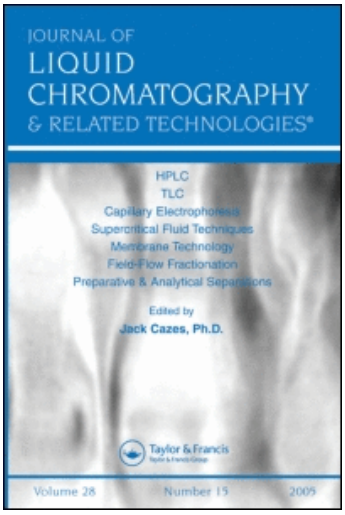

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Comparison of Total Lipid Composition in Gb3-Positive and Gb3-Deficient Burkitt's Lymphoma Cells

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Abstract: Thin-layer chromatography was used to compare the glycolipid, phospholipid, and neutral lipid compositions of the globotriaosylceramide (Gb3)-positive Burkitt's lymphoma Daudi cell line and the Gb3-deficient Daudi-derived VT500 cell line. Glycolipid compositions were determined by co-migration of lipids with glycosphingolipid standards and staining with orcinol. Differences in glycolipid composition of the two cell lines, in addition to Gb3 content, included differences between expression of multiple cerebroside bands and more complex glycolipids. The composition of neutral lipids was similar in the two cell lines. Neutral lipids co-migrated with triacylglycerol, diacylglycerol, and fatty acid standards. In addition, cholesterol appeared to be the major neutral lipid component in both cell lines. Phospholipid compositions were analyzed by developing chromatograms in two dimensions and staining with molybdenum blue reagent and ninhydrin. As determined by co-migration with standards and staining properties, phospholipids expressed similarly in both cell lines were identified as lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. Phosphatidic acid and cardiolipin were present in trace amounts. Phospholipids comigrating with phosphatidylinositol and sphingomyelin were strongly expressed in VT500 cells relative to their expression in Daudi cells.

Keywords: Thin layer chromatography, Glycolipids, Phospholipids, Neutral lipids, Burkitt's lymphoma

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INTRODUCTION

Lipids are essential components of cells, performing structural and signal transduction functions. Globotriaosyl ceramide, or Gb3, has long been known as the blood group pk antigen,[1,2] the Shiga toxin receptor,[3,4] a marker for the germinal center stage of B cell development (CD77),[5,6] and as an antigen associated with the B cell-derived Burkitt's lymphoma.[7] Subsequent studies have demonstrated roles for Gb3 in interferon-alpha (IFN) signaling,[8–10] CD19-mediated adhesion,[11] apoptosis,[6,12] and potentially, antigen presentation[13] in Burkitt's lymphoma cells.

Much of this work was performed by comparing cell signaling and expression of related proteins in Gb3-positive and Gb3-deficient Burkitt's lymphoma cell lines. Differences in signaling, related to IFN-alpha, apoptosis and CD19-mediated adhesion,[6,8–12] and in expression of the proteins CD19,[9] and HLA-D (MHC class II proteins)[13] have been documented. However, differences in lipid composition beyond Gb3 expression in these Burkitt's lymphoma cells have not previously been investigated. Such differences could provide data relevant to regulation of lipid synthesis and signal transduction pathways in Gb3-positive and Gb3-deficient cell lines.

Lipids relevant to signaling pathways include not only glycolipids, but phospholipids such as sphingomyelin and phosphatidylinositol, and neutral lipids such as diacylglycerol (DAG) and free fatty acids. In addition, cell cholesterol, glycolipid and phospholipid content has a dramatic effect on lipid raft/caveolae formation and inclusion in these rafts of protein receptors and associated signaling molecules such as kinases.[14–19] A comparison of the composition of glycolipids, phospholipids, and neutral lipids was performed on the Gb3-positive Burkitt's lymphoma cell line Daudi and the Daudi-derived Gb3-deficient VT500 cell line using thin-layer chromatography.

EXPERIMENTAL

Cell Culture

Burkitt's lymphoma cell lines, Daudi and VT500 cells, were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and gentamicin. Prior to lipid extraction in 20 volumes of 2 : 1 chloroform:methanol, cells were washed three times in PBS. Lipids were separated by Folch partition,[20] the lower phase was dried by flash evaporation and resuspended in minimal 2 : 1 chloroform:methanol (approximately 5 μL per million cells extracted).

Thin-Layer Chromatography

Lipid corresponding to extract from approximately 2 million cells was applied per lane of TLC plate. Lipid standards were purchased from Matreya, Inc.

(Pleasant Gap, PA) except for triacylglycerol and diacylglycerol (tristearin and distearin) and sphingosine which were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Standards were spotted at approximately 5 μg of individual lipid standards per lane. Lipid analysis was performed as previously described.[21] Polyester-backed silica gel thin layer plates (PE Sil G plates, 250 um) were purchased from Whatman, Ltd. (Maidstone, Kent, England). For neutral lipid analysis, TLC plates were developed using the solvent system hexane/diethyl ether/formic acid (80:20:2, by vol.). For glycolipid analysis, TLC plates were developed using chloroform/methanol/water, (65:25:4, by vol.). Phospholipid analysis was performed by developing plates in two dimensions, first with chloroform/methanol/28% ammonium hydroxide, (65:25:4, by vol.; solvent system A), drying plates and subsequently developing in chloroform/acetone/methanol/acetic acid/water (30:40:10:10:1, by vol.; solvent system B). Lipid spots on thin-layer chromatograms were visualized by spray reagents. Neutral lipids were visualized by spraying with charring or phosphomolybdic acid reagent (Sigma-Aldrich, Inc., St. Louis, MO). Glycolipids were visualized by spraying with orcinol reagent[22](orcinol in 70% sulfuric acid). Phospholipids were visualized using molybdenum blue reagent spray for phosphate groups or ninhydrin spray (Sigma-Aldrich, Inc.) for phospholipids containing free amino groups.

RESULTS AND DISCUSSION

In the present study, lipids of Gb3-positive Daudi and Gb3-deficient VT500 cell lines were extracted and glycolipids, phospholipids, and neutral lipids were analyzed by thin-layer chromatography. TLC is useful as an initial screen for changes or differences in lipid composition in cells and tissues. The analysis is inexpensive in terms of supplies and does not require specialized equipment such as an HPLC system and associated detectors. Not surprisingly, the majority of lipids appear to be shared between the Daudi and Daudi-derived VT500 cell lines. However, some differences in lipid composition beyond Gb3 expression were observed. Lipid identification by co-migration with standards is not definitive. This requires more complex structural analysis, such as by mass spectroscopy. The study is also not designed to be strictly quantitative in nature in that quantitative analysis as by densitometry was not available. Despite these limitations, the observed differences in lipid composition between Daudi and VT500 cell lines suggest that Gb3 or CD77 expression affects expression of other lipids in addition to the previously documented effects on protein expression and related signal transduction pathways.

Differences in the expression and migration of several glycolipid bands were observed in extracts of VT500 and Daudi cells (Figure 1). As has been well documented in previous studies, VT500 cells are deficient in Gb3 expression relative to the parental Daudi cell line.[9,23] Glycolipids

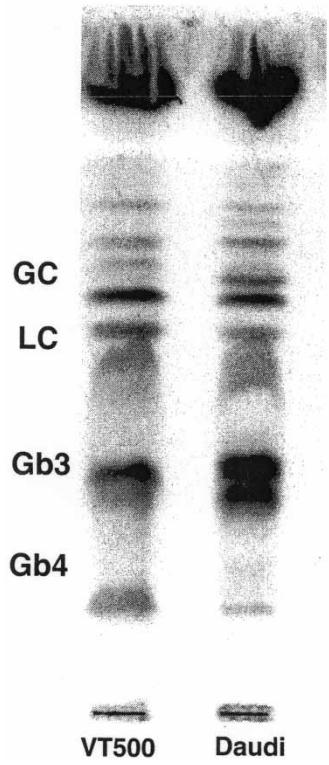

Figure 1. TLC analysis of Daudi and VT500 cell glycolipids. Chromatogram was developed in chloroform/methanol/water (65:25:4, by vol.) and glycolipids were visualized using orcinol spray reagent. Standards: GC, galactosyl ceramide; LC, lactosyl ceramide; Gb3, globtriaosyl ceramide; Gb4, globotetraosyl ceramide (globoside).

co-migrating with the lactosyl ceramide standard did appear to be more strongly expressed in VT500 cells than in Daudi cells. A glycolipid band migrating below the globoside (Gb4) standard was also expressed to a much greater extent in VT500 cells. Obvious differences in expression of cerebrosides (ceramide monohexosides) exist between the cell lines. Glucosyl and galactosyl ceramide are the most common cerebrosides of human tissues with glucosyl ceramides typically migrating above the galactosyl ceramides in TLC analysis, although fatty acid chain length somewhat affects migration of the lipids on TLC plates. An increase in expression of the Gb3 precursors glucosyl ceramide and lactosyl ceramide might be expected if the galactosyl transferase activity responsible for Gb3 synthesis was decreased in VT500 cells. This is consistent with increased expression of lipid co-migrating with lactosyl ceramide (ceramide dihexoside) in VT500 cell extracts. However, the multiple differences in expression and migration of cerebroside bands indicate that the observed glycolipid differences are

not simply due to the accumulation of the Gb3 precursors glucosyl ceramide and lactosyl ceramide. Furthermore, the increase in expression in VT500 cells of glycolipid migrating below the Gb4 standard indicates changes in more complex glycolipids. Galabiosylceramide also migrates in the region of lactosyl ceramide, although this lipid is only a very minor component of Daudi cells and has not been reported in VT500 cells.[4]

Phospholipid analysis required development of TLC plates in two dimensions for adequate separation of phospholipids (Figure 2). Molybdenum blue spray reagent identified spots as phospholipids, and ninhydrin spray identified the phospholipids that contained free amino groups. Based on staining properties and co-migration with phospholipid standards, lipids in both VT500 cells and Daudi cells were identified as lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, and cardiolipin. Lipid migrating in the area of phosphatidylinositol was observed in VT500 cells. Although sphingomyelin was present in both cell lines, expression was stronger in VT500 cells. In contrast, phosphatidic acid appeared to be more strongly expressed in Daudi cells, along with phospholipid migrating just below the phosphatidylserine standard. Unidentified phospholipid migrating near the origin was present in both cell lines. Differences in phospholipid

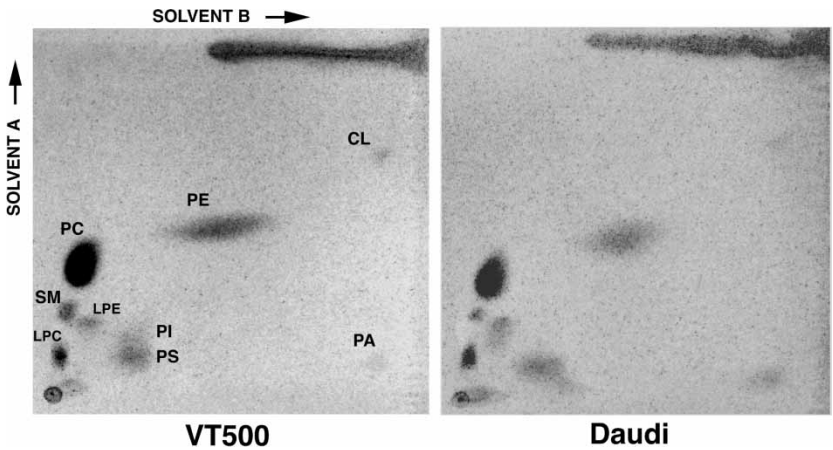

Figure 2. TLC analysis of Daudi and VT500 cell phospholipids. Chromatogram was developed in two directions with chloroform/methanol/28% ammonium hydroxide, (65:25:4, by vol.; solvent system A), then in chloroform/acetone/methanol/acetic acid/water (30:40:10:10:1, by vol.; solvent system B). Phospholipids in the chromatogram shown were visualized using molybdenum blue spray reagent. Standards: LPC, lysophosphatidyl choline; LPE, lysophosphatidyl ethanolamine; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol; PA, phosphatidic acid; CL, cardiolipin; SM, sphingomyelin.

composition, such as in sphingomyelin and phosphatidylinositol content, could relate to differences in signaling pathways relating to apoptosis or B cell activation.[18,24–27]

Neutral lipids (Figure 3) migrating in the area of the cholesterol standard were present in both cell lines. This lipid is almost certainly cholesterol, as it stained red following spraying with charring reagent, indicating the presence of sterol.[28] The cell lines also contained faint phosphomolybdic acid-staining bands migrating with the solvent front. Although cholesterol ester standards migrate at, or just below, the solvent front in this solvent system (not shown), the cellular material did not stain for sterols and remains unidentified. Both cell lines possessed neutral lipids that co-migrated with methyl oleate (MFA), tristearin (TG), and lauric acid (dodecanoic acid, FA). Triacylglycerols previously have been reported to be minor components of lymphocytes and Burkitt's lymphoma cells.[29,30] The identity of lipid co-migrating with the methyl oleate standard is less certain. While free fatty acids may be present in human cells, it should be noted that the sphingosine standard also

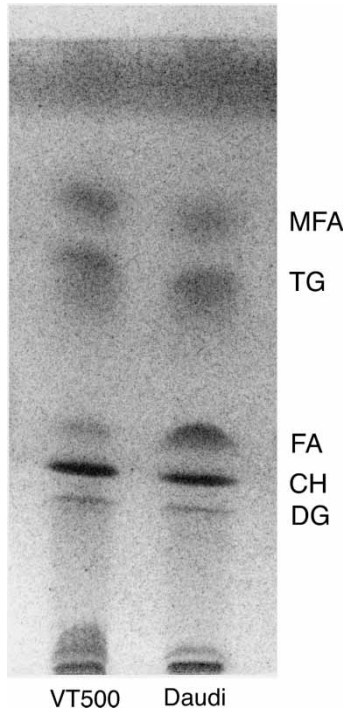

Figure 3. TLC analysis of Daudi and VT500 cell neutral lipids. Chromatogram shown was developed in hexane/diethyl ether/formic acid (80:20:2, by vol.) and neutral lipids were visualized using phosphomolybdic acid reagent. Standards: MFA, fatty acid methyl ester (methyl oleate); TG, triacylglycerol (tristearin); FA, free fatty acid (lauric acid); CH, cholesterol; DG, diacylglycerol (distearin).

co-migrated with the lauric acid standard on the chromatograms. Sphingosines and related compounds are amongst the sphingolipid metabolites important in cell regulatory pathways.[31–34] The neutral lipid band present below the cholesterol standard co-migrated with diacylglycerol (distearin). Diacylglycerol can function as a second messenger following its generation through degradation of complex lipids such as phophatidylinositol. The presence of diacylglycerol, an activator of protein kinase C, could indicate that a constitutive PKC signaling pathway is active in VT500 and Daudi cells.[35,36]

Changes in lipid composition can alter membranes in several ways. Lysolipids and fatty acids can destabilize membranes. Fatty acids serve as components of or precursors to other lipids including not only glycerol-based lipids and sphingolipids, but also signaling molecules such as prostaglandins. Changes in lipid composition also alter lipid raft formation. Raft or microdomain formation has been shown to play a major role in the immune system with respect to the function/clustering of cell surface receptors and associated signal proteins such as kinases.[14,37,38] Perhaps association of IFNAR-1 and CD19 with Gb3[11,39] is necessary for inclusion of these proteins in lipid rafts and associated growth inhibition, adhesion and other signaling pathways[9,10,40,41] at the Gb3-positive, germinal center stage of B cell development.[5,16,42,43] Differential expression of other lipids in these cell lines could contribute to the exclusion from or inclusion of receptors and associated proteins in lipid rafts. Although the cholesterol content of VT500 cells and Daudi cells appears similar,[44] their sphingolipid compositions, glycosphingolipids and sphingomyelin, differ beyond the previously described divergence in Gb3 expression.

The influence of lipid composition on cell protein composition and cell-signaling pathways can be complex and far-reaching. VT500 cells were selected to be resistant to both IFN growth inhibition and Shiga toxin (verotoxin) cytotoxicity (at doses up to 500 ng/ml of media).[23] Additional investigations have shown that Gb3 expression in Daudi cells is variable and Daudi-derived cells can be subcloned that are more sensitive or more resistant to IFN than typical cells of the parental line.[9] The degree of sensitivity correlates with Gb3 expression: highly sensitive lines express high levels of Gb3 and resistant lines express low Gb3 levels. Reconstitution of VT500 cells specifically with Gb3 restores sensitivity to IFN type I-induced growth inhibition,[9] anti-CD19 antibody-induced cell adhesion[11] and Shiga toxin-induced cytotoxicity.[45,46] Gb3 expression also appears to modulate expression of known or suspected Gb3-binding proteins in Burkitt's lymphoma cells including CD19, IFN alpha receptor subunit 1(IFNAR-1) and MHC class II proteins.[9,13] Apoptosis pathways are also influenced by Gb3 expression.[6,12,40]

The results of the present investigation demonstrate the complexity of regulation of lipid expression in cells. Selecting for change in composition of a single glycosphingolipid such as Gb3 could result in changes in cell expression of not only other glycolipids but of phospholipids and

neutral lipids, as well. The interaction between lipid and protein as it relates to expression, function within signal transduction pathways and physical interaction within lipid rafts and other membrane domains is certain to be an active area of research well into the future.

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