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Globotriaosyl ceramide modulates interferon-α-induced growth inhibition and CD19 expression in Burkitt's lymphoma cells

Mark D. Maloney[1]*, Beth Binnington-Boyd[2] and Clifford A. Lingwood[2,3]

[1]*Department of Biology, Spelman College, Atlanta, Georgia,* [2]*Research Institute, Hospital for Sick Children, Toronto, Ontario, and* [3]*Department of Laboratory Medicine and Pathology, University of Toronto, Toronto, Ontario*

Previous studies have indicated that globotriaosyl ceramide (Gb3 or CD77) plays a role in α-interferon signal transduction and CD19-mediated homotypic adhesion in B cell lines derived from Burkitt's lymphoma. These roles for Gb3 may involve the proteins IFNAR-1 (subunit 1 of the interferon-α receptor) and CD19, respectively, both of which have potential Gb3-binding sites in their extracellular domains which resemble those of the verotoxin (Shiga toxin and Shiga-like toxin) B subunit. The majority of this work was performed using wild-type Daudi cells and a single, Gb3-deficient mutant cell line, VT500. In the present investigations, these and additional Daudi-derived cells with varying degrees of sensitivity to interferon-α were examined for Gb3 expression, interferon-induced growth inhibition and CD19 expression. The degree of interferon-induced growth inhibition and CD19 expression correlated with Gb3 expression in the various cell lines tested. In addition, reconstitution of the VT500 cell line with Gb3 but not other glycolipids partially restored the sensitivity of cells to IFN-induced growth inhibition. The degree to which reconstitution restored sensitivity to growth inhibition was similar to the results of previous studies in which Gb3 reconstitution restored sensitivity to verotoxin-induced cytotoxicity. These results demonstrate that Gb3 is specifically required for IFN-induced growth inhibition in Daudi cells and provide further evidence of a role for Gb3 in CD19 expression and function in these cells.

Keywords: **B lymphocytes, Cell Surface Molecules, Glycosphingolipid, Cytokines, FACS, CD77**

Introduction

Globotriaosyl ceramide (Gb3) or CD77 is a marker for the germinal center stage of B cell development [1,2]. Gb3 is also known as the Burkitt's lymphoma associated antigen [3,4] due to its high expression on these cells which are phenotypically similar to germinal center B cells [5]. Gb3 has been implicated as playing a role in interferon-α (IFN-α) signal transduction [6–8], CD19-mediated homotypic adhesion [9] and CD19 and Gb3-mediated apoptosis in Gb3-positive B cells [10–12]. The B subunits of the verotoxin (VT) family of toxins, which includes Shiga toxin as well as Shiga-like toxins, target cells by binding to surface Gb3 molecules and, potentially, other glycosphingolipids containing terminal Galα1-4Gal residues [13,14]. The A subunit possesses N-glycanase activity and mediates the cytotoxic action by cleaving a specific adenine residue on ribosomal RNA, thus inhibiting protein synthesis [15]. Early evidence of a role for Gb3 in interferon signal transduction focused on the finding that, in the Burkitt's lymphoma cell line Daudi and a Daudi-derived mutant cell line, IFN-induced growth inhibition correlated with susceptibility to VT (16). Recent work using wild-type Daudi cells and the Gb3-deficient mutant VT500 cells indicated a potential role for Gb3 in IFN-signal transduction, specifically IFN-stimulated gene factor 3 ($ISGF_3$) formation [7]. Evidence also has indicated a role for Gb3 in both spontaneous and monoclonal antibody-induced CD19-mediated adhesion [9] and CD19-mediated apoptosis [12] in these cells. CD19 is a core member of a complex containing CD19, CD21 (CR2), CD81 (TAPA-1) and Leu-13 [17] which functions as a coreceptor complex modulating signal transduction through the B cell antigen receptor (18–20). The roles of Gb3 in IFN and CD19 signaling are most likely mediated through interaction of Gb3 with verotoxin-like regions on CD19 and the IFNAR-1 protein subunit of the IFN-α receptor [6,9]. The

*To whom correspondence should be addressed: Dr. Mark Maloney, Department of Biology, Spelman College, 350 Spelman Lane SW, Atlanta, Georgia 30314-4399. Tel: 404-223-7694; Fax: 404-223-1449; E-mail: mmaloney@spelman.edu

proposed Gb3-binding region on both these proteins appears to be most similar to the site 1 Gb3-binding site identified by modeling studies of verotoxin B-subunit structure [21–23]. Direct ligation of Gb3 on the surface of these cells by VT B-subunit or anti-Gb3 antibody also leads to apoptosis [10,11]. In the current investigations, wild-type Daudi cells, VT500 cells and additional Daudi-derived subclones were examined in order to further define the roles of Gb3 in IFN-α-induced growth inhibition, CD19 expression and VT-induced cytotoxicity in these Burkitt's lymphoma-derived B cell lines.

Materials and Methods

Daudi and Daudi-derived cell lines

Cells were maintained at 37°C, 5% CO_2 in RPMI 1640 media/10% fetal bovine serum. Daudi cells were obtained from the ATCC. Gb3-deficient VT500 cells were selected from the parental Daudi line as previously described [16]. The Daudi-S and Daudi-R subclones were originally selected following screening for high sensitivity and resistance, respectively, to IFN-α-induced growth inhibition [24]. We further selected the Daudi-R subclone for VT resistance by culturing cells with 500 ng/ml VT1 for 1 week. The resulting cells were designated Daudi-RVT.

IFN-induced growth inhibition and VT-induced cytotoxicity

For growth inhibition assays, 3.5×10^5 cells/ml were treated with 10-fold serial dilutions of IFN-α2b and counts of viable cells determined 4 days post-treatment as described [25]. For VT-induced cytotoxicity assays, cells were treated with 10-fold serial dilutions of VT1 and counts of viable cells were determined 4 days post-treatment. Results are presented as percent cell counts relative to untreated controls (100%).

Surface expression of Gb3 and CD19

Flow cytometry analyses of the surface expression of Gb3 and CD19 were performed in double-labeling experiments using VT1 conjugated to FITC [26] and anti-CD19 mAb (clone B4) conjugated to phycoerythrin (Coulter Scientific) at 10 ug/ml. Cells were stained on ice for 1 hr, washed in cold PBS and fixed with 1% formaldehyde in PBS. Analyses were performed using an Epics Profile Analyzer (Coulter Scientific).

Reconstitution of VT500 cells with glycolipids and treatment with IFN-α

Gb3-deficient VT500 cells were reconstituted with Gb3, Gb4 or digalactosyl diglyceride (DGDG or Galα1-6Gal diacyl glycerol) incorporated into fusigenic liposomes, washed with PBS and maintained according to established procedures [25,27]. Reconstituted cells were treated with 100 units/ml of IFN-α2b and growth inhibition was determined at 4 days post-treatment as above. Growth inhibition was calculated as percent decrease in viable cells relative to untreated controls.

Thin-layer chromatography (TLC) overlay assay

Lipids were extracted from cells and analyzed by thin-layer chromatography according to established procedures (28). Briefly, lipid extracts were separated by TLC and glycolipids visualized by spraying with orcinol reagent. Alternatively, Gb3 was identified by use of the VT-TLC overlay procedure [28,29]. Lipid extracts from cells and Gb3 standard were spotted on TLC plates which were developed in chloroform/methanol/water, 65 : 25 : 4 (by volume). The plates were dried, blocked with gelatin in tris-buffered saline (TBS), and incubated sequentially with TBS containing VT, anti-VT monoclonal antibody (clone PH-1), goat anti-mouse immunoglobulin-peroxidase conjugate and 4-chloro-1-naphthol as substrate.

Results

The results of the VT1 cytotoxicity studies on Daudi and Daudi-derived cell lines are given in Figure 1. Daudi-S cells were extremely susceptible to VT-induced cytotoxicity although the original Daudi cell line also exhibited a high degree of sensitivity. VT500 and Daudi-RVT cells were highly resistant to VT cytotoxicity and Daudi-R cells were intermediate in their susceptibility. Similar results were obtained in the IFN-induced growth inhibition assays (Figure 2). Daudi-S cells were most susceptible to growth inhibition at the lowest dose of IFN tested although the original Daudi cell line was also highly susceptible to growth inhibiting effects of IFN. In contrast, Daudi-RVT and VT500 cells were highly resistant to IFN-induced growth inhibition. Daudi-R cells were again intermediate in their susceptibility. IFN-induced significantly more growth inhibition in Daudi-R cells than in Daudi-RVT and VT500 cells at the most physiologically relevant IFN doses below 1000 U/ml. Analysis of Gb3 surface expression by flow cytometry (Table 1) indicates that a correlation exists between the expression of VT surface receptors and sensitivity to VT 1-induced cytotoxicity and susceptibility to IFN-induced growth inhibition. The relative surface expression of CD19 in the Daudi-derived cells was similar to the relative surface expression of VT receptors in the Daudi-derived cells (Daudi-S cells > Daudi wild-type > Daudi-R > VT500 > Daudi-RVT), indicating a correlation exists between Gb3 and CD19 expression, as well. TLC analysis of Gb3 content in Daudi-S, Daudi-R and Daudi-RVT also shows a substantial decrease in whole cell Gb3 content between Daudi-S and Daudi-R cells and between Daudi-R and Daudi-RVT cells (Figure 3). Reconstitution of the Gb3-deficient VT500 cells with glycolipids resulted in the restoration of susceptibility to IFN-induced growth inhibition in the cells reconstituted with Gb3, but not in those treated with exogenous Gb4 or DGDG (Figure 4). These results indicate that there is a specific requirement for Gb3 in the IFN-signaling pathway for growth inhibition. Results shown are representative of experiments performed in triplicate.

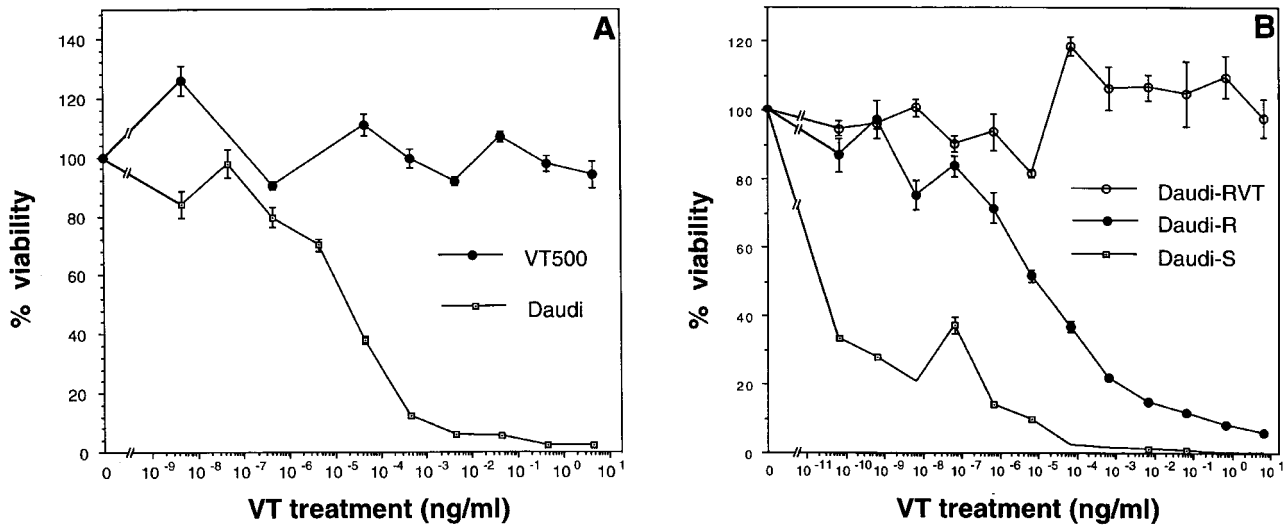

Figure 1. VT-induced cytotoxicity of Daudi-derived cells. Daudi-derived cells (3.5×10^5 cells/ml) were treated with 10-fold serial diluations of VT1. Values represent the mean ± standard error of at minimum triplicate counts of viable cells performed 4 days after VT1 treatment. Percent viability of cells was calculated relative to the average of untreated controls. A) Cytotoxicity of Daudi and VT500 cells. B) Cytotoxicity of Daudi-S, Daudi-R and Daudi-RVT cells.

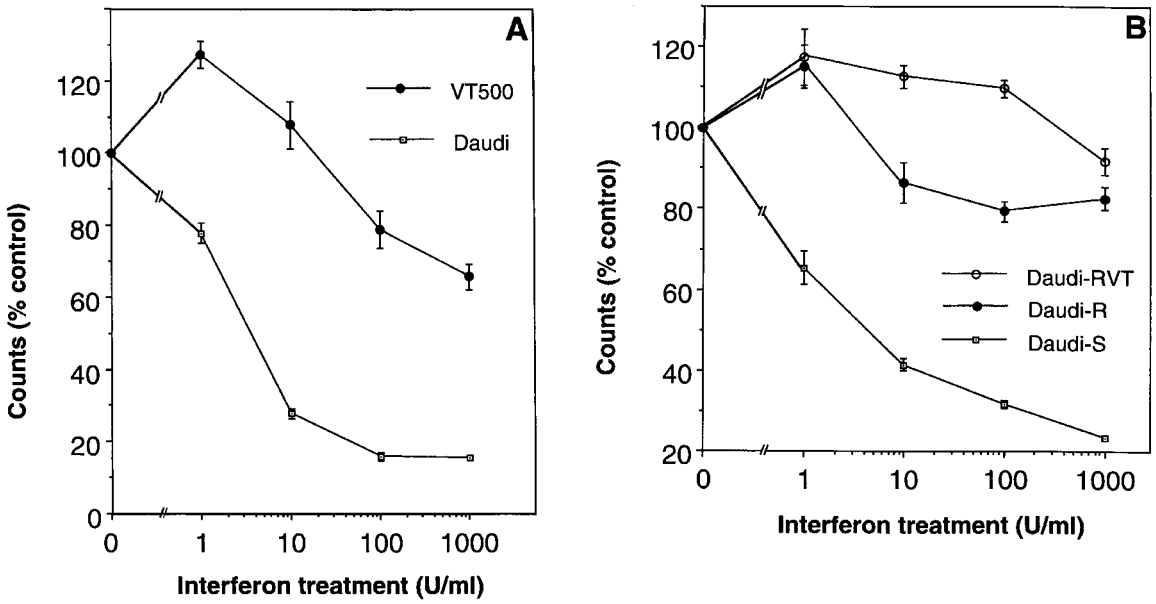

Figure 2. IFN-induced growth inhibition of Daudi-derived cells. Daudi-derived cells were treated with ten-fold serial dilutions of IFN-α2b. Values represent the mean ± standard error of (at minimum) triplicate counts of viable cells performed 4 days after VT1 treatment. Cell growth was calculated as cell number relative to the average of untreated controls. A) Growth inhibition of Daudi and VT500 cells. B) Growth inhibition of Daudi-S, Daudi-R and Daudi-RVT cells.

Discussion

Gb3 or CD77 has been identified by monoclonal antibodies as a marker for both germinal center B cells and Burkitt's lymphoma cells [1–4]. Gb3-mediated or modulated functions are likely to be of major importance in the development of humoral immune responses because the germinal centers of lymphoid tissue are sites of a number of events relating to B cell development and antibody production including somatic mutation, apoptosis, isotype switching and generation of memory B cells [30–33]. Investigations using the Burkitt's lymphoma line Daudi as a model for germinal center B cells and VT as a probe for Gb3 function have indicated a role for this glycosphingolipid in IFN-α signal transduction, CD19-mediated homotypic adhesion and apoptosis in these cells [6–12]. Comparisons of IFN responses in wild-type Daudi cells

Table1. Quantitative analysis of cell surface CD19 and Gb3 expression in Daudi subclones using flow cytometry

	Mean Fluorescence Intensity	
	CD19	*Gb3*
Daudi-S	40.25	29.97
Daudi	38.28	25.65
Daudi-R	32.77	18.09
VT500	27.09	8.08
Daudi-RVT	15.89	4.10

Flow cytometry was performed using an Epics Profile Analyzer. Cells were stained for CD19 or Gb3 expression using phycoerythrin-labeled anti-CD19 monoclonal antibody and VT 1-FITC conjugate, respectively.

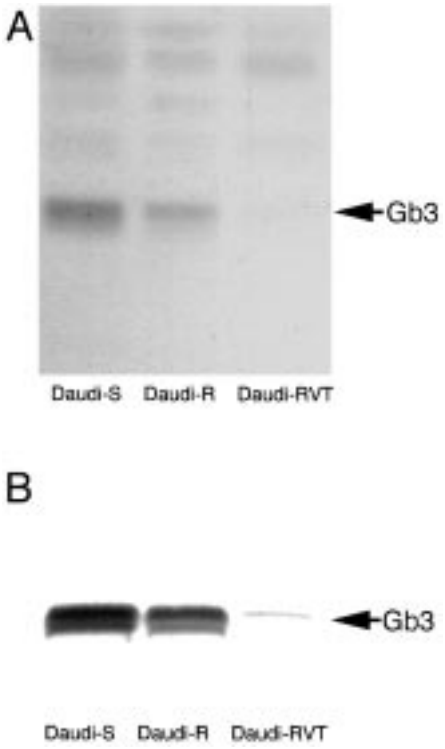

Figure 3. TLC analysis of total cell Gb3 content of Daudi-S, Daudi-R and Daudi-RVT cells. A) TLC analysis of whole cell glycolipid content as visualized using orcinol reagent and analyzed by densitometry. B) Whole cell Gb3 content as determined by TLC overlay analysis. Thin-layer chromatograms were blocked with gelatin and incubated sequentially with VT, anti-VT monoclonal antibody, goat anti-mouse immunoglobulin-peroxidase and 4-chloro-1-naphthol as substrate.

and a Gb3-deficient Daudi line suggested a role for Gb3 in growth inhibition [16] and $ISGF_3$ formation [7]. Treatment of Daudi cells with PDMP (1-phenyl-2-(deanoylamino)-3-morpholino-1-propanol), an inhibitor of glycosphingolipid synthesis, also implicated glycosphingolipids, although not specifically Gb3, as having a role in these IFN-mediated

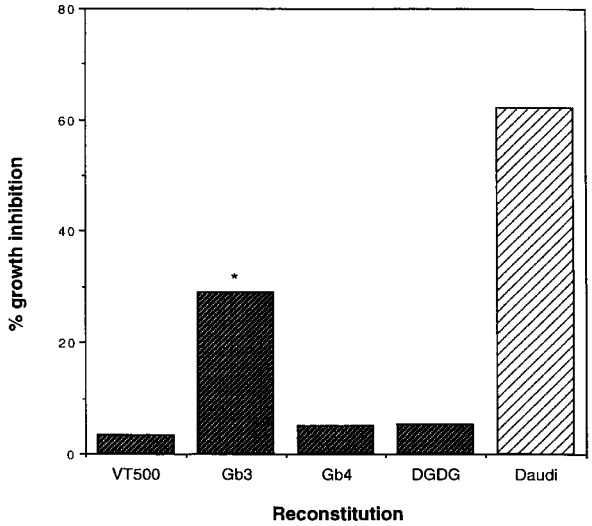

Figure 4. Reconstitution of growth inhibition in interferon-resistant cells by addition of exogenous Gb3. Gb3-deficient VT500 cells were reconstituted with Gb3, Gb4 or DGDG, and treated with 100 U/ml of IFN-α 2b. Growth inhibition was calculated as percent reduction in mean counts of reconstituted cells relative to control VT500 cells. Results represent the mean percent calculated from triplicate counts of viable cells performed 4 days post-treatment. *Significant at $P < 0.002$ using student's t-test.

responses [7]. Recent evidence indicates that Gb3 plays a role in IFN-α-induced antiviral mechanisms, as well [8]. Comparisons of CD19 function in Daudi and VT500 cells indicated a role for CD19/Gb3 interaction in both the spontaneous homotypic adhesion of Daudi cells in culture and the anti-CD19 monoclonal antibody-mediated adhesion [9] involving signal transduction, the integrin system and potentially other strong mechanisms of adhesion (34–37). A specific role for Gb3 in the anti-CD19 antibody-induced adhesion pathway was demonstrated by the reconstitution of this pathway in VT500 cells using liposomes containing Gb3 but not other glycolipids [9]. These roles for Gb3 in adhesion and IFN-signaling likely involve interactions with VT B subunit-like regions in the extracellular domains of CD19 and the IFNAR-1 subunit of the IFN-α receptor complex, respectively [6,9].

The current investigations expand upon the results of previous studies by investigating the ability of Gb3 reconstitution of Gb3-deficient Daudi cells to restore IFN-induced growth inhibition, and by comparing cells other than Daudi wild-type and VT500 cells with regard to Gb3 expression, CD19 expression and IFN-signaling. The results of VT cytotoxicity assays, IFN-induced growth inhibition assays and flow cytometry analysis of Gb3 surface expression on the Daudi-derived cell lines are given in Figures 1–2 and Table 1, respectively. Chromatographic analyses of lipid extracts from the Daudi-derived cells [38] (Figure 3) show differences in total cell Gb3 levels in these cells which correlate with both Gb3 surface expression and VT-induced cytotoxicity. There was, overall, very good correlation between Gb3 expression, VT susceptibility and IFN-induced growth inhibition in the

various Daudi cell lines tested. Daudi-S cells and Daudi-R cells were originally selected as subclones solely on the basis of susceptibility and resistance, respectively, to IFN-alpha-induced growth inhibition. Daudi-S cells, which have the highest expression of Gb3, were extremely sensitive to VT cytotoxicity and the most sensitive to IFN-induced growth inhibition at the lowest dose tested. This degree of sensitivity may relate to the high levels in these cells of the Gb3 lower band isoform which has been associated with retrograde transport of toxin to the nucleus and enhanced cytotoxicity [39,40]. The parental Daudi cell line which expresses high Gb3 levels was also very susceptible to both VT cytotoxicity and growth inhibition by interferon treatment. Daudi-R cells with their intermediate levels of Gb3 were somewhat resistant to IFN-induced growth inhibition. However, they were significantly more susceptible to growth inhibition than the severely Gb3-deficient Daudi-RVT cells and VT500 cells, at least in the physiologically relevant range of IFN concentrations below 1000 units (U)/ml[41]. Daudi-R cells were also intermediate in their susceptibility to VT-induced cytotoxicity between the highly sensitive Daudi-S cells and the highly resistant Daudi-RVT and VT500 cells (Fig. 1). Rarely, cell types have been reported in which Gb3 surface expression does not correlate with VT-induced cytotoxicity which may relate to a deficiency in the retrograde transport pathway of internalized toxin [40,42]. However, with the observed correlation of Gb3 expression and VT cytotoxicity, this does not appear to be the case in the Daudi cell lines. CD19 expression also correlated strongly with Gb3 expression in the Daudi-derived cell lines (Table 1) with VT500 cells and Daudi-RVT cells possessing low levels of Gb3 and CD19 expression relative to the wild-type Daudi cells and Daudi-S cells, and Daudi-R cells possessing intermediate levels of both Gb3 and CD19. These results support an intracellular function for Gb3 in CD19 expression in addition to the role of CD19/Gb3 binding in intercellular homotypic adhesion [9]. We have also investigated the surface expression of CD19 on Daudi and VT500 cells using other anti-CD19 antibody clones (results not shown). While CD19 expression was consistently higher in Daudi than in VT500 cells, different clones showed both higher and lower differences in CD19 expression than the B4 clone. This suggests that the differences in CD19 expression could be due to a conformational rather than quantitative change in CD19 expression when Gb3 is present. When performing such assays, care must be taken to ensure that Gb3-deficient cells do not revert to Gb3-positive status as can occur spontaneously in culture with a concomitant increase in CD19 surface fluorescence [9].

Reconstitution of VT500 cells with glycolipids in liposomes demonstrated that Gb3 can restore growth inhibition in response to IFN-α (Figure 4), thus conclusively demonstrating a role for Gb3 in this pathway of IFN signal transduction. The degree to which cells responded to IFN following reconstitution with Gb3 (approximately 30% growth inhibition) is similar to the responsiveness of Gb3-reconstituted VT500 cells to VT-induced cytotoxicity [25]. Differences in the degree of growth inhibition observed in Figure 2A and the reconstitution results of Figure 4 are attributable to the high cell density required for the reconstitution procedure [27] which also applied to culture of the untreated VT500 and Daudi control cells in Figure 4 but not to cells in assays of Figure 2. Results of reconstitution experiments are especially important as they demonstrate that Gb3 is the component specifically required for IFN-induced growth inhibition rather than other potential differences in the Daudi-derived cells. Previous experiments using glycolipid synthesis inhibitors showed that glycosphingolipids were important to IFN-α signaling and that $ISGF_3$ formation was impaired in VT500 cells [7], but these studies did not conclusively demonstrate that Gb3 expression was the critical deficiency. One toxin in the VT family of toxins, VT2e, binds to both Gb3 and globotetraosyl ceramide (Gb4 or globoside) on cell surfaces [25]. The VT B subunit-like domain on the IFN-αreceptor subunit IFNAR-1 has an amino acid sequence with similarities to the Gb3-binding VT's as well as to VT2e [43]. Possibly, IFNAR-1 binds only terminal Galα1-4Gal residues as do VT1 and 2, or both terminal and subterminal Galα1-4Gal residues are recognized, but only glycosphingolipids such as Gb3 with terminal Galα1-4Gal can function in the IFN-induced growth inhibition pathway. Although CD19 has been identified as the mediator of spontaneous adhesion of Daudi cells to both Gb3 and Gb4 matrices, only Gb3 reconstitutes the anti-CD19 monoclonal antibody-induced adhesion pathway [9]. In addition to resistance to growth inhibition [24,44,45], Daudi-R cells have been shown to be resistant to the IFN-induced inhibition of mu heavy chain expression [24] and deficient in IFN-induced Leu-13 expression and homotypic adhesion [46] relative to Daudi and Daudi-S cells. The present results, therefore, indicate a potential role for Gb3 in these processes, as well.

We have previously described roles for Gb3 as a component of CD19-mediated signal transduction and adhesion pathways and as a potential modulator of CDl9 expression [9]. Previous investigations also have shown that direct ligation of Gb3 by VT, VT B subunit or anti-Gb3 antibodies can induce apoptosis in Burkitt's lymphoma cells [10,11]. We have recently described a role for Gb3 in a CDl9-mediated apoptotic pathway requiring retrograde transport of CD19 through the Golgi apparatus to the endoplasmic reticulum and nuclear membrane [12]. This is significant as VT also undergoes this relatively unique retrograde transport upon internalization [42,47], suggesting a role for Gb3-binding in the pathway. The current investigations expand upon these previous results by linking Gb3 expression, CDl9 expression, VT-induced cytotoxicity and IFN-induced growth inhibition in additional Daudi subclones. Importantly, while previous studies implicated Gb3 or a related glycosphingolipid as having a role in IFN-induced growth inhibition, the results of the current reconstitution studies (Figure 4) comprise the first evidence to conclusively demonstrate that Gb3 specifically functions as an essential component of this growth inhibition pathway.

Recent investigations have indicated important roles for Gb3 in additional signal transduction and activation pathways. A role for Gb3 in modulating the activity of the Src family kinase Yes in a renal cell carcinoma line has been reported [48]. Gb3 expression has been shown to be tightly regulated in fibroblasts even following treatment with inhibitors of glycosphingolipid synthesis [49]. Although Gb3-related cell functions were not identified, this regulation may also relate to the reported association of kinases with glycosphingolipid-enriched microdomains [50,51]. Previously, we proposed a role for Gb3 in antigen presentation [52]. Recently, VT B-subunit fused to a tumor antigen was shown to target the recombinant protein to professional antigen presenting cells (B cells and dendritic cells) and promote processing of the exogenous protein antigen for MHC class I-restricted presentation [53]. A role for other glycosphingolipids in modulating cell growth and signal transduction also has been established. For example, glucosyl ceramide and the ganglioside GM3 are involved in the regulation of neuronal growth and development [54], and GM3 has been shown to regulate epidermal growth factor (EGF) signaling by preventing dimerization of the EGF receptor subunits [55]. Activation of B cells through surface immunoglobulin or CD44 may also require association of these proteins with glycosphingolipid domains containing Src family kinases [56,57]. Together with the results of the current study, these investigations have established the importance of Gb3 and other glycosphingolipids as essential components of a number of signal transduction pathways. Certainly with regard to IFN-α and CD19-mediated pathways in B cells, the status of Gb3 expression must be considered a potential factor in any observed modulation of signal transduction in addition to the relevant protein receptors, kinases, transcription factors and other proteins associated with the signaling pathway.

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