Induction of HIV-1 resistance: cell susceptibility to infection is an inverse function of globotriaosyl ceramide levels

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To examine the role of the glycosphingolipid (GSL), globotriaosylceramide (Gb3, CD77, pk blood group antigen) in HIV-1 infection, we have pharmacologically modulated Gb3 metabolism in an X4 HIV-1 infectable monocytic cell line (THP-1) that naturally expresses Gb3 and in a Gb3-expressing glioblastoma cell line (U87) transfected to express both CD4 and CCR5 to permit R5 HIV-1 infection. THP-1 and U87 cells were treated with either a competitive inhibitor of α-galactosidase A, 1-deoxygalactonojirimycin (DGJ) to induce Gb3 accumulation, or a glucosylceramide synthase inhibitor, phenyl-2-palmitylamino-3-pyrrolidino-1-propanol (P4) to deplete cells of Gb3. HIV susceptibility was determined via measurement of p24* antigen production by ELISA. In addition, total cellular Gb3 content was determined using thin layer chromatography followed by Verotoxin1 overlay binding. The cell surface expression of Gb3 was verified by FACS analysis. We found that DGJ significantly decreased THP-1 and U87 cell susceptibility to HIV-1 infection, respectively, at a concentration of approximately 100 µM. In contrast, P4 (2 µM) substantially increased cellular susceptibility to HIV-1 infection. Total cellular GSL analysis verified increased Gb3 expression in cells treated with DGJ and considerable reduction of Gb3 in P4-treated cells as compared to controls. These results show a reciprocal relationship between Gb3 expression and infection with either X4 HIV-1 or R5 HIV-1. These results support previous studies that Gb3 provides resistance to HIV infection. Variable Gb3 expression may provide a natural HIV resistance factor in the general population, and pharmacological manipulation of Gb3 levels may provide an approach to induction of HIV resistance.

Keywords: glycosphingolipids/gp120/lipid rafts/pk blood group antigen

Introduction

Many studies have implicated glycosphingolipids (GSLs) in the mechanism of HIV pathogenesis (Viard et al. 2004). The HIV adhesin, gp120, has been shown to bind several distinct GSLs in vitro (Bhat et al. 1993; Delezay et al. 1997; Mylvaganam and Lingwood 1999) including Gb3. GSLs are primary components of lipid microdomains or “rafts” and such cholesterol-enriched membrane domains are central to the HIV life cycle (Fantini et al. 2000; Manes et al. 2000; Liao et al. 2001) both in terms of entry and exit of the virus (Campbell et al. 2001). GSLs are strongly implicated in HIV infection of CD4-negative cells, such as those of the brain (Harouse et al. 1991), reproductive epithelium (Yeaman et al. 2003), or GI tract (Ullrich et al. 1998). The GSL, globotriaosylceramide, has been particularly implicated in the mechanism of virus/host cell fusion (Puri et al. 1998a, 1998b; Puri et al. 1999; Hug et al. 2000) and Gb3 metabolism is aberrant in HIV patients (Fantini et al. 1998). We have shown that a soluble analog of Gb3 synthesized in our laboratory (Mylvaganam and Lingwood 1999) is a high-affinity receptor for gp120 in monolayer insertion studies (Mahfoud, Mylvaganam et al. 2002). Adamantyl Gb3 proved to be an effective inhibitor of HIV infection in vitro, irrespective of tissue tropism, HIV strain, or drug resistant status (Lund et al. 2006). Furthermore, we found that PBMCs from patients with Fabry disease, who accumulate Gb3 due to a defect in the α-galactosidase responsible for Gb3 breakdown, are resistant to R5 HIV-1 infection in vitro (Lund et al. 2005). Recently, we have examined HIV susceptibility in relation to genetic polymorphisms in Gb3 metabolism, i.e., within the P blood group system (Spitalnik P and Spitalnik S 1995). PBMCs from individuals lacking Gb3 (p phenotype) were found to be hypersusceptible to both X4 and R5 HIV-1, whereas PBMCs from the rare Pk phenotype, in which Gb3 accumulates due to a defect in the conversion of Gb3 to Gb4, are highly resistant to both X4 and R5 HIV-1 infection (Lund et al., submitted). Thus, we have proposed Gb3 as a natural resistance factor against HIV infection. While Gb3 is considered a B cell differentiation antigen (Mangeney et al. 1991), we have shown that Gb3 synthesis is induced in PHA/IL2-activated PBMCs (Lund et al. 2006), suggesting that Gb3 could play a protective role in primary T cell infection.

To further validate this hypothesis, we have now attempted to pharmacologically mimic the genetically defined Gb3 expression phenotypes within the P blood group, in an X4 HIV-1 susceptible monocytic cell line (THP-1) that naturally expresses CD4, CXCR4, and Gb3 (Schols et al. 1997) and in a glioblastoma cell line (U87) naturally expressing Gb3 and transfected to express both CD4 and CCR5 so that it is susceptible to R5 HIV-1 (Puri et al. 1999). Our studies confirm an inverse correlation between HIV-1 infection and Gb3 expression to provide additional rationale for the study of Gb3 in the pathogenesis and prevention of HIV infection.
Material and methods

Phenyl-2-palmitylamino-3-pyrrolidino-1-propanol (P4) was a generous gift from Dr. J. Shayman, University of Michigan. 1-Deoxygalactonojirimycin (DGJ) was from Sigma (St. Louis, MO).

**DGJ/P4 treatment of cells**

THP-1 cells were cultured in complete RPMI1640 medium (In-vitrogen Canada, Burlington, Ontario) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 10 µM gentamicin antibiotics. Human monocyteic cells (THP-1) expressing Gb3 were obtained from ATCC and Gb3-negative THP-1 cells and CD4, CCR5-transfected U87 glioblastoma cells were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD) and cultured in DMEM (In-vitrogen Canada) supplemented with 15% fetal bovine serum (FBS), 1 µg/mL puromycin, and 300 µg/mL G418. Approximately 5 × 10^5 cells were plated in triplicate in 12-well plates, and then treated with the DGJ (50, 100, 200 µM) or P4 (2 µM) for 5 days. Control samples did not receive any treatment. After 5 days, cells were infected with HIV-1MB or HIV-1Bar-L, and cell susceptibility was measured using p24^{Gag} antigen production by ELISA.

**Viruses and in vitro infection**

X4 HIV-1MB stocks (NIH AIDS Reference & Reagent Program) were grown in Jurkat C, and multiplicity of infection (m.o.i.) was calculated using MT-4 cell infection (NIH AIDS Reference & Reagent Program) based on total p24^{Gag} levels (Branch et al. 2002) and infections carried out at an m.o.i. of 0.1. The R5 HIV-1Bar-L stock was grown in PBMCs, and an infectious dose was calculated based on total p24^{Gag} levels (Branch et al. 2002). Infections were carried out in a level 3 containment facility (University of Toronto) as described previously (Branch et al. 2002) with minor modifications. Approximately 5 × 10^5 THP-1 or U87 cells (treated with DGJ/P4 or untreated) were infected at 37°C for 1 h with HIV-1MB or HIV-1Bar-L, respectively. After infection, the cells were washed extensively with phosphate buffered saline (PBS) lacking MgCl2/CaCl2 (Princess Margaret Hospital Media Facility, Toronto, Ontario) and cultured in complete RPMI1640 or DMEM medium. Aliquots of culture supernatant were taken 2 h after initial viral infection and each day thereafter up to 7 days. To determine viral production, ELISA was used to measure p24^{Gag} antigen levels (ELISA, ZeptoMetrix, Buffalo, NY).

**VT1/TLC overlay**

At least 2 × 10^6 cells were centrifuged, washed with PBS lacking MgCl2/CaCl2, and stored at −20°C to disrupt the cell pellet. GSLs were extracted from cells by vigorously shaking them overnight in chloroform:methanol (C:M, 2:1, vol/vol). The mixture was filtered through glass wool and lipids collected were dried under nitrogen gas. Extracts were saponified to isolate the GSL fraction by resuspending in 1 M NaOH in methanol for 1 h at 37°C. After incubation, the sample was neutralized with an equivalent volume of 1 N HCl to achieve a slightly basic pH. Volumes were adjusted to form a Folch partition, C:M:W, 8:4:3, and centrifuged to separate the upper and lower phases. The upper phase was removed, discarded, and replaced with an equal volume of C:M:W, 1:47:48. After another round of centrifugation, the upper phase was discarded and the lower phase was dried down as described previously. The dried extract was resuspended in C:M, 2:1, to approximately 10^6 cells/20 µL for direct analysis by silica gel chromatography. Equivalent aliquots were applied to TLC precleared by C:M, 98:2. GSLs were then separated by TLC (C:M:W, 65:25:4), and the GSL species were detected either by development with orcinol spray (Sigma) at 110°C for 10 min or by verotoxin-1 (VT1) binding using TLC overlay (Nutikka et al. 2003).

For VT1 overlay, the TLC plate was run in the appropriate solvent (C:M:W, 65:25:4) and then dried completely in a fume hood. The plate was blocked with 1% bovine gelatin and after incubation at 37°C for 1 h, the plate was washed with 50 mM TBS, pH 7.4. The plate was incubated for 45 min at room temperature with purified VT1, diluted to a concentration of 1 µg per 10 mL in TBS. After washing, the plate was incubated for 45 min with a monoclonal anti-B subunit (Boyd et al. 1994; Nutikka et al. 2003) diluted 1/2000, and then with HRP-conjugated goat anti-mouse IgG (diluted 1/2000; BioRad). The plate was developed for 1–10 min with 3 mg/mL solution of 4-chloro-1-naphthol (4-CN) in methanol freshly mixed with 5 volumes of TBS and 1/2000 dilution of 30% H2O2. ImageJ software was used to determine pixel density of the bands in the overlays.

**Flow cytometry**

For surface receptor expression, cells were harvested and incubated for 20 min at 4°C with 10% human AB serum in a FACS buffer (PBS, 2% FBS, 0.1% sodium azide, 5 mM EDTA). Cells were resuspended in a 100 µL FACS buffer, and 1.5 µg monoclonal mouse anti-Gb3 (Seikagaku Corp., Tokyo, Japan) was added, followed by incubation for 30 min at 4°C in the dark. After one wash with the FACS buffer, 5 µg/mL APC-labeled goat anti-mouse IgG (Invitrogen Molecular Probes) was added and cells were incubated for 30 min at 4°C in the dark. Following one wash and a 20 min block with 10% mouse serum, 5 µL anti-CD4-FITC and 5 µL anti-CXCR4-PE were added. After an additional 30 min incubation at 4°C and one wash, a 500 µL FACS buffer was added. Data were collected via Becton Dickinson FACS Calibur and analyzed by Cell Quest.

**Statistical analysis**

The means and standard error of the mean (SEM) of the results from several independent experiments were determined and analyzed statistically. Student’s t-tests were used for statistical analysis (P < 0.05 was considered significant).

**Results**

**THP-1 cells highly expressing Gb3 (DGJ treated) are less susceptible to infection with HIV-1 in vitro**

We first assessed the susceptibility of Gb3-expressing THP-1 cells on HIV-1MB infection after treatment with DGJ and P4. Using an m.o.i. of 0.1 to permit reliable evaluation of infection via p24 expression after 5 days, we found that increasing doses of DGJ resulted in a substantial inhibition of THP-1 cell infection in vitro (Figure 1A). The 100 µM dose was chosen as the representative dose for all other experiments since this dosage led to significant inhibition of HIV-1 MB. In contrast, at this same dose
Fig. 1. Effect of DGJ or P4 on HIV-1IIIB THP-1 cell infection. HIV-1IIIB was incubated with THP-1 cells after treatment with DGJ or P4 for 5 days. The p24<sup>gag</sup> antigen expression level was monitored for 3–5 days after infection. (A) DGJ-treated THP-1 cells infected with HIV-1IIIB in triplicate and are representative of two similar experiments (*P < 0.05, 100 µM dose of DGJ in comparison with untreated cells (0 µM)). (B) P4-treated THP-1 cells infected with HIV-1IIIB in triplicate and are representative of three similar experiments (**P = 0.03, 2 µM dose of P4 in comparison with untreated cells (0 µM)).

m.o.i., increasing doses of P4 resulted in a significant increase in HIV-1IIIB infection of THP-1 cells in vitro (Figure 1B). Two micromolar P4, effective to deplete cellular GSLs without toxicity (Lee et al. 1999), was used in subsequent experiments. DGJ had no effect on cell viability <300 µM (data not shown).

A comparison of the DGJ- (100 µM) and P4- (2 µM) treated Gb3-expressing THP-1 cells shows an opposite response to HIV-1 infection (Figure 2A). Total Gb3 was determined in the DGJ- or P4-treated cells, via VT1/TLC overlay (Figure 2B). Cell cultures treated at all concentrations of DGJ show a significant increase in Gb3 expression. In contrast, P4-treated cells show a major reduction in Gb3 expression. The expression of HIV-1 infection as a function of the THP-1 cell Gb3 concentration shows a linear inverse relationship (Figure 2C).

DGJ treatment only inhibits HIV infection of THP-1 cells that naturally express Gb3

We obtained two THP-1 subclones: one expresses Gb3 and one does not. CD4 expression was greater on the Gb3 +ve subclone. Both subclones were cultured with 100 µM DGJ for 5 days. FACS analysis showed a significant increase in Gb3 only in the Gb3 +ve THP-1 subclone (Figure 3A and B). DGJ-treated cells were infected with HIV-1IIIB, and, again, only in the THP-1 cell line expressing Gb3 was there inhibition of HIV-1IIIB infection (Figure 3C), even though the expression of both CD4 and CXCR4 was unaffected (Figure 3A). This indicates that only the increase in Gb3 mediated the reduced HIV-1 infection and not any other cellular effect.

U87 cells express CD4, CCR5, & Gb3 and are HIV-1<sub>Ba-L</sub> susceptible

U87 cells showed a substantial expression of CD4, Gb3, and CCR5 via FACS analysis and showed robust exponential infection with HIV-1<sub>Ba-L</sub> (unlike THP-1 cells which express CD4 and CXCR4 but not CCR5 coreceptor) (data not shown). Therefore, U87 cells were used to assess the effects of pharmacological manipulation of Gb3 on R5 HIV-1 infection.

Increased U87 cell Gb3 increases resistance to R5 HIV infection in vitro

Following DGJ treatment of U87 cells, both cellular and cell surface Gb3 levels were assessed. Cells treated at all concentrations of DGJ showed a significant increase in Gb3 expression.
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Fig. 3. FACS analysis of surface receptors in two THP-1 subclones. The cell surface expression of CD4, CXCR4, and Gb3 on Gb3-expressing (A) and non-Gb3-expressing (B) THP-1 cells. Mean fluorescence intensity (MFI) was calculated based on isotype controls to determine expression levels of receptors detected. (C) HIV-1IIIB was incubated with the two THP-1 sub lines (±Gb3 expression) after treatment with DGJ for 5 days. The p24 gag antigen expression level was monitored for 3 days after infection. (*P < 0.05).

Discussion

Our present data on modulation of Gb3 levels in HIV susceptible cell lines support studies suggesting that increased Gb3 can reduce susceptibility to HIV infection. The Fabry lysosomal storage disease results from the accumulation of Gb3 due to defective α-galactosidase activity. Lymphocytes from Fabry patients are resistant to R5 HIV-1 infection (Lund et al. 2005). Pk1 individuals accumulate Gb3 due to defective globotetraosylceramide synthase and their lymphocytes are resistant to both X4 and R5 HIV-1 infection (Lund et al., submitted) p blood group individuals have no Gb3 due to defective globotriaosylceramide synthase and p lymphocytes are hypersusceptible to both X4 and R5 HIV infection (Lund et al., submitted).

We have attempted to pharmacologically mimic these Gb3 polymorphisms in cultured cells to further verify the protective effect of Gb3 against HIV infection. Our results indicate that increasing Gb3 expression using DGJ inhibition of α-galactosidase in Gb3-positive THP-1 cells and U87 cells leads to a significant inhibition of infection with HIV-1IIIB and HIV-1Ba-L, respectively. Conversely, decreasing Gb3 levels through the use of P4 GSL inhibition induced THP-1 and U87 cells to become more susceptible to HIV-1 infection. Cellular GSL analysis via VT1/TLC overlay and FACS indicated that Gb3 expression was increased in cells treated with DGJ, while when treated with P4,
Gb3 expression dramatically decreased. Surface expression did not always fully reflect the total change in cellular Gb3 content but cell surface Gb3 is most likely to modulate HIV susceptibility. Our results show a linear inverse relationship between cellular Gb3 levels and infection with either HIV-1IBH or HIV-1Ba-L. Thus, when Gb3 levels are high, HIV infection is at a minimum.

DGJ had little effect on the infection of Gb3-negative THP-1 cells and FACS analysis indicated Gb3 levels remained negative after DGJ treatment. This indicated that the difference in HIV infection was due to an increase in Gb3 and not because of another cellular affect and DGJ was nontoxic to THP-1 cells. We, therefore, conclude that pharmacologically increasing Gb3 is an effective and novel means to prevent HIV infection in vitro. Our results support our previous studies suggesting a protective role for Gb3 expression in HIV-1 infection.

Previous studies on the effect of inhibition of GSL synthesis on HIV infection in vitro showed GSL depletion-inhibited infection (Hug et al. 2000; Ablan et al. 2006) and this could be reversed in glucosyl ceramide synthase knockout cells by CD4 and coreceptor overexpression (Rawat, Eaton et al. 2004). However, inhibition studies have not been performed in Gb3-expressing cells and the inhibitor of glucosyl ceramide synthase used was not as selective as P4 (Lee et al. 1999). These studies showed a facilitative GSL function, even for exogenous Gb3 in terms of membrane fusion (Puri et al. 1998a, 1998b; Puri et al. 1999). It is clear that inhibition/depletion of glucosyl ceramide synthase does not have a selective effect on any given GSL. Thus, the role for GSLs is complex and may be concentration and/or lipid heterogeneity dependent. We have recently found that gp120 binding to Gb3 can be dependent on the fatty acid moiety (Mahfoud et al. in press). Both facilitative (Hug et al. 2000) and inhibitory (Rawat, Gallo et al. 2004) roles for GM3 ganglioside in HIV infection have been reported.

In order to understand the way in which Gb3 inhibits HIV infection, it is important to recognize the role of lipid rafts. In the absence of HIV-1, CD4 and the coreceptors are not physically associated in the membrane (Jones et al. 1998). However, studies have indicated that CD4 and the coreceptor must interact with lipid rafts for HIV–host cell fusion to occur and lead
to infection (Fantini 2000; Liao et al. 2001). Specifically, it has been shown that CD4 and CCR5 may interact within lipid rafts containing GM3 and Gb3 (Sorice et al. 1997; Hammache et al. 1998; Manes et al. 2001). Even though CXCR4 is not initially associated with rafts, it is recruited later for membrane fusion after HIV-gp120 interacts with CD4 (Sorice et al. 2001; Nguyen et al. 2005). Currently, Fantini et al. suggest that gp120 binds GSLs in rafts and this interaction facilitates the migration of CD4-gp120 complexes to the appropriate chemokine coreceptor (Fantini 2000). Specifically, the initial gp120-C2 loop CD4 contact causes the V3 loop to undergo a conformational change exposing the Gb3 (GSL) and chemokine coreceptor binding motif (Delezay et al. 1996; Xiao et al. 1998). Indeed, the binding motif, XXXXPGRFXXX (Delezay et al. 1996), within the V3 loop for Gb3 and other GSLs overlaps with the consensus binding motif, S/GXXXPGXXXXXXE/D (Xiao et al. 1998), for chemokine coreceptor. The V3 loop then interacts with GSLs, and subsequent contact with chemokine coreceptor leads to HIV–host cell fusion (Delezay et al. 1996; Sakaida et al. 1998; Mahfoud, Garmy et al. 2002; Cormier and Dragic 2002). Since Gb3 can bind to HIV-gp120 (Mylvaganam and Lingwood 1999; Mahfoud, Mylvaganam et al. 2002), it is not difficult to surmise that the overexpression of Gb3 could increase the potential for HIV-gp120–Gb3 interactions. In our model, this would lead to sequestering of the virus and if enough Gb3 is present, may compete to prevent further coreceptor interactions. In addition, Gb3 could restrict the lateral movement of HIV gp120–CD4 within the plasma membrane, making contact with a coreceptor less frequent. Reduced CD4 lateral motility inhibits infection (Rawat et al. 2008).

In our experiments, the differential expression of Gb3 as a result of treatment with DGJ or P4 may cause changes in the composition of lipid rafts. Particularly, it is possible that high levels of Gb3 induced by DGJ or via genetic manipulation alter the patterns of raft versus nonraft associated interactions with the CD4 receptor and/or coreceptor. Furthermore, the DGJ-treated THP-1 cells did not become more sensitive to VT1 (data not shown), indicating that this increase in Gb3 may be in the nonraft fraction (Falguieres et al. 2001). To support this, we have found that gp120 can bind Gb3 monolayers primarily at surface pressures typical of nonraft domains (Mahfoud et al. in press).

Thus, the inhibitory role of Gb3 may be in the nonraft component and a facilitative role in the raft fraction. The protective effect of Gb3 against HIV infection may also relate to the role proposed for Gb3 in α2-interferon signal transduction (Lingwood and Yiu 1992; Khine and Lingwood 2000).

In addition, it is well established that lipid rafts are utilized for trafficking to intracellular compartments for a number of viruses, including HIV-1 (Manes et al. 2003). In our experiments, the use of DGJ resulted in differences in intracellular Gb3 expression levels. These differences in expression may influence lipid raft composition of intracellular compartments within target cells as well as at the level of the plasma membrane. Because lipid rafts are critical in processes including assembly and budding of HIV (Nguyen and Hildreth 2000), an increase in any individual GSL, like Gb3, may cause alteration or disruption of raft composition and/or function. In the case of HIV, increases in Gb3 may negatively impact viral infectivity and budding. Perhaps, the lack of Gb3 creates more favorable raft associations with viral proteins and/or localization of raft components, leading to increased infectivity. Therefore, variable Gb3 expression may provide a natural HIV risk factor in the general population. These findings may lead to future therapeutic and/or prevention strategies to combat HIV/AIDS.

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Conflict of interest statement
None declared.

References


