma supernatant from the Developmental Studies Hybridoma Bank maintained by the Dept. of Biology at the Univ. of Iowa, Iowa City, IA under contract no. N01-HD-2-3144. MoAbs 1B2 and SH34 were purchased from ATCC as a hybridomas. MoAb TE5 was a kind gift of Dr. Eric Holmes, Pacific Northwest Research Foundation, Seattle, WA. Rabbit polyclonal anti-Gg3 and MoAb Gal01 were purchased from Matreya, Pleasant Gap, PA. Recombinant B19 empty capsids were a kind gift from MedImmune (Gaithersburg, MD).
Anti-B19 (MoAb 829) was purchased from Chemicon International (Temecula, CA). Shiga holotoxin (Stx) from *Shigella dysenteriae* and polyclonal rabbit anti-shiga toxin antibody were a kind gift of Dr. Donohue-Rolfe, Tufts University, MA. All MoAbs used were mouse IgM except anti-B19 (mouse IgG). The antibody specificity of MoAbs and Stx are shown in Table 4.

### Table 4. Immunologic Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Epitope</th>
<th>Family</th>
<th>Specificity</th>
<th>Ref</th>
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</thead>
<tbody>
<tr>
<td>MoAb TE5</td>
<td>GlcNAcβ1-R</td>
<td>lacto/neolacto</td>
<td>Lc₃, Lc₅, etc</td>
<td>57</td>
</tr>
<tr>
<td>MoAb 1B2</td>
<td>Galβ1-4GlcNAcβ1-R</td>
<td>neolacto</td>
<td>lactosaminyl</td>
<td>55</td>
</tr>
<tr>
<td>MoAb Pk002</td>
<td>Galα1-4Galβ1-4Glc/GlcNAc-R</td>
<td>globo</td>
<td>Gb₃, P₁</td>
<td>53</td>
</tr>
<tr>
<td>MoAb MC631</td>
<td>(R)-GalNAcβ1-3Galα1-4Gal-R</td>
<td>globo</td>
<td>Gb₄</td>
<td>54</td>
</tr>
<tr>
<td>MoAb Gal-01</td>
<td>Galβ1-1Cer</td>
<td>gala</td>
<td>GalCer</td>
<td>Matreya</td>
</tr>
<tr>
<td>Stx</td>
<td>Galα1-4Gal-R</td>
<td>gala/globo</td>
<td>Gal₂Cer, Gb₃, P₁</td>
<td>21,59</td>
</tr>
<tr>
<td>anti-Gg₃</td>
<td>GalNAcβ1-4Galβ1-4Glc-R</td>
<td>ganglio</td>
<td>Gg₃</td>
<td>Matreya</td>
</tr>
<tr>
<td>MoAb SH34</td>
<td>Galβ1-3GalNAcβ1-4Gal-R</td>
<td>ganglio</td>
<td>Gg₄</td>
<td>56</td>
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<tr>
<td>B19 capsid</td>
<td>(R)-GalNAcβ1-3Galα1-4Gal-R</td>
<td>globo</td>
<td>Gb₄, Forssman</td>
<td>18,58</td>
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<tr>
<td></td>
<td>Galβ1-4GlcNAc-R</td>
<td>neolacto</td>
<td>lactosaminyl</td>
<td>25</td>
</tr>
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</table>

**High Performance Thin Layer Chromatography.**

HPTLC was performed using aluminum-backed HPTLC plates (E. Merck, Darmstadt, Germany). Approximately 40 µg neutral GSL was spotted per lane and then developed in a solvent of chloroform-methanol-water 60:30:5 (C-M-W, v/v). GSLs were detected by spraying with diphenylamine reagent (DPA) or by immunostaining as described below. GSL bands were characterized by intensity (% total staining density) and relative mobility (Rₚ) by scanning densitometry at 580 nm (Shimadzu Instruments, Columbia, MD). Error in mobility measurements
(SD) was $\pm 0.10$ unless stated otherwise. GSL standards were purchased from Matreya and Sigma.

**HPTLC immunostaining**

Air-dried, solvent-developed plates were dipped in a hexane solution of 0.2% polyisobutyl-methacrylate (Polysciences, Warrington, PA) for 60 sec and air-dried. HPTLC-immunostaining with MoAbs was performed as described using an avidin-linked alkaline phosphatase detection method (Vector, Laboratories, Burlingame, CA). HPTLC-immunostaining with B19 capsids and Stx was performed as described. All experiments were performed in triplicate.

**High Performance Liquid Chromatography.**

Underivatized neutral GSLs from an ANLL M1 was separated by HPLC (Shimadzu LC-610) using evaporative laser light scattering detection (Varex ELSD II, Burtonsville MD). The sample (30.0 mg) was applied to a preparative silica gel column (1.0 x 25 cm, 8 µm; Ranin, Woburn, MA) and eluted with a gradient of C-M (95:5) to C-M-W (60:40:20, v/v), applied at a flow rate of 5 mL/min over 2 hrs. Fractions were collected every 2 mins and analyzed by DPA and HPTLC-immunostaining. Because two major GSL bands co-eluted, fractions 31-36 were pooled and re-chromatographed over an analytical column (5 x 100 mm, Spherisob SI-80, 3 µm; ES Industries, West Berlin, NJ) using a gradient of hexane-isopropanol-water 68:30:2 to 5:90:5 (v/v) at a flow rate of 1 mL/min over 25 min.

**Characterization of carbohydrate structure.**

For $^1$H-nuclear magnetic resonance spectroscopy (NMR), underivatized GSL samples were exchanged against D$_2$O by repeated lyophilization and then dissolved in dimethylsulfoxide-$d_6$ containing 2% D$_2$O and 1% tetramethylsilane (Aldrich, Milwaukee, WI). NMR spectra were acquired at 600 MHz on a Bruker AMX-600 spectrometer (Karlsruhe, Germany; Dept. of Chemistry, University of Iowa) in the Fourier-transformed mode with quadrature detection at a probe temperature of 303K. Integrated, 1-D spectra were typically obtained with a sweep width of
Cooling-13

6000 Hz (10 ppm), 4.01 sec cycle time and 200-400 scans. Homonuclear 2-D NMR (COSY, TOCSY, NOESY) spectra were obtained essentially as described.\textsuperscript{63-65} For COSY experiments, 256-400 time proportional phase increments (TPPI) were collected with 32 transients per \( t_1 \), a pulse delay of 4 sec and a sweep width of 6000 Hz over 4K data points in \( t_2 \). For TOCSY and NOESY experiments, 400 TPPI were collected with 48 transients per \( t_1 \), a pulse delay of 4 sec and a mixing time of 100 ms and 500 ms, respectively.

Fast atom bombardment mass spectroscopy (FAB-MS) was performed on an Autospec mass spectrometer (Micromass, Inc., Beverly, MA; Dept. of Chemistry, University of Iowa) using triethanolamine/tetramethylurea as the matrix.\textsuperscript{66} For compositional analysis, GSLs were hydrolyzed with dry methanolic hydrochloric acid, followed by trimethylsialylation and gas chromatography (Shimadzu) over a DB-5 column (J&W Scientific, Folsom, CA) at 150°C for 2 min, then 4°C/min to maximum 230°C.\textsuperscript{67} Individual sugars were identified by their retention time and peak areas relative to trimethylsialyl standards (Sigma).

**Statistics.** Statistical correlations were performed using the Pearson-Product Moment Correlation and student t-test.\textsuperscript{68} A \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Decreased Neutral GSL Expression in ANLL**

Neutral GSL content was determined by direct measurement of the dried, purified neutral GSL and reported as \( \mu g \) dry weight GSL per 100 million leukocytes collected (Table 5). In general, the total neutral GSL yield from mature granulocytes and CML cells were comparable to those obtained by other investigators (114-139 \( \mu g/10^8 \) cells).\textsuperscript{24,30,37,69} In contrast, the neutral GSL content of ANLL cells was 8-10 fold less then mature neutrophils, ranging from 6.2-19.4 \( \mu g/10^8 \) cells (mean 9.8 ± 4 \( \mu g/10^8 \) cells). Among monocytic leukemias (M5), the neutral GSL yield was very similar to mature peripheral blood monocytes (11 \( \mu g/10^8 \) cells).\textsuperscript{24}
Table 5. Distribution of Neutral GSLs Extracted from ANLL, CML and Normal Peripheral Blood Granulocytes*

<table>
<thead>
<tr>
<th>Neutral GSL Bands*</th>
<th>Relative Distribution Neutral GSL (% Total) per Sample†</th>
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<tbody>
<tr>
<td></td>
<td>Size</td>
</tr>
<tr>
<td>GSL1</td>
<td>0.72</td>
</tr>
<tr>
<td>GSL2</td>
<td>0.57</td>
</tr>
<tr>
<td>Ratio: GSL2/GSL1</td>
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<tr>
<td>GSL3</td>
<td>0.45</td>
</tr>
<tr>
<td>GSL4</td>
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<tr>
<td>(%GSL4)</td>
<td></td>
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<tr>
<td>0.23 f</td>
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<tr>
<td>(% GSL4)</td>
<td></td>
</tr>
<tr>
<td>Total GSL(µg/10⁸ cells)</td>
<td>8.8</td>
</tr>
</tbody>
</table>

*GSLs were identified by the size of the oligosaccharide moiety, relative mobility (R_f) and immunostaining characteristics (Figure 1).

Abbreviations: GSL1, monoglycosylceramide; GSL2, diglycosylceramide; GSL3, triglycosylceramide; GSL4, tetraglycosylceramide. Solvent was C-M-W 65:30:5 (v/v).

†Percent distribution of major neutral GSL species, as determined by scanning densitometry of DPA-stained HPTLC plates. Numbers in parenthesis indicate the % total GSL4 as band e (Figure 1A, R_f 0.27) or band f (doublet, R_f 0.23,). Results reported are the mean of seven, independent sample determinations, SD < 3.0%.
Relative Expression of Neutral GSL in Myeloid Leukemia

To examine the expression of individual GSL species, neutral GSL samples were separated by HPTLC and then chemically stained with DPA reagent. Based on relative mobilities (R_f, Table 5 and Figure 1A), leukemic and normal peripheral blood cells expressed predominantly mono- (GSL1, R_f 0.72), di- (GSL2, R_f 0.57), tri- (GSL3, R_f 0.45) and tetra-glycosylceramides (GSL4, R_f 0.23 and 27). On densitometry, GSL2 was the major GSL species expressed by most myeloid cells except sample 12, where GSL4 constituted nearly 65% of the total. In general, neutral GSL expression in ANLL differed from normal granulocytes by a relative increase in GSL1 and decreased GSL2/GSL1 ratio. In addition, most ANLL samples appeared to express at least two GSL4 species, with R_f similar to Gb_4 (R_f 0.27, lanes R, L, P) and nLc_4 (R_f 0.23, lane N).

Myeloid Leukemia Cells Express Neolacto-series GSLs

Neolacto-series GSLs are a characteristic feature of mature granulocytes and have been reported in ANLL and CML. To screen for neolacto-family GSLs, samples were immunostained with MoAbs TE5 and IB2 (Table 4; Figures 1B, C). As shown in Figure 1B, MoAb TE5 recognized Lc_3 (GSL3, R_f 0.45) in all myeloid cells. In samples 1, 8 and 12, a second TE5+ band (R_f 0.18) was also identified, which may represent nLc_5 (Table 1). Likewise, MoAb 1B2 binding was observed to nLc_4 (GSL4, R_f 0.23) in granulocytes and all myeloid leukemias. Additional 1B2+ GSL bands (R_fs 0.08, 0.01) were noted in samples 1, 2, 3, 5, 6, 8, 12, and 13 and may represent the extended neolacto-series GSLs, nLc_6 and nLc_8, respectively.

Globo-series Expression in ANLL

Globo-GSLs (Gb_3, Gb_4; Table 1) are major neutral GSLs in red cells, platelets, lymphocytes and monocytes, but are reportedly absent from mature peripheral blood neutrophils. To screen for Gb_3 and Gb_4, samples were immunostained with MoAbs Pk002 and MC631, respectively (Figures 1D, E). MoAb Pk002 binding to Gb_3 (R_f 0.45) was observed in all three positive controls (lanes R, P, L) and 9/11 ANLLs, although staining was weak in 2/3 ANLL M5 samples.
Faint or no binding was observed in ANLL M4, CML and mature neutrophils. Likewise, MoAb MC631 binding to Gb$_4$ (R$_f$ 0.27) was observed in 9/11 ANLLs. A faint MC631 band was also observed in the CML-B/M2 sample (lane 12). No Gb$_4$ was identified in samples 8, 9, 13 and normal granulocytes. Nonspecific binding to nLc$_4$ was noted in sample 12 (brackets).

**Gala-series GSL are not expressed in Myeloid Leukemia**

The gala-series GSL, galactosylceramide (GalCer), is reportedly expressed by the myeloid leukemia cell lines K562 and KG1, and is a receptor for HIV. A second gala-series GSL, galabiosylceramide (Gal$_2$Cer), is a receptor for Stxs and has been reported to be a marker of mature neutrophils. To screen for GalCer and Gal$_2$Cer, samples were immunostained with MoAb Gal 01 and Stx from *Shigella dysenteriae*, respectively (Figures 1F, G). Positive controls included GalCer (R$_f$ 0.72) and human kidney GSLs (Gal$_2$Cer and Gb$_3$). Faint GalCer was identified in one ANLL (M5b, lane 11) and platelets. Stx binding to Gal$_2$Cer (triplet, R$_f$ 0.57) was observed only in the kidney control (Figure 1G, lane K). Stx also recognized a GSL3 band (R$_f$ 0.45), consistent with Gb$_3$, in 9/11 ANLL samples, platelets, red cells, lymphocytes, and kidney. Stx binding was also observed to Gb$_4$ in many samples and is an artifact associated with polyisobutylmethacrylate-fixation.

**Myeloid leukemias do not express ganglio-series neutral GSLs**

Ganglio-series GSLs have been reported in the promyelocytic cell line HL-60, K562 cells, murine myelogenous leukemia, and some human acute lymphoblastic leukemias. To screen for ganglio-series GSLs, GSL samples were immunostained with a polyclonal anti-Gg$_3$ and MoAb SH34, an anti-Gg$_4$. Authentic Gg$_3$ and Gg$_4$ were included as positive controls. Neither Gg$_3$ (R$_f$ 0.40) nor Gg$_4$ (R$_f$ 0.21) were identified in any sample tested (data not shown).
Figure 1. Immunologic detection of neolacto- and globo-family GSLs, including Gb3, in acute myeloid leukemia. Neutral GSLs from ANLL (lanes 1-11), CML (lanes 12 and 13), and peripheral blood neutrophils (N), lymphocytes (L), platelets (P), and red cells (R) were separated by HPTLC and chemically stained with DPA or immunostained with carbohydrate-specific MoAbs and lectins (Table 4). Panel A, DPA spray; Panel B, MoAb TE5; Panel C, MoAb IB2; Panel D, MoAb Pk002; Panel E, MoAb MC631; Panel F, MoAb Gal01; Panel G, Stx. Lane numbers for ANLL and CML refer to individual sample numbers shown in Tables 2-4. Numbers to left of figures refer to the relative mobility (Rf) of specific GSLs. GSL controls included galactosylceramide (GalC, Fig 1F) and kidney neutral GSLs (K, Fig 1G). Bracketed bands (Panel E, lane 12) represent nonspecific binding. Solvent was C-M-W 60:30:5 (v/v).
Isolation and characterization of major neutral GSLs in ANLL

To verify that ANLL blasts express globo- and neolacto-series GSLs, the total neutral GSL fraction from an ANLL M1 (sample 3) was separated by HPLC. Isolated GSLs were identified by HPTLC, based on their mobility and immunologic reactivity (Figure 2A). A total of six major DPA bands (a-f) were identified and tentatively identified as GlcCer (band a), LacCer (b), Gb3 (c, Stx+, Pk002+), Lc3 (d, TE5+), Gb4 (e, MC631+) and nLc4 (f, IB2+). Two bands (d and e) co-eluted in our initial separation and were subsequently isolated by re-chromatography on an analytical HPLC column (Figure 2B).
Figure 2. HPLC isolation and purification of the six major neutral GSLs in ANLL.

Panel A) The total neutral GSL fraction from an ANLL M1 (sample 3) was separated by HPLC, followed by HPTLC of individual fractions (fraction numbers 7 through 47). Six major neutral GSLs (bands a-f) were identified. Panel B) HPTLC of the isolated and purified major neutral GSLs (bands a-f) in ANLL. The total neutral GSL fractions from an ANLL (sample 3) and CML (sample 13) were included as controls. Lane S, commercial GSL size standards ranging from mono- (GSL1, GlcCer), di- (GSL2, LacCer), tri- (GSL3, Gb3), tetra- (GSL4, Gb4) and pentaglycosylceramides (GSL5, Forssman). HPTLC solvent, C-M-W 65:25:4 (v/v). Stain, DPA reagent.
Isolated GSLs were further characterized by $^1$H-NMR. \textsuperscript{62,63} Characteristic proton signals for the ceramide lipid moiety were identified in all six bands (Table 6). \textsuperscript{63} Signature proton resonances for the oligosaccharide moiety were identified in the anomeric proton region at 4-5 ppm (Figure 3). Based on the number, chemical shift (ppm) and $J_{1,2}$ coupling constants of the anomeric protons, \textsuperscript{63} bands a-f were identified as GlcCer, LacCer, Gb$_3$, Lc$_3$, Gb$_4$ and nLc$_4$ (Table 6). \textsuperscript{62-65,74,75} The chemical shifts of the remaining methine and methylene protons were also consistent for GlcCer, LacCer, Lc$_3$, Gb$_4$ and nLc$_4$ (data not shown). \textsuperscript{62-65,75} Band c was not subjected to 2D-NMR due to insufficient sample.
Figure 3. $^1$H-NMR 1-D spectra between 4.0 and 5.0 ppm, showing the anomeric protons for ANLL GSL bands a-f. Panels A-F) 1-D NMR spectrum for GSL bands a-f, respectively. The size and composition of the carbohydrate moiety of each GSL band was determined by the number and chemical shift (ppm) of their anomeric or H-1 protons, which are present as split peaks in the NMR spectrum between 4.0 and 5.0 ppm. The anomeric linkage ($\alpha$, $\beta$) of each carbohydrate was determined by the coupling constant or difference in frequency ($J_{1,2}$; Hz) between each set of H-1 peaks. The numbering of individual anomeric protons (I-1, II-1, III-1, IV-1, V-1, VI-1), and their subsequent identification, based on published standards and 2-D NMR, is shown in Table 6. Note that the H-1 proton of glucose (I-1, Table 6) in bands C and E is adjacent or split by the H-5 proton of galactose (III-5), which is consistent with the published NMR spectra of Gb$_3$ and Gb$_4$, respectively. $^{64,74,75}$
Table 6. Proton chemical shifts (\(^{1}\)H, ppm) and \(J_{1,2}\) coupling constants (Hz) of bands a-f

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<tr>
<th>Band</th>
<th>VI</th>
<th>V</th>
<th>IV</th>
<th>III</th>
<th>II</th>
<th>I</th>
<th>R(^{1,2})</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>NH-</th>
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<td>(7.9)</td>
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* Due to small sample, \(J_{1,2}\) coupling constant and R\(^{1}\)\(a\) not resolved.
Bands c, e and f were also analyzed by mass spectrometry (FAB-MS) and compositional analysis by gas chromatography (data not shown). As shown in Figure 4, band c possessed a trihexose oligosaccharide, composed of galactose and glucose (ratio 2.3:1.0), consistent with a Gal-Gal-Glc-Cer or Gb₃. Similarly, band e was a HexNAc-Hex-Hex-Hex-Ceramide, composed of Gal:Glc:GalNAc (ratio 2.3:1.0:0.74), consistent with Gb₄. In contrast, band f possessed a Hex-HexNAc-Hex-Hex-Ceramide sequence, composed of Gal:Glc:GlcNAc (ratio 1.73:1.0:0.51), consistent with the neolacto-GSL, nLc₄. In summary, the major myeloid neutral GSLs in ANLL were identified and confirmed as GlcCer, LacCer, Gb₃, Lc₃, Gb₄ and nLc₄ by immunologic, chromatographic and spectroscopic methods.
Figure 4. Confirmation of Gb3, Gb4 and nLc4 by fast atom bombardment mass spectrometry.

FAB-MS of ANLL bands c, e and f showing molecular (M-H) and sequence ions. Congenic peaks, representing heterogeneity in ceramide fatty acid content, are identified in samples C and F. Note bands c and e both possess a trihexosyl-ceramide sequence characteristic of all globo-GSLs. The FAB-MS of bands c, e and f are consistent with the structures of Gb3, Gb4 and nLc4, respectively (Table 1).
ANLL characteristics associated with the expression of globo-GSLs

The expression of globo-GSLs and the %Gb₄ (% total GSL4) were compared by F.A.B. subclass, cytogenetic and lineage-specific immunophenotypic markers (Tables 2 and 5). In general, there was no association between the expression of globo-GSLs and a specific F.A.B. subclass. Globo-GSLs were identified in all myeloblastic ANLLs (F.A.B. M0-M2) and 4/5 “differentiated” ANLLs (F.A.B. M3 and M5) but not ANLL M4, CML and mature granulocytes. There was no association between GSL expression and cytogenetic abnormalities: The t(15:17) and inv(16) in samples 7 and 8 are common chromosomal abnormalities in ANLL M3 and M4, respectively.76 There was a slight positive correlation between globo-GSL expression and expression of CD13 (P<0.05). A weak association was also noted between increased Gb₄ (% total) and expression of lymphoid (P<0.10) or early hematopoietic markers, (CD34 and TdT, P<0.10; Pierson Product Moment Correlation).

Parvovirus B19 binds both Gb₄ and nLc₄ on myeloid blasts

Gb₄ is the receptor for parvovirus B19 on red cells and platelets.18,23 Parvovirus B19 is also reported to bind mature neutrophils, which lack Gb₄, possibly through binding nLc₄ on neutrophils.25,77 To investigate whether B19 recognized either Gb₄ and/or nLc₄ on myeloblasts, GSL samples were subjected to HPTLC-immunostaining with recombinant B19 empty capsids as described.18,25 B19 bound Gb₄ in red cells, platelets, lymphocytes and 5/6 ANLLs (Figure 5). In addition to Gb₄, B19 also recognized nLc₄ in all myeloid cells tested. A comparison of B19 binding to Gb₄ and nLc₄ was compared using the ratio of B19 binding to DPA-staining as determined by scanning densitometry (B19/DPA, mm²). In general, B19 binding to Gb₄ was twice that observed for nLc₄ (B19/DPA ratio: 0.81 ± 0.28 vs 0.37 ± 0.06, n=6; P<0.01, t-test).
Figure 5. Parvovirus B19 binds both Gb₄ and nLc₄ in ANLL. Panel A) The total neutral GSL fraction from human platelets (P) and normal granulocytes (N), separated by HPTLC and chemically stained with DPA. Panel B) Neutral GSLs immunostained with parvovirus B19 capsids. GSL samples include the total neutral GSL fractions from ANLL (samples 1, 3, 5, 6, 8, 11; Tables 2-4), CML (sample 13), neutrophils (N), lymphocytes (L), platelets (P) and red cells (R). Numbers on left indicate the mobility of Gb₄ (Rₚ 0.27) and nLc₄ (Rₚ 0.23). Solvent, C-M-W 65:30:5 (v/v).
DISCUSSION

The last decade has seen increased interest in the expression, differentiation, regulation and biological role of cell surface carbohydrates. On granulocytes and monocytes, carbohydrate antigens have been shown to facilitate vascular adhesion, cell activation, and phagocytosis.\textsuperscript{14-16,78,79} Cell surface carbohydrates may also play a role in myelopoiesis, as evidenced by changes in N-glycan and ganglioside expression with neutrophilic and monocytic differentiation.\textsuperscript{8,23,80-83} In HL60 cells, granulocytic and monocytic differentiation are accompanied, and induced by, distinct changes in ganglioside synthesis and expression.\textsuperscript{8,82,83} Our studies suggest that myelomonocytic differentiation, particularly granulocytopenesis, may also be accompanied by changes in the expression of neutral GSLs.

Using ANLL cells as a model for neutral GSL differentiation in early myelopoiesis, we observed both quantitative and qualitative differences in the expression of neutral GSLs. Quantitatively, myeloblasts expressed significantly less neutral GSL then mature granulocytes or CML cells. Similar findings have been reported by others and suggest a dramatic increase in total neutral GSL content with neutrophilic maturation.\textsuperscript{30,31,81} Neutrophilic maturation also appears to be associated with a relative and absolute increase in LacCer content, as evidenced by the \%LacCer and LacCer/GlcCer ratio. Biologically, high LacCer expression on mature neutrophils may play a role in inflammation and infection. A receptor for several bacteria and fungi,\textsuperscript{3,84,85} LacCer may facilitate microbial adhesion to neutrophils. In addition, LacCer activates neutrophils, stimulating NADPH oxidase activity and upregulating CD11b/CD18 and ICAM expression on neutrophil membranes.\textsuperscript{14,15,86}

ANLL samples were also screened for qualitative differences in globo-, gala-, neolacto- and ganglio-family GSL expression. Contrary to earlier reports, we found no evidence that Gal\textsubscript{2}Cer is a marker of neutrophilic maturation.\textsuperscript{29,38} With one exception, neither GalCer nor Gal\textsubscript{2}Cer were identified in any myeloid cell tested. Nor did we find evidence of ganglio-series GSLs in human myeloid cells. Early reports of Gg\textsubscript{4} expression on HL60 cells might reflect the known crossreactivity of anti-Gg\textsubscript{4} antibodies with LacCer and neolacto-GSLs.\textsuperscript{56,72} In agreement with our
findings, Yohe et al recently reported an absence of ganglio-series gangliosides in monocytes. In contrast, both ANLL and CML expressed neolacto-series GSLs, a hallmark feature of mature peripheral blood neutrophils. Several extended neolacto-series GSLs, including an unusual intermediate (nLc₅), were also identified in ANLL, CML and mature neutrophils. Earlier studies have suggested that ANLL cells lack the necessary “elongating” glycosyltransferases necessary for synthesis of long-chain neolacto-GSLs. Our data, however, indicates that early myeloid cells are capable of synthesizing a host of neolacto-GSLs, including GSLs with Le xa activity (data not shown). Neolacto-GSLs (nLc₄, nLc₆, nLc₈) are reported to serve as receptors for Neisseria meningitidis and Haemophilus influenzae whereas Le xa and sialyl-Le xa GSL may serve as potential receptors for human granulocytic ehrlichiosis and E-selectin.

Unlike CML or mature neutrophils, most ANLL samples also expressed globo-family GSLs. Gb₃ and Gb₄ were identified in 9/11 ANLL samples by immunologic and physical methods and did not appear to reflect RBC, platelet or lymphocyte contamination. As shown in Table 3, platelet contamination in ANLL samples was minimal relative to our normal granulocyte control. In addition, all samples were differentially centrifuged and washed to remove residual platelets prior to lipid extraction. Likewise, lymphocyte contamination was <5% with leukemic blasts accounting for >85% of all nucleated cells extracted. We also found no correlation between the %Gb₄, % lymphocytes and platelet/leukocyte ratios in our ANLL samples (P>0.10, Pearson-Product Moment Correlation). To eliminate RBC contamination, all leukocyte samples were repeatedly washed with NH₄CO₃ to osmotically lyze residual RBCs prior to lipid extraction. If Gb₃ or Gb₄ were the result of residual RBCs in our leukocyte pellets, they should be uniformly present in all leukocyte samples, including the normal granulocyte control, which was not observed.

The common expression of globo-GSLs in our ANLL samples agrees with several small early studies. Previously, Macher et al reported Gb₄ in 6/6 clinical ANLLs, with Gb₄ accounting for 20-75% of the total GSL4 present. Gb₃ and/or Gb₄ were also identified in KG1, THP-1 and K526 cells, three human ANLL cell lines. Finally, Gb₄ has been reported on the membranes of some clinical ANLL samples by immunofluorescence microscopy and flow cytometry.
contrast, Kyogashima et al failed to observe either Gb$_3$ or Gb$_4$ in 3/3 ANLLs, including two cases of acute monocytic leukemia, which might be expected to express globo-GSLs.$^{24,32,83}$ Overall, 17/20 or 85% of all clinical ANLL samples published to date have been shown to express globo-GSLs, suggesting that globo-GSL are commonly expressed in ANLL. In general, globo-GSLs are expressed by myeloblastic (M0-M2) and monoblastic (M5) leukemias, and in our study, weakly associated with the expression of either early hematopoietic or lymphoid markers. In contrast, globo-GSLs are weak or absent on more “differentiated” neutrophilic leukemias (HL60, CML) and mature granulocytes.$^{37,38,72,81}$ The unusually strong expression of globo-GSLs in our hypogranular ANLL M3, and a second clinical hypogranular M3 sample reported by Macher et al,$^{31}$ may indicate phenotypic and maturational differences between the hypogranular and hypergranular (HL60) variants of ANLL M3.

Based on our results and those of others,$^{24,27-32}$ we propose an updated model of neutral GSL differentiation during human myelopoiesis (Figure 6). As originally suggested by Buehler et al,$^{27}$ early myeloblasts express GlcCer, LacCer, globo- and neolacto-series GSLs. With increasing neutrophilic maturation, there is a marked increase in total neutral GSL with increased LacCer and neolacto-GSL synthesis, accompanied by a loss in globo-GSL expression. Although it is unclear at what point globo-GSL synthesis ceases, the absence of globo-GSLs in HL60 cells suggests it may occur at or after the promyelocytic stage of differentiation.$^{8,27,81}$ Interestingly, trace Gb$_3$ was recently identified in neutrophil secretory granules, but not azurophilic and primary granules, which arise later in neutrophilic maturation.$^{59}$

Unlike granulocytes, monocytic differentiation does not appear to be associated with either an increase or dramatic shift in neutral GSL synthesis. Both monoblasts and mature circulating monocytes express globo- and neolacto-GSLs, averaging 6-11 µg/neutral GSL per 10$^8$ cells.$^{24}$ Monocytes, therefore, maintain a GSL pattern very similar to that observed in early myeloblasts and possibly even committed progenitor cells such as CFU GM, CFU-GEMM and CFU Meg/E:$^{26}$ GlcCer, LacCer, globo- and neolacto-GSLs have been identified in HEL cells, an erythroblastic cell line; HUT7, an erythro-megakaryoblastic cell line; and K562 cells, a pleuripotent cell line capable
of erythroid, monocytic, granulocytic and megakaryocytic differentiation.\textsuperscript{25-28} Overall, neutral GSL differentiation in human myelopoiesis differs significantly from human lymphopoiesis, which is characterized by neolacto-expression in early pro- and pre-B cells, followed by a switch to globo-GSLs with increasing lymphoid maturation.\textsuperscript{39,40} It also differs from murine myelopoiesis, which is characterized by globo- and ganglio-series expression in early myeloid cells, ganglio-GSLs in mature neutrophils and increased globo-GSL expression in monocytes.\textsuperscript{23,41}

Figure 6. Modified model of neutral GSL expression in human myelomonocytic differentiation. Globo- and neolacto-GSLs are expressed by CFU-GM, early myeloblasts and monoblasts. During granulocytic maturation, globo-GSL synthesis ceases, accompanied by a marked increase in total neutral GSL content. In contrast, globo- and neolacto-GSLs are expressed by early and late monocytes, with no apparent increase in total neutral GSL content with increasing monocytic differentiation. Modified from Buehler et al, 1985.\textsuperscript{27}
The expression of globo-GSLs on early myeloid precursors and other blood cells may have important implications for autologous stem cell transplantation.\textsuperscript{20,22} Recently, LaCasse et al proposed using Stx1 for ex vivo purging of autologous stem cell collections. In preliminary studies, Stx1 binding and cytotoxicity was demonstrated against lymphoma, multiple myeloma and breast cancer but not HPC, with no discernable loss in HPC numbers and normal hematopoietic differentiation in vitro.\textsuperscript{20} Likewise, Stx1 effectively purged lymphoma cells from murine marrow prior to transplantation in SCID mice.\textsuperscript{22} In fact, Stx1 could be used as an immunotoxin for a large number of malignancies: Globo-GSLs are commonly expressed on tissues arising from embryonic mesoderm, and are highly expressed on many epithelial tumors including those demonstrating P-glycoprotein-mediated multidrug resistance.\textsuperscript{7,25,91}

Given the potential clinical applications for Stx1 in transplantation, it is imperative to discern the expression and regulation of globo- and other GSLs in human hematopoiesis. Our data, and those of other investigators, suggest that globo-GSLs might be expressed by early myelomonocytic precursors and possibly late committed pleuripotent progenitors, such as the CFU-GM, CFU-GEMM, and CFU-Meg/E. As a consequence, Stx treatment could potentially delay early cellular recovery following autologous transplantation. This may explain the dose-dependent decrease in CFU (CFU-GM and CFU-Meg/E) observed after Stx treatment of murine bone marrow, which expresses globo- and ganglio-series GSLs.\textsuperscript{22,23} In humans, Stx1 treatment also decreased early CFU formation but only at high Stx1 doses.\textsuperscript{22} The latter may reflect the relatively low levels of Gb\textsubscript{3} in early myeloid cells when compared to lymphoma and other Stx-sensitive tissues.\textsuperscript{21} In contrast, myelomonocytic precursors might be quite sensitive to Stx2e, which recognizes Gb\textsubscript{4} or P blood group antigen.\textsuperscript{19} Stx1 and Stx2e binding and toxicity have been shown for THP-1, a Gb\textsubscript{3}-positive ANLL cell line. Interestingly, THP-1 differentiation is associated with a 50\% decrease in Gb\textsubscript{3} and resistance to Stx1.\textsuperscript{92}

Globo-GSL expression on normal peripheral blood elements may also have major implications for Stx1-mediated tumor purging. Gb\textsubscript{3} is a major GSL on human red cells, platelets and lymphocytes, representing a natural “sink” for Stx1 in stem cell and marrow collections. As a result,
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significantly higher Stx1 concentrations may be required, possibly approaching levels toxic to late committed progenitors.\textsuperscript{20,22} This has not been a problem in murine transplantation models since murine erythrocytes, lymphocytes and neutrophils express predominantly ganglio-GSLs.\textsuperscript{23,93,94} Because GSL expression and differentiation is often tissue and species-specific, transplantation protocols using Stx1 for ex vivo purging in animal models may require considerable modification prior to use in human transplantation. For example, a combination of CD34 selection, followed by ex vivo purging with Stx1 (or Stx2e), could minimize Stx toxicity of CD34+ myeloid progenitors by removing peripheral blood elements, thus decreasing the final Stx concentration necessary for tumor purging.

Finally, Gb\textsubscript{4} expression on myeloblasts may explain the neutropenia sometimes observed after parvovirus B19 infection. The etiologic agent of “fifth disease”, parvovirus B19 frequently causes a mild to severe anemia, often accompanied by mild decreases in platelets and leukocytes.\textsuperscript{35} B19 has also been linked to chronic neutropenia of childhood and aplastic anemia.\textsuperscript{33,34} Several studies have shown that both B19-induced anemia and thrombocytopenia reflect primary viral infection of marrow erythroblasts and megakaryocytes, which express Gb\textsubscript{4}, the B19 receptor.\textsuperscript{25,95,96} Our data suggests that early myeloid precursors also express Gb\textsubscript{4} and are potentially susceptible to B19 infection. Although myeloid cells are reportedly resistant to B19 in vitro,\textsuperscript{95} B19 infection of early myeloid precursors with decreased myeloid progenitors and inhibition of CFU-GM formation have been demonstrated in vivo.\textsuperscript{32-35} As previously reported, weak B19 binding to nLc\textsubscript{4} may indicate a second, lectin-binding site on the B19 capsid.\textsuperscript{25} B19 binding to nLc\textsubscript{4} and structurally similar glycans on myeloid glycoproteins may explain why B19 viral replicative forms are preferentially isolated in the neutrophilic fraction of blood.\textsuperscript{25,77,97}

In summary, human myeloid differentiation appears to involve changes in both neutral GSL and ganglioside expression. Changes in the expression and concentration of specific GSLs may play a role in neutrophil function by binding microorganisms\textsuperscript{3,84,85,88} as well as potentiating neutrophil activation, endothelial adhesion and phagocytosis.\textsuperscript{14-16,86} Globo-GSL expression on myeloblasts also suggest the potential susceptibility of early myeloid precursors to parvovirus B19
and Stx1. With the recent cloning of several GSL glycosyltransferases, including Gb3- and Gb4-synthase,\textsuperscript{98,99} it may become possible to study the physiologic role and regulation of GSLs during hematopoiesis.
ACKNOWLEDGMENTS

The authors wish to thank Dr. Lynn Teesh, Director of the High Resolution Mass Spectrometry Facility and John Snyder of the High Field Nuclear Magnetic Resonance Facility, Dept. of Chemistry, The University of Iowa, for their help in FAB-MS and NMR analysis.
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Glycosphingolipid expression in acute nonlymphocytic leukemia: common expression of shiga toxin and parvovirus B19 receptors on early myeloblasts

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