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α1,4Galactosyltransferase activity and Gb_3Cer expression in human leukaemia/lymphoma cell lines

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We have used two methods to evaluate the level of expression of Gb_3Cer in several human leukaemia/lymphoma cell lines representative of the myeloid (K562, KG-1, HL-60, and THP-1) and lymphoid (Reh, Daudi, Raji, RPMI 8226, CCRF-CEM, MOLT-4) lineages blocked at varied stages of differentiation. TLC immunostaining of glycolipid extracts with a monoclonal antibody, 12-101, and FACS analysis with the same antibody were used to demonstrate that the expression of Gb_3Cer in neoplastic myeloid and lymphoid cells is both lineage and differentiation dependent. As a possible control point in the regulated expression of Gb_3Cer we have investigated the first committed step in the synthesis of globo series glycosphingolipids that involves UDP-Gal:LacCer α(1,4)-galactosyltransferase (α1,4GalT). We present the first characterization of this enzyme in a human myeloid cell line using an ELISA-based assay, which was subsequently used to measure α1,4GalT activity in the human leukaemia/lymphoma cell lines. In general, there is a positive correlation between the levels of endogenous Gb_3Cer and the level of the α1,4GalT activity. However, in two cases (KG-1 and CCRF-CEM) the level of enzyme activity did not correspond to the level of Gb_3Cer expression.

Keywords: α-galactosyltransferase, globotriaosylceramide, human leukocytes, regulation of glycosphingolipid biosynthesis

Introduction

The glycosphingolipid, globotriaosylceramide (Gb_3Cer) is of biological interest for many reasons. Expression of Gb_3Cer is known to be elevated in various lymphomas [1, 2], leiomyosarcoma [3], seminoma [4] and familial dysautonomia [5]. Gb_3Cer has been identified as a ligand for Shiga [6, 7] and Shiga-like [8, 9] toxins. Its presence in human milk [10] is thought to exert a protective effect against infantile diarrhoeas caused by these toxins. Gb_3Cer has also been identified as the P^k blood group antigen [11]. This antigen is expressed on many types of human blood cells (erythrocytes [11], lymphocytes [12] and platelets [13]). Gb_3Cer has been identified as a differentiation antigen for B lymphocytes (i.e. CD77, see ref. [14]), was proposed as a marker for apoptosis of germinal centre B-cells [15] and was recently shown to induce apoptosis in Burkitt lymphoma cells [16]. Thus Gb_3Cer has been implicated as an important functional ligand in several biological processes.

Our interest in the expression of this compound centres around its presence or absence at various stages of leukocyte differentiation along the myeloid and lymphoid pathways. Previous studies have shown that immature myeloid cells [17] and all lymphoid cells [18–20] express globo series compounds. Therefore, it has been established that human leukocytes express different families of glycosphingolipids depending on their lineage and maturity. The regulatory events controlling the expression of these compounds in various populations of human leukocytes have not been determined.

Gb_3Cer is synthesized in a stepwise fashion: Cer $\rightarrow$ GlcCer $\rightarrow$ LacCer $\rightarrow$ Gb_3Cer. This glycosphingolipid is also a substrate for Gb_4Cer synthesis. Therefore the level of expression of Gb_3Cer may be controlled by one or more of the enzymes involved in its synthesis or turnover. The enzyme, UDP-Gal:galactose α(1,4)-

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galactosyltransferase (α1,4GalT), plays a key role in the biosynthetic pathway for glycosphingolipids. It catalyses the first committed step in the biosynthesis of globo series glycosphingolipids:

$$Gal\beta 1\text{-}4Glc\beta 1\text{-}1Cer(LacCer) + UDP\text{-}Gal \rightarrow Gal\alpha 1\text{-}4Gal\beta 1\text{-}4Glc\beta 1\text{-}1Cer\ (Gb_3Cer) + UDP$$

This galactosyltransferase has been partially purified from rat liver [21]. A recent report described the characteristics of a CF-54 extract of this enzyme from human placenta [22]. However, there is little information available regarding the characteristics of this enzyme in human leukocytes. We describe in detail in this report the chracteristics of α1,4GalT from a human monocytic leukaemia (THP-1) cell homogenate.

Several studies have shown that there is a direct correlation between the level of α1,4GalT activity and the expression of Gb_3Cer in human tissues. Decreased levels of globo series glycosphingolipid expression and α1,4GalT activity were found [23] after chemically induced differentiation of TERA-2 cells. Other studies with fibroblasts [24] and lymphoid cells [1, 25] have shown that the level of Gb_3Cer expression directly correlates with the level of α1,4GalT activity in those cells. However, in two studies [26, 27] it was shown that the level of Gb_3Cer expression did not correlate with the level of α1,4GalT activity. We have investigated whether or not Gb_3Cer expression correlates with α1,4GalT activity in human leukocytes in the current study.

Recent studies [1, 2, 25, 26] on the expression of Gb_3Cer in human leukocytes have focused only on cells of the lymphoid lineage. In one case [2] only glycosphingolipid extracts from B-cell neoplasms were analysed. In two cases [1, 28] both glycosphingolipid extracts and cell surface expression of this glycosphingolipid were examined. In only one of the latter cases [1] was the α1,4GalT activity also measured. Thus a more complete study was necessary to begin to understand what factors regulate expression of Gb_3Cer in human leukocytes.

We present here the results of such a study using a series of human leukaemia/lymphoma cell lines, which are representative of various stages of leukocyte (myeloid and lymphoid) differentiation. We have examined Gb_3Cer levels in glycosphingolipid extracts and on the cell surface and measured the activity of α1,4GalT for each of the cell lines. TLC immunostaining, FACS analysis, and a microtitre ELISA-based enzyme assay were used to show that there is a positive correlation between the expression of Gb_3Cer and α1,4GalT activity. Minor quantitative variations were found between Gb_3Cer expression levels and enzyme activity. Interestingly, we found that this correlation did not hold for one of the T-cell lines (CCRF-CEM) and one of the myeloid cell lines (KG-1).

Materials and methods

Materials

Neutral glycosphingolipids were prepared from human myeloid cells [17], as previously described. The cell lines were obtained from ATCC (Rockville, MD) or the cell culture facility at University of California, San Francisco, CA. UDP-galactose, γ-galactonolactone, CDP-choline, bovine serum albumin (BSA), irrelevant IgM antibody (MOPC-104E) and p-nitrophenylphosphate (Sigma 104 phosphatase substrate) were purchased from Sigma (St Louis, MO). Sodium cacodylate was purchased from Aldrich (Milwaukee, WI). The monoclonal antibody, 12-101 [29], was purchased from Accurate Chemical & Scientific (Westbury, NY). R-Phycoerythrin conjugated goat anti-mouse IgM anti sera ($F(ab')_2$) was obtained from TAGO (Burlingame, CA). Biotin-conjugated goat anti-mouse IgM, biotinylated alkaline phosphatase, and the TLC immunostain substrate were obtained from Vector Laboratories (Burlingame, CA) as part of the Alkaline Phosphatase Standard Vectastain ABC Kit. UDP-[^{14}C]Gal (257 mCi mmol^{-1} and [^{14}C]Gal (55.7 mCi mmol^{-1}) were obtained from American Radiolabelled Chemicals, Inc. (St. Louis, MO) and Amersham (Arlington Heights, IL), respectively. Hydrofluor liquid scintillation cocktail was obtained from National Diagnostics (Manville, NJ). Protein assay reagent was obtained from Bio-Rad (Richmond, CA). All other reagents were of the highest grade available.

Cell culture

The cell lines used (classified according to Saito [30]) were: K562, myeloid stem cell; KG-1, myeloblast; HL-60, promyelocyte; Reh, lymphoid stem cell; CCRF-CEM, T-blast I; MOLT-4, T-blast II; Daudi and Raji, B-blast I; and RPMI 8226, plasma cell. The human monocyte cell line, THP-1 [31], was also used. All cell lines were grown in suspension at 37 °C in a humidified atmosphere with 5% CO_2. The medium was RPMI 1640 supplemented with 20% (KG-1 and RPMI 8226) or 10% (all others) fetal calf serum and 1% penicillin/streptomycin.

Cell homogenates

Homogenates from each cell line were prepared for use in the enzyme assay. Cells were collected and washed three times with 50 mM cacodylate buffer (pH 6.7) and finally suspended in the same buffer (2–6 mg protein per ml). Aliquots of each suspension were used fresh or centrifuged and stored as pellets at −70 °C. Frozen cell pellets were thawed and resuspended in 50 mM cacodylate buffer, pH 6.7 to a protein concentration of 2–6 mg ml^{-1}. The suspensions were kept at 0 °C and homogenized with an Omni 5000 homogenizer. These crude preparations were used as the source of α1,4GalT

on the day of preparation. To ensure that all homogenates prepared from frozen cells contained active enzymes, β1,4GalT activity was measured as previously described [32], and found to be high in all cell lines. Protein concentrations were determined by a microassay procedure (Bio-Rad Protein Assay booklet) based on the method of Bradford [33], with bovine serum albumin as a standard.

TLC immunostaining of endogenous glycosphingolipids in cell lines

TX-100 was added to the cell homogenate to obtain a final concentration of 0.4%. An aliquot (300 μl; 0.6–1.6 mg protein) of the resulting cell preparation was added to 2 ml theoretical upper phase (TUP, chloroform: methanol:0.1 M KCl (3:48:47), see ref. [34]). The sample mixture and tube rinses (2 × 1 ml TUP) were passed through a C_{18} Sep-Pak and then reapplied twice. Distilled, deionized water (DDW, 10–20 ml) was used to elute the water soluble components. Methanol (10 ml) was used to elute the glycolipids. The organic phase, containing the glycosphingolipids, was dried under a stream of nitrogen, dissolved in 500 μl of chloroform: methanol (1:1) and dried again. The sample was subsequently brought up in 40 μl of chloroform:methanol (1:1) and spotted in its entirety on an aluminum backed HPTLC plate (Silica Gel 60, EM Separations). Chromatographic separation was done with C/M/W 60:35:8 (v/v/v). The immunostaining procedure previously described [35] was used to identify endogenous Gb_3Cer on the TLC plate. Briefly, the TLC plate was soaked in the following solutions in the order listed: 5% BSA/PBS (0.5 h, room temperature (RT)), primary antibody, 12–101 (2 h, RT or 16 h, 4 °C), biotinylated secondary antibody (biotinylated goat anti-mouse IgM, 1–2 h, RT), avidin-linked biotinylated alkaline phosphatase (1–2 h, RT), substrate (SK-5200 from Vector Labs; 5–10 min, RT). The plate was rinsed with PBS and soaked for 5 min in PBS before soaking in each solution listed. This entire procedure was done twice to ensure the reproducibility of the results.

TLC densitometry

After the glycosphingolipid bands were visualized on the TLC plate by immunostaining, the plate was scanned on a flatbed greyscale scanner (HP ScanJet IIc) that was interfaced to a Macintosh LC II. A digitized image of the TLC plate was obtained with DeskScan II and subsequently imported into Image 1.41 (NIH, public domain). The Image Gel Plotting Macro was used to obtain a chromatographic profile of the band that comigrated with standard glycosphingolipid in the appropriate lanes on the TLC plate image. For each lane on the image of the TLC plate containing immunostained endogenous Gb_3Cer the corresponding peak in the chromatogram was integrated to obtain a numerical value for the intensity of the immunostained band. Each intensity value was then divided by the protein amount for its respective cell line to facilitate comparison with the other cell lines. A TLC plate containing varied amounts (0.05–1.3 nmol) of the standard, Gb_3Cer, was immunostained under the same set of conditions and used to verify the linearity of the densitometric measurements.

Fluorescence activated cell sorting (FACS) analysis of cell surface Gb_3Cer expression

Cells (~5×10^5) were rinsed twice with staining media (Dulbecco's PBS containing 10 mM HEPES (pH 7.3), 0.1% azide, and 2% heat inactivated fetal calf serum) and incubated with an irrelevant IgM mouse monoclonal antibody (MOPC-104E, as a control) or with a mouse monoclonal antibody that binds to Gb_3Cer (12–101) for 0.5 h on ice. Following two washes with staining media the cells were incubated with R-phycoerythrin conjugated goat anti-mouse IgM anti-sera as above. Following two washes with staining media the cells were analysed on a FACSort flow cytometer (Becton Dickenson). Each cell line was subjected to FACS at least twice to ensure the reproducibility of the profiles.

ELISA-based α1,4galactosyltransferase assay

Microtitre wells (Falcon 3912 Micro Test III Flexible assay plate) were incubated with 150 μl 5% BSA/PBS, washed three times with distilled, deionized water, and allowed to dry at room temperature. Subsequently, the glycosphingolipid acceptor (2.5 nmol LacCer in 10 μl of 85% ethanol) was allowed to dry in the microtitre wells at room temperature. A set of three to six wells was used for each set of reaction conditions. Each well, with immobilized glycosphingolipid acceptor, was incubated with a cell homogenate (20–50 μg total protein) in a reaction mixture (total volume 65 μl) that contained 8 mM $MnCl_2$, 1.2 mM UDP-Gal, 75 mM cacodyate buffer (pH 7.0), 8 mM γ-galactonolactone, 8 mM CDP-choline. As a control a set of three to six wells, which contained immobilized glycosphingolipid acceptor and the complete reaction mixture minus UDP-Gal, was used for each set of reaction conditions. All solutions and the microtitre plate were kept on ice during the preparation of the reaction mixture. Each well, containing the glycosphingolipid acceptor, was incubated with the enzyme reaction mixture at 37 °C for 3–16 h. The reaction was terminated by removing the enzyme reaction mixture from each well and rinsing the microtitre wells with PBS (150 μl, three times).

The ELISA procedure, previously described [32], was used to identify and quantify the product. Briefly, the wells were incubated with each of the following reagents

(100 μl per well) in the order listed: 1% BSA/PBS (0.5 h, room temperature (RT)), primary antibody (12-101, 2–6 h, RT), secondary antibody (biotinylated goat anti-mouse IgM, overnight, 4 °C), and enzyme complex (biotinylated alkaline phosphatase plus avidin, 1–2 h, RT). Between steps the wells were rinsed three times with 150 μl PBS. The primary antibody, 12-101, was diluted 1:75 with 1% BSA/PBS. Finally, the substrate, *p*-nitrophenylphosphate, was dissolved in 0.1 M Tris buffer, pH 9.5, and added to each well (100 μl per well). The absorbance at 405 nm was measured at several time intervals with an EIA Reader (BioRad Model 2550). Absorbance was converted to amount of product by comparison to a standard binding curve for the primary antibody, 12-101. The standard curve was prepared by conducting the ELISA with various amounts of Gb_3Cer, which were incubated with a complete reaction mixture minus the nucleotide sugar.

TLC immunostaining of reaction products

A microtitre plate was used as a reaction vessel to generate the reaction products as described above (in the ELISA-based assay procedure). However, instead of reacting the products with monoclonal antibodies, the reaction products were recovered from the microtitre wells as follows: the reaction mixture from each well was transfered into a tube containing 1–2 ml TUP and the well was rinsed with TUP. The reaction mixtures and TUP rinses from a total of six wells were pooled for each set of reaction conditions. The products and unreacted glycolipid acceptor were separated from the other components of the reaction mixture by C_{18} Sep-Pak chromatography and immunostained as described under TLC immunostaining of endogenous glycosphingolipids. This procedure was done for qualitative verification of the formation of the enzyme product, Gb_3Cer.

UDP-galactose hydrolysis

The ELISA-based assay was done in a microtitre well as described above with the addition of 0.1 μCi UDP-[^{14}C]Gal to the well. The reaction was terminated by spotting the enire reaction mixture from one well onto a 50 cm strip of Whatman 1 paper. Descending paper chromatography was done using authentic UDP[^{14}C]Gal and [^{14}C]Gal as standards on separate strips. After development for 19 h in the solvent system (95% EtOH:1 M ammonium acetate (pH 3.8), 75:30, see ref. [36]), the strips were dried and cut at 1 cm intervals. Each square, suspended in Hydrofluor (containing 10% DDW), was counted in a liquid scintillation counter (Beckman LS 3801). A chromatogram for each strip was reconstructed by plotting radioactivity vs. position (cm). The peak areas in the chromatogram were used to quantify the amount of UDP-Gal hydrolysis.

Results

Endogenous Gb_3Cer in human leukaemia/lymphoma cells

Cell lines which contain Gb_3Cer were identified by TLC immunostaining with the antibody, 12-101 (Galα1-4Gal specific). Glycosphingolipid extracts from four of the ten human leukaemia/lymphoma cell lines contained a band that co-migrated with Gb_3Cer and was immunostained by 12-101 (see Fig. 1). Gb_3Cer was found in three of the lymphoid cell lines: Daudi, Raji, RPMI 8226 (Panel A, lanes 1–3). In addition, Gb_3Cer was found in the monocytic myeloid cell line, THP-1 (panel B, lane 4). Densitometric analysis of the immunostained plate revealed that Daudi and THP-1 had the largest amounts of endogenous Gb_3Cer (Table 1). The Gb_3Cer band for the Raji cells was 33% as intense as that for the Daudi cells. A recent report by Taga *et al.* [25] also showed that Gb_3Cer content varies among different Burkitt lymphoma cell lines. The endogenous Gb_3Cer in RPMI 8226 cells was 30% of the amount found in Daudi cells. The low level of Gb_3Cer in the RPMI 8226 cell line may result from its conversion to Gb_4Cer. Kalisiak previously reported [2] the presence of this compound in this cell line and we have detected an orcinol stained band that co-migrates with Gb_4Cer in a lipid extract from this cell line (unpublished results). Neither the pre-B cell line (Reh; data not shown), the T-cell line (CCRF-CEM, MOLT-4; Panel A, lanes 4,5) nor the myeloid cell line (K562, KG-1 and HL-60; Panel B, lanes 1–3) extracts contained visible bands that co-migrated with Gb_3Cer. However, densitometric analysis did reveal trace amounts of this compound in the CCRF-CEM and KG-1 extracts (Table 1).

Upon examination of the positively immunostained bands in Fig. 1 it is clear that most of the cell lines express Gb_3Cer that appeared as a doublet. In all of the

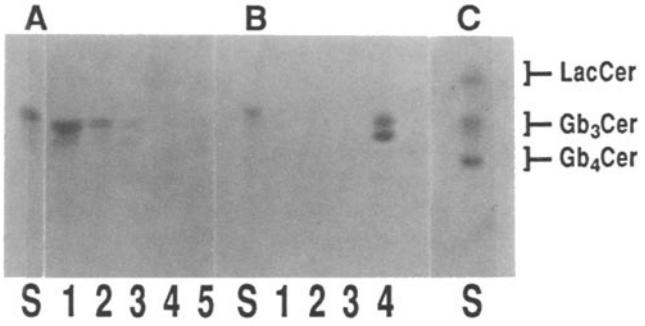

Figure 1. Endogenous glycosphingolipids in human leukaemia/lymphomas immunostained with 12-101. Panels A and B were immunostained with 12-101 and the bands in plate C were visualized with orcinol spray. Lane S, glycolipid standards as indicated on the right. Panel A-lymphoid cell extracts immunostained with 12-101: lane 1, Daudi; lane 2, Raji; lane 3, RPMI 8226; lane 4, CCRF-CEM; and lane 5, MOLT-4. Panel B-myeloid cell extracts immunostained with 12-101: lane 1, K562; lane 2, KG-1; lane 3, HL-60; and lane 4, THP-1.

Table 1. Summary of α1,4GalT activity and Gb_3Cer expression in human leukaemia/lymphoma cell lines.

Lineage	Cell line	Gb_3Cer expression		α1,4GalT Activity (pmol mg^{-1} h^{-1})
		Extract	Surface	
Myeloid				
	THP-1	+ (82)[a]	+ (100)[b]	133 (73)[c]
	K562	–	–	ND[d]
	KG-1	trace	+ (14)	ND
	HL-60	–	–	ND
Lymphoid				
	Reh	–	–	ND
	CCRF-CEM	trace	+ (9)	ND
	MOLT-4	–	–	ND
	Daudi	+ (100)	+ (74)	182 (100)
	Raji	+ (33)	+ (23)	18 (10)
	RPMI 8226	+ (30)	+ (31)	74 (41)

[a]+, indicates positive TLC immunostaining; –, indicates no staining. Values in parentheses are percentages relative to the intensity per mg of the band from the Daudi extract.
[b]Values in parentheses are percentages relative to mean fluorescence intensity/background of the THP-1 cells, as determined by FACS analysis.
[c]The amount of product Gb_3Cer was determined by subtracting the amount of Gb_3Cer in the control wells from the amount of Gb_3Cer in the wells that contained a complete reaction mixture. Enzyme activities are the average of three or more separate ELISA-based assays between which the typical standard deviation was 10–15%. Values in parentheses are percentages relative to the activity of the Daudi cells.
[d]ND, not detected.

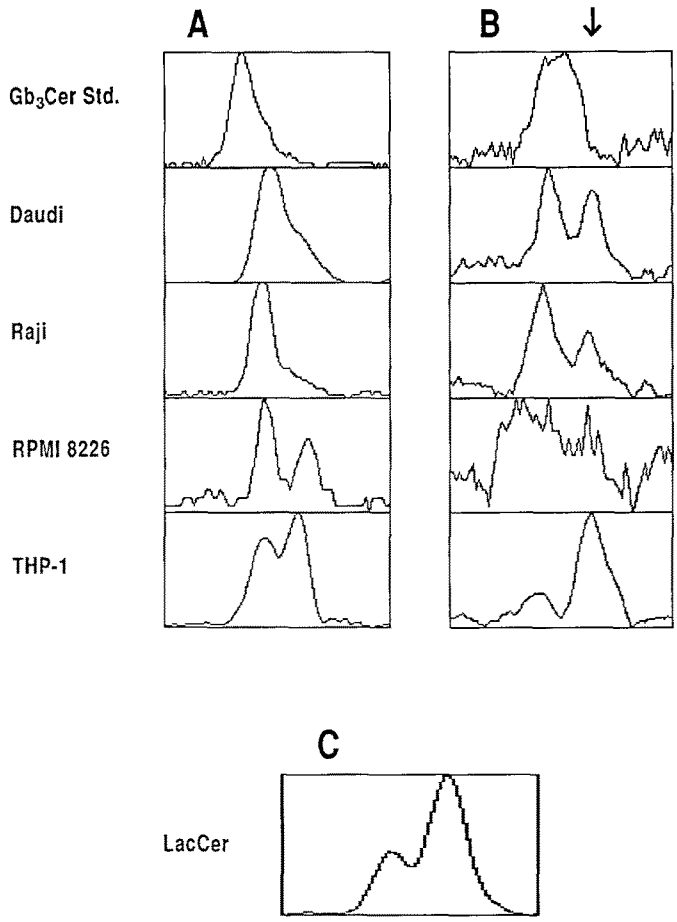

Figure 2. Chromatographic profiles of the TLC immunostained endogenous glycosphingolipid and reaction products from mixtures containing LacCer and human leukaemia/lymphoma cell homogenates. The procedures described under TLC immunostaining and TLC densitometry were used to obtain the profiles shown. Panel A shows the profiles for endogenous Gb_3Cer and panel B shows the profiles for reaction product Gb_3Cer; both TLC plates were immunostained with 12-101. The amount of glycosphingolipid material applied to the TLC plate for the product immunostain was 10–30 fold less than the amount used for the endogenous Gb_3Cer immunostain. The arrow (Panel B) denotes the position of the Gb_3Cer product band. Panel C shows the profile for the LacCer acceptor visualized by reaction with orcinol reagent. For each profile the top of the TLC plate is at the left side.

cell lines except THP-1 there was a predominant upper band (see chromatographic profiles in Fig. 2A). Gb_3Cer isolated from THP-1 cells appeared as a doublet in which the bands were of similar intensity. The presence of a doublet is probably due to a difference in the ceramide moiety. We have observed that the difference in the ceramide moiety of glycosphingolipids isolated from human leukocytes is usually associated with a difference in the length of the fatty acid chain and not the sphingosine base. We previously observed that glycosphingolipids containing long chain fatty acids run higher on the TLC plate than the equivalent glycosphingolipids containing short chain fatty acids. This suggests that the predominant band in lymphoid cells and the upper band in the THP-1 cells contain long chain fatty acids. It should also be noted that the standard Gb_3Cer (prepared from porcine kidney) ran higher on the plate than the predominant band for lymphoid cells (see profile in Fig. 2A). This chromatographic behaviour is most likely a result of the standard Gb_3Cer having a different ceramide composition than that found in human leukocytes.

FACS analysis of surface Gb_3Cer expression in human leukaemia/lymphoma cells

To evaluate the cell surface expression of Gb_3Cer each of the human leukaemia/lymphoma cell lines was analysed by FACS. The results for the FACS analysis are shown in Fig. 3. No staining with the irrelevant IgM antibody (MOPC-104E) was observed (solid profile, Figure 3). The results with the primary antibody, 12-101, are also shown in Fig. 3 (outline profiles). From the outline profiles in Fig. 3 it can be seen that six of the cell lines (Daudi, Raji, RPMI 8226, CCRF-CEM, KG-1, and THP-1; panels A-D, F and H, respectively) were stained with this antibody and thus express Gb_3Cer on their cell surface. The narrow shape of the peaks obtained for

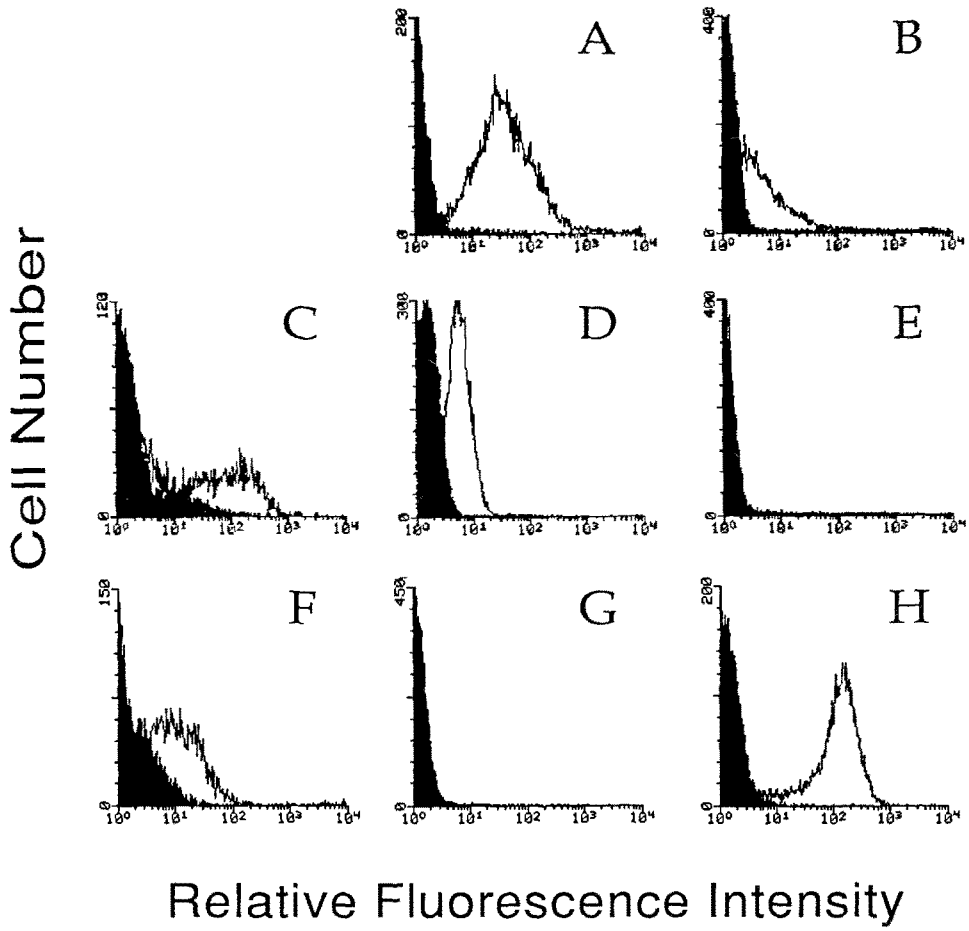

Figure 3. FACS analysis of human leukaemia/lymphoma cell lines with 12-101. Approximately 5×10^5 cells were used with the monoclonal antibody, 12-101, in the procedure described under FACS analysis to obtain the data shown. Panel A, Daudi; panel B, Raji, panel C, RPMI 8226; panel D, CCRF-CEM; panel E, MOLT-4; panel F, KG-1; panel G, HL-60; and panel H, THP-1. Solid profiles were obtained with an irrelevant mouse IgM antibody (MOPC-104E) and outline profiles were obtained with a mouse monoclonal antibody that binds to Gb_3Cer (12-101).

CCRF-CEM and THP-1 indicate that these cell populations were more homogenous with respect to the relative amount of Gb_3Cer they express than those in the other four cell lines. The broad profile for RPMI 8226 cells suggests that these cells are more heterogeneous with respect to the relative amount of Gb_3Cer they express than the other five cell lines. RPMI 8226 cells appeared to have a mixed staining pattern. A portion of the cell population was stained strongly, whereas the remaining portion was stained weakly or not at all. The staining intensity for Daudi and THP-1 cells was high whereas the level of staining for Raji cells was low (summarized in Table 1). FACs analysis also showed that CCRF-CEM and KG-1 cells were weakly stained by 12-101 (Fig. 3: Panels D and F, respectively). This low level of fluorescence intensity (see Table 1) reflects the trace amount of Gb_3Cer that was detected by densitometry of these two immunostained cell extracts on the TLC plate. Thus six of the ten cell lines examined had detectable cell surface expression of Gb_3Cer. The remaining T-cell line (MOLT-4), lymphoid stem cell line (Reh; data not shown) and myeloid cell lines (K562, data not shown; HL-60) were not stained by 12-101 (Fig. 3: Panels E and G, respectively) and thus did not have detectable levels of cell surface of Gb_3Cer.

α1,4galactosyltransferase in THP-1 cells

As described in the introduction there are several steps in the biosynthetic pathway for Gb_3Cer. Therefore the level of Gb_3Cer expression may be controlled by one or more of the enzymes involved in its synthesis or turnover. The most straightforward possibility for a point of regulation in this pathway is the step in which α1,4GalT converts LacCer to Gb_3Cer. The ELISA-based enzyme assay described in the Materials and methods section was used to measure this α1,4GalT activity. Initial studies were performed with THP-1 cells to establish the optimal conditions for the assay. Pre-blocking the plate with 5% BSA/PBS before the addition of the glycosphingolipid acceptor was necessary to eliminate high background

levels from non-specific binding during the ELISA procedure. The use of TX-114 and anionic detergents eliminated the enzyme activity, whereas the use of Tween 20 resulted in high background levels and an activity equivalent to that obtained with an homogenate made without detergent. An enzyme activity equivalent to 75% of that obtained with detergent-free homogenate was obtained with 0.01% TX-100. However, the addition of greater amounts of TX-100 (0.05–0.8%) resulted in loss of activity. This result is different than that reported [1] for the Daudi enzyme, which was found to have an optimal detergent requirement of 0.3% TX-100.

The α1,4GalT in THP-1 cells had a pH optimum at 7.0. The Mn^{2+} requirement was absolute and no activity was observed with other cations (Co^{2+}, Ca^{2+}, Zn^{2+}, Ni^{2+}). UDP-Gal hydrolysis was less than 9%. The K_m values for UDP-Gal and LacCer were 0.52 mM and 1.3 μM, respectively. High concentrations of UDP-Gal resulted in decreased enzyme activity, thus an optimal concentration (1.2 mM) of UDP-Gal was used in subsequent experiments. The reaction was linear with time to 16 h and with protein concentration to 0.8 $\mu g\,\mu l^{-1}$.

α1,4galactosyltransferase in human leukaemia/lymphoma cells

The ELISA-based assay was used to measure α1,4GalT activity in homogenates prepared from cells that were either freshly collected or stored at −70 °C. With only one exception there was no difference in activity between homogenates made from cells that were freshly collected or from cells stored at −70 °C (data not shown). However, the α1,4GalT activity in one cell line (RPMI 8226) was destroyed by storage at −70 °C. Therefore, for this cell line, fresh cell homogenates were used as an enzyme source.

All ten human leukaemia/lymphoma cell lines were tested for α1,4GalT activity. Among the myeloid lineage cells (K562, KG-1, HL-60 and THP-1) only the monocytic leukaemia cell line, THP-1, had detectable α1,4GalT activity. Among the lymphoid lineage cells (Reh, RPMI, 8226, Daudi, Raji, MOLT-4, and CCRF-CEM) only the lines representing committed B-cells (RPMI 8226, Daudi and Raji) had detectable α1,4GalT activity. Enzyme activity was lacking in the Reh homogenate (Table 1). This is consistent with the observation made by Taga *et al.* [25] that only cell lines representing B-cells later in the differentiation pathway express α1,4GalT activity. Cells (Daudi, Raji, RPMI 8226 and THP-1) that express Gb_3Cer have a correlative level of α1,4GalT activity. For example (see Table 1), Daudi had intense staining of endogenous Gb_3Cer (100%) and surface Gb_3Cer (74%) that was complemented by a high level of α1,4GalT activity (100%). RPMI 8226 cells had a moderate level of staining (extract, 30%; surface, 31%) and a concommitant moderate level of enzyme activity (41%). Likewise, the Raji cells had a slightly lower level of staining (extract, 33%; surface, 23%) and a lower level of α1,4GalT activity (10%).

Reaction product immunostain results

To confirm the identity of the reaction products they were extracted from the microtitre wells (as described in the Materials and methods section) and immunostained with 12-101. The chromatographic profiles of TLC immunostained products are shown in Fig. 2. Each of the cell homogenates that had detectable α1,4GalT activity also yielded a product that was immunostained by 12-101 (Daudi, Raji, RPMI 8226 and THP-1; panel B). The lack of a product that stained with 12-101 in the other cell lines (Reh, CCRF-CEM, MOLT-4, K562, KG-1, HL-60; data not shown) is consistent with the absence of α1,4GalT activity in those cell homogenates (see Table 1).

As described above (in results from TLC immunostaining of endogenous Gb_3Cer) immunostained endogenous Gb_3Cer in human leukocytes appeared as a predominant upper band with a lower band that was of similar or lower intensity (or is non existent). The profiles of immunostained Gb_3Cer product are shown in Fig. 2B. The profile for the standard Gb_3Cer in panel B is slightly broader than the profile for the standard in panel A of Fig. 2. Since the two TLC plates were developed on different days the slight change in chromatographic behaviour may have resulted from very slight changes (e.g. relative humidity) in chromatographic conditions. Overall, the baseline is noisier in the product profiles than in the endogenous profiles since much less (10–30-fold) glycosphingolipid material was used for the product immunostain. Comparison of the endogenous Gb_3Cer profiles (Fig. 2A) with the product Gb_3Cer profiles (Fig. 2B) reveals that upon product formation a lower migrating band was present, which resulted in the formation of a doublet for all cell lines. The lower migration position of this product band is most likely a result of the fact that the LacCer used in these experiments was taken from a fraction that had been enriched in short chain fatty acids (see profile in Fig. 2C). Examination of the THP-1 profiles reveals a distinct shift in Gb_3Cer distribution: upon product formation the upper band is greatly diminished and the lower band is concurrently intensified. Each of the other cell lines showed a similar pattern. In one case (Raji) only the predominant upper band was present in the immunostain of endogenous Gb_3Cer, whereas a distinct doublet was detected in the immunostain of the products.

Thus the immunostained product bands were, in some cases, a combination of endogenous Gb_3Cer and product from the enzyme reaction. To further confirm that product was actually made by each of the cell lines that had substantial amounts of endogenous Gb_3Cer an addi-

tional experiment was done. The incorporation of radiolabelled Gal from UDP-[^{3}H]Gal was measured for all ten cell lines using storage phosphor technology on a TLC plate containing the radiolabelled products (data not shown, see ref. [37]). All of the cell line homogenates that generated a product which was immunostained by 12-101, also generated a radioactive product that co-migrated with Gb_3Cer. Additionally, none of the remaining cell line homogenates generated a radioactive product (detection limit in fmol range) that co-migrated with Gb_3Cer.

Discussion

Gb_3Cer is an important molecule in many biological processes. For example, it has been shown [38] that TNF-α and IL-1 induce human endothelial cells to express significantly higher levels of Gb_3Cer and thus increase the verotoxin (VT-1) binding to these cells. It is well known that this glycosphingolipid is a ligand for Shiga and Shiga-like toxins as well as bacterial adhesins [39]. Gb_3Cer also serves as a blood group antigen and a differentiation marker for human lymphoid B-cells. Previous studies of the expression of globo series compounds have shown that their expression is also lineage dependent. The importance of this antigen in human leukocyte differentiation prompted us to study the expression of this antigen in a variety of human leukocytes.

We have presented here the first characterization of the enzyme that catalyses the conversion of LacCer to Gb_3Cer in human myeloid cells. The properties of the α1,4GalT activity in THP-1 cells are different from those reported for the lymphoid enzyme characterized [1] in Daudi cells. The major difference is the detergent requirement. In our hands no enzyme activity was detectable with 0.1–0.8% TX-100 in either the ELISA-based assay or the traditional radioactive assay (unpublished results), whereas the Daudi enzyme had optimal activity with 0.3% TX-100. It is unlikely that the myeloid and lymphoid enzymes are different proteins since we were also able to measure high α1,4GalT activity in Daudi cells using the conditions established for measuring this activity in THP-1 cells. Part of the difference may have arisen from the fact that we used a crude homogenate and the previous characterization of the Daudi enzyme utilized a Golgi-rich membrane preparation.

Previously the study of Gb_3Cer expression in human leukocytes has focused on its expression in lymphoid cell lines and in only one case [1] has there been an attempt at establishing whether or not there is a correlation between enzyme activity and antigen expression (both at the cell surface and in total cell glycolipids). Our study is the first to provide information regarding enzyme activity and the expression of Gb_3Cer in human leukocytes of both myeloid and lymphoid origin. We used several human leukaemia/lymphoma cell lines which are representative of human leukocytes (myeloid and lymphoid) blocked at various stages of differentiation. We established the distribution pattern of Gb_3Cer by TLC immunostaining of glycosphingolipid extracts and FACS analysis of cell surface staining. We also measured the α1,4GalT activity in each of the cell homogenates. As described in the following paragraphs the results from each of the analyses have allowed us to make several conclusions regarding Gb_3Cer expression and α1,4GalT activity in human leukocytes.

We have investigated the distribution of Gb_3Cer in a variety of human leukaemia/lymphoma cell lines and shown that the expression of this compound is differentiation and lineage dependent. All of the B-lymphoid cell lines (Daudi, Raji, and RPMI 8226) had readily measurable quantities of Gb_3Cer in the cell extracts and on the cell surface, whereas the lymphoid stem cell line (Reh) had neither enzyme activity nor detectable levels of Gb_3Cer. Our results confirm that the expression of Gb_3Cer occurs after the pre-B stage in the B-cell differentiation pathway as described by others [2, 25, 28]. The T-lymphoid cell lines were found to contain very little (CCRF-CEM) or no (MOLT-4) Gb_3Cer either in the cell extract or on the cell surface. Thus our results indicate that the expression of Gb_3Cer in lymphoid cells is differentiation dependent: B-lymphoid cells express relatively large amounts of Gb_3Cer compared to T-lymphoid cells. This provides supporting evidence for the results obtained in a study by Cohen *et al.* [40] in which several human lymphoid cells were screened and only B-lymphoid cells were found to be sensitive to verotoxin (VT-1), which binds to Gb_3Cer.

The expression of Gb_3Cer in myeloid cells was also found to be differentiation dependent. Three cell lines used in this study are representative of the myeloid stem cell (K562), myeloblast (KG-1) and promyelocyte (HL-60) stages of myeloid differentiation (see ref. [17]). TLC immunostaining and FACS analysis confirmed our previous finding [17] that cells of the myeloid lineage (HL-60) that represent a later stage of differentiation do not express any globo compounds. We previously identified [17] Gb_3Cer in KG-1 cells by radiolabelling studies with [^{14}C]Gal. Our immunostain of the glycosphingolipid extracts from these cells and the low level of surface staining with 12-101 in the FACS analysis of these cells confirmed that this compound is present in trace amounts. K562 cells lacked detectable Gb_3Cer expression. Our earlier findings [17] suggested that these cells had a trace amount of this compound which was detected by radiolabelling studies. In contrast it has also been found that verotoxin (VT-1) did not bind to K562 cells (personal communication with Dr C.A. Lingwood), which indicates the absence of Gb_3Cer on the cell surface. Therefore this cell line appears to have little or

no Gb_3Cer. However a clone of the K562 cell line was reported [41] to have detectable amounts of Gb_3Cer. With the exception of this K562 clone, myeloid cells along the granulocytic pathway express very little or no Gb_3Cer.

THP-1 was used as a cell line representative of the monocyte pathway of myeloid differentiation. Our previous study [20] with AMoL and AMMoL patient samples showed that monocytic leukaemia cells expressed Gb_3Cer. Thus it was anticipated that the THP-1 cell line would have a glycosphingolipid profile similar to these cells. We have established by TLC immunostaining and FACS that THP-1 cells do express Gb_3Cer. This is in agreement with the work of Sherwood and Holmes [42] who found that a similar monocytic cell line, U937, also expresses Gb_3Cer. These findings confirm that monocytic cell lines express Gb_3Cer and model their normal counterparts, which were found by Kiguchi *et al.* [43] to express Gb_3Cer. Thus, in myeloid leukaemia cells the pattern of Gb_3Cer expression is differentiation dependent: monocytic cells express relatively large amounts of this compound, whereas granulocytic cells express very little or none.

It is possible that this differentiation dependent distribution of Gb_3Cer in human leukocytes may be a direct result of the enzyme activity responsible for its biosynthesis. Others have found such a direct dependence of globo series glycosphingolipid expression on α1,4GalT activity. In a study by Chen *et al.* [23] TERA-2-derived human embryonal carcinoma cells were chemically induced to differentiate. This resulted in a marked reduction in expression of globo series glycosphingolipids and a concomitant reduction in α1,4GalT activity. Likewise human fibroblasts [24] which had the p blood group phenotype (total lack of globo series antigens) were shown to lack α1,4GalT activity. Wiels *et al.* [1] and more recently Taga *et al.* [25] also reported a positive correlation between Gb_3Cer expression and α1,4GalT activity in cells representative of various stages of B-cell differentiation. Each of these studies supports the notion that Gb_3Cer expression is directly related to α1,4GalT activity.

It is also possible that this differentiation dependent distribution of Gb_3Cer in human leukocytes may not be a direct result of the enzyme activity responsible for its biosynthesis; this compound is a precursor for Gb_4Cer synthesis and can also be degraded by α-galactosidase. In fact, other studies have indicated that the regulation of Gb_3Cer expression is complex. For example, Iizuka *et al.* [27] found that lymphoblastoid cells with the p phenotype (and thus lack Gb_3Cer) do have α1,4GalT activity. It was recently demonstrated by Mobassaleh *et al.* [44], that the dynamic balance between α1,4GalT and α-galactosidase activities can regulate Gb_3Cer expression. Pudymaitis *et al.* [26] found that a Daudi mutant (VT20) selected for its resistance to verotoxin (VT-1) had greatly reduced expression of Gb_3Cer on the cell surface but no reduction in its α1,4GalT activity. Thus based on glycosphingolipid expression and enzyme activity measurements the effect of α1,4GalT activity in controlling the expression of Gb_3Cer in human cells is different for various cell types.

Our results suggest that Gb_3Cer expression in human leukocytes correlates with α1,4GalT activity in most but not all cases. This conclusion is based on a comparison of the results from TLC immunostaining and FACS analysis to the measured level of α1,4GalT activity. For example, only committed B-lymphoid cells (Daudi, Raji and RPMI 8226) and monocytic cells (THP-1) had detectable levels of Gb_3Cer and α1,4GalT activity, whereas one T-lymphoid cell line (MOLT-4) and two myeloid cell lines (K562 and HL-60) had neither Gb_3Cer nor α1,4GalT activity. Others have reported [1, 25] a similar finding for lymphoid cells, but this is the first report of the correlation of Gb_3Cer expression and α1,4GalT activity in myeloid cells.

Minor variations in the level of enzyme activity (10%) relative to Gb_3Cer expression (33%) were found in the Raji cells. This may be a result of using a crude homogenate instead of a purified golgi preparation of the enzyme. A similar finding for lymphoid cells was reported by Taga *et al.* [25]. Two exceptions (KG-1 and CCRF-CEM) to the direct correlation of Gb_3Cer expression and enzyme activity were found. FACS analysis revealed low levels of surface Gb_3Cer in two cell lines (KG-1 and CCRF-CEM; 9% and 14% of highest value, respectively) and the glycosphingolipid extracts from these two cells also showed trace amounts of Gb_3Cer. Surprisingly, no α1,4GalT activity was detected in either of these cell lines. Since a whole cell homogenate was used in our enzyme assay it is possible that α1,4GalT activity was present and made a product, but that this product was subsequently degraded by an endogenous α-galactosidase activity. Further studies are underway to determine whether this or other factors are involved.

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