AGLYCONE MODULATION OF HIV GP120 BINDING TO GLYCOSPHINGOLIPID (GSL) DETERGENT-RESISTANT MEMBRANE (DRM) CONSTRUCTS

by

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Aglycone modification of HIV gp120 binding to glycosphingolipid (GSL) detergent-resistant membrane (DRM) structures

Master of Science, 2008

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ABSTRACT

HIV gp120 binds CD4+ cells within plasma membrane lipid rafts inducing a conformational change in gp120 that exposes its V3 loop that binds to a chemokine co-receptor, also within lipid rafts, and initiates fusion. Glycosphingolipids (GSLs) may also be bound by gp120. Lipid rafts, enriched with GSLs and cholesterol, are required for HIV entry and therefore the binding of gp120 to GSL-containing vesicles has been studied. Most of the GSL-structures were within the theoretical raft fraction on a discontinuous sucrose gradient while gp120 binding occurred outside of this fraction where a minority of structures migrated. Gb3 fatty acid content modulated binding. Gp120 bound preferentially to structures depleted of cholesterol and binding was enhanced by treating gp120 with CD4. Two water-soluble mimics of Gb3 inhibited gp120 binding to the different structures. The results demonstrate that the aglycone modulation of GSLs alters their receptor function and that the soluble mimics inhibit binding.
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<td>ABC</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>AIDS</td>
<td>Autoimmune Disease Syndrome</td>
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<td>AMEM</td>
<td>Alpha Minimal Essential Media</td>
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<td>ARV</td>
<td>Antiretroviral</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CNS</td>
<td>Central Nervous System</td>
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<td>CT</td>
<td>Choleratoxin</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<td>DIM</td>
<td>Detergent Insoluble Membrane</td>
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<td>DRM</td>
<td>Detergent Resistant Membrane</td>
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<tr>
<td>EC</td>
<td>Extracellular</td>
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<td>EMEM</td>
<td>Eagle’s Modified Minimal Essential Media</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>FA</td>
<td>Fatty Acid</td>
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<td>GalCer</td>
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<td>HUS</td>
<td>Hemolytic uremic syndrome</td>
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<td>MBCD</td>
<td>Methylbetacyclodextrin</td>
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<td>MDCK</td>
<td>Mandin Darby canine kidney</td>
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<td>Multidrug Resistance 1</td>
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<td>MES</td>
<td>2-(N-Morpholino)ethanesulfonic acid</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<td>NNRTI</td>
<td>Non-nucleoside Reverse Transcription Inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside Reverse Transcription Inhibitor</td>
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<td>Protein Kinase R</td>
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<td>RT</td>
<td>Reverse Transcriptase</td>
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<tr>
<td>RT</td>
<td>Room Temperature</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>SGC</td>
<td>Sulfogalactosylceramide</td>
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<tr>
<td>Tat</td>
<td>Trans Activator of Transcription</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered Saline</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>VSV</td>
<td>Vesicular Stomatitis Virus</td>
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<td>VT</td>
<td>Verotoxin</td>
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CHAPTER 1: INTRODUCTION

1.1. HIV/AIDS

1.1.1 Epidemic and History

Since the earliest described cases of HIV/AIDS in 1981 [8], the disease has become a leading global health risk. It is currently classified as a pandemic[9]. It was estimated that in 2007 33 million people were living with HIV with 2.7 million of those people having newly acquired the virus. Furthermore, an estimated 2.8 million people died of AIDS in 2006 [10]. The virus has exerted its greatest effects in sub-Saharan Africa as well as in the rest of the developing world. The HIV virus and AIDS has certainly proven to be one of the greatest menaces, rivaling malaria, to the human race having claimed over 25 million lives since 1981[10].

This immunodeficiency syndrome was first reported in 1981 in Haitians living in the United States who were presenting cases of opportunistic infections and Kaposi’s sarcoma [11]. The causative agent was retrospectively identified by Robert Gallo as well as by Luc Montagnier [12, 13] and later named the human immunodeficiency virus (HIV) [14]. Twenty-seven years later a vaccine still does not exist.

AIDS is the result of the depletion of the body’s immune system. Infected individuals become increasingly susceptible to opportunistic infections as well as to cancer. The opportunistic infectious agents can be bacteria, viruses, fungi or parasites and the specific species are dependent on the geographical prevalence of such organisms. The increased risk of cancer comes in the form of Kaposi’s sarcoma as well as cervical
cancer and lymphomas and systemic symptoms such as fever, sweats, swollen glands, weakness and weight loss are also prevalent [15].

Extensive research has been performed in order to better understand the viral life cycle and ideally to develop methods to prevent and/or cure infection. This resulted in the first anti-retroviral (ARV) drug azidothymidine (AZT, zidovudine), in 1987 and to subsequent generations of antiretroviral drugs. AZT is a nucleoside reverse transcription inhibitor (NRTI) that acts by being an available nucleoside that is incorporated into the growing strand of DNA by RT but is a dead end in this process in that no further nucleosides can be added to it [16]. Other RT inhibitors include the non-nucleoside reverse transcription inhibitors (NNRTIs) that inhibit RT non-competitively by binding to it and inhibiting its enzymatic activity[17]. The first NNRTI was nevirapine [18]. These drugs inhibit the replication of the virus by selectively inhibiting reverse transcription leading to a decrease in viral replication. The protease inhibitors (PIs) inhibit viral replication by serving as analogues for the viral protease enzyme, thus, they inhibit the cleavage of viral proteins within newly formed viral particles making them non-infectious [19]. Unfortunately, these ARVs do not completely inhibit viral replication and the high capacity to mutate gave rise to resistant strains [20, 21]. In 1996 a new treatment strategy, Highly Active Anti-Retroviral Therapy (HAART), was introduced. It consists of a combination of ARVs and leads to a decrease in mutation and an increased patient survival [22]. HAART, however, is also not fully suppressive to viral replication and therefore viral mutation. Also, a lack of patient compliance has lead to suboptimal results. There is an obvious need for better strategies to fight this disease, both by treatment it and by preventing the transmission of the virus. Some of the current research
and development is focusing on prevention of viral transmission by using microbicides as well as vaccines. Previous attempts at vaccine development have failed and current prospects lack promise [23].

1.1.2. The Virus

The HIV virus belongs to the Retroviridae family and Lentivirus subfamily of viruses. Being a retrovirus, its genomic material comprises two plus (+) sense single strands of RNA that amount to ~9.2 kilobases of genomic material. The viral RNA must be reverse transcribed into DNA in the host cell cytoplasm and then integrated into the host cell genome with the help of the viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN) which are packaged into the virion. The virus also contains an envelope as well as a protein core. The envelope is made up of a lipid bilayer that is derived from the host cell plasma membrane during the budding of newly formed virions. Glycoprotein 120 (gp120) is the adhesin on the outer surface of the virus that is responsible for recognizing host cells and initiating host cell entry. Gp120 was co-crystallized with its host cell receptor CD4 and a monoclonal anti-gp120 antibody fragment in 1998 [4]. Gp120 is found primarily in a trimeric form where each monomer is complexed with the transmembrane protein gp41. Gp41 mediates viral/cell membrane fusion allowing the virus to gain access to the cytoplasm of host cells. Together, gp120 and gp41 are known as gp160. A schematic representation of an HIV virion is depicted in fig. 1: HIV Virion.
1.1.3 HIV Entry

HIV virions are able to gain access to their host cells by way of viral host-cell membrane fusion [24]. HIV envelope glycoprotein gp120 first recognizes and binds to its primary receptor on host cells, CD4 [25]. This interaction gives rise to a conformational change in gp120 which exposes its third variable loop (V3) which then

Figure 1: HIV Virion is a simplified schematic representation of an infectious HIV virion. Two single-stranded RNA sequences are found within the capsid along with the viral enzyme reverse transcriptase (RT). The capsid is surrounded by the matrix and together they are encapsulated by a lipid membrane containing the transmembrane gp41s attached to the envelope gp120 forming gp160 which are found as trimers. [3]
binds to a seven transmembrane-spanning chemokine co-receptor [26]. The conformations of CD4-bound and unbound gp120 are shown in fig. 2: **CD4-Induced Gp120 Conformational Change.** A subsequent conformational change leads to the gp41 mediated fusion of the viral and host cell membranes [27]. At this stage the viral genome has gained access to the host cell cytoplasm where it goes on to be reverse transcribed and integrated into the host genome. A schematic representation of these early steps of the HIV life cycle (along with a proposed role of GSLs (discussed in section 1.2) are shown in fig. 3: **HIV Host Cell Entry.**

![Fig.2 CD4-Induced Gp120 Conformational Change.](image)

**Fig.2 CD4-Induced Gp120 Conformational Change.** Shows the conformational change in gp120 when bound to CD4. Panel a shows the unliganded conformation while panel b shows the liganded conformation and panels c and d are the same conformations as a and c respectively but reoriented in order to show the likely linkage of gp120 to gp41[1].
The chemokine co-receptor that the V3 loop of gp120 binds determines the tropism of the viral strain. Two chemokine co-receptors predominate. The α-chemokine receptor CXCR4 is bound by “X4” strains of HIV and is found on T-cells. The β-
chemokine co-receptor CCR5 is bound by “R5” strains of the virus and is expressed on monocytes, macrophages, DCs, activated T cells and memory T-cells [28]. HIV is also able to infect certain cells that do not express CD4 including neurons, epithelial cells, spermatozoa, renal epithelial cells, cardiomyocytes, CD4⁻ lymphocytes and thymocytes, and CD8⁺ T-cells [29-35]. It has been suggested that susceptibility in CD4⁻ cells is due to chemokine co-receptors being sufficient for infection, at least in CD4⁻CCR5⁻CXCR4⁺ pre-T cells [36]. However, this is not always the case. Another suggestion is that CD4⁻ cells become CD4⁺ infectable cells by the selective transfer of CD4 to them by neighbouring cells [37]. It has also been suggested that another cell surface molecule, VPAC-1, may act as an alternate receptor for HIV entry. Sequence similarity between HIV-1 gp120 and the natural ligand of VPAC-1, VIP, prompted investigation of the possible role of VPAC-1 in HIV-1 infection. Indeed, VPAC-1 expression was shown to significantly affect HIV-1 infection of different T cell lines expressing similar levels of CD4 and chemokine co-receptors. Also, manipulating VPAC-1 expression in host cells (by way of cDNA transfection) showed a positive correlation between VPAC-1 expression and infection [38]. VPAC-1 may, therefore, be able to replace CD4 as the primary cell surface receptor for HIV by enabling the same conformational changes in gp160 required for fusion.

The lack of understanding of why some receptor-lacking cells are susceptible to infection as well as unexplained differential susceptibility to infection across the population suggest that other players are involved and underscore a need for a better understanding of these players and their role.
1.1.4 Gp120

HIV gp120 is a highly glycosylated envelope glycoprotein responsible for recognizing and binding to host cells during the course of infection. Gp120 contains five constant domains (C1-C5) as well as five variable domains (V1-V5). The conserved region C2 is responsible for binding to CD4 while the third variable region, or the V3 loop, binds to the chemokine co-receptors and determines the tropism of the viral strain [39]. Cryoelectron microscopy tomography has recently shown that approximately 14 Env spikes exist per viral particle [40].

Figure 4: Crystal Structure of gp120 Gp120 was co-crystallized along with its host cell receptor CD4 and a monoclonal anti-gp120 antibody fragment in 1998 [4].
1.1.5. Gp120 Associated Pathology in the CNS

HIV-1 attacks the CNS of infected individuals and causes motor and cognitive complications as well as HIV-1-associated dementia (HAD) [41]. Gp120 on its own, shed from the virus and from gp41, has been shown to be toxic to neuronal cells [42]. Specifically, gp120 has been proposed to damage the CNS via direct and indirect pathways. Neurons and glial cells of the CNS express CD4 as well as CXCR4 and CCR5 [43, 44]. Other cell types of the CNS also express the two classical chemokine coreceptors on their cell surface and may contribute to gp120-mediated neurological dysfunction [45]. Gp120 has been shown to directly engage CXCR4 and CCR5 on neuronal cells leading to intracellular signaling events resulting in apoptosis [46] [43]. In neuroblastoma cells, gp120 exposure causes a rapid increase in intracellular calcium causing increased cyclooxygenase and 5-lipoxygenase activity, increased membrane lipoperoxidation and increased mitochondrial uncoupling. The end result of these events is cell death by necrosis. Interestingly, this gp120-mediated toxicity was enhanced in cholesterol-depleted cells and negligible in cholesterol-enhanced neuroblastoma cells [47]. This contrasts the role of cholesterol in HIV infection that is discussed later (see section 1.4.2 and 1.4.4.). Other studies have shown that gp120 disrupts intracellular calcium levels specifically by deregulating the plasma membrane and ER-mediated regulatory systems [48, 49]. One study shows that gp120 exposure leads to increased neuronal expression of PKR causing protein kinase signaling leading to neuronal apoptosis. This particular study was able to show that inhibition of PKR signaling alone inhibited gp120-mediated neurotoxicity and therefore they claim that PKR is a critical
player in gp120-mediated neuronal cell death [45]. Indirectly, gp120 mediates neuronal cell death by activating macrophages and microglia which in turn cause the hyperactivation of NMDA receptors on neurons ultimately leading to ER stress and intracellular calcium release [50].

1.1.6 HIV Nephropathy

HIV is able to infect renal epithelial cells [51] and HIV-associated nephropathy (HIVAN), characterized by collapsing focal segmental glomerulosclerosis, tubular dilation, and interstitial inflammation, is a leading complication of HIV infection though seen almost exclusively in patients of African descent [52]. Interestingly, the expression level of Gb3, a glycosphingolipid highly expressed in the human kidney [53], is elevated in PBMCs during HIV infection [54].
1.2 Glycolipids

1.2.1 Composition and Synthesis

Glycosphingolipids (GSLs) are carbohydrate-lipid conjugates found on the outer leaflet of the plasma membrane of eukaryotic cells. Their hydrophobic, water-insoluble moiety is ceramide, which consists of a fatty acid chain linked to a sphingosine base. The fatty acid chain is variable in carbon chain length, saturation and hydroxylation [55]. The ceramide is linked to a hydrophilic, water-soluble core sugar sequence. Most GSLs belong to one of four groups defined by their carbohydrate composition. The groups are the globo- (Gal\(\alpha1\)-4Gal), lacto- (Gal\(\beta1\)-3GlcNAc), neolacto- (Gal\(\beta1\)-4GlcNAc) and ganglio- (Gal\(\beta1\)-3GalNAc) series. A minor Gala series with a base galactosyl ceramide also exists [6].

GSL synthesis begins in the ER [56] where the condensation of serine and palmitoylCoA give rise to 3-keto-sphinganine which is then reduced to sphinganine and acylated with a long chain fatty acid by ceramide synthase to give dihydroceramide. Finally, ceramide desaturase inserts a 4,5-\textit{trans} double bond forming ceramide which is able to flip between the inner and outer leaflets of the ER membrane and is trafficked to the Golgi both vesicularly and non-vesicularly [57]. At the cytosolic face of the Golgi, glucose is added to ceramide by glycosyl transferase giving rise to glycosyl ceramide which then flips into the Golgi lumen primarily via the ABC transporter MDR1 (or pgp) which also functions as a drug efflux pump[5]. In the Golgi lumen lactosyl ceramide synthase converts glucosyl ceramide into lactosyl ceramide, which serves as the major precursor for the biosynthesis of glycolipids in the Golgi.
1.2.2 Gb3

Alpha1-4galactosyl transferase converts lactosyl ceramide into globotriaosyl ceramide (shown in fig.6 Gb3), the first GSL of the globo series. Gb3 is also known as the pK blood group antigen as well as cell differentiation marker 77 (CD77) [58]. Gb3 is the substrate for beta1-3GalNac transferase which forms Gb4 (or P antigen), the major neutral GSL of most cells [59]. Importantly, Gb3 is the cell surface receptor of E. coli derived verotoxins [60]. It is expressed in humans in the prostate and kidneys, in the ducts of sweat glands, as well as on monocytes, lymphocytes and granulocytes [53].
Cellular Gb₃ accumulation leads to a lysozomal storage disease Fabry’s disease [61].

**Fig.6 Gb₃** The chemical structure of Gb₃ which is composed of a hydrophilic carbohydrate portion consisting of three sugar residues (Galα1-4Galβ1-4Glc) linked to the hydrophobic ceramide consisting of sphingosine molecule branched to a fatty acid of variable carbon chain length, saturation and hydroxylation. [5]
1.2.3 Glycolipids Normal Cellular Function

In addition to playing an important structural role on the outer leaflet of the plasma membrane of eukaryotic cells, GSLs also have physiological roles in membrane trafficking, signaling, and cell polarization [62]. The different roles of GSLs are generally related to their location within lipid rafts, which are discussed later in section 1.4.1.

1.2.4 Glycolipids and Disease

Originally GSLs were believed to simply protect cells from harmful substances by forming mechanically stable and chemical resistant barriers on the outer cell surface. It is now known that GSLs play important and varied roles in different diseases. Neutral GSLs are recognized by natural killer cells in cancer and auto-immune diseases [63]. Many different glycolipids accumulate in lysosomal storage diseases including Gb\textsubscript{3} in Fabry’s Disease and GlcCer in Gaucher’s disease [61] [64]. GSLs are also cell surface receptors for extracellular toxins including GM1/CT, Gb\textsubscript{3}/VT [65] [66].

1.2.5 Gb\textsubscript{3}-Binding Pathogens

Gb\textsubscript{3} is the cell surface receptor for \textit{E. coli} derived verotoxins [67]. Importantly, VT-1 and VT-2 are the pathogenic verotoxins that cause hemolytic uremic syndrome (HUS) and haemorrhagic colitis, primarily in infants [68] [69] [70] [71]. Verotoxins are \textit{AB}\textsubscript{5} toxins that consist of a pentamer of B subunits responsible for host cell recognition and entry and a single catalytic A subunit that causes the pathology by interfering with the ribosome. VT binding to Gb\textsubscript{3} leads to endocytosis of the toxin. The B subunit of
verotoxin recognize the terminal alphaGal1-4 ofGb3 which is primarily located in DRM fractions on the cell surface [72][73][74]. Other pathogens that utilize cell surface Gb3 as a receptor are B19 parvovirus, *Streptococcus suis* and enteropathogenic *E. coli* [75][76][77].

1.2.6 Cholera Toxin and GM1

GM1 is the glycolipid cell surface receptor for cholera toxin which is another AB5 toxin and is derived from the bacterium *Vibrio cholerae* [66]. CTs catalytic activity is to ADP-ribosylate the Gs alpha subunit of the G protein heterotrimer leading to a constitutively active cAMP production. This leads to a disregulation of ion concentrations and rapid dehydration [78]. Importantly, CT binds to GM1 located in DRMs or lipid rafts and this interaction is the gold standard for studying such ligand-receptor interactions (CT binding is used as a marker for lipid rafts) [79]. However, the use of GM1 as a raft marker is still debated [80]. Studies in GUVs have found that CTB initially binds GM1 only in the l_d and subsequently is recruited to the l_o phase [81].

1.2.7 Influenza and GM3

The influenza virus is another virus that has binding affinity for glycolipids, specifically GM3. Influenza recognizes host cells with its surface adhesin molecule, hemaglutinin (HA), which recognizes sialic acid containing molecules on the cell surface. HA shows specific affinity for the sialic acid containing ganglioside N-Acetylaneraminyllactosylceramide, GM3-NeuAc [82]. The specific sugar residue
linkages of GM3 also play a role in the strain specific binding of influenza to GM3. One study showed that an avian strain of influenza had a higher affinity for a Neu5Acalpha2-3Lc4Cer (2,3GM3) than for Neu5Acalpha2-6Lc4Cer (2,6GM3) while a human strain of the virus bound equally well to both analogs. This particular study also found that an avian strain recognized GM3 with a shortened fatty acid chain while their human strain did not [83]. An adamantyl2,6GM3 compound, where the fatty acid chain of the glycolipid is replaced with an adamantane frame, has been made. It is water-soluble mimic of 2,6GM3 that is in the same family as AdaGb3 described in section 1.3.3.

1.2.8 VSV and Glycolipids

Gangliosides have been shown to be a component of the receptor complex of VSV-susceptible cells and gangliosides were also able to inhibit VSV infection [84].

1.2.9 Glycolipid Heterogeneity

GSLs show heterogeneity in their fatty acid composition. Variances are seen in chain length, saturation and alpha-hydroxylation. Originally this was thought to lack any functional importance as no specific enzyme was known and it was believed that availability was the only factor determining fatty acid composition. Since a family of ceramide synthases with fatty acid specificity (Lass1-6) was identified it is now believed that this heterogeneity has functional importance[85, 86]. The role of this heterogeneity is not well understood but it has been suggested that it is important in terms of the roles that GSLs have in extracellular ligand recognition and intracellular trafficking [87] with an underlying importance on membrane order [88].
The major fatty acid-dependent isoforms of human renal Gb₃ are C16 (21%), C18 (5%), C22 (15%), C24 (27%) and C24:1 (23%) [89]. The lipid moiety of Gb₃ is required for its verotoxin receptor function as the lipid-free sugar shows little VT binding [90] [91]. Also, the fatty acid chain length of Gb₃ determines the intracellular trafficking of the VT-bound glycolipid [92].

It is believed that changes in fatty acid carbon chain length and degree of saturation affect the partitioning of these analogues in cellular plasma membranes. Specifically, these fatty acid properties affect the order or fluidity of the microenvironment. The shape of the conjugates may determine the local membrane curvature that they exist in on the plasma membrane. It has been suggested that lipids with long, saturated acyl chains will partition into highly ordered/rigid domains with comparable hydrophobic thickness and that those with short and unsaturated acyl chains partition preferentially into more fluid/less ordered plasma membrane microenvironments [93].

The fatty acid moiety of GM1 has been shown to affect the incorporation of the isoforms into detergent-resistant membrane microdomains on human leukemia HL-60 cells. Three unsaturated FA-containing isoforms (C18,22,24) were all found in lesser quantities in the DRM than their saturated counterparts. C8 isoforms were isolated in the lowest quantity in DRMs while the C14,16,18,22,24 containing FAs were all isolated in similar quantities from DRMs [80].
1.3 HIV and Glycolipids

1.3.1 Gp120 and Glycolipids

Gp120 contains a highly conserved glycolipid binding motif in its V3 loop, GPGRAF, which binds to several glycolipids including GalCer, SGC, GM3 and Gb₃ [94]. The V3 peptide SPC₃ (containing eight V3 consensus GPGRAF motifs radially branched on a neutral polyLys core matrix) is able to bind to these same glycolipids, does not bind to CD4 and inhibits HIV-1 infection [95]. The affinity of this motif is low-moderate for Gb₃ and high for GalCer, depending on the viral strain [96]. Fantini et al. showed that gp120 or the V3 loop alone increases the surface pressure of monolayers of GalCer, Gb₃ or GM3 via insertion in a specific manner [97]. Gp41 has also been shown to bind to GalCer [98].

Originally, it was believed that glycolipids aided viral entry either by acting as co-receptors or by being able to replace CD4 as the primary surface receptors in CD4⁺ HIV permissive cells (specifically neurons) [99, 100]. GSLs are thought to play an indirect role in viral entry by being required for the fusion process [101, 102]. A trimolecular complex of GM3 (the monogangliosides GM1 and GM3 are the major GSLs expressed on CD4⁺ cells [103]) along with CD4 and gp120 supports the hypothesis that glycolipids function as co-factors in viral fusion. However, at least one study shows that this GM3 co-localization is not required for HIV entry by inhibiting GM3 expression [104]. One study found that the addition of Gb₃ or GM3 only to GSL-depleted host cells rescues CD4/CXCR4-dependent fusion [102, 105]. Also, studies using GSL deficient cells showed that when these cells had low env receptor levels there was no fusion whereas fusion was unimpaired with normal receptor levels suggesting that GSLs may be
required for receptor clustering/organization only when they are poorly expressed [106]. Additionally, cells expressing elevated levels of GM3 are resistant to HIV-1 infection but are rendered susceptible upon treatment with PPMP which correlated with down-regulated GM3 levels [107].

Other evidence suggests that GSLs play an inhibitory role in HIV infection. Fabry cells which accumulate Gb3 are resistant to R5 HIV-1 infection, although this elevation of Gb3 is accompanied by a decrease in CCR5 expression [108]. Also, pre-treatment of HIV-1 with a soluble semi-synthetic analogue of Gb3, AdaGb3, inhibits fusion, suggesting that a Gb3-bound V3 loop inhibits HIV-1 viral entry [109]. We hypothesize that this is the result of the overlap between the glycolipid and chemokine co-receptor binding motifs on the V3 loop such that a glycolipid occupied V3 loop is unable to bind to its chemokine co-receptor, preventing viral entry. The consensus binding motif for CCR5 binding to the V3 loop of gp120 is S/GXXXGPGXXXXXXXE/D [110] and the binding motif for Gb3 (XXXGPGRAFXXX) [95] is within the CCR5 binding domain. Ongoing studies involving biochemical manipulation of Gb3 expression levels show that there is an inverse relationship between Gb3 levels and infectivity [111]. While it is clear that GSLs are able to act as co-factors in HIV infection, their exact role is unclear. It is expected that different GSLs, and their combinations, play different roles, varying amongst cell types. It is also possible that the relationship between GSL expression level and infectivity is not linear and that a certain level of expression is required for infection whereas over expression might lead to a loss of susceptibility.
The expression of GSLs is altered at the early stages of HIV-1 infection. Such changes are characterized by increases in Gb₃ and GM3 in the PBMCs and increases in glucosylceramide in the PBMCs and CD4⁺ lymphocytes of infected patients [54]. It is unclear whether this phenomenon is a host-mediated response to try and resist HIV replication or a HIV-mediated hijacking of lipid metabolism in order to facilitate its spread. Also, HIV-associated neuropathology (HAD) is associated with upregulated ceramide and sphingomyelin in the brain tissue and CSF of affected individuals [112].

1.3.2 GSL Biosynthesis Drugs vs. HIV

Many different drugs that interfere with different stages of the GSL synthesis pathway have been studied in terms of their effect on HIV infection. L-cycloserine inhibits serine palmitoyltransferase (the rate-limiting step in sphingolipid synthesis) as well as HIV-1 infection in a HIV-susceptible T lymphocyte cell line [113]. PPMP, which inhibits UDP:glucosyl transferase and therefore GSL synthesis, inhibits HIV-1 entry in multiple cell lines [101, 105, 114]. NB-DNJ also interferes with glucosyl ceramide synthesis and was shown to inhibit HIV-1 infection as well as syncitium formation [115] [116]. This drug reached phase 2 clinical trials as a treatment of HIV-1-positive patients but proved ineffective [117].

1.3.3 GSL Analogs vs. HIV

Different GSL analogues have been synthesized with the hope that these gp120-binding molecules may effectively inhibit HIV infection. Analogues of GalCer containing altered carbohydrates and lipid chain length, as well as introduced glycol
spacers between the amphipathic and amphiphilic portions of the molecule have been made [118, 119]. Galactosyl amphiphiles containing amino groups, hydroxyls or anionic groups causing their clustering are able to bind to gp120 and inhibit HIV-1 infection [120, 121]. A water-soluble polymerizable analogue of betaGalCer was also synthesized [122]. A multivalent neoglycodendrimer mimic of GalCer is able to bind to gp120 with high affinity and inhibits HIV infection at similar levels to the previously characterized potent inhibitor, dextran sulfate [123]. C-glycoside analogs of GalCer have also shown the ability to inhibit HIV/host cell fusion of X4 and R5 viruses [124].

Our laboratory has made a semi-synthetic, water-soluble mimic of Gb₃, AdaGb₃ (shown in fig. 6 AdaGb₃), by replacing the fatty acid residue of the GSL with an adamantane frame. AdaGb₃ (originally made to inhibit VT) binds to gp120 with a 1000-fold greater affinity than Gb₃ and inhibits X4 and R5 HIV-1 infection in the micromolar range [109]. Importantly, this compounds water-soluble nature makes it a very interesting drug candidate. It is currently being tested in a mouse model (using a VSV/pseudoenveloped HIV model) as a vaginally or rectally applied microbicide prophylactic against the viral transmission of the virus.

Our laboratory has also collaborated with Dr. Steve Henry (Auckland, New Zealand), testing the inhibitory effects of FSLGb₃ on HIV infection as well as VT toxicity. FSLGb₃, functional spacer linker Gb₃, is another soluble Gb₃ mimic with a spacer group inserted between the glycone and aglycone portions of the molecule. The spacer contains nitrogen and phosphate groups as shown in fig.8 FSLGb₃.
**Fig. 7 AdamantylGb₃** The chemical structure of adamantylGb₃ where the fatty acid of Gb₃ has been replaced with an adamantane frame (shown on the left) rendering the molecule water-soluble.

**Fig. 8 FSLGb₃** The chemical structure of functional spacer linker Gb₃ shows the insertion of nitrogen and phosphate-containing groups between the glycone and aglycone portions of the molecule.
1.4 Membranes

1.4.1 Lipid Rafts

In 1972 Singer and Nicolson described the fluid mosaic model in which the cell membrane was composed of a liquid crystalline, $L_c$, portion of disordered phospholipids and a dense liquid ordered, $L_o$, phase uniquely on the outer leaflet of the membrane enriched with sphingolipids and cholesterol [125]. In 1992 Brown and Rose first introduced the idea of lipid rafts and Simons and Ikonen first isolated them in 1997 by separating these detergent-resistant membrane microdomains from the rest of the detergent-soluble membrane [126, 127]. Lipid rafts are controversial in their composition, physiological role and even in their existence in cell membranes [128]. Evidence that lipid rafts are too transitory to have a physiological role has been presented. Another source of controversy stems from the difficulty in studying lipid membranes and specifically lipid rafts. Until recently, the only way to isolate and study lipid rafts was to treat cells with a cold, non-ionic detergent and separate the cellular components on a sucrose density gradient, under the assumption that only the raft microdomains of the cell are detergent-resistant and that the proteins and lipids that float to low density fractions are raft-associated [129]. For this reason, it is more accurate/responsible to refer to the structures isolated in such a manner as detergent resistant membranes (DRMs) or detergent insoluble membranes (DIMs) rather than lipid rafts. Recently, the relative order of membranes can be better visualized by use of a new class of dyes including Laurdan, that exchange their fluorescent properties with the liquid ordered phase of cell membranes which can then be visualized microscopically. Also,
non-invasive methods (such as 2 photon microscopy and fluorescent correlation spectroscopy) have demonstrated the importance of plasma membrane domain microheterogeneity [130, 131]. As defined by the 2006 Keystone Symposium on Lipid Rafts and Cell Function, ‘Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions.’ [132]. They are involved in a number of cellular processes including apoptosis, proliferation and stress responses [133]. Molecules such as Src, Lck, Fyn, Lyn, Hck, Grb-2, Ras, GPI-anchored proteins, and GTP-binding proteins involved in processes of metabolism, development, and immunity through intracellular signaling are associated with lipid rafts [134-137]. Lipid rafts are concentrated at the apical surface of polarized cells whereas they are randomly distributed throughout non-polarized cells [138].

1.4.2 Lipid Rafts, Cholesterol and HIV

Although the precise role that lipid rafts play in the infectious cycle of HIV is not clearly understood, it is clear that such membrane microdomains are capable of altering HIV infection in vitro and they likely do play a role in viral entry and egress. It is well accepted that CD4 is associated with lipid rafts in T cell signaling and that a marker of this event is activation of the intracellular membrane-anchored protein Lck [139]. In 2001 it was reported that the HIV protein Gag, associated with viral production at the cell membrane, associates with cholesterol-enriched microdomains at the host cell membrane and that depletion of cellular cholesterol significantly decreases virion production [140].
In 2002, Popik et al. showed that treatment of cells with MBCD resulted in a relative decrease in raft fraction cholesterol content as well as in the raft-associated GM1 and CD48. Importantly, this lipid raft disruption coincided with a 10-fold decrease in the entry of an HIV-1 viral strain into Jurkat T cells as compared to untreated cells without significantly altering surface expression of CD4 and CXCR4 [141]. Interestingly, while CD4 and CCR5 are appreciably enriched in raft domains, most CXCR4 is found in non-raft fractions [141]. This may be the result of CCR5 constitutively co-localized with CD4 while CXCR4 is not [142]. Another study made CD4 partitioning mutants and found that mutants that associated with non-raft domains on the cell surface had a considerably diminished ability to internalize HIV-1 (R5 and X4) and to mediate Lck activation while this mutant was still able to bind gp120 suggesting that it is specifically the fusion step that requires a cholesterol-enriched ‘lipid raft’ environment [143].

Conversely, some studies have found that HIV entry does not depend on lipid rafts. These studies highlight the fact that the commonly used means of depleting cholesterol, MBCD, have additional affects on host cells. One study using functional mutants of CD4 that did not localize to lipid rafts found that viral entry levels were equivalent to those using wild type CD4-containing cells [104]. Another study claimed to prevent CD4 incorporation into lipid rafts by preventing its palmitoylation and interaction with p56\textsuperscript{lek} which did not result in suppressed HIV-1 entry while MBCD treatment of these mutant cells did inhibit infection suggesting that entry is dependent on cholesterol in a lipid raft-independent fashion [144]. A recent study, using a confocal fluorescence resonance energy transfer-based approach, found that CD4, associated with
rafts, is constitutively associated with CCR5 yet the ternary interaction between these two receptors and gp120 occurs outside of membrane rafts [145].

Cholesterol is a common molecule that is thought to play an important role in HIV lipid raft mediated entry. Cholesterol is a sterol that is found in the plasma membrane of cells. It is made up of the characteristic four fused rings of a steroid linked to an alcohol. Cholesterol forms lipid rafts along with GSLs. It interacts with sphingolipid hydrocarbon chains and as a glue maintaining lipid rafts [146]. It also decreases the fluidity of the plasma membrane and therefore promotes ordered microdomains [47]. Membrane cholesterol determines the order within the membrane (the phase transition) and is a function of the hydrophobic mismatch between the hydrophobic moieties of neighbouring molecules. Cholesterol-GSL membrane microdomains also function as platforms that bind lipid-modified proteins including GPI-anchored proteins and src-family tyrosine kinases involved in signaling [62]. The interaction of phospholipids with cholesterol varies with acyl chain length (C16->20) and saturation [91, 147]. While one study shows an inverse relationship between cholesterol content and gp120 binding to CHP100 cells, others show that depleting HIV-susceptible cells with low concentrations of co-receptors of cholesterol decreases the ability of the virus to mediate fusion [47]. Direct treatment of HIV-1 with MBCD destabilizes the HIV-1 virion structure resulting in smaller, denser, non-infectious particles [148].

GalCer enriched domains at the apical surface of the epithelial cell barrier of the gastrointestinal tract are believed to be responsible for the CD4-independent transcytosis of HIV across this barrier. GalCer is known as an alternate receptor for gp120 [100]. This interaction does not lead to the internalization of HIV by the epithelial cells which
instead transcytose the virus to the basolateral membrane giving them access to HIV susceptible mononuclear cells [149, 150] [149].

Lipid rafts are also postulated to play a role in host cell signal transduction within HIV-1-infected cells. Specifically, the HIV-1 protein Nef is associated with lipid rafts [151]. Expression of this protein coincides with high viral load and pathogenicity [152]. One study showed that Nef aids in viral budding from lipid rafts, which corresponds with an increase in GM1 in viral envelopes when Nef is present. These effects resulted in an increased infectivity of HIV. Interestingly, this study also found that the well-studied effects of altering cellular cholesterol only effected HIV infectivity when Nef was present suggesting that cholesterol’s importance may lie in it’s effect on Nef recruitment to lipid rafts [153].

Binding of an anti-CD4 antibody to CD4 on Jurkat T cells results in the recruitment of adhesion molecules (LFA-1, integrin alphaL, VLA-4, alpha4 integrin, beta1 integrin), cholesterol, CCR5 and CXCR4 to CD4 and membrane cholesterol was required for the co-localization of CD4 and the chemokine receptors [154].

The existing data regarding HIV and lipid rafts would suggest that cholesterol rich membranes do play a role in the replication cycle of the virus. While some studies have found that raft-associated CD4 is required for efficient viral entry, other studies dispute this requirement. What does seem to be clear is that cholesterol is required for both viral entry and complete replication. It is entirely possible that cholesterol is acting independently of lipid ordered microdomains. It is imperative that a difference between cholesterol enriched membranes, or microdomains, be distinguished from lipid rafts, which has certainly led to some confusion/contention in the past. In order to discriminate
between these two things, and to circumvent the possible ambiguous effects of MBCD, future studies should make use of lipid order targeted dyes such as Laurdan.

1.4.3 Lipid Rafts and Other Infectious Agents

CT and VT bind to the glycolipids GM1 and Gb3, respectively, within lipid rafts [65, 66] and other bacteria are able to secrete pore-forming toxins that are targeted to raft-associated molecules [155]. The scrapie isoform in prion disease and the beta-amyloid protein in Alzheimer’s disease require caveolae (subdivisions of glycolipid membrane rafts [156]) and cholesterol in order to convert into their pathogenic forms [157]. Many enveloped viruses use host cell cholesterol and/or lipid rafts to different extents during their replication cycle. These viruses include influenza, measles, VSV, Semliki forst virus (SFV), simian virus 40, sindbis virus and retroviruses such as HIV [158] [159] [148, 160].

1.4.4 Radius of curvature

It is known that lipids, specifically cholesterol and cone-shaped lipids with spontaneous negative curvature such as the phospholipid PE, modulate membrane curvature. Cholesterol and lipids with cone shape (PE) promote a negative membrane curvature which promotes fusion by allowing the hemifusion state in which only the outer leaflets of the viral and cell membranes are fused and an hourglass structure is formed wherein a very highly negative radius of curvature exists shown in figure 9 [161] [7]. Specifically, membrane cholesterol is redistributed and enriched in areas of high curvature during the fusion process. It is postulated that the spontaneous negative
curvature of cholesterol allows membranes to reach the required levels of curvature despite the high energy of these transient structures [162].

A recent study showed that Verotoxin B subunit interaction with Gb₃ induces tubule-mediated membrane invaginations on the cell membrane. These invaginations result in a negative curvature that drives subsequent Gb₃/VT interactions and ultimately the internalization of the toxin [163]. While this specific process results in an endocytosed toxin, rather than a viral fusion event, it does provide insight on the role that glycolipids play in membrane organization and internalization. Also, while VT is internalized via Gb₃ membrane recognition, it appears that Gb₃ plays an inhibitory role towards HIV entry. In another study, it was shown that when erythrocyte exvaginations (increased positive curvature) and invaginations (towards negative curvature) are induced, the cholera toxin receptor GSL GM1 is enriched on these areas of altered membrane curvature [164]. This provides further evidence that induced membrane curvature plays a role in cellular toxin accumulation and that not only does a post-toxin

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**Fig. 9: Viral/Host early membrane fusion.** A schematic representation of the initial steps of fusion. After the initial apposition of the membranes (left), the hemifusion step occurs where only the outer leaflets of each membrane are fused and where cholesterol is enriched in these areas of high negative curvature (right) [7].
binding remodeling of membranes allow internalization but that the curvature of receptor-containing microdomains on the cell membrane may also play a role in the ability of ligands to bind to these receptors, especially GSL receptors.
1.5 Hypothesis:

HIV gp120 binds GSLs via its highly conserved glycolipid-binding motif within its V3 loop [2]. While the role that GSLs play in HIV infection is controversial, it is known that the accumulation of Gb3 in host cells leads to HIV resistance [61]. GSLs are enriched in lipid ordered microdomains on the outer leaflet of the plasma membranes [62]. Lipid ordered domains are thought to play an integral role in HIV entry into host cells [143]. Cholesterol is another molecule that is enriched in lipid ordered domains and it has been suggested that its presence on host cell membranes is required for HIV infection [146]. Membrane order therefore appears to modulate the ability HIV to enter host cells. We therefore hypothesize that the modulation of the aglycone moiety, and the membrane environment, of GSLs modulates HIV gp120 to the carbohydrate portion of GSLs and that this subsequently affects the involvement of GSLs in HIV infection.

1.6 Objectives

1. To investigate the binding of monomeric gp120 to glycosphingolipid detergent-resistant membrane constructs separated on a discontinuous sucrose gradient.
2. To determine the effect of fatty acid composition of the different isoforms of Gb3 on their ability to bind gp120.
3. To determine the affect of cholesterol levels in the constructs on their ability to bind gp120.
4. To determine the effect of gp120 treatment with water-soluble Gb3 on its ability to bind to the GSL DRM constructs.
CHAPTER 2: MATERIALS & METHODS

Glycolipids:

Gb$_3$ and Gb$_4$ were purified in the Lingwood lab from human kidney using silica column chromatography [165]. Briefly, human kidney from the Pathology Department at Toronto General Hospital (stored at -20°C) was minced into ~0.5cm pieces, mixed with an equivalent volume of PBS and blended to homogeneity. The homogenate was added to 20 volumes of CHCl$_3$:MeOH (2:1, vol/vol) and stirred vigorously for 18 hours. The extract was filtered and water was added to it to obtain a Folch partition (CHCl$_3$:MeOH:H$_2$O, 2:1:0.6, vol/vol/vol) which was shaken vigorously and allowed to separate overnight. The lower (organic) phase was collected and dried using a rotating evaporator. The sample was dissolved in a minimal volume of CHCl$_3$:MeOH (98:2, vol/vol) and run through a batch elution silica column to isolate the neutral glycolipids (neutral glycolipids elute with acetone:methanol (9:1, vol/vol)). The elutant was saponified (2-5mg lipid/mL 1M NaOH in MeOH and pH adjusted to ~8 with HCl) where lipid contaminants (base-labile phospholipids and other glycerolipids) were removed as they partition into the upper phase of a Folch partition. The lower phase was isolated and run through a DEAE-Sephadex A-25 column to remove sulfated glycolipids and finally the sample was loaded onto a second silica column to fractionate remaining GSLs and isolate Gb$_4$ and Gb$_3$. Purity was determined when a single band (corresponding to a known standard) appeared on a TLC sprayed with orcinol when an excess (~10µg) of the sample was loaded. GalCer (bovine brain, type2 (hydroxylated)) and SGC were obtained from Sigma.
AdamantylGb₃ was prepared as described previously [166]. Dimethylformamide was added to a solution of oxalyl chloride in dichloromethane. Adamantane acetic acid was then slowly added over 30 minutes. After stirring at room temperature for 2 hours, oxalyl chloride in excess and solvent were removed under a stream of N₂, and residual adamantane acetic acid was dissolved in dichloromethane. LysoGb₃, prepared by hydrolysis of Gb₃, was suspended in dichloromethane and pyridine, and then 2 aliquots of the adamantane acetic acid solution were added at 30-minute intervals. After the reaction, the mixture was dried under N₂ (TLC; chloroform:methanol:water, 80:20:2 (v/v/v)) and the product purified on a mini silica column. The same was done for GalCer.

FSLGb₃ was obtained from Dr. Steve Henry’s laboratory (Dr Stephen Henry, Glycoscience Research Centre, Auckland Institute of Technology, New Zealand). This compound consists of globotriose with a spacer group (containing nitrogen and phosphate groups) inserted between the glycone and aglycone moieties. The chemical structure of FSLGb₃ is shown in figure 7: FSLGb₃ (section 1.3.3).

Gb₃ Fatty Acid Isoforms The fatty acid composition of purified renal Gb₃ was determined by FAB MS. Gb₃ homologues corresponding to the fatty acid isoforms were made by deacylation of renal Gb₃ and reacylation of lyso-Gb₃ with a single fatty acid as previously described [167].

Verotoxins:
VT1 was purified from recombinant *E. coli* strain JB28 (previously described, Nutikka, Binnington-Boyd et al. 2003). VT2 was purified from recombinant *E. coli* strain R82pJES1-20DH5a, kindly provided by Dr. J. Samuel (Texas A&M University, college Station, TX) [168]. Toxin purity was verified by SDS-PAGE and toxicity was tested on Vero cells.

**Gp120 sample strains**

All of the gp120 samples used were R5 strains obtained from the NIH AIDS Research Reference and Reagents Program. Catalog #4961, used predominantly throughout the experiments, is a recombinant HIV-1BaL strain. Catalog #7363 is an HIV-1SF162 gp120. Catalog #2968 is an HIV-1 gp120 CM external envelope protein is a full-length recombinant protein derived from the *env* gene of HIV-1. Catalog #3234 is the *env* gene product from HIV-1 93TH975.15 that was cloned into a baculovirus vector. Catalog #10080 is a recombinant HIV-196ZM651 gp120.

**Anti-gp120 Ab Panel**

Five different anti-gp120 antibodies were incubated with the 4961 gp120 sample in order to confirm that the protein was intact and that there was reasonable means of detecting it. Five-fold dilutions of the gp120 sample, starting at 100ng, were immobilized on a nitrocellulose membrane, which was then blocked in 1% skim milk for 1 hour at 37°C. The membranes were then incubated with the antibodies individually, as well as with anti-VT1 as a negative control, for 1 hour at RT before being washed (3 times for 3 minutes on a shaking table with TBS). The ligands were detected with secondary HRP-
conjugated goat antibodies followed by visualization with 4-chloronapthol. All antibodies were obtained from the NIH Reference and Reagents Program. Ab1 is a human monoclonal anti-HIV-1 V3 (257-D IV) antibody. Ab2 is a human monoclonal antibody recognizing HIV-1 V3 (268). Ab3 is a human monoclonal anti-HIV1 antibody that recognizes the V3 loop of gp120. Ab4 is human monoclonal anti-gp120 antibody and importantly is a conformation-specific antibody that only recognizes gp120 in its tertiary structure and recognizes an epitope made up of sequences of the proteins V1, V3 and CD4 binding domains [169]. Ab5 is a human HIV-1MN V3 Antiserum solution.

**Anti-GSL antibodies:**

Anti-GM1 antibody was generously donated by the Mahuran lab at SickKids. The rat IgM anti-Gb3 antibody (mAb 38.13) was grown in the Lingwood Lab. Briefly, the 38.13 clone [170] was used to produce the mAb anti-Gb3 (raised against cell-incorporated immunogenic Gb3) which was purified from culture supernatent using affinity column chromatography [92]. Mouse IgG anti-Gb3 was obtained from the Seikagaku Corp., Tokyo, Japan.

**VSV-G:**

The VSV-G protein and the anti-VSV-G polyclonal rabbit antibody were obtained from the Gaudin laboratory (Molecular and Structural Virology, Gif sur Yvette, France). The VSV-G protein was used at a concentration of 3µg/mL in the discontinuous sucrose gradient and the antibody was used at a dilution of 1:1000 in TBS during immunodetection.
**Miscellaneous:**

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit, goat anti-human and goat anti-mouse was purchased from Sigma. Monosialoganglioside (GM1), cholesterol, sucrose, MES, Triton X-100, sphingomyelin, phosphatidylethanolamine and phosphatidylserine, cholera toxin, anti-CT antibody produced in rabbit, and methyl-beta-cyclodextrin were obtained from Sigma. HRP-cholera toxin B subunit, $[^3]$H cholesterol were from ICN. Organic solvents were all from Caledon. Tris buffer was from BioShop. Sodium chloride was from EMD. Rabbit anti-VT1B 6869 antibody was raised in the Lingwood lab using purified VT1B subunit as antigen. ECL reagents were from Pierce.

**Media and Cell Culture:**

Vero cells were used for the verotoxin cytotoxicity assays and MDCK cells were used for the influenza experiment. Vero cells are kidney epithelial cells expressing high levels of Gb$_3$. These cells were grown in Eagle’s modified Minimum Essential Medium (EMEM) (from Wisent) supplemented with 5% fetal calf serum and 1% penicillin/streptomycin (Wisent). MDCK, Mandin-Darby canine kidney cells, were cultured in Alpha Minimal Essential Medium (AMEM, from Wisent) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Both cell lines were split at 1:5 every 3-4 days with 0.05% trypsin/EDTA (Wisent) (2 minute incubation at 37°C post 2X PBS washings).
**Protein Assay:**

The concentration of the gp120 sample used was confirmed using a Bradford reagent, BCA protein assay. Briefly, a standard curve of absorption versus protein concentration was made using standard dilutions of BSA. This standard curve, along with the absorbance of the gp120 sample incubated with the BCA reagent was used to determine the concentration of the sample.

**Non-Reducing SDS-PAGE:**

Gp120 samples (~1µg each) were mixed with sample buffer and heated to a boil before being loaded and run on a non-reducing 8% SDS-PAGE gel at a constant current of 35mAmp/gel. The molecular weight marker included was the Fermentas Life Sciences Page Ruler Prestained Ladder (#SSM0671). Proteins were visualized using Comassie Brilliant Blue protein stain.

**Vesicle Construction:**

In order to study the binding of gp120 to GSLs in a raft-like environment, detergent-resistant membrane (DRM) constructs consisting of glycolipid and cholesterol were made in a detergent-containing non-ionic buffer (1% Triton in MES, w/vol). A 2:1 (50µg:25µg or 0.69mol:1mol) ratio of glycolipid to cholesterol were placed in a glass tube and dried under nitrogen flow, resuspended in excess ethanol and re-dried under nitrogen in order to remove residual water. The samples were suspended in 750µL of a 1% Triton containing MES buffer (pH 7), vortexed for 1 minute, sonicated in a water for 1 minute
and placed in a 55°C water bath for 5 minutes. 750µl of 70% sucrose in MES buffer (w/vol) is then added to the sample (giving a 35% sucrose solution) and the samples are let to sit for 1 hour at RT.

**Discontinuous Sucrose Gradient:**

The vesicles, now in 5mL Beckman ultra-clear ultracentrifuge tubes, were then overlaid with a discontinuous sucrose gradient (1.5mL 35%, 2mL 30%, 1.5mL 5%) containing 3µg/mL of gp120 (or 1µg/mL VT-1/CT, or 3µg/mL VSV-G, or 1µg/mL anti-Gb3, or 1µg/mL anti-GM1) in the first 1ml 30% fraction. The gradient includes the sucrose density corresponding to DRMs, which is found at the 5%/30% sucrose interface [146]. The vesicles are separated on the sucrose gradient by ultracentrifugation (72h, 20°C, 34,000rpm, SW55Ti swinging bucket rotor) and 10 0.5ml fractions were collected from the top of the gradient and analyzed for gp120 content (indicating DRM binding) by immunodetection. Although cell DRMs are often isolated at 4°C, we found that separation was not altered at RT.

**Unseparated Vesicles:**

Alternatively, the DRM constructs are not separated on a sucrose gradient and the unseparated vesicles, made in MES buffer without sucrose or Triton, were directly loaded onto a nitrocellulose membrane and overlaid with gp120 (3µg/mL, 2hours to overnight at RT/cold room) prior to immunodetection.
**Immunoblot analysis of DRM fractions**

Aliquots of the gradient fractions were loaded onto nitrocellulose using a slotblot apparatus (Schleicher & Schuell Minifold I microsample filtration manifold). The samples were washed with 50mM TBS, 150mM NaCl containing 1% skim milk blocking solution and left at 37°C for 1 hour. The membrane was rinsed with TBS and incubated with anti gp120-Mab F425 B4a1 or mAb anti VT1 (1µg/mL) for 2 hrs at room temp. After washing, bound antibody was detected using an appropriate HRP conjugated anti-species antibody. Visualization was achieved either by addition of a 4-chloronaphthol solution (20mL TBS: 4 mL 4-CN: 10µL H2O2) (2-10 minutes at RT) which reacts with HRP to give a purple color or by a 1 minute incubation with a 1:1 (vol/vol) ratio of the ECL reagents followed by exposure of a film.

**Glycolipid Extraction and Detection by TLC Overlay**

Gb3 was extracted from gradient fractions according to the Folch procedure [171]. Gb3 was separated from the organic phase by thin-layer chromatography (TLC) in chloroform, methanol, water, 60:40:8 (v/v/v). The plates were dried and blocked with 1% fish skin gelatin in 50 mM Tris-buffered saline (TBS) at 37°C for 1 hour. Then they were washed three times 5 min, with 0.01% gelatin TBS and incubated with 1 µg.mL-1 VT1 or VT2 for 1 hour at RT. After, plates were incubated with corresponding antibodies (rabbit anti-VT1B 6869 at 1:2000 in TBS); and finally the corresponding horseradish-peroxidase-conjugated goat anti-rabbit at 1: 2000 in TBS. Each step consisted of 1 hour incubation followed by three times 5 min washing with TBS. After final wash with TBS, toxin binding was visualized with 4-chloro-1-naphthol peroxidase substrate [172]. Similar plates were prepared and stained with orcinol carbohydrate spray for comparison.
The distribution of GM1 within the gradient was determined by solid phase extraction of fractions, TLC separation and orcinol detection. Aliquots of the GM1/cholesterol structure fractions were loaded onto a reverse phase (C18) column in water and washed with water in order to remove the sucrose. The methanol elutants were then dried down under nitrogen, redissolved in 2:1 (C:M,v:v) solvent and loaded and separated on TLC with 60:40:9 (C:M:W, v:v:v) solvent. GM1 distribution was visualized by orcinol spray of the TLC plate.

**Methyl-beta-cyclodextrin (MBCD) Treatment:**

Nitrocellulose immobilized and blocked vesicles were treated with 10mM methyl-beta-cyclodextrin in TBS for 45 minutes at 37°C. After extensive washings with TBS the membrane was then overlaid with gp120 as previously described.

**Soluble CD4 (sCD4):**

Prior to Gb3 DRM exposure, gp120 (3µg/mL) was treated with 150µM soluble CD4 in TBS at 37°C for 1 hour. The gp120/sCD4 solution was then overlaid on membrane-immobilized vesicles as previously described.

**Vero Cell Verotoxin Cytotoxicity:**

Cells grown to confluency were trypsinized and 100 µL of $1.5 \times 10^5$/mL cell suspension was seeded in 96-well cell culture plates and grown for 24 h at 37°C prior to the experiment. Then, 100uL of 5pg/mL VT1 or VT2 in complete media was added and cell culture plates were further incubated at 37°C for another 72 h. In some experiments, toxins were pre-incubated with 2-fold serial dilutions of AdaGb3 or FSLGb3 starting at
100 µM for 1 hour at 37°C. At the end of the incubation period, the cells were fixed with 2% formaldehyde in PBS and stained with crystal violet as described [173]. The percentage of live cells was calculated from absorbance of destained cells read at 570 nm using a microtiter plate reader (Dynatech Laboratories, Chantilly, VA).

**Inhibition of Gp120 Binding to Vesicles:**
Prior to incubation with the immobilized vesicles, gp120 was incubated with either AdaGb₃ or FSLGb₃. Briefly, the mimics were dried down under nitrogen flow and re-dissolved in TBS to give concentrations of 300 µM. Gp120 was added to the solution at a concentration of 3 µg/ml. This solution was incubated at 37°C for 1 hour prior to its incubation with the vesicles composed of Gb₃, GalCer, Gb₄, cholesterol, or SGC for 2 hours at room temperature or overnight at 4°C. Finally, the solution was washed away (3x3min. with TBS) and bound gp120 was detected immunologically as previously described (see immunodetection).

**Immunofluorescent microscopy:**
Vero cells were grown, in which media on 12mm glass of cover slips overnight in 24 well plates. The media was removed and the cells were then incubated with fresh media along with VT-1 (1 µg/mL) along with AdaGb₃ (200 µM, 400 µM), FSLGb₃ (400 µM), or neither overnight at 4°C. The soluble compounds used had been pre-incubated with VT-1 for 1 hour prior to incubation with cells. The supernatants were washed away and fluorescent immunodetection of bound VT-1 was performed using anti-VT-1 (6869) followed by AlexaFluor488-green goat anti-rabbit secondary. Control coverslips were
prepared where incubation with VT1 was omitted. The coverslips were then washed again using media and the cells were fixed with a 4% formaldehyde solution. The coverslips were then placed in dH2O briefly (1 min.) before mounting with a fluorescent mounting medium (DAKO) and allowed to dry 5 minutes prior to visualization. The fluorescently-labelled cells were then visualized using a Leica DMIRE2 fluorescent microscope and images were acquired with a digital camera.

**Post-embedding immuno-electron microscopy:**

**Preparation of vesicles**
Gb₃:chol (2:1, m/m) vesicles were created by drying down Gb₃ and cholesterol in glass tubes under nitrogen. The reagents were resuspended in ethanol before being dried down once again under nitrogen flow. The sample was then re-suspended in MES buffer (0.2 mg/mL), vortexed for 1 minute, sonicated for 1 minute, and placed in a 55°C heat bath for 5 minutes. The vesicles were then left at RT for at least one hour. The vesicles were then concentrated by adding MES buffer to the solution and spinning down the vesicles at 34,000 rpm for 30 minutes (Beckman SW55Ti Rotor, Beckman 5ml ultracentrifuge tubes) at 20°C. In order to aid in the sectioning of the vesicles, the vesicles were stained with toluene blue dye for 1 minute before excess MES buffer was added, the solution was vortexed and centrifuged at 20,000 rpm for 30 minutes at 20°C. The pelletted vesicles were then air dried for at least 1 hour at RT. Vesicles were infiltrated in gelatin (20% in PBS) before cryo-freezing. Thin sections were cut using a Leica cryomicrotome and mounted on grids at the Advanced Bioimaging Centre, Hospital for Sick Children.
**Immuno-gold labeling of the sections**

For immunogold labeling, section grids were first washed by floatation on a drop of PBS. Sections were blocked with 1% BSA in PBS and then incubated with a 1:300 dilution of VT-1 stock (1.5mg/mL) in PBS for 1 hour at room temperature followed by washing then incubation with anti-VT1 (6869) at 1:200 dilution in PBS. After washing, bound antibodies were detected with a 1:50 dilution of 10 nm gold-conjugated protein A. Sections were post-fixed in 2% uranyl acetate in water for 15 min at room temperature before treatment with methyl cellulose. To determine the level of background labeling, vesicles which were not incubated with VT-1 were labeled with either anti-sera followed by protein A-gold or just by A-gold. Stained grids were imaged using a JEM2001 transmission electron microscope.

**Influenza infection assay:**

MDCK cells were grown on coverslips in 24 well plates in AMEM with 2% fetal calf serum (FCS) and trypsin (because it is beneficial to influenzaB growth) overnight to confluency. Prior to infection, a clinical isolate of Influenza B was pre-incubated, for 1 hour at 37°C, with Ada2-6GM3 (300µM), AdaGb3 (300µM), adamantane, or with just media. Cells were washed with buffer and incubated with the virus (+/- adamantyl compound) for 72 hours. Cells were washed again before detection of influenzaB, indicating infection, was done using the direct fluorescent antibody (DFA) technique [174]. Briefly a rhodamine-labelled anti-influenzaA/B monoclonal antibody was used to detect influenzaB that had infected the cells. The reagent for detection of the virus was SimulFluor® Flu A/Flu B and the protocol was completed according to the
manufacturer's recommendations [175]. These experiments were done at the department of Virology at the Hospital for Sick Children in Toronto, Canada.

**Gel Filtration Column Chromatography**

The 4961 gp120 sample (1µg/mL) was first dialyzed against the TBS (50mM tris-HCl, 150mM NaCl, pH 7) buffer before 110µL was loaded onto a Superdex 200 HR 10/30 column that had been washed with 2 column volumes of TBS. The column size exclusion limit is 1.3x10^6 M_r, the flow rate was 0.35mL/min and post column detection of collected fractions at 280nm was done by a connected HPLC system. Peak retention times were converted into molecular weight by comparing with a calibration curve prepared using proteins of known molecular weight. This experiment was carried out at the Rini laboratory at the University of Toronto.

**ImageJ:**

ImageJ software, a free image-processing program developed at the NIH, was used to quantify relative binding intensities visualized on TLC and nitrocellulose blots. The pixel density of bands were integrated and the background signals, obtained from appropriate regions of the blot, were subtracted in order to compare binding intensities as a function of the intensity of bands.
CHAPTER 3: RESULTS

3.1. Gp120 Characterization

The gp120 predominantly used in the experiments was characterized in order to ensure its functionality. This particular strain, obtained from the NIH AIDS Research & Reference Reagent Program, is an R5/BaL recombinant strain of gp120. Specifically, we wanted to determine whether the protein is monomeric/oligomeric, that it binds CD4, and its tertiary structure was maintained.

3.1.1 Non-Reducing Gel Electrophoresis

The sample was run on a non-reducing 8% SDS-PAGE gel and stained with Coomassie blue to show that the protein is 116 kDa molecular weight as indicated by the NIH AIDS Research & Reference Reagent Program from where it was obtained. A non-reducing gel was used in order to confirm that there are no free/un-paired sulfhydryl bonds on the protein that might allow it to crosslink to other monomers. The 4961 strain migrated to the 116 kDa position demonstrating a lack of disulfide bonds and that the protein was intact (fig. 9). Other R5 strains of gp120 were run on the non-reducing gel and three strains showed un-detectable bands in the 120 kDa region while one other strain (catalogue #7363) sample had a band just below the 130 kDa marker (fig. 10). Four of the samples had bands at the top of the gel and they may have been gp120 trimers in the 360 kDa area but the lack of a marker in this region prohibits the accurate determination of their mass. The functionality of the strains lacking detectable bands in the 120 kDa vicinity is further explored in section 3.2.2.
Fig. 10 SDS PAGE. Different R5 strains of gp120 obtained from the NIH AIDS Reference and Reagents Program were run on an 8% non-reducing SDS PAGE gel along with a lane of standard molecular weight markers.
3.1.2. Gel Filtration Column

The sample was separated on a high-resolution gel filtration column in order to confirm the oligomeric status of the protein under non-denaturing conditions. One peak was seen in the UV readout indicating that the sample was monomeric (fig. 11A). Importantly, there was no peak in the void volume indicating that none of the protein had aggregated which may have lead to non-specific binding to Gb3 and skewed protein quantitation. When the read-out peak was analyzed against a standard curve (known MW vs. volume or time of elution post-injection), the molecular weight of the protein was determined to be 165.8kDa (fig. 11b). The extra mass of the protein can be accounted for by glycosylation (the protein is recombinant). This shows that the sample used is indeed monomeric gp120.
Time post-injection = 37.8min – 2.16min -1.5min = 34.14 min.
Volume post-injection = 34.14min (0.35ml/min) = 11.95 mL
Std. curve trendline equation: y=10.4X +41.5
X=11.95, y=165.8

**Fig.11 Gel Filtration Column.** The 4961 strain of gp120 was run through a Superdex 200 HR 10/30 gel filtration column. The elution peak time (A) was converted into a volume based on the flow rate of 0.35mL/min and the volume was converted into a molecular weight based on a standard curve (B) for the column created using proteins of known molecular weights obtained from Bio-Rad.

### 3.1.3 Protein Assay

The concentration of the protein sample was also determined using a Bradford assay using BCA standards. A shortcoming of this assay is that detection levels are a result of only aspargine and the hydrophobic amino acids of the protein and differences in the amino acid composition between the standard protein and the sample protein lead to error. Nevertheless, the sample was found to be at a stock concentration of 1.2mg/mL (fig.12), which is near the indicated 1mg/mL. For the purpose of this study the exact
concentration of the protein is not of vital importance. Of importance are the qualitative findings leading to a better understanding of the interaction between gp120 and membrane-bound Gb₃.

### 3.1.4 Antibody Panel

To ensure that the protein was detectable in a specific manner a panel of anti-gp120 antibodies was directed towards the protein loaded onto a nitrocellulose membrane. All five antibodies tested recognized the strain and allowed for the convenient visualization of the protein (fig.13). An anti-verotoxin antibody did not recognize the protein. Importantly, Ab4 is a conformation specific antibody that only
recognizes gp120 in its tertiary structure and recognizes an epitope made up of sequences of the proteins V1, V3 and CD4 binding domains [169].

**Fig.13 Anti-gp120 Antibody Panel.** 5-fold dilutions of the 4961 gp120 strain were loaded onto a nitrocellulose membrane and incubated with 5 different anti-gp120 antibodies (1ug/ml) as well as VT1. Antibody binding was detected using HRP-conjugated secondary antibodies and visualized by 4-CN exposure. All 5 antibodies were bound to the protein at the 100ng level while no VT1 binding to gp120 was detected.
3.2 Gp120 Binding to Glycolipid cholesterol DRMs separated on a sucrose gradient

To study gp120 binding to Gb$_3$ within a lipid raft-like environment, vesicles composed of glycolipid and cholesterol were made in a 1% Triton-containing non-ionic MES buffer ensuring the membranes formed are detergent-resistant. The structures are separated by density centrifugation on a discontinuous sucrose gradient containing the theoretical raft fraction (‘fraction 5’ at the 5%/30% sucrose interface) where cell extracted DRMs migrate [176] [129].

3.2.1 Gp120 Binding to Glycolipid:Cholesterol DRMs

Previous studies show that while the majority of vesicles migrate to the raft fraction area, binding of VT-1 is only detected in fraction 1 +/- 2 (hereafter referred to as fraction A) (fig.5id). This same profile was also found for gp120 binding to Gb$_3$:chol, GalCer:chol, and SGC:chol vesicles, while Gb$_4$:chol vesicles did not bind gp120 (fig.14). Specifically, 18.7% of detected gp120 was found in fraction A whereas no binding was found in the fractions 3,4,5 and 6 (hereafter referred collectively to as fraction B) where 7%, 20%, 21% and 20% of total extracted Gb$_3$ was found. The remainder of the detected gp120 was in the densest fractions and was presumed to have been unbound gp120 that fell to the bottom of the tubes during ultracentrifugation. The majority of the cholesterol was also found in fraction B corresponding with the distribution of Gb$_3$. Gp120 also bound to GalCer in fraction A and not in the ‘theoretical raft fractions’. Binding in fraction A to GalCer was greater than that to Gb$_3$, corresponding to 22.2% of total gp120 detected (once again the remainder of gp120 was detected in the densest fractions) and extracted GalCer was found most in fractions 3(22%), 4(34%) and 5(19%). Gp120
binding to SGC:chol vesicles was the strongest with 25.1% of detected gp120 in fraction A while once again no binding was seen in fraction B where all of the extracted SGC was seen. Gp120 binding to Gb₄ vesicles in fraction A was negligible at 3.92%. The results were quantified using ImageJ pixel density integration software. Gp120 detected in the bottom (densest) fractions was considered not to have bound to any glycolipid structures and presumably it remained at the bottom of the tubes due to the high density of proteins. The lower bands in iv are residual sucrose.

Fig. 14 R5 HIV gp120 binding to GSL/cholesterol DRM constructs. DRM's were separated by gp120 containing sucrose gradient centrifugation and immobilized on nitrocellulose for gp120 immunodetection. i) gp120 binding to a-Gb₃, b-GalCer, c-Gb₄, d-SGC, gradient fractions, e- VT1 binding to Gb₃ sucrose gradient separated DRMs, ii) Gb₃ gradient fractions extracted and Gb₃ content determined by VT1/TLC overlay, iii) GalCer gradient fractions extracted and GalCer content determined by TLC/orcinol, iv) SGC gradient fractions extracted and SGC content determined by TLC/orcinol, v) detection of cholesterol after TLC of fraction extracts.
3.2.2 Different Gp120 Strains

Different strains of R5 gp120 were also tested for binding to the vesicles on the gradient. While only one of four other strains tested showed detectable binding (also only in fraction1), the non-binding strains were those that had no bands in the 120kDa area on the SDS-PAGE non-reducing gel (see fig. 8) suggesting that these strains had either formed aggregates or denatured into proteins which are unable to bind to the DRMs (fig.15). These findings aid in showing the specificity in monomeric gp120 binding to GSL DRM constructs.

![Image of binding patterns](image_url)

**Fig.14 Binding of different R5 gp120 strains to Gb3/cholesterol DRM constructs.** 4 different strains of gp120 (catalogue #s 4961, 7363, 3234, and 2968 all obtained from the NIH) were included in the separation of Gb3/cholesterol DRMs by centrifugation upon which fractions were immobilized on nitrocellulose and the different gp120s were immunodetected and visualized using ECL.

3.2.3 CT Binding to GM1:Cholesterol DRMs

This binding profile was also seen when the experiment was repeated using GM1:cholesterol vesicles and their ligand CT. CT binding to GM1 is well-characterized
and is known to occur in lipid rafts (it is the gold standard for ligand binding to glycolipid receptors within DRMs) [79]. Once again, the majority of the glycolipid and the cholesterol were found in the fraction B while binding was only detected in fraction A (fig.16). Specifically, all bound CT was found in fraction 1 despite 36% of the total GM1 being located in fraction B.

Fig.16 Cholera toxin binds a minor subfraction of GM1/cholesterol DRM constructs. Cholera toxin was added to GM1/cholesterol DRM constructs and vesicles separated by ultracentrifugation on a discontinuous gradient. After separation, fractions were tested for cholera toxin (upper panel) a,b,c increasing aliquot sizes, and extracted and GM1 separated by TLC (lower panel) and detected by orcinol. CT is only found in fraction 1. GM1 is distributed in the gradient but accumulates in fractions 5 and 6. Most GM1 is ‘invisible’ to CT.
3.2.4 AntiGSL Ligands

The Gb3/cholesterol DRM gradient assay was repeated with different glycolipid ligands to determine whether any ligand could bind GSL in fraction B and thereby whether the basis of non recognition is an asymmetric distribution of the glycolipids in these fractions (where they are found only on the inner leaflet of the membrane) or whether they are present on the outer leaflet and by some means not recognized by the ligands previously investigated. While an anti-GM1 antibody also bound only in fraction A and a mouse antiGb3 IgG antibody also bound only in fraction A, a rat anti-Gb3 IgM (38.13) antibody was found to bind in fraction A as well as in fraction B (fig.17) indicating that, at least for the Gb3 DRMs, the glycolipids are present on the outer leaflet but are presented in a manner that does not allow for the binding of gp120 or VT-1.

Fig. 17 Anti-GSL Antibody binding to separated GSL/cholesterol DRM constructs. The mouse monoclonal antiGb3 IgG BGS23 was compared with the rat monoclonal antiGb3 IgM (38.13) for binding sucrose gradient separated Gb3/cholesterol vesicles. The mouse IgG antiGb3 bound exclusively bound exclusively in fraction1. The rat IgM antiGb3 bound vesicles in fractions 4-6, corresponding to the majority of total Gb3, as well as fraction1. An anti-GM1 antibody also only bound in fraction1.
3.2.5 VSV-G Protein Binding to Glycolipid:Cholesterol DRMs

Gb₃/cholesterol DRM binding by VSV G-protein, the vesicular stomatitis virus adhesin protein, was also assessed. This protein, the adhesin on VSV that is responsible for its cell entry, was shown to bind to Gb₃ and GalCer as well as to Gb₄ (the one glycolipid that gp120 does not bind to) (fig.18). This demonstrates VSVs binding ability to glycolipids and may have implications on the life cycle of VSV as well as for other viruses of the same family.

**Fig.18 VSV surface adhesin protein G binding to GSL/cholesterol DRM constructs.** DRMs were separated by VSV-G sucrose gradient centrifugation and immobilized on nitrocellulose for VSV-G immunodetection. 75uL per fraction was loaded and anti-VSV-G Ab was used at 1µg/mL. VSV-G bound to all GSL/cholesterol DRM constructs tested (Gb₃, Gb₄, GalCer) and no VSV-G was detected in samples lacking VSV-G or replaced with gp120.

3.2.6 The Triton Effect

To determine the necessity of Triton in forming GSL undetectable vesicles, a VT-1 binding to Gb₃:cholestrol structures separated on the sucrose gradient was performed as
previously except that the buffer in which the structures were formed did not contain Triton. The lack of Triton did affect the binding pattern of VT-1 to the structures on the gradient. Significant binding was detected with decreasing intensity from fraction 1 to fraction 10 (fig.19). The lack of Triton also resulted in a redistribution of total Gb3 in the gradient. The extracted Gb3 was detected only in fraction 1 but presumably undetectable decreasing amounts were present in fractions 2-10 (fig.19). Triton, therefore, is necessary in order to promote the type of structure that migrates to the theoretical raft fraction densities.

**Fig.19 The Triton Effect.** Gb3/cholesterol DRM constructs with (+Triton) and without (-Triton) were separated by ultracentrifugation and immobilized on nitrocellulose prior to overlay with VT-1, which was immunodetected. The lack of Triton resulted in a redistribution of total extracted Gb3 that moved from fractions 3,4,5 (+Triton) to fraction 1 (-Triton) and a redistribution of VT-1 binding which moved from fraction 1 mainly (+Triton) to decreasing levels from fraction 1 onward (-Triton).
3.2.7 cryoIEM

In order to visualize the binding of VT-1 in fraction A and to further explore the lack of detected binding to the major structures extracted from fraction B, cryoimmuno EM was performed on sections of the fractions. Specifically, previous preliminary data showing differences in immunogold labeling to fraction A and fraction B was confirmed using the whole sample of structures prior to separation on a sucrose gradient. We were able to differentiate between the different structures because the preliminary data had clearly shown that all of the vesicles that migrate to fraction A are smaller, with diameters in the range of 100 nm, than those that migrate to fraction B which have diameters of approximately 250nm and also contain smaller internal vesicles [177]. The first major finding, which supports the binding pattern of intact vesicles, is that the gold labeled VT-1 was found mostly on the outside of the unilamellar structures (of ~100nm) typical of fraction A while few fraction B vesicles were labeled externally on their outer bi-lamellar membrane but were labeled internally on both their inner leaflet and internal vesicles. This lack of surface labeling agrees with the binding experiments on the sucrose gradient and suggests that Gb3 is somehow “absent” from the outer leaflet of the membrane or that more likely, the Gb3 on the outer leaflet is unavailable for VT-1 or gp120 binding. Furthermore, these larger fraction B vesicles were labeled on their inner leaflet which challenges the random nature of their construction. Also, smaller vesicles were found inside these larger fraction B vesicles and were labeled. Importantly, nothing in the fraction B vesicles was externally available for ligand binding which supports the binding experiments. Figure 20 shows a panel of the structures.
Fig. 20 Post embedding VT1 cryoimmunoelectron microscopy of Gb₃ DRMs.
VT1 immunogold labeling of sucrose gradient fraction B vesicles (panel A), fraction A vesicles (panel B) and unfractionated Gb₃/cholesterol vesicles (panels C-I).
Vesicles which are VT labeled internally only are panels A and C. Vesicles with surface labeling only B,E,G and H. Both vesicle types are in panels D,F and I. The VT1-antibody-gold complex is 50 Angstroms and thus surface binding is occurring only when gold is seen external to the vesicle limiting membrane. A 100nm-bar is shown for panels A and B.
3.2.8 Gb₃:cholesterol Ratios

The Gb₃:cholesterol ratios of fraction A and fraction B were quantified using radiolabeled cholesterol and by quantifying the Gb₃ content per fraction using the imageJ program on TLCs (VT1 overlays) of the extracted Gb₃. While this specific approach of determining the Gb₃ content lacks great precision, the results of pooled experiments showed very small error and showed that the ratio in fraction 1 (1.31) and fraction 5 (1.23) were too similar to account for the difference in binding or density (fig.21). The difference in density is likely explained by the fact that the larger, denser vesicles contain internal GSL/cholesterol vesicles inside while the smaller less dense vesicles are empty.

**Fig. 21 Gb₃:cholesterol ratios in fraction 1 and fraction 5.** The ratios of Gb₃ to cholesterol (mass:mass) for fractions A and B were determined using pooled results of the radiolabeled cholesterol counts and the integrated values of Gb₃ content using ImageJ on the TLC of extracted Gb₃.
3.3 Fatty Acid Heterogeneity

3.3.1 Fatty Acid Isoforms of Gb₃

The different naturally occurring isoforms of Gb₃, varying in fatty acid chain length and saturation, were isolated (describe in M&M) and used as a template to generate isoform specific vesicles of Gb₃:chol in the same manner as previously described [167]. The renal Gb₃ extracted from human kidney was de-acylated to lysoGb₃ (lacking the fatty acid) before coupling with specific fatty acids. The fatty acid composition of Gb₃, while not affecting the hydrophilic sugar head group, greatly affected its binding of gp120 (this was also previously found with VT-1 [178]). While the saturated C16 and C22 containing isoforms bound gp120 in the gradient spin at similar levels as renalGb₃ (once again all in fraction A) the saturated C18 and C20 containing isoforms did not bind gp120 and the C24 containing isoform bound gp120 in fraction1 at a decreased intensity. Furthermore, the unsaturated C24:1 containing isoform also did not bind gp120 at all (fig.22). This fatty acid dependency on binding was for the most part the same as was seen previously for VT-1 binding [178].
Fig. 22 R5 HIV gp120 binding to Gb3 fatty acid isoform/cholesterol DRM constructs. GSL/cholesterol DRMs were constructed using Gb3 fatty acid homologues separated on sucrose gradients in the presence of gp120. i) Aliquots of the gradient fractions (1-10) were immobilized on nitrocellulose and gp120 detected immunologically: a) renalGb3, b) C16Gb3, c) C18 Gb3, d) C20Gb3, e) C22Gb3, f) C24Gb3, g) C24:1Gb3, h) Gb4 vesicles, i) renalGb3 without gp120 in gradient. ii) The binding intensity in fraction 1 was quantified by integrating the positive signals using ImageJ software and the results are represented as the binding intensity relative to renalGb3 (ia) which showed the most intense binding.
3.3.2 Dominant negative and positive

Different mixtures of fatty acid dependent isoforms were combined in order to gain insight on the cause of this fatty acid dependent binding effect. Originally, when the natural frequency of each isoform was added to reconstitute the composition of renalGb3, the unsaturated C24:1 was omitted because unsaturated fatty acids are less likely to incorporate into ordered structures and may hinder their formation [179]. Surprisingly, C24:1 was necessary for VT-1 binding as the removal of this isoform eliminated all binding [178]. The same result was found with gp120 binding. The Gb3 isoforms reconstituted to their natural human renal frequency (Rec.), therefore corresponding to ‘renalGb3’ bound the same as renalGb3. When only the unsaturated isoform was removed (recon – C24:1, MIX) there was no gp120 binding indicating that C24:1 was required and suggesting that the fluidity is required. In the VT-1 experiments, it was found that the additional removal of either of the other non-binding isoforms (C18 and C20) compensated for the lack of C24:1 and restored binding [178]. This was not the case for gp120 binding where the removal of C24:1 along with either C18 or C20 did not induce binding. However the removal of C18 and C20 (in addition to C24:1) restored the binding of gp120 to fraction A. Interestingly, when only C18 and C24:1 are used to make the vesicles (along with cholesterol) (both isoforms do not bind separately) binding was restored to a level near renalGb3 (summation in fig.23).
Fig. 23 R5 HIV gp120 binding to different combinations of Gb3 fatty acid isoform/cholesterol DRM constructs. GSL/cholesterol DRMs were constructed using Gb3 fatty acid homologues separated on sucrose gradients in the presence of gp120. Aliquots of the gradient fractions (1-10) were immobilized on nitrocellulose and gp120 detected immunologically: renalGb3, reconstitutedGb3, MIX –C18Gb3 –C20Gb3 and C18Gb3 +C24:1Gb3 were all strongly bound by gp120 in fraction 1 while no binding was detected for Rec. –C24:1Gb3, MIX –C18Gb3 and MIX –C20Gb3.
3.4 Gp120 Overlay Binding

An assay was developed in order to monitor gp120 binding to Gb₃-containing membranes outside of the sucrose density ultracentrifugation process. This assay allows us to rapidly monitor gp120 binding in a system allowing the manipulation of conditions and treatment of the vesicles and of gp120. Furthermore, such a system alleviates the requirement for a 72-hour spin of the vesicles prior to detection.

3.4.1 Gp120 binding to un-separated vesicles on a nitrocellulose membrane

Detection of gp120 binding to Gb₃ vesicles (2:1 Gb₃:chol) loaded directly onto a nitrocellulose membrane and incubated with gp120 was achieved. A dose dependent response was seen when 3µg/mL of gp120 was overlaid on a membrane containing 40,20 and 10µg of glycolipid. Gp120 also bound to GalCer vesicles and did not bind to Gb₄-containing vesicles. There was no background when the primary anti-gp120 antibody was omitted (fig.24). Triton was not added to the vesicle preparation facilitating gp120 detection. Although 40 or 20µg are large quantities of GSL loaded in order to detect gp120 binding, it is assumed that this is necessary because only a fraction of the total Gb₃ is available on the vesicle surface.
3.4.2 Vesicles with and without cholesterol

Vesicles were made in the same manner as previously described except cholesterol was omitted. When the vesicles are composed of solely the glycolipid (Gb₃ or GalCer), stronger gp120 binding was detected compared to the 2:1 ratios of GSL:cholesterol (fig.25). Binding to Gb₄ vesicles with or without cholesterol was negligible.
3.4.3 Methylbetacyclodextrin (MBCD) Treatment

In order to strengthen the finding that the presence of cholesterol in the glycolipid vesicles inhibits gp120 binding, the effect of MBCD treatment of the vesicles was assessed. MBCD is a cholesterol-depleting agent commonly used in the field of lipid/membrane biology to deplete membranes of cholesterol [180]. When 2:1 vesicles of Gb₃:cholesterol were treated with 10mM MBCD for 45 minutes at RT, a significant increase in gp120 binding to these cholesterol depleted vesicles as compared to the untreated vesicles was observed (fig. 26).
3.4.4 sCD4 pre-treatment

Using this system, the effect of soluble CD4 pretreatment (150nM) of gp120 on binding to the vesicles was determined. Gp120 changes its conformation when it is CD4-bound in a manner that makes its V3 loop accessible for binding to chemokine co-receptors [39]. When gp120 was treated with 150nM sCD4 at 37°C for 1 hour an increased detection of gp120 binding to the vesicles at the 40µg and 20µg Gb₃-loaded level was seen (fig.27).

Fig.26 R5 HIV gp120 binding to Gb₃/cholesterol DRM constructs treated with MBCD. The constructs were treated with 10mM of the cholesterol-depleting agent for 45 minutes prior to gp120 overlay and subsequent gp120 immunodetection. MBCD (i.e. cholesterol depletion) enhanced the binding of gp120 to the Gb₃/cholesterol DRM constructs.
This was not surprising as it is also the V3 loop of gp120 which contains the glycolipid binding motif, GPGRAF [94]. This finding shows that gp120 has an increased affinity for Gb₃ when CD4-bound. However, gp120 binding to Gb₃, unlike binding to the chemokine co-receptors, is significant before gp120 changes conformation. Gb₃ is much smaller than CCR5 and CXCR4 and therefore it has access to the V3 loop pre-conformational change. Furthermore, the fact that Gb₃ is able to bind to gp120 and therefore the HIV virus before it binds to CD4 has considerable implications. This suggests that GSLs may act as alternate receptors for HIV infection on CD4⁻ cells since

**Fig. 27 R5 HIV gp120 pre-treated with sCD4 binding to Gb₃/cholesterol DRM constructs.** Gp120 was incubated with 150nM soluble CD4 for 1 hour at 37°C prior to overlay on Gb₃/cholesterol DRM constructs. This treatment enhanced the binding of gp120 to the constructs.
the conformational change in gp120 is not necessary for binding to the GSLs. One must then consider whether GSLs are able to promote the fusion and internalization of the virus into the host cell. It is possible that certain glycolipids, such as GalCer which has been suggested as an infection promoting GSL, promote the internalization of the virus whereas others inhibit infection and that a specific GSL-bound virion on the host cell surface either simply does not lead to internalization on CD4\(^+\) cells or sequesters the virion in a cell surface microenvironment where it cannot access pro-fusogenic receptors on CD4\(^+\) cells. The results of section 3.3 could also suggest that different fatty acid isoforms of Gb\(_3\) may be pro-fusogenic while others are anti-fusogenic and this could play a role in HIV infection (specifically in variable susceptibility).
3.5 Soluble Mimic Studies

Different soluble GSL mimics were tested for their ability to inhibit the binding of gp120 and of other ligands to their corresponding GSL receptors. AdaGb$_3$ and FSLGb$_3$ are known to inhibit HIV infection \textit{in vitro} and the following experiments aim to determine if they specifically inhibit gp120 binding to GSLs. Another aim was to determine the effect of FSLGb$_3$ on verotoxin-mediated toxicity and to compare it to AdaGb$_3$. Finally, another water-soluble adamantylGSL compound, Ada2,6GM3 was assessed for its ability to inhibit influenza infection.

3.5.1 VT-1 Binds to FSLGb$_3$

First, the ability of the semi-synthetic water-soluble mimic of Gb$_3$, FSLGb$_3$ (shown in intro fig. 7) to bind VT-1 was assessed. FSLGb$_3$, Gb$_3$, and other glycolipid standards were separated by TLC. Indeed, VT-1 does bind to FSLGb$_3$ as indicated by TLC overlay where VT-1 was detected immunologically. In fact, VT-1 binds with an appreciably higher affinity to FSLGb$_3$ than Gb$_3$ as indicated by the intensity of the bands. The control glycolipids were not bound by VT-1.
3.5.2 Soluble Mimic inhibition of gp120 binding to un-separated vesicles

AdaGb₃ and FSLGb₃ are semi-synthetic water-soluble analogues of Gb₃ that bind gp120 and inhibit infection when pre-treated with the virus [109]. These soluble mimics of Gb₃ should inhibit binding of gp120 to the Gb₃ containing membrane vesicles. This experiment is important in order to show both that gp120 remains bound to these soluble mimics and to determine whether or not the gp120-bound Gb₃ analogues can insert into the vesicle membrane. If this were the case the binding of gp120 by the analogues may not inhibit binding to the Gb₃ on the vesicles as the gp120 would not be removed. These two possibilities would be indistinguishable in our assay. Conveniently, this was not an issue. AdaGb₃ or FSLGb₃ pre-treatment of gp120 inhibited gp120 binding to the vesicles. The water-soluble compounds were used at 300µM (the
concentration shown to completely inhibit HIV infection of cells *in vitro* [109]) and were incubated with gp120 for 1 hour at 37°C prior to incubation of the mixture with the vesicles. Gp120 treatment with either compound did inhibit binding to the Gb3 vesicles with FSLGb3 demonstrating a greater inhibitory effect. Negligible binding was detected to Gb4 and to cholesterol only. This finding shows that occupancy of the glycolipid binding motif of gp120 inhibits further binding through this motif to the same sugar head group. Then, the effect of pre-treating gp120 with the water-soluble mimics of Gb3 on binding to other glycolipids (GalCer and SGC) that gp120 is able to bind to was examined. This is important in terms of characterizing the glycolipid binding motif of the V3 loop of gp120. Specifically, if a Gb3 occupied site is unable to inhibit binding to other glycolipids this would corroborate evidence that the same motif specifically binds to most glycolipids. Indeed, pre-treatment of gp120 with 300µM of either FSLGb3 or AdaGb3 for 1 hour at 37°C inhibits gp120 binding to GalCer and to SGC vesicles loaded on a nitrocellulose membrane compared to un-treated gp120 binding to the same vesicles. Once again FSLGb3 had a greater inhibitory effect than AdaGb3 and as previously shown [181], SGC was the best receptor for gp120 followed by GalCer and then Gb3. Gb4 was poorly bound by gp120.
Fig. 29 Soluble Gb3 mimic treated R5 HIV gp120 to GSL DRM constructs. gp120 was treated with 300uM of either FSLGb3 or AdaGb3 for 1hr at 37°C prior to overlay on GSL/cholesterol constructs and immunodetection of gp120. Untreated gp120 bound best to SGC constructs followed by GalCer then Gb3. Binding was negligible to cholesterol and to Gb4. Binding intensities to all were decreased with treatment of either compound and in general FSLGb3 had a greater inhibitory effect.
These results shed great insight on the mechanism of Gb₃-mediated inhibition of HIV infection. It has been suggested that GalCer and other glycolipids aid in the binding and fusion of HIV virions and therefore Gb₃, on the cell surface or AdaGb₃, might inhibit infection by inhibiting the interaction between gp120 and those other pro-infection GSLs. Perhaps more importantly, these results provide support for the hypothesis that Gb₃ inhibits HIV infection by competing for the chemokine co-receptor binding site on the V3 loop of gp120. The GPGRAF motif is found within the chemokine co-receptor binding motif on the V3 loop and therefore it is hypothesized that occupancy of the GPGRAF sequence inhibits binding of the chemokine co-receptors. The results demonstrate that occupancy of the GPGRAF motif by Gb₃ inhibits the subsequent binding of other V3 loop ligands and it is only reasonable to believe that this would also apply to the chemokine co-receptors, required for HIV infection in the majority of cases. Naturally, a future experiment is to test this hypothesis by specifically determining if Gb₃ bound gp120 is able to bind to the chemokine co-receptors.

3.5.3 FSLGb₃ Preventing Verotoxin induced Vero cell cytotoxicity

The possibility of the FSLGb₃ compound inhibiting verotoxin-induced Vero cell cytotoxicity was examined. It had previously been well characterized that AdaGb₃ is able to inhibit VT-1 and VT-2 mediated cell death [182] as this was the initial intended use for the semi-synthetic compound. Indeed, FSLGb₃ also inhibits VT-1 and VT-2 induced Vero cell death. VT-1 or VT-2 pre-treated with FSLGb₃ in the nano-picomolar range was able to inhibit Vero cell death in a dose-response sigmoidal curve (fig.30). Once again, FSLGb₃ proved to be a better inhibitor than AdaGb₃.
3.5.4 Soluble mimic inhibiting VT-1 binding

AdaGb3 and FSLGb3 pretreatment of VT-1 also both inhibit binding of VT-1 to Vero cells (fig.30). Vero cells were incubated with 1µg/mL of VT-1 for 16 hours at 4°C (retarding internalization of the toxin) and binding was detected by immunodetection.
with a fluorescently tagged anti-VT1 antibody and visualized using a fluorescent microscope. When the toxin was pre-incubated (1 hour at RT) with 200µM AdaGb3 the intensity of the green fluorescence significantly decreased indicating an inhibition of binding. At 400µM of either AdaGb3 or FSLGb3 there was no fluorescence and therefore binding to the cells was completely inhibited by the compounds. The contrast/background images show that intact cells, comparable to the untreated samples, were present in the treated slides and therefore the lack of binding was not due to cell death/disturbance.

**Fig.31 Soluble Gb3 mimic inhibition of VT1 binding to Vero cells.** To investigate the effect of the compounds specifically on binding of VT1 to Vero cells, the compounds were pre-incubated with VT1 at room temperature for 1 hour prior to a 16 hour incubation with the cells at 4 degrees to hinder internalization. Bound VT1 was detected immunologically and visualized using a fluorescently-labeled secondary antibody. AdaGb3 decreased the intensity of the green fluorescence, correlating with VT1 binding, at 200µM and no binding was seen at 400µM of either the AdaGb3 or FSL-Gb3 treated VT1 samples.
### 3.3.5 AdaGM3 Inhibition of Influenza Infection

Since another GSL virus interaction exists between sialoconjugates and influenza virus and since our lab had previously synthesized an Adamantyl-2,6GM3 compound known to bind to HA of the human virus, pre-incubation of this adamantyl compound was tested for its ability to inhibit flu virus infection. Pre-incubating virus with Ada-2,6-GM3, which binds to influenza virus adhesin molecule hemagglutinin inhibits infection of MDCK cells with a clinical FluB sample of the virus.

**Fig.32 Ada2,6GM3 inhibition of influenza virus host cell entry.** Prior to incubation with confluent MDCK cells, a clinical strain of influenzaB was treated with 300uM Ada2,6GM3 at 37°C. Internalized virus was visualized with a green fluorescence-tagged anti fluB antibody. Untreated virus was able to enter the cells (A) while the Ada2,6GM3 treated virus was unable to enter the cells (C). AdaGb₃ had no effect on internalization (B).
CHAPTER 4: DISCUSSION

4.1 Invisible Gb₃

It is apparent that a major portion of the total vesicular Gb₃ constructs (fraction B) is unavailable for gp120 binding. This is also the case for gp120 binding to GalCer and SGC composed vesicles as well as CT binding to GM1 and VT1 binding to Gb₃. Initially, two possible explanations should be considered; either that these glycolipids were somehow absent from the outer leaflet of the outer membrane or that they are present but in a conformation/presentation wherein they are not recognized by their ligands. The latter was considered more likely as even if the glycolipids were not present on the outer leaflet of the membrane, cholesterol alone in the outer leaflet should not be able to form the vesicles [183] which are clearly seen in the cryoIEM images. Either way, Gb₃ is now known to be present on the outer membrane due to the finding that the rat anti-Gb₃ antibody binds in fraction B. A current experiment is to obtain cryoIEM images of the rat antibody binding to the vesicles and this ligand will likely be bound to the outer leaflet of the outer membrane of fraction B, unlike VT-1 which only labeled such vesicles internally.

The requirement for different hydroxyl groups within the Gb₃ carbohydrate moiety for binding to Mab38.13 as well as VT1 were previously compared. It was shown that Mab38.13, but not VT1 or VT2, bound Gb₃ when the 3OH of the beta-galactose and the 3OH of the glucose residue were deleted [92]. Therefore, if this region of the molecule was obscured by cholesterol in fraction B this could explain why only Mab38.13 bound. Both the 3OH of the β-galactose and the 3OH of the glucose impinge on the rotation around the α/β galactose and the galactose/glucose glycosidic linkages
respectively. This suggests that the deoxy analogues could rotate more freely to prevent VT1 binding. It is possible that an altered conformation of Gb$_3$ and cholesterol in fractionB results from an altered rotation around the glycosidic bonds ultimately preventing gp120 and VT1/2 binding but still permitting Mab 38.13 binding.

It should be noted that our protocol differs from most other DRM preparations in that the 72 hours of spinning is longer than the typical 18-20 hours and our protocol uses 5mL ultracentrifuge tubes as opposed to the typical preparations which use 10mL tubes [129]. When the experiments were repeated at the 10mL tube scale we did not achieve the separation of the different types of structures. It is possible therefore that in most other preparations of density gradient separated DRM constructs that the two different types of structures that are found are not separated due to a longer migrational distance (due to the different tube sizes) and/or a shorter ultracentrifugation period.

One obvious question is what is the cause of the difference in density between the different structures as this may help in determining the cause in their differential binding capabilities. Since our model only includes glycolipid, cholesterol, and Triton (which is found equally distributed throughout the gradient) the ratio of Gb$_3$ to cholesterol was examined. As seen in the results section, the ratios were too similar between fractions 1 and 5 for this to be the cause. The difference in density may be the result of the internal structures that are found in fraction B increasing their density as opposed to the fractionA vesicles that are empty. Also, the differences in hydration state of the different structures may be affecting their density. Another difference is the radii of curvature between the two structures as the fraction B vesicles are larger than the fraction A vesicles. A future experiment will examine Laurdan staining on fraction A and fraction B isolated vesicles in order to determine possible differences in membrane order between the two structures [184].
A similar, but less drastic, difference between the maximal Gb3 content and the maximal VT-1 binding fractions is seen with cell extracted detergent-resistant membranes, which obviously have more components compared to the model Gb3 DRMs. Likely, some of these additional components are responsible for decreasing the discrepancy in density between the two forms of DRM Gb3. Nevertheless, the fact that this phenomenon is also seen in cells supports our model raft-like vesicles as a means of studying ligand binding to low density raft-like structures. Due to the fact that gp120 is more permiscuous in its GSL binding than VT-1, together with the fact that the chemokine co-receptor CCR5 is abundantly expressed, cell DRM experiments were not repeated with gp120 as the results could be ambiguous and since, as already stated, the VT1 data supports our artificial vesicle model for gp120 binding.

The findings of this section of experiments have broad-spectrum implications on the biochemistry of cell membranes and glycosphingolipids therein. In the past it was believed that GSLs are found on the outer leaflet of the plasma membrane and no studies known have challenged either this belief or that all of the membrane GSL is available to bind its ligand. Future studies are required in order to better understand these findings and will certainly involve more electron microscopy and Laurdan in order to better visualize the structures (detect hydration) and determine the cause of the Gb3 unavailability on the larger vesicles. It is possible that the GSLs do not arrange in a manner typical of the fluid mosaic model where the hydrophilic moieties line up on the extracellular-vesicular side and the hydrophobic tails align uniformly facing inwards. Perhaps the GSLs in fraction B are twisted somehow such that they do not align ideally and their carbohydrate binding sites become unavailable for ligand binding.
Regarding HIV infection, these results show that gp120 does in fact bind to low-density ‘raft-like’ membrane microdomains. They support the model system for further use for studying such interactions. Future studies should aim to examine X4 strains of gp120 as well as begin to add additional cellular factors (lipids, etc.) to the vesicles and different mixtures of glycolipids thought to play a role in HIV infection. Once again, the broad-spectrum implications of glycolipid membrane biochemistry might shed some light on the role of Gb3 and glycolipids in HIV infection.

Previous data has supported the hypothesis that Gb3 inhibits HIV infection and ongoing studies show an inverse relationship between cellular Gb3 expression and infection [111]. Along with the findings that gp120 binds to Gb3 within low-density microdomains it can be hypothesized that unexplained differences in susceptibility throughout the population may be due to variable Gb3 expression and more specifically the amount of expressed Gb3 found in the different microdomain structures represented by fractions A and B that could be representative of similar domains on cell membranes.

4.2 Fatty Acid Heterogeneity

Changes in fatty acid chain length of as little as 2 carbons can effectively reduce gp120 binding from maximal (renalGb3) levels to undetectable and that an unsaturated FA does not bind at all whereas the corresponding saturated FA binds at near maximal levels.

It is known that unsaturated fatty acids promote a more fluid, disordered membrane [185] and this may be why the C24:1 containing isoform alone does not bind. The reason that the C18 containing isoform does not bind alone and that the C20
containing isoform binds weakly may be due to their parity with their neighbouring sphingosine molecule, which is 18 carbons and contains a double bond. This parity may result in a highly rigid tightly packed membrane that does not allow for the ligands to recognize the sugar head group of Gb₃ and bind to the structures. A balance between a highly ordered (C18 and C20) and highly disordered (C24:1) membrane arrangement is required in order for gp120 to bind to Gb₃ where perhaps there is enough order to permit recognition and lateral mobility to allow clustering of Gb₃ for gp120 to bind. The best support for this argument is certainly the sample containing only the non-binding C18 and C24:1 containing isoforms which together are able to bind gp120 highly effectively.

Future experiments will aim to prove that the C18 and C20 containing isoforms promote highly rigid membranes, and that this is due to parity between them and their adjacent sphingosine molecule. In order to determine the relative order of the membranes of the different isoform-containing DRMs, Laurdan staining will be performed to see differences in its incorporation. Laurdan is a chemical that exchanges its fluorescent properties uniquely with membrane microdomains of increased order (ie. lipid rafts) [184]. In order to determine whether the C18 and C20 isoforms do not bind is because of their parity with sphingosine creating ‘too rigid’ a structure, the length of the sphingosine molecule on the different fatty acid containing isoforms of Gb₃ will be biochemically manipulated. If our hypothesis is correct, decreasing or increasing the length of sphingosine on a C18 or C20 fatty acid containing isoform should restore binding whereas decreasing or increasing the sphingosine chain length on C16 or C24 fatty acid containing isoforms should abrogate their binding to gp120. Laurdan staining can be performed on these newly created isoforms in order to confirm that the effect of
hydrophobic chain length on ligand binding is in fact due to changes in membrane organization and therefore Gb$_3$ head group presentation/availability.

Another future aim is to investigate whether these isoform dependent binding affinities translate into differential infection by adding individual isoforms of Gb$_3$ to HIV susceptible cells by liposomal fusion. This was previously achieved using renalGb$_3$ where fusion of the Gb$_3$-containing liposome with the previously Gb$_3$-lacking cells inhibited HIV infection [109]. Alternatively, this can be achieved by transfecting cells with a family of Lass ceramide synthase genes that have fatty acid selectivity [86] [85]. The gp120 binding isoforms may inhibit HIV infection proportionally to their binding affinities and that the non-binding isoforms may have no effect on infection.

In general, these results have relevance in regards to membrane GSL biochemistry and the role that this might play in HIV infection, specifically variable susceptibility in the general population. Although fatty acid chain length of membrane molecules affects membrane organization the mechanism has not been proven. The future studies proposed would aid in proving the relationship between fatty acid heterogeneity and membrane order. Once this relationship has been established fatty acid dependent isoforms of glycolipids (and possibly other fatty acid conjugated membrane molecules) might be used as tools to alter membrane order. The results demonstrate the impact that GSL isoform composition in cells may have on infection. Changes in isoform expression may alter GSL incorporation into lipid ordered domains, which may alter their ability to bind gp120. Furthermore, Gb$_3$ isoforms may affect HIV infection, without binding gp120, based on their variable presence within lipid ordered domains as their presence may be required for the changes in membrane curvature during fusion. Variable expression of
Gb₃, and other glycolipid isoforms may represent an unknown variable in HIV susceptibility whereby the specific expression levels of the different isoforms may be a determinant of their susceptibility via membrane order.

4.3 Gp120 Overlay

The gp120 vesicle overlay studies represent an appropriate model for studying the binding of gp120 to glycolipid-enriched membrane microdomains. In this protocol, gp120 binds preferentially to Gb₃ vesicles without or with low levels of cholesterol. This contrasts with reports that productive HIV infection requires cholesterol perhaps due to its promoting liquid ordered domains [141]. Gp120 binding to more fluid, non-raft Gb₃ could provide the basis for our demonstration that Gb₃ can be an HIV resistance factor [111]. This inhibitory role of Gb₃ in HIV infection may due in part to pre or post-CD4 binding of gp120 to Gb₃ sequestering HIV virions in membrane microdomains lacking the required cholesterol levels (and associations with the co-receptors that accompany high cholesterol) for infection.

Also, the finding that gp120 is able to bind to Gb₃ through its V3 loop without the CD4-induced conformational change would suggest that if indeed there is a competition between Gb₃ and the chemokine co-receptors for gp120 that Gb₃ would have a competitive advantage in that it is able to bind to gp120 before the conformational change, unlike CCR5 and CXCR4, likely due to the fact that it is an appreciably smaller molecule than the protein co-receptors and therefore has access to the somewhat protected V3 loop domain of gp120.
4.4 Inhibition of binding by soluble mimics

The soluble mimic studies effectively provide more knowledge on the mechanism whereby AdaGb₃ and FSLGb₃ inhibit HIV infection. Pre-treatment of gp120 with these compounds decreases their binding to Gb₃ and if it is assumed that V3 loop occupancy by glycolipids inhibits the further binding of gp120 to the chemokine co-receptors than logically the inhibition of viral replication, at the fusion step, is in part due to a decreased binding capability of gp120 to the chemokine co-receptors [109], although the carbohydrate-bound protein is likely still able to attach to host cells via CD4 which binds a different domain on gp120.

In the future these ideas will be further explored by investigating the effect of these compounds on the binding ability of gp120 to CD4 and to the chemokine co-receptors. This will require an assay in which such interactions can be monitored in a membrane-like format which may prove to be more challenging than the current DRM model because it is not known how these proteins will incorporate into the vesicles and whether the increase in density will allow us to study them on the discontinuous sucrose gradient.

The finding that these Gb₃ analogs inhibit gp120 binding to other gp120-binding glycolipids (GalCer SGC) supports the existence of a single glycolipid binding motif in the gp120 V3 loop which in turn aids the argument that occupancy of this motif, found within the chemokine co-receptor binding motif, inhibits subsequent binding of gp120 to its co-receptors. This better understanding of the mechanism whereby these soluble Gb₃ mimics inhibit HIV infection should aid in further elucidating the role of GSLs in infection and may reveal possible ways of optimizing their use against infection.


