SENSITIVITY OF HIV-1 SUBTYPE C VIRUSES TO GRIFFITHSIN, CYANOVIRIN-N AND SCYTOVIRIN: POTENTIAL HIV-1 MICROBICIDES

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A thesis submitted to the Faculty of Medicine, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy.

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DECLARATION

I declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Kabamba Bankoledi Alexandre

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ABSTRACT

The majority of HIV-1 infections around the world occur via sexual intercourse and women, especially in developing countries, are disproportionately affected. Recently a number of strategies have been proposed to control the spread of HIV, among these the use of microbicides to prevent the sexual transmission of the virus. A clinical trial of 1% tenofovir gel that conferred up to 39% protection provided a proof-of-concept that an anti-HIV microbicide is feasible. Various other compounds, acting at different stages of HIV-1 life cycle, are also being investigated as potential microbicides. These include the lectins Griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN). GRFT was isolated from the red algae *griffithsia sp*. while CV-N and SVN were isolated from the blue green alga *Nostoc ellipsosporum* and the cyanobacterium *Scytonema varium*, respectively. These lectins bind mannose-rich glycans found on the surface of HIV-1 envelope and act as entry inhibitors. Although HIV-1 subtype C is the main cause of infections around the world, almost all studies conducted with GRFT, CV-N and SVN are based on subtype B viruses. The Chapter Two sought to establish the neutralization sensitivity of HIV-1 subtype C viruses to the three lectins, using both a cell line and primary cells, and compared this sensitivity to subtype B. This Chapter also examined mannose-rich glycans on HIV-1 that are involved in GRFT, CV-N and SVN binding. The conclusion from this study was that the neutralization of subtype C viruses by these lectins is similar to subtype B and that the 234 and 295 mannose-rich glycans were involved in their interaction with the virus. In general these data supported further studies on the use of GRFT, CV-N and SVN for prevention of HIV-1 subtype C sexual transmission. In Chapter Three, the ability of GRFT to expose the CD4 binding site

(CD4bs) on HIV-1 gp120 is explored. I found that this exposure resulted in the enhancement of HIV-1 binding to plates coated with anti-CD4bs antibodies b12 and b6 or the CD4 receptor mimetic CD4-IgG2. This lectin also synergized with b12 and HIV-positive plasma containing antibodies to the CD4bs to neutralize the virus. Furthermore, the glycan at position 386, which shields the CD4bs, was shown to be involved in both GRFT enhancement of HIV-1 binding to b12 and b6 and in the synergistic interaction between the lectin and these antibodies. The importance of this study is that it investigated in details the effect of GRFT binding on HIV-1 envelope and also suggests this lectin can be used in combination with anti-HIV-1 antibodies to synergistically enhance the anti-viral activity. In Chapter Four I investigated GRFT, CV-N and SVN inhibition of the virus binding to the DC-SIGN receptor and their inhibition of the DC-SIGN transfer of HIV-1 to target cells. These lectins only moderately inhibited the virus binding to the receptor while they potently inhibited its transfer to target cells. However, the inhibition of transfer was stronger when the virus bound the lectins after binding the DC-SIGN receptor compared to when it bound the lectins prior to binding the receptor. These three lectins can, therefore, inhibit the sexual transmission of HIV-1 since the DC-SIGN-mediated transfer of the virus to susceptible cells is pivotal to this mode of transmission. Chapter Five is an investigation of the ability of HIV-1 subtype C to escape GRFT, CV-N and SVN, which involved growing the virus under escalating concentrations of these compounds. This was to know how this virus behaves under conditions of continuous exposure to the lectins. I found that HIV-1 subtype C became increasingly resistant to the lectins and viral envelope sequence analysis showed that this was associated with the deletion of mannose-rich glycans on gp120. Furthermore, of the

11 potential mannose-rich glycosylation sites on gp120 seven (230, 234, 241, 289, 339, 392 and 448) were involved in GRFT, CV-N and SVN resistance. Thus, the conclusion was that although these three lectins are potent inhibitors of HIV-1 infection, the virus is also able to escape their neutralization by deleting mannose-rich glycans on its envelope. However, the fact that escape to these lectins involved multiple deglycosylation and was only partial suggests that HIV-1 subtype C escape from GRFT, CV-N and SVN in a microbicide formulation may not be an easy process. We discuss the implications of these findings in Chapter Six and suggest future studies that could complement data presented in this thesis. Overall our data show that GRFT, CV-N and SVN can prevent the sexual transmission of both free and DC-SIGN associated HIV-1 particles and supports further development of these lectins as microbicides against HIV-1.

PUBLICATIONS FROM THIS THESIS

Kabamba B. Alexandre, Elin S. Gray, Bronwen E. Lambson, Penny L. Moore, Isaac A. Choge, Koleka Mlisana, Salim S. Abdool Karim, James B. McMahon, Barry R. O'Keefe, Rachel Chikwamba and Lynn Morris. Mannose-rich glycosylation patterns on HIV-1 subtype C gp120 and sensitivity to the lectins, griffithsin, cyanovirin-N and scytovirin. 2010. Virology 402: 187-196.

Kabamba B. Alexandre, Elin S. Gray, Ralph Pantophlet, Penny L. Moore, James B. McMahon, Ereck Chakauya, Barry R. O'Keefe, Rachel Chikwamba and Lynn Morris. Binding of the mannose-specific lectin, griffithsin, to HIV-1 gp120 exposes the CD4-binding site. 2011. Journal of Virology 85 (17): 9039-9050.

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Kabamba B. Alexandre, Penny L. Moore, Elin S. Gray, Ereck Chakauya, James B. McMahon, Barry R. O'Keefe, Rachel Chikwamba and Lynn Morris. The mechanism of HIV-1 subtype C escape from griffithsin, cyanovirin-N and scytovirin. In preparation.

OTHER PUBLICATIONS

Morris L., M. Coetzer, E.S. Gray, T. Cilliers, **K.B. Alexandre**, P.L. Moore and J.M. Binley. Entry inhibitors of HIV-1 subtype C isolates, in Entry Inhibitors in HIV Therapy. J.D. Reeves, C.A. Derdeyn, editors. 2007. Pages 119-131. Birkhäuser, Germany.

Hazel Tumelo Mufhandu, Elin Solomonovna Gray, Maphuti Carol Madiga, Nancy Tumba, Thandeka Khoza, **Kabamba Bankoledi Alexandre**, Kurt Wibmer, Lia Rotherham, Lynn Morris and Makobetsa Khati. UCLA1 a Synthetic Derivative of a gp120 RNA Aptamer Inhibits Entry of Human Immunodeficiency Virus Type 1 Subtype C Isolates (In press in the Journal of Virology).

PRESENTATIONS AT MEETINGS

Kabamba Alexandre, Elin Gray, Penny Moore, Natasha Taylor, Rachel Chikwamba, Barry O'Keefe, James McMahon and Lynn Morris.
Sensitivity of HIV-1 subtype C viruses to Griffithsin and Scytovirin: potential HIV microbicides.
Poster presentation at the 2006 International HIV Microbicide Conference.

Kabamba Alexandre, Elin Gray, Penny Moore, Natasha Taylor, Rachel Chikwamba, Barry O'Keefe, James McMahon and Lynn Morris.
Sensitivity of HIV-1 subtype C viruses to Griffithsin and Scytovirin: potential HIV microbicides
Oral presentation at the 2006 National Institute for Communicable Diseases Research Day.

Kabamba Alexandre, Elin Gray, Penny Moore, Natasha Taylor, Rachel Chikwamba, Barry O'Keefe, James McMahon and Lynn Morris.
Sensitivity of HIV-1 subtype C viruses to Griffithsin and Scytovirin: potential HIV microbicides.
Poster presentation at the 2007 National (South Africa) AIDS Conference.

Kabamba Bankoledi Alexandre, Elin Gray, Ralph Pantophlet, Rachel Chikwamba, James McMahon, Barry O'Keefe and Lynn Morris.
IgG1b12 binding of HIV-1 is increased in the presence of the mannose-binding lectin, Griffithsin.
Poster presentation at the 2008 National Institute for Communicable Diseases Research Day.

Kabamba Bankoledi Alexandre, Bronwen Lambson, Elin Gray, Rachel Chikwamba, Koleka Mlisana, Salim Abdool Karim, James McMahon, Barry O'Keefe and Lynn Morris.
Entry inhibition of HIV-1 subtype C from blood and vaginal mucosa by the lectins Griffithsin, Cyanovirin-N and Scytovirin: potential HIV microbicides.
Poster discussion talk (short oral presentation) at the 5th International AIDS Society Conference on HIV Pathogenesis, Treatment and Prevention in 2009.

Kabamba Bankoledi Alexandre, Elin Gray, Penny Moore, Ralph Pantophlet, Rachel Chikwamba, James McMahon, Barry O'Keefe and Lynn Morris.
Binding of the mannose-specific lectin, Griffithsin, to HIV-1 gp120 exposes the CD4-binding site.
Oral presentation at the University of the Witwatersrand Research Day in 2010.

Kabamba Bankoledi Alexandre, Elin Gray, Penny Moore, Ralph Pantophlet, Rachel Chikwamba, James McMahon, Barry O'Keefe and Lynn Morris.

Binding of the mannose-specific lectin, Griffithsin, to HIV-1 gp120 exposes the CD4-binding site.
Poster presentation at AIDS Vaccine 2010 Conference.

Kabamba B. Alexandre, Elin Gray, Ralph Pantophlet, Penny L. Moore, Bronwen E. Lambson, Isaac A. Choge, Koleka Mlisana, Salim S. Abdool Karim, James B. McMahon, Rachel Chikwamba, Barry R. O'Keefe and Lynn Morris.
The anti-HIV lectin griffithsin: inhibition of clinical isolates, enhancement of anti-CD4bs antibody binding, and oligosaccharide-specific effects on potency.
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Kabamba B. Alexandre, Elin S. Gray, Hazel Mufhandu, Penny L. Moore, Ereck Chakauya, James McMahon, Barry O'Keefe, Rachel Chikwamba and Lynn Morris.
The C-type lectins Griffithsin, Cyanovirin-N and Scytovirin inhibit HIV-1 interaction with the DC-SIGN receptor.
Oral presentation at the 5th South African AIDS Conference.

Kabamba B. Alexandre, Elin S. Gray, Hazel Mufhandu, Penny L. Moore, Ereck Chakauya, James McMahon, Barry O'Keefe, Rachel Chikwamba and Lynn Morris.
The C-type lectins Griffithsin, Cyanovirin-N and Scytovirin inhibit HIV-1 interaction with the DC-SIGN receptor.
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Kabamba B. Alexandre, Penny L. Moore, Elin S. Gray, Ereck Chakauya, James B. McMahon, Barry R. O'Keefe, Rachel Chikwamba and Lynn Morris. The mechanisms of HIV-1 subtype C resistance to GRFT, CV-N and SVN. Oral presentation at the 2012 International Microbicide Conference.

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ABBREVIATIONS

UNAIDS	United Nations Programme on HIV/AIDS
HIV-1, HIV-2	Human Immunodeficiency Virus Type-1, -2
SIV	simian immunodeficiency virus
AIDS	Acquired Immunodeficiency Syndrome
gp41, gp120	glycoprotein 41 KDa, 120 KDa
CD4	cluster differentiation 4
CD4bs	CD4 binding site
CD4i	CD4 induced
Env	envelope glycoprotein
V1, V2, V3, V4 and V5	variable regions 1-5
RNA	ribonucleic acid
CCR5	C-C chemokine receptor type 5
CXCR4	chemokine (C-X-C motif) receptor 4
P24	Gag protein 24KDa
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin
CDR	complementarity determining region
GRFT	griffithsin
CV-N	cyanovirin-N
SVN	scytovirin
MAb	monoclonal antibody
µL	microliter
µg	microgram
mg	milligram
CAPRISA	Centre for the AIDS Program of Research in South Africa
CAP	CAPRISA
WT	wild type
IgG	immunoglobulin G
MPER	membrane proximal external region
$TCID_{50}$	50% tissue culture infectious doses
IC_{50}	50% inhibitory concentration
ID_{50}	50% inhibitory dilution
PBMC	peripheral blood mononuclear cells
DMEM	Dulbecco Modified Eagle's Media
RPMI medium	Roswell Park Memorial Institute medium
DEAE-dextran	diethylaminoethyl-dextran
PBS	phosphate buffered saline
ELISA	enzyme linked immunoabsorbent assay
CBA	carbohydrate binding agent
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
sCD4	soluble CD4

CHAPTER 1
INTRODUCTION

1.1. Background

According to the 2009 World Health Organization report on the global AIDS epidemic there were 33 million people living with HIV in 2008 (http://www.unaids.org). The same report estimated the number of new HIV infections to be 2.7 million during the same year with the overall AIDS death rate reaching 2 million. HIV is mainly transmitted through sexual intercourse that accounts for ~ 80% of infections around the world (http://www.unaids.org).

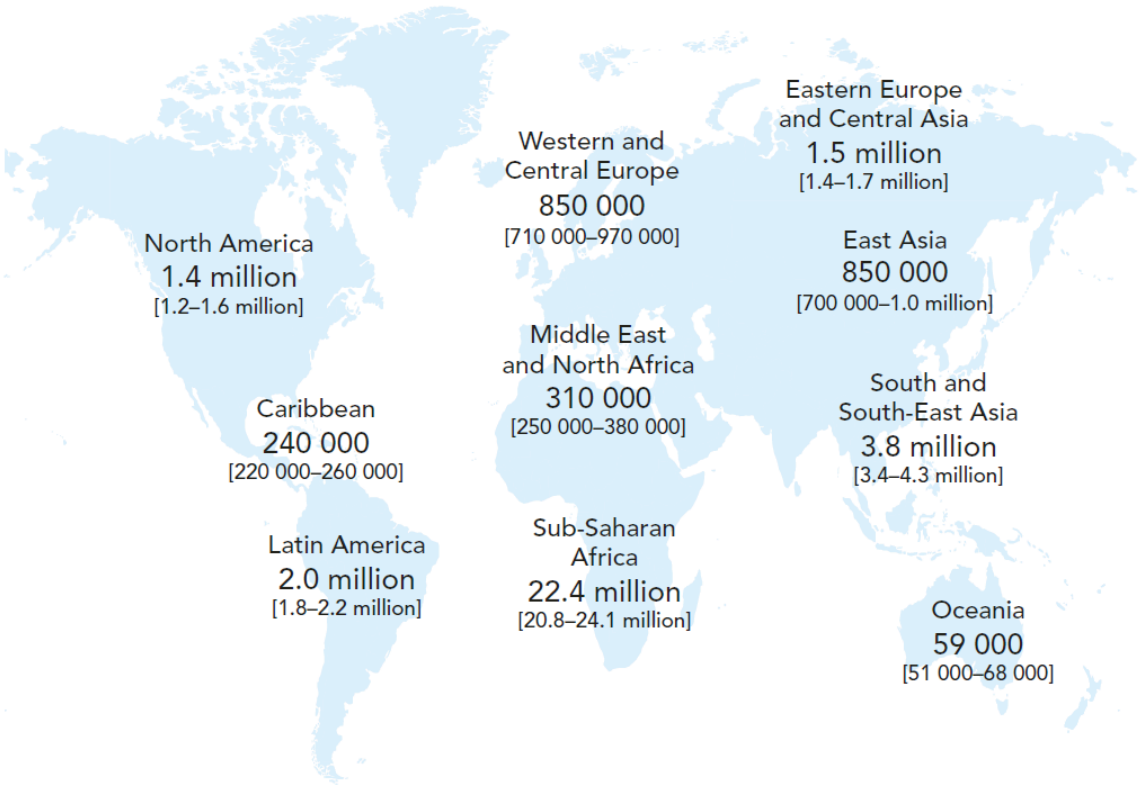


Figure 1.1: The estimated number of people living with HIV/AIDS in 2008 by region. The geographic distribution of the world population living with HIV (adult and children combined) as published by the World Health Organization report on global AIDS (http://www.unaids.org).

Globally sub-Saharan Africa remains the most affected region (Figure 1.1) with an estimated 22.4 million people infected with HIV. In 2008 ~ 75% of all deaths

caused by the virus occurred in this region. However, the HIV epidemic is most severe in Southern Africa with a prevalence ranging from 10-28% in the adult population (Figure 1.2).

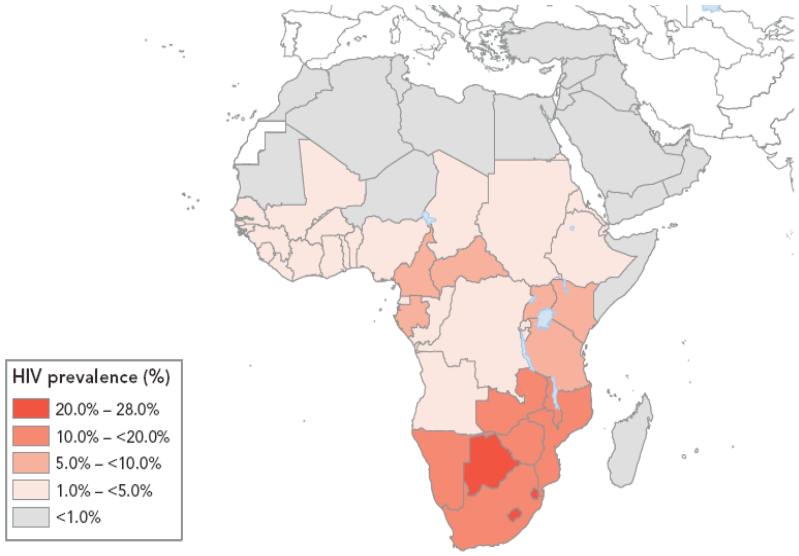


Figure 1.2: HIV mortality and prevalence in Africa. Geographic distribution of HIV prevalence in the adult population in Africa published in 2007. Warmer colour indicate higher prevalent of the virus (http://www.unaids.org).

Compared to men, women are more affected by the HIV epidemic and this is more pronounced in Sub-Saharan Africa where women constitute ~ 60% of all cases of HIV infection (83). Furthermore, the proportion of females infected with HIV compared to males is higher in the 15 to 24 years old age category as a study conducted in a number of Sub-Saharan African countries revealed (Figure 1.3).

The discovery of an effective vaccine against HIV-1 remains the main focus of HIV prevention strategies (94). However, the genetic diversity of HIV poses a serious challenge to this effort which is exacerbated by the fact that the correlates of protection for an effective HIV-1 vaccine are unknown (49, 73, 94). Due to this, many compounds with the ability to block HIV-1 entry and replication in target cells are being proposed as potential microbicides to prevent the sexual transmission of HIV. Amongst HIV-1 microbicide candidates are found non-specific inhibitors such as

buffering agents that inactivate viruses by maintaining the acidic pH in the vaginal tract; moderately specific inhibitors such as anionic polymers that neutralize the virus by binding to its positively charged envelope glycoproteins; antiretroviral agents such as tenofovir that target HIV-1 reverse transcriptase (39); and lectins such as griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN) that bind to HIV-1 envelope to inhibit its entry into target cells (17, 22, 103).

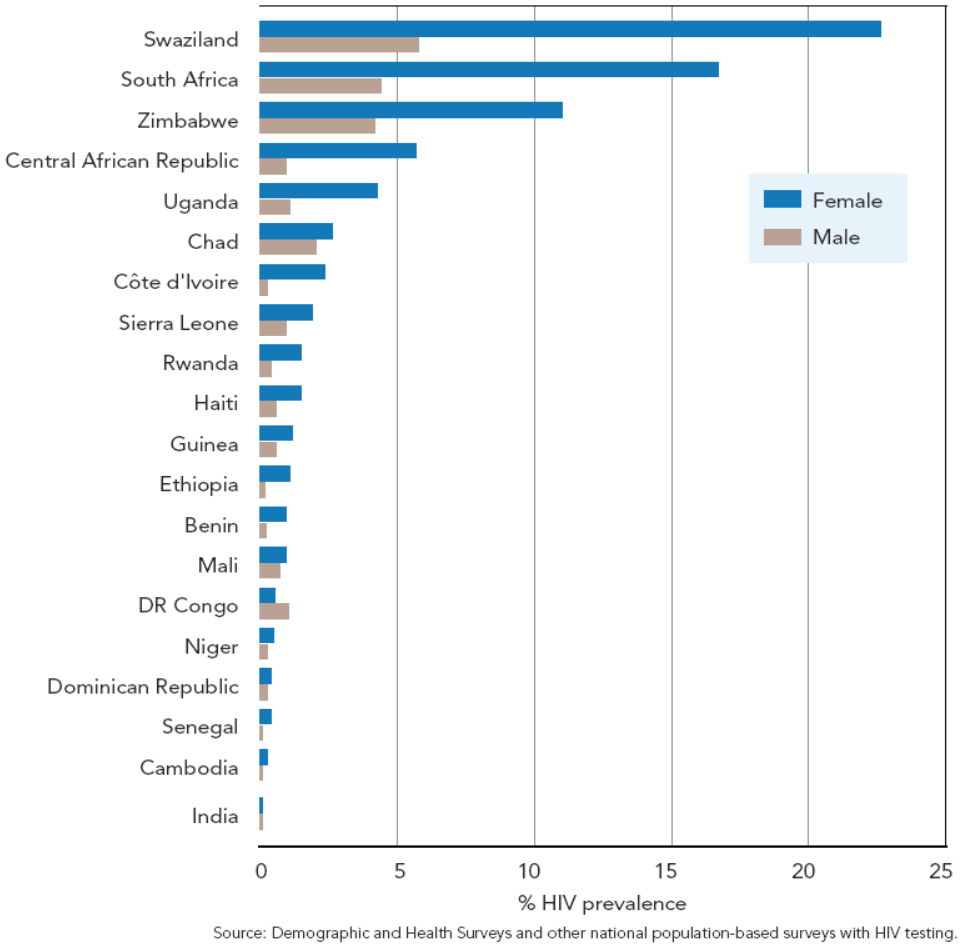


Figure 1.3: Comparison of HIV prevalence between males and females in the 15 to 24 years old age category. Young females were found to be disproportionately more infected by HIV than young males in all 20 countries selected for the study except the Democratic Republic of Congo and India (http://www.unaids.org).

1.2. HIV structure, genetic diversity and life cycle

HIV belongs to the genus lentivirus and family *Retroviridae* (76). A mature HIV particle is made of an outer lipid bilayer (envelope) (Figure 1.4) that is derived from the host cell during the budding step of the viral life cycle (64). Protruding outside the envelope is the gp41-gp120 glycoprotein complex that occurs as both trimer and monomer on the viral surface (101, 121, 160, 164). The interaction between the trans-membrane gp41 and gp120 is non-covalent. An HIV particle contains a core consisting of the p24 protein (64). Inside the core are found the viral enzymes protease, reverse transcriptase and integrase that are essential for HIV replication and the two RNA molecules that constitute the viral genome. The core is separated from the envelope by a matrix made of the p17 protein.

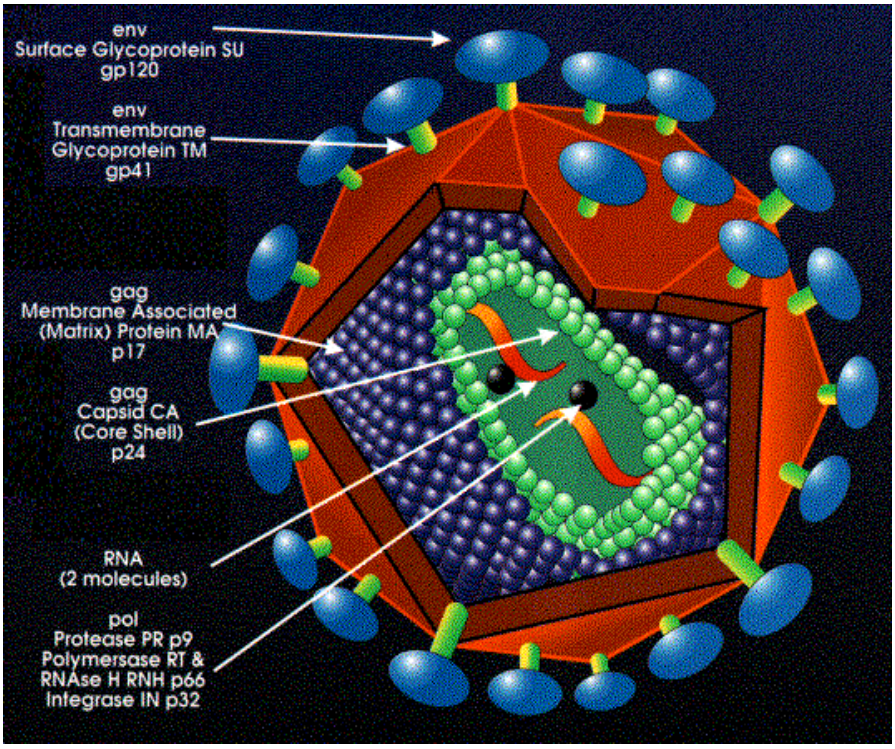


Figure 1.4: Structural arrangement of an HIV particle. An idealized computer generated structure of an HIV particle. In maroon is the outer lipid bilayer that constitutes the viral envelope. All other major components of the virus are indicated in the Figure (http://www.washington.edu/.../columns/dec00/cells.4.htm).

HIV is classified as HIV type 1 (HIV-1) and HIV type 2 (HIV-2) (93). HIV-1 was isolated in 1983 and is responsible for the global pandemic while HIV-2, discovered two years later, is less pathogenic and less transmissible. This virus is mainly limited to West Africa (76).

HIV-1 is divided into groups named group M, O, and N (25, 26). The group M is further divided into subtypes also known as clades. These subtypes are referred to alphabetically as A, B, C, D, F, G, H, J and K (25, 76). Subtype A and F are in turn divided into sub-subtype A1 and A2 and sub-subtype F1 and F2, respectively (26). Recombinant viruses, made from the recombination of genomes from different subtypes, also form part of group M viruses and they are more common in regions where there are different subtypes in circulation (26).

The high genetic diversity displayed by HIV-1 is partly attributed to its error prone reverse transcriptase enzyme (64). More precisely this enzyme introduces one error per genome with every round of replication. In addition to the high replication rate and the frequent recombination events between different viral genomes, the host immune pressure also plays an important role in HIV-1 diversity.

The global HIV-1 epidemic is characterized by a disproportionately high prevalence of a given subtype or recombinant virus in a particular region. For example HIV-1 infection in the North and Central America is mainly caused by subtype B viruses (Figure 1.5). This subtype is also the most prevalent in Western Europe. However, in Southern Africa HIV-1 infection is mainly caused by the subtype C virus that is also responsible for over 50% of HIV infections around the world (26).

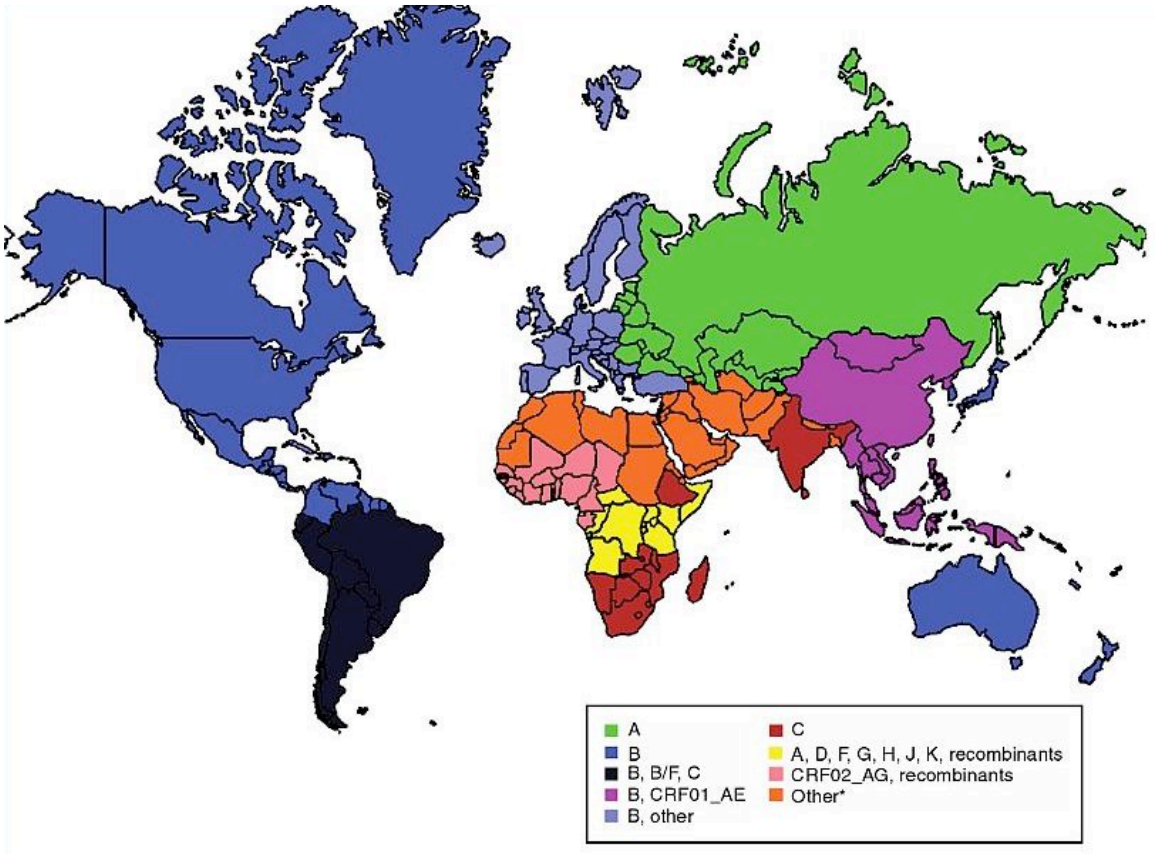


Figure 1.5: The global HIV-1 prevalence by subtype. The Figure shows the spread of HIV-1 subtypes around the world. Each subtype is colour coded and the countries where it is found are indicated. This Figure was taken from the website (http://img.medscape.com/article/708/915/708915-fig1.jpg).

The HIV-1 life cycle begins with the adsorption of the viral particle onto the surface of a target cell via the interaction of gp120 and the CD4 receptor (63). However, the CD4 receptor promoted infection requires the presence of a co-receptor which is, most commonly, the CCR5 chemokine receptor although the CXCR4 chemokine receptor is also used (65). HIV-1 that use the CCR5 co-receptor are referred to as R5 viruses and those that use the CXCR4 co-receptor are referred to as X4 viruses (136). The binding of the virus to the CD4 receptor results in a major conformational change of the gp41-gp120 trimer that exposes the co-receptor binding site (88). This is followed by the fusion of the viral envelope and the cell membrane (63), a process mediated by gp41 (56). After the cell-virus fusion, the viral genomic RNA is released into the cellular cytoplasm where HIV-1 reverse transcriptase

mediates its conversion into cDNA that later translocates to the nucleus to integrate into the host cell chromosome as proviral DNA. This process is mediated by the viral enzyme integrase (64). Subsequent to the integration step is the transcription of the integrated viral DNA to synthesize RNA and proteins. The latter (protease, integrase and reverse transcriptase) are further processed to their final active form by HIV-1 protease (65). The synthesized RNA and proteins are then packaged into new viral particles that bud from the cell to infect other cells.

1.3. HIV-1 gp120-gp41 glycoprotein complex

1.3.1. Native structure

HIV-1 has evolved from chimpanzees simian immunodeficiency virus (SIV) (135) and both viruses still share many essential genotypic and phenotypic characteristics (34). Thus much of what we know about the structure of HIV-1 gp41-gp120 complex (Env) is derived from studies conducted with SIV Env.

The structure of SIV and HIV native Env (gp41-gp120 trimer) has been visualized by three research groups using cryoelectron tomography. Zhu *et al.* (164) proposed that the SIV gp41-gp120 trimer is made of a gp120 'head', comprising the three gp120 units, attached to three transmebrane gp41 proteins projecting outward like "tripod legs". In this structure the head is made of a primary mass and two secondary lobes while the visible part of the leg is composed mainly of the membrane proximal external region (MPER) of gp41 located between the membrane spanning domain (MSD) and the C-terminal heptad repeat 2 (HR2) of the glycoprotein. In the same study Zhu *et al.* also reported that HIV-1 Env spikes are not evenly spread on the viral surface. Instead, they form clusters in which protein complexes are separated from their nearest neighbour by ~15 nm (Figure 1.6). In addition, Zhu *et al.* studied

the structure of HIV-1 native Env using the same technique they used for SIV (165) which revealed that HIV-1 Env was also made of a 'head' attached to tripod-like legs.

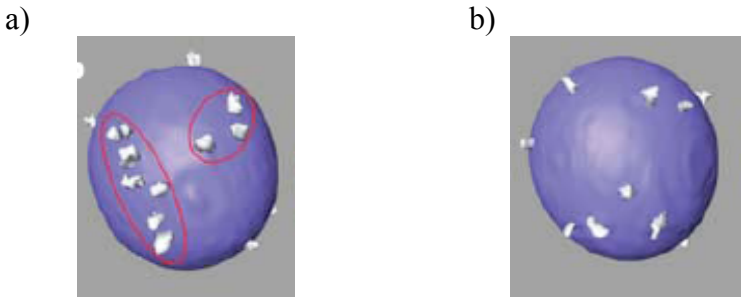


Figure 1.6: The distribution of gp41-gp120 trimers on HIV-1 envelope. Top (a) and bottom (b) views of an HIV-1 particle showing clusters of Env circled in red. This Figure is an adaptation from Zhu *et al.* (164).

Contrary this, Zanetti *et al.* showed that the native SIV Env is made of a three lobbed 'head', constituting the gp120 trimer, attached to three tightly packed gp41 monomers resulting in a lolly pop-like structure (160).

However, Liu *et al.* proposed a native Env structure that was markedly different from those of the two previous groups (88). One of the most striking differences in this structure was the distinctive hole in the gp120-gp120 interface (Figure 1.7). However, the gp41 arrangement of this structure bore similarities to that of Zanetti *et al.* The Liu *et al.* model is now widely believed to be the correct envelope trimer structure.

a) b)

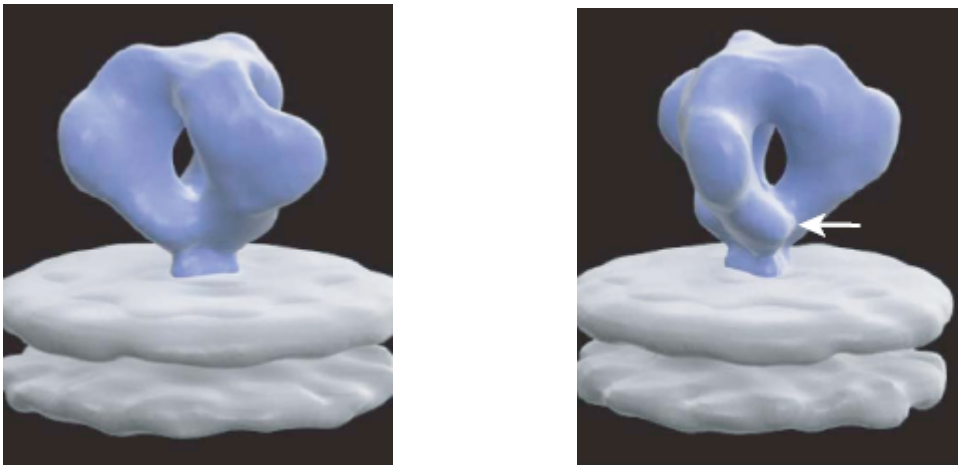


Figure 1.7: Cryo-electron tomography structure of HIV-1 native Env proposed by Liu *et al*. (a) and (b) side views of HIV-1 gp41-gp120 trimer taken from two different angles. The arrow in (b) shows the location of gp41. This Figure is an adaptation from Liu *et al.* (88).

1.3.2. HIV-1 Env glycosylation

HIV-1 gp41 and gp120 are encoded by the Env gene in the viral genome (65) and are synthesized in the rough endoplasmic reticulum as a single molecule that then undergoes extensive glycosylation with N-linked glycans to form gp160 (51). This is followed by gp160 oligomerization i.e. assembly into a gp41-gp120 trimer. All N-linked glycans on gp160 are initially mannose-rich before being either fully processed to form complex glycans or partially processed to form hybrid glycans (Figure 1.8). This processing takes place in the Golgi-apparatus. Mannose-rich glycans are made of a stretch of 7 to 9 terminal mannose residues, complex glycans contain terminal sialic acid residues while hybrid glycans are a mixture of both (30, 85). Once the gp160 trimer is cleaved into gp41 and gp120, by the host cell proteases (43, 67), it is exposed on the cell membrane from which the virus derives its envelope during the budding process.

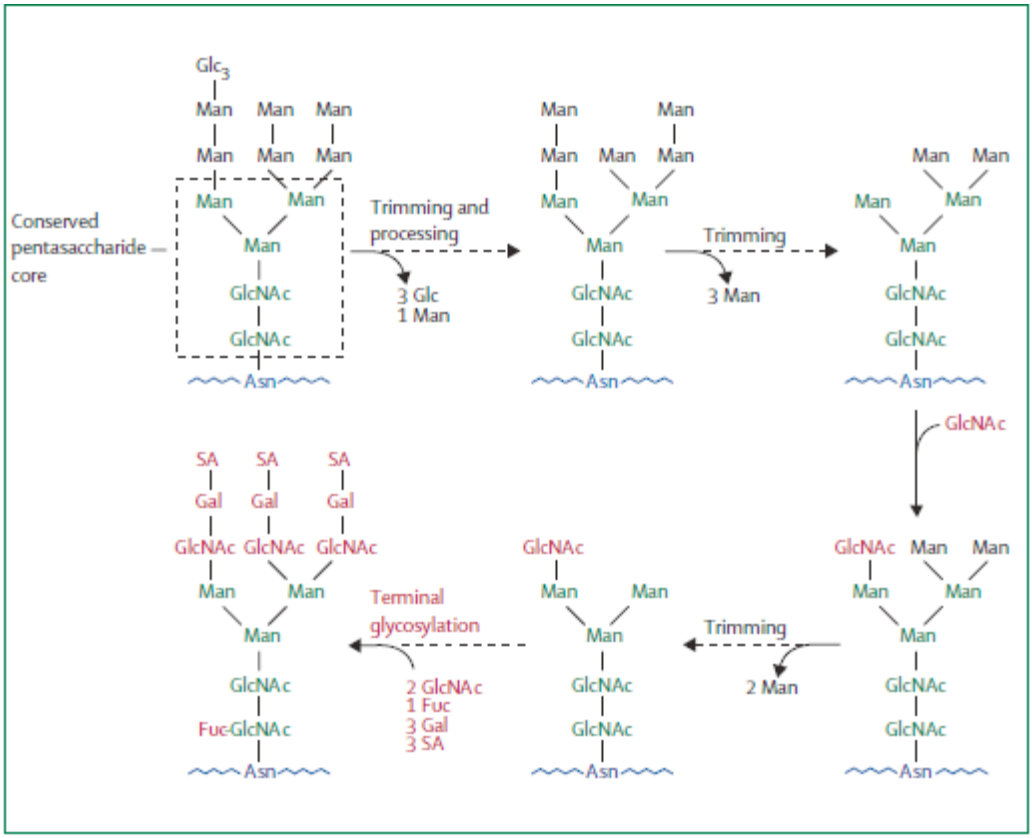


Figure 1.8: A diagram of the processing of HIV-1 gp160 N-linked glycans. The processing of N-linked glycans on gp160 begins with mannose-rich glycans shown on the top left of the Figure and ends with complex glycans show at the bottom left. Glc, Man, SA, GlcNAc, Gal, fuc and Asn stand for glucose, mannose, sialic acid, N-acetyl-glycosamine, galactose, fucose and asparagine, respectively. This Figure was taken from an article by Balzarini (6).

Glycans on HIV-1 gp120 account for ~50% of its molecular mass (82, 85) (Figure 1.9). However, while mannose-rich and hybrid glycans are found exclusively in the conserved regions of this glycoprotein, complex glycans are located in its variable regions. On average there are ~24 potential N-linked glycosylation sites on gp120. Complex oligosaccharides are found on 13 of these sites and the remaining 11 are occupied by hybrid and/or mannose-rich glycans (85, 166). Although Leonard and co-workers reported that all these sites are glycosylated, a study by Go *et al.* suggested that this is not always the case (58, 85). This being said, it is important to note that recently Doores *et al.* reported that 98% of glycans on native HIV-1

envelope (Env) are barely processed beyond $Man_5GlcNAc_2$ i.e. five mannose residue glycans (48).

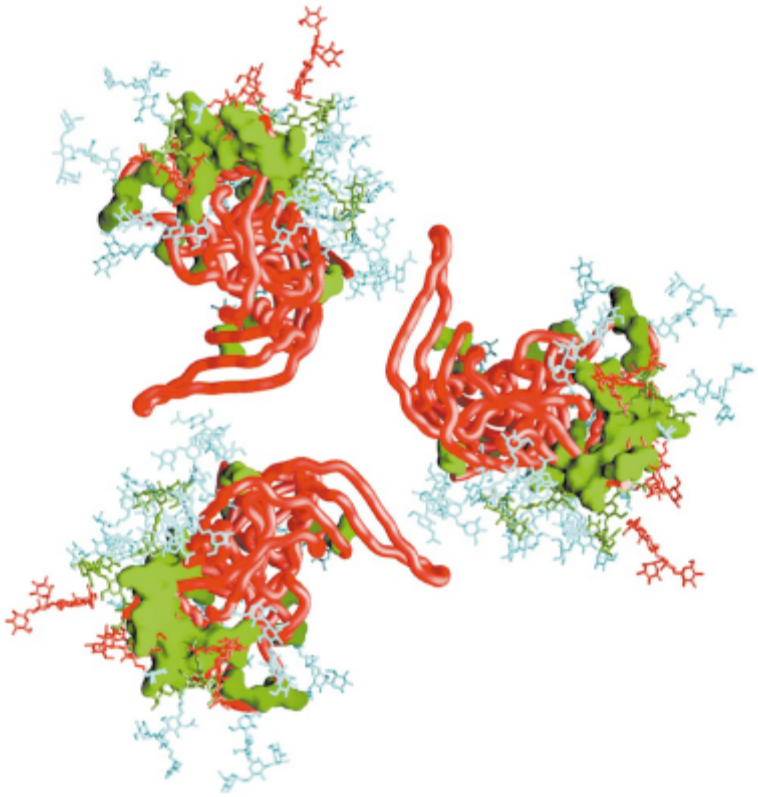


Figure 1.9: N-linked glycans on HIV-1 gp120. N-linked glycans on the surface of gp120 are shown in cyan, red and green and their positions are based on HIV-1 viruses HXBc2 and YU2. This Figure was taken from Kwong *et al.* (80).

Glycans on HIV-1 envelope play an important role in mediating the virus interaction with key cellular receptors such as the DC-SIGN receptor (57, 119, 120). This receptor, expressed on dendritic cells, enables the virus to be transported from the sites of mucosal transmission to the lymph nodes to infect $CD4^+$ T cells (117, 120). In addition, HIV-1 glycans promote the proper folding of the virus glycoproteins (86), determine the virus antigenicity and sensitivity to envelope inhibitors as well as enable the virus to develop resistance to neutralizing antibodies (16, 59, 155). However, HIV-1 glycosylation patterns vary between subtypes as can be seen by comparing viruses from subtypes A, B and C (161). For example, 75% of

subtype A and B envelopes are glycosylated at position 295 while only 20% of subtype C are glycosylated at this site (Figure 1.10); and at position 230 there are 5% of subtype A and 20% of subtype B that are glycosylated compared to over 80% of glycosylation for subtype C. Furthermore, at position 289 less than 10% of subtype A are glycosylated and also almost none of these viruses is glycosylated at position 392. However, for both subtype B and C the level of glycosylation at positions 289 and 392 is above 65%.

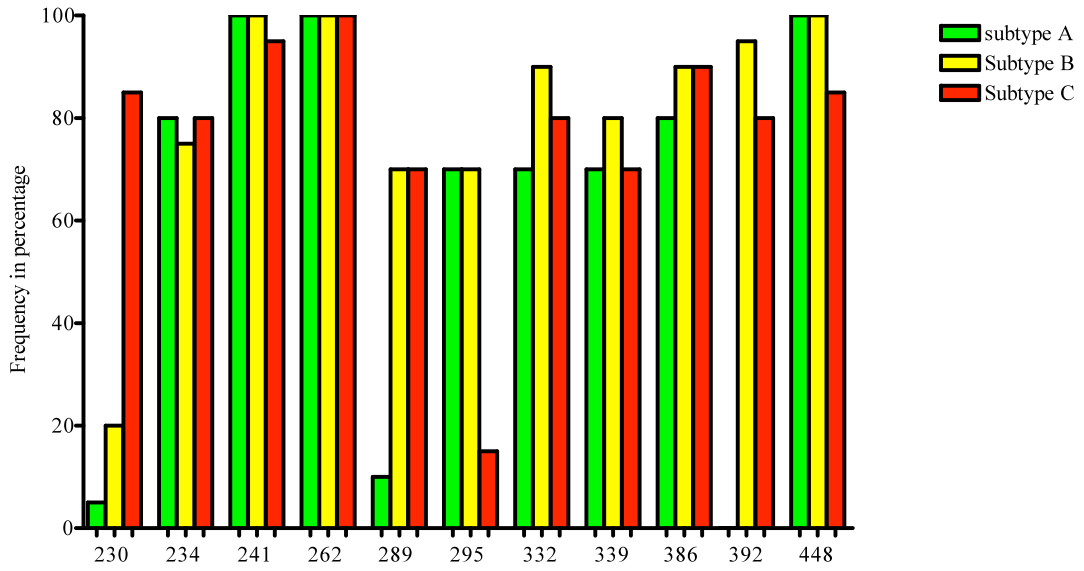


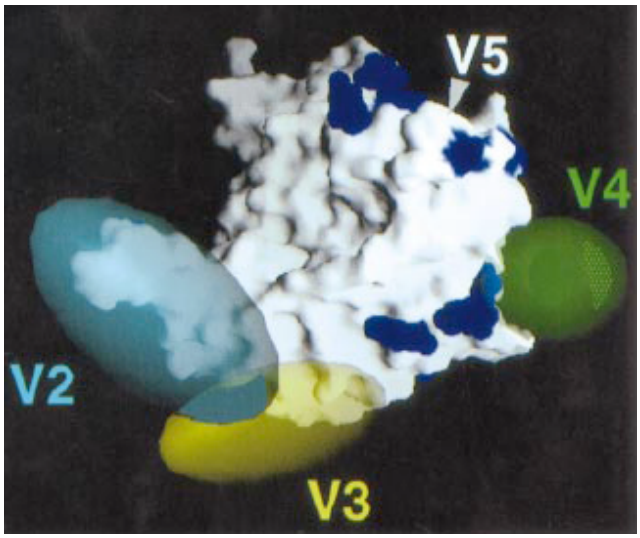
Figure 1.10: HIV-1 subtype A, B and C mannose-rich glycosylation pattern. This Figure is an adaptation from Zhang *et al.* (161). The position of mannose-rich glycans is the same as published by Leonard *et al.* (85).

1.3.3. Molecular structure of HIV-1 gp120

Generally speaking HIV-1 gp120 is made of four faces which are: the variable face composed of variable regions; the non-neutralizing face that is mainly located at the gp120 interface of the Env trimer; the heavily glycosylated silent face and the

neutralizing face that includes the receptor binding site and the region exposed to neutralizing antibodies (Figure 1.11a and b) (79).

a)



b)

Figure 1.11: Spacial arrangement of the four HIV-1 gp120 faces. (a) The positions of the four variable loops that constitute the variable face are shown. The Figure does not show the variable loop 1; however, it is in close vicinity of the variable loop 2 (see Figure 1.12 and 1.13). The positions occupied by N-linked glycans are shown in blue. (b) The non-neutralizing face (maroon), the silent face (yellow) and the neutralizing face (green) of gp120. This Figure is an adaptation of Wyatt and Sodroski (157).

HIV-1 gp120 has five variable regions or loops made of amino acid sequences that are highly variable between subtypes (Figure 1.12) (85, 92). The arrangement of these regions is such that adjacent variable loops are separated by a conserved region, made of relatively conserved amino acid sequences and there are five conserved regions on gp120. The five variable loops are exposed on the outside of gp120 while the conserved regions are protected by folding inside the glycoprotein (157). Together with the glycosylation of gp120, this special arrangement of variable and conserved regions protects the virus from the host immune pressure.

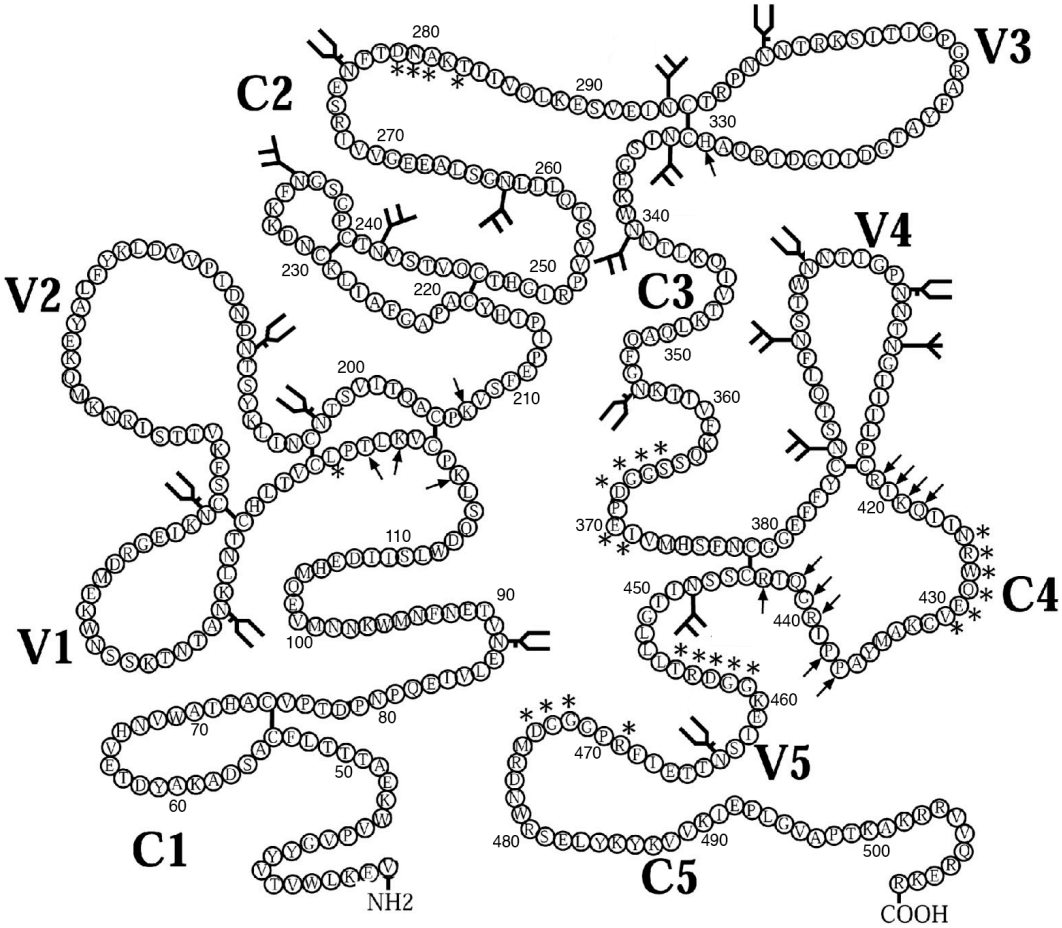


Figure 1.12: HIV-1 gp120 amino acid sequence and linear arrangement of variable and conserved regions. Two-dimensional structure of HIV-1 gp120; the small numbers stand for the positions of the numbered amino acids according to the sequence of HIV-1 HXB2. The C stands for constant region and the V stands for variable region. The location of mannose-rich glycans is indicated by tri-branched antennae and that of complex glycans by U-shaped antennae. This Figure was adapted from McCaffrey *et al.* (92).

However, besides its division into the variable, non-neutralizing, neutralizing and silent face, HIV-1 gp120 can also be seen as a two domains glycoprotein and these domains are the outer domain, in which are found most of gp120 glycans, and the less glycosylated inner domain (Figure 1.13) (163). Between these two domains is found a sheet of four anti-parallel β-strands known as the bridging sheet. In the gp41-gp120 complex the inner domain is sandwiched between gp41 and the outer domain (88). The crystal structure of HIV-1 gp120, in complex with a two-domain fragment of the human CD4 receptor and the Fab fragment of the CD4 induced epitope antibody 17b, showed that the inner domain is made of seven β-strands and two α helixes while the outer domain is made of two barrels, the termini distal barrel containing seven anti-parallel β-strands and the proximal barrel made of six mixed-directional β-strands surrounding an α helix (Figure 1.13) (81). The two barrels are connected by a barrel-barrel juncture.

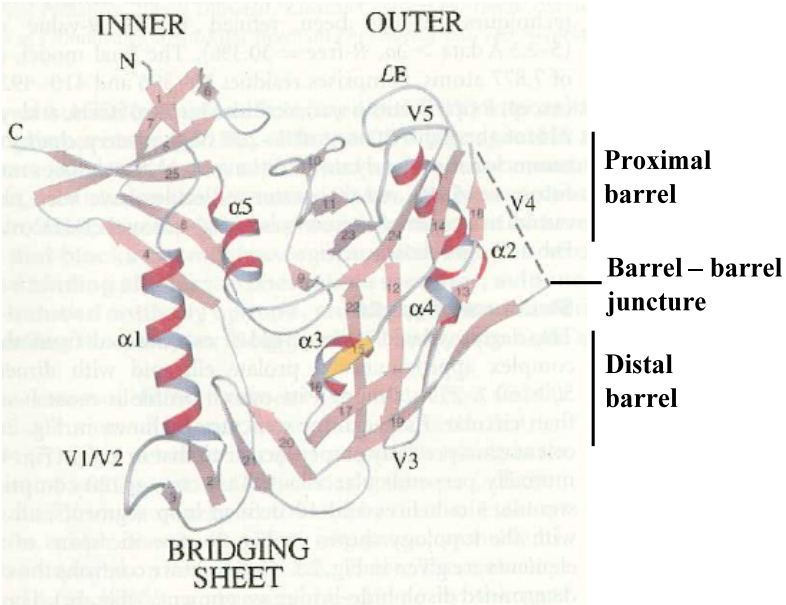


Figure 1.13: The ribbon structure of HIV-1 gp120. (a) The Figure shows the structural arrangement of the major components of gp120 with numbered strands and the positions of the variable loops indicated. This Figure is an adaptation from Kwong *et al.* and Zhou *et al.* (81, 163).

1.3.4. The CD4 and co-receptor binding sites

The CD4 receptor is the main receptor used by HIV-1 to infect cells (81, 157). Its binding site is made of a discontinuous region located on the outer surface of gp120 comprising conserved amino acid sequences shielded from the immune pressure by variable loops (28). More precisely, the CD4 receptor binds gp120 at a site between the inner domain, the outer domain and the bridging sheet (Figure 1.14) (http://www.hiv.lanl.gov/content/immunology/pdf/1998/REVIEWS/sodroski.pdf).

The gp120-CD4 interface has two cavities: a shallow cavity occupied by water molecules and a deep cavity extending 10 Å inside gp120 (157). The opening of the deep cavity is occupied by the CD4 residue Phe43 that has been shown to be critical for gp120-CD4 interaction. However, Asp368 of gp120 that interacts with Arg59 of CD4 together with other gp120 residues, important for CD4 binding, are also found around this cavity.

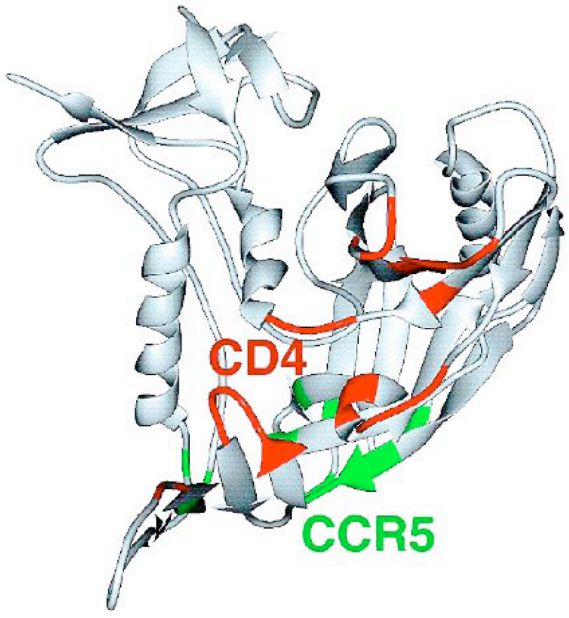


Figure 1.14: The CD4 receptor binding site on gp120. The red patches show the CD4 binding site while the green patches show the CCR5 co-receptor binding site on gp120. This Figure was taken from Wyatt *et al.* (http://www.hiv.lanl.gov/content/immunology/pdf/1998/REVIEWS/sodroski.pdf).

Studying the crystal structure of SIV gp120, Chen *et al*. (34) showed that in both unliganded and CD4 bound state the outer domain has essentially the same structure. However, upon CD4 binding major changes take place in the inner domain that involve a four-turn α helix, a β ribbon, a three-stranded β sheet and a α-helix at the outer-domain/inner-domain junction (Figure 1.15). The CD4 receptor binding to gp120 also induces the formation of the four-strand bridging sheet located between the outer and inner domain.

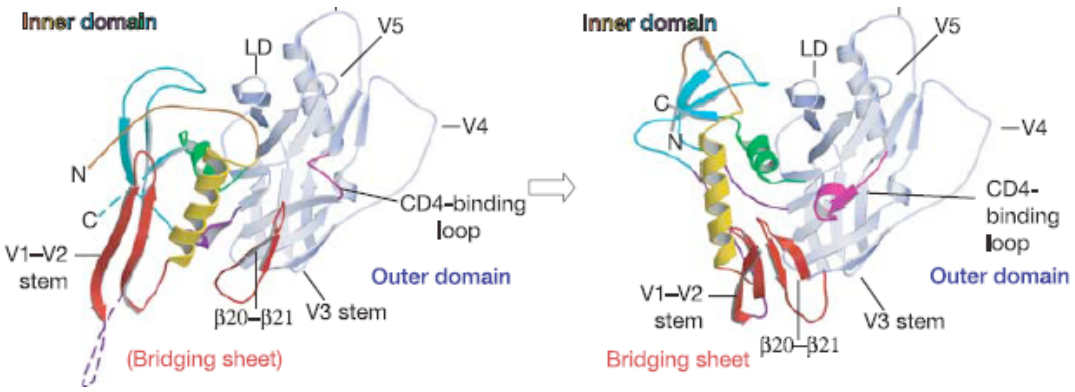


Figure 1.15: Change of conformation induced in SIV gp120 upon the CD4 receptor binding. SIV gp120 in its unliganded conformation is shown on the left hand side and the CD4 bound conformation is shown on the right hand side (34).

Besides being shielded by variable loops, access to the CD4 binding site is also impaired by conformational masking of this site; an effect caused by the size of the antibody foot print on gp120, the orientation of the antibody-gp120 bonds imposed by Env glycans and the required change in the gp120 conformation upon antibody binding (79). These factors combine to make the change in entropy of certain antibody-CD4 binding site interaction largely negative. Here it should be noted that the more negative the entropy of a reaction the less it is thermodynamically favoured or likely to occur (52). Using isothermal titration calorimetry, Kwong *et al*. showed that antibodies that bind to the receptor-binding-site epitope had large

negative entropies compared to antibodies that didn't bind to this site (79). Their study also showed that the binding of the CD4 receptor to gp120 has large negative entropy. However, Kwong *et al*. suggested that the simultaneous binding of CD4 receptors to gp120 in the same or different Env trimers provides sufficient avidity to overcome the conformational masking (79).

1.3.5. The co-receptor binding site

HIV-1 use of either the CCR5 or CXCR4 co-receptor is a major determinant of viral pathogenesis and disease progression (100). Most HIV-1 infections begin with viruses that utilise the CCR5 co-receptor and in many cases the emergence of strains that use the CXCR4 co-receptor occurs in later stages of the disease (100). The bridging sheet and the V3 loop in gp120 constitute the co-receptor binding site (Figure 1.14 shows the CCR5 binding site which overlaps the CXCR4 binding site) (157) and signature amino acids sequences in the V3 loop determine the co-receptor specificity (100, 125).

1.4. HIV-1 sexual transmission

1.4.1. The female genital tract

The female genital tract comprises the vaginal tract and the cervix. This genital tract is covered by a stratified squamous epithelial cell lining starting in the vagina and ending at the ectocervix (Figure 1.16) (38, 136). This lining is divided into five zones namely; the cornified, the condensation, the clear, the parabasal and the basal zone (Figure 1.17). However, the epithelial cell lining in the endocervical canal only has one layer, a structure that makes it more vulnerable to disruptions such as those caused by sexual intercourse induced microtrauma (99).

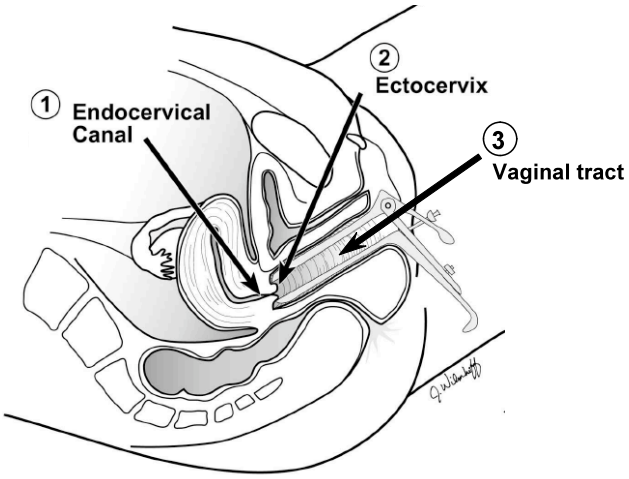


Figure 1.16: Female genital tract anatomy. A cross section of the female genital tract showing the vagina and the cervix composed of the endocervical canal and the ectocervix. This Figure was adapted from Coombs *et al.* (38).

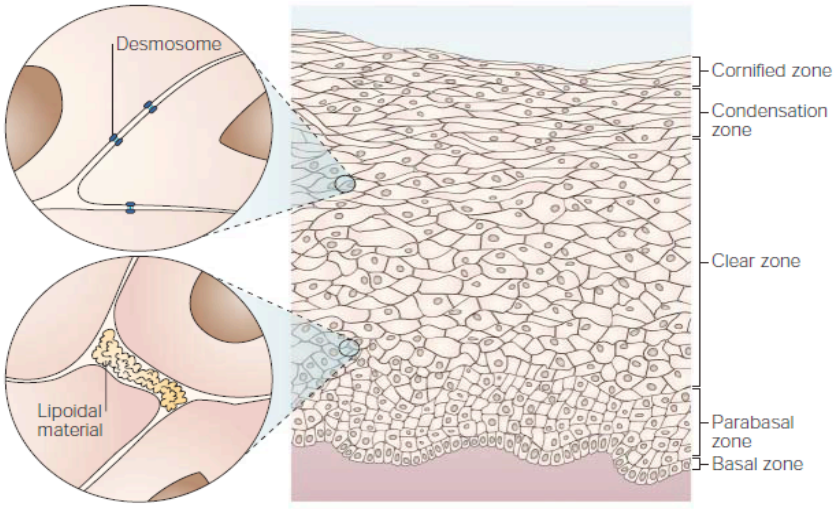


Figure 1.17: The composition of the vaginal and cervical squamous epithelial cell lining. The stratified structure of the squamous epithelial cell lining showing its five layers; the lipoidal material and dermosomes found between its constituent cells are also shown. This Figure was taken from Shattock and Moore (136).

Many HIV-1 target cells are found in both the vagina and the cervix. For example $CD4^+$ mononuclear lymphocytes and inflammatory cells are present in the stroma of the cervix (38) while Langerhans cells (LCs), that play an important role in mediating HIV-1 crossing of the vaginal mucosa, are located inside the epithelial cell lining. However, it should also be noted that recent reports suggested that LCs interaction with HIV may block its transmission (41). Lastly, the subepithelium and

the lamina propria contain a significant population of macrophages, $CD4^+$ T cells and dendritic cells that promote HIV-1 infection (83, 99, 136).

1.4.2. HIV-1 infection via the genital tract

In females HIV-1 infection by heterosexual intercourse begins when viral particles, free or cell associated, released into the genital tract infect their target cells (83, 139). Although the precise location of HIV-1 infection in the female genital tract has yet to be pinpointed, cervical ectopy, caused by damage in the epithelial cell lining, has been associated with increased risk of HIV-1 infection (98). However, Kell *et al*. reported HIV-1 infection of a woman suffering from the Meyer-Rokitansky-Küster-Hauser syndrome, a congenital disorder that results in the lack of a uterus (74). This was supported by Miller *et al*. who showed that the inoculation of cell-free SIV into the vaginal tract of rhesus macaques resulted in a systemic infection (97). These reports suggest that HIV-1 can be transmitted via both the cervix and the vagina.

Different mechanisms have been proposed to explain how HIV-1 released in the vaginal tract crosses the epithelial cell lining to infect cells in the lamina propria and subepithelium. Amongst these mechanisms, the breach of the genital mucosa during sexual penetration is widely accepted to be one of the main causes of male to female transmission of HIV-1 (38, 99, 136). The loss of the genital mucosa integrity can result in viral particles crossing the epithelial cell lining to infect $CD4^+$ T cells and macrophages in the lamina propria and subepithelium (83, 147) (Figure 1.18). In fact it has been suggested that the breach of the epithelial cell lining integrity exposes intraepithelial LCs to HIV-1, though it has to be noted that these cells can also get into contact with the virus through their dendritic processes that are exposed in the vaginal lumen (99). Infected LCs leave their initial site of exposure to disseminate the

virus to other sites such lymph nodes. This is supported by a study in macaques that showed SIV to interact with dendritic processes of LCs in the epithelial cell lining enabling it to cross this barrier (137). It is also believed that the breach of the epithelial cell lining integrity allows viral particles to interact with dendritic cells in the subepithelium an interaction that also promotes their dissemination (116, 119, 120). However, besides the sexual intercourse induced vaginal epithelial microtrauma that is present in > 60% of all cases of consensual sex (109), pre-existing infection of the genital tract caused by bacteria and viruses can also enhance the risk of contracting HIV-1 due to lesions and inflammation they cause (37, 38, 134). In addition, direct infection of epithelial cells and transcytosis have been proposed as mechanisms that facilitate HIV-1 crossing of the vaginal mucosa (136). However, some studies have demonstrated that primary cervical and vaginal epithelial cells do not transcytose infectious HIV-1 particles and that they also do not get infected by the virus (44, 62).

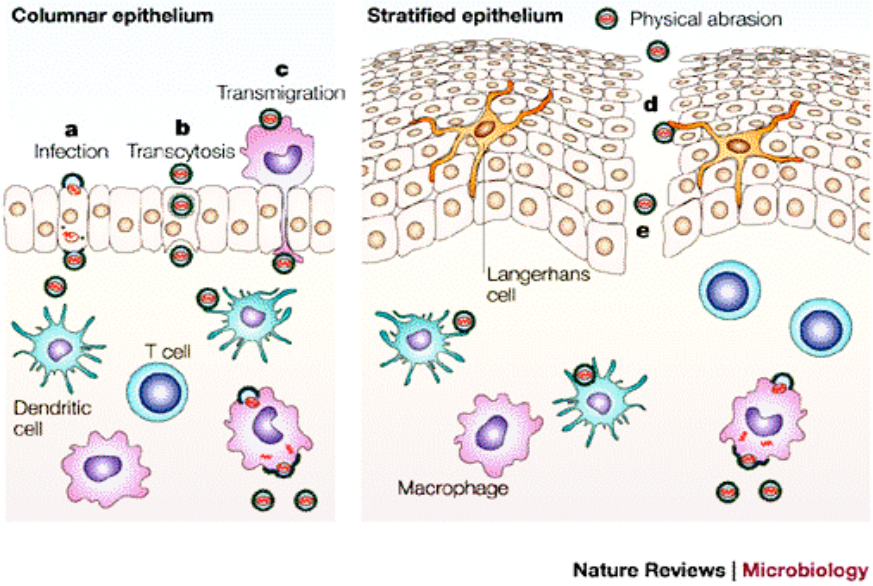


Figure 1.18: Mechanisms that facilitate HIV-1 crossing of the cervico-vaginal epithelial cell lining. On the left hand side of the Figure are shown mechanisms that facilitate the crossing of the columnar epithelium, (a) HIV-1 infection of epithelial cells; (b) HIV-1 transcytosis through these cells and (c) transmigration of infected cells between epithelial cells. On the right hand side are shown mechanism that promote the crossing of the stratified epithelial cell lining; (d) and (e) are steps involved in the physical abrasion promoted HIV-1 crossing of the vaginal mucosa. This Figure was taken from Shattock and Moore (136).

1.4.3. HIV-1 interaction with the DC-SIGN receptor

The DC-SIGN receptor is a C-type lectin, Ca^{2+} dependent, receptor that may play a critical role during both heterosexual and male to male transmission of HIV-1 by mediating the virus adsorption to dendritic cells (DCs), macrophages and endothelial cells (13, 21, 100, 117, 119, 136). The DC-SIGN receptor is a tetrameric type II transmembrane protein and its binding to HIV-1 has been shown to involve glycans found on the surface of gp120 (69, 70). HIV-1 binding to DC-SIGN enhances the efficiency of viral transmission to cells that are susceptible to infection such as $CD4^+$ T cells (13, 105, 119, 159). A study by Pohlmann and co-workers showed that the DC-SIGN receptor expressed on 293-T cells is able to bind X4 and R5 viruses and this binding results in an efficient transfer of the virus to co-cultured T-cells (120).

HIV-1 bound to the DC-SIGN receptor can also be internalized by dendritic cells and this may help the virus escape surveillance by the immune system (117, 120). When dendritic cells come into contact with $CD4^+$ T cells the virus resurfaces in the zone of contact between the two cells to form viral synapses that lead to an increased rate of infection and replication of the virus (Figure 1.19). However, Burleigh *et al*. (27) reported that the DC-SIGN receptor mediates the infection *in cis* of immature dendritic cells and this plays a critical role in the long term transfer of newly made viruses to T cells. Similarly, the DC-SIGN receptor on macrophages is also believed to mediate their infection *in cis* (119).

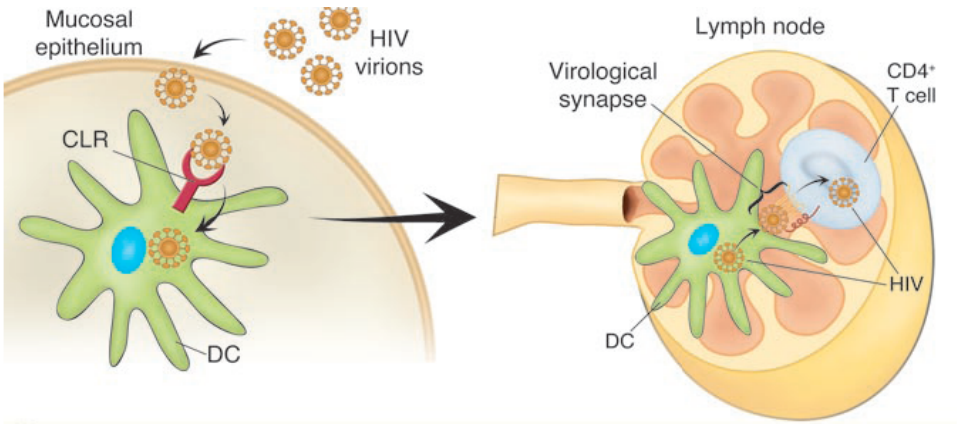


Figure 1.19: Mechanism of HIV-1 transfer to target cells via C-type lectin receptors. The Figure shows HIV-1 capture by means of a C-type lectin receptor (CLR) and internalization into a dendritic cell (DC). The arrow indicates the migration of the cell to the lymph node to transfer the virus to a $CD4^+$ T cell. This Figure was taken from Piquet and Sattenteau (117).

1.4.4. Female genital tract natural defence against HIV-1

There are several elements in the female genital tract that constitute natural barriers to HIV-1 infection. Amongst them is the stratified structure of the epithelial cell lining and the acidic pH of the genital environment (38, 99). A study in rhesus macaques showed that the thickening of the vaginal epithelium with esterogen protects against vaginal challenge with SIV while the thinning of this lining with progesterone decreases this protection (90, 137). In addition, the mucus secreted by epithelial cells

in the endocervix can provide protection against HIV-1 by constituting a physical barrier to infected cells. This mucus also contains anti-viral proteins such as the secretory leukocyte protease inhibitors, lactopherin, human-β-defensin (HBD-1) and lysozyme (147). Lastly, endocervical cells express the CXCR4 ligand SDF-1 that can present a chemical barrier to X4 viruses (99).

1.5. HIV-1 microbicides

1.5.1. Introduction

1.5.1.1. Overview

The developing world remains the epicentre of the HIV epidemic with more women infected compared to men (http://www.unaids.org). The higher rate of HIV-1 infection amongst women in the developing world may be explained by their economic dependence on men, domestic violence, high incidence of rape, cultural norms and their inability to negotiate safe sex practices such as the use of condom with their male partners (53). This supports the need for a female-controlled HIV-1 microbicide i.e. a cervico-vaginally applied product that can prevent the male to female transmission of the virus.

Many agents have been proposed as microbicide candidates to prevent HIV-1 infection at the site of mucosal transmission. Some of these agents are specific inhibitors of HIV-1 infection while others are non-specific. Amongst HIV-1 microbicides candidates are found compounds that modify the cervico-vaginal environment (vaginal milieu protectors) such as buffering agents, those that interact with the positively charged viral envelope such as polyanionic polymers, envelope inhibitors such as lectins and reverse transcriptase inhibitors. Some of these

compounds have already been tested in clinical trials (Table 1.1) while others are still in developmental stage.

Table 1.1. Overview of HIV-1 microbicides history.

	Advantages	Disadvantages	Examples in class	Type of trial	Year of completion	Outcome
Selected non-specific microbicide agents						
Surfactants	Have a broad inhibitory activity against a wide range of pathogens	Can be toxic to host cells	Nonoxinol 9	Phase III	1996 and 2000	Failed
			C31G	Phase III	2005 and 2006	Halted due to low incidence of infection
Vaginal milieu protectors/acidifying agents	Inhibitory to HIV, HSV and C trachomatis	Not known	BufferGel	Phase II/phase IIb		Ongoing
Entry inhibitors: anionic polymers	Can be inhibitory to other STI	Different subtypes will respond differently to these compounds	PRO2000	Phase III	2009	Failed
			Carraguard	Phase III	2008	Failed
Selected specific microbicide agents						
Entry inhibitors: CCR5 inhibitors	Bind to specific ligands	Not inhibitory to other STI	PSC-RANTES	Tested in macaques	2004	Protective against [a] SHIV
Reverse transcriptase inhibitors	Active in different cell types	Not inhibitory to other STI	Tenofovir	Phase III	2010	Protective against HIV

This Table is an adaptation from Cutler and Justman (39).
[a] SIV/SHIV chimeric virus

1.5.2. Vaginal milieu protectors

The low pH of female genital tract has virucidal effect against HIV-1 (147) and this pH is due to the production of lactic acid and hydrogen peroxide (H_2O_2) by *lactobacillus* species, such as *lactobacillus acidophilus* and *lactobacillus crispatus*, that are part of the resident microbial ecology of a healthy vaginal tract (39, 77, 106). However, semen resulting from sexual intercourse render this environment favourable to HIV-1 by neutralizing the vaginal acidity (141). BufferGel ^{TM} or Carbopol 974 (BufferGel, ReProtect, Baltimore, MD, USA), a formulated carbopol polymer with a buffering agent that has the ability to buffer twice its volume of semen to a pH $\leq$ 5, is a microbicide candidate that acts by maintaining the acidic pH of the vaginal tract.

Although, clinical trials in India, Thailand, Malawi and Zimbabwe showed that the use of BufferGel ™ destroyed the bacterial populations that caused vaginosis (147) this formulation does not affect the genital *lactobacillus* species population. BufferGel ™ has already passed two phase I safety trials (39, 91, 150) and also proved to be safe and accepted amongst HIV-1 infected and uninfected men during a penile tolerance study (140).

Like BufferGel ™, Acidform ™ is also a buffering agent that was originally designed for use as a sexual lubricant (39, 106). This formulation has already been subjected to two phase I safety trials and one penile tolerance trial (2, 3, 132). In addition to the two buffering agents there are also "probiotics" in development aimed at achieving the same goal (106).

1.5.3. Surfactants

Surfactants render bacteria and viruses non-infectious by solubilising their membrane (147). However, a major limitation with surfactants is that they are non-specific i.e. they also target host cells. C31G also known as SAVVY® (Cellegy Pharmaceuticals, Quakertown, PA, U.S.A) is one of the best known surfactants. This formulation consists of cetyl betaine and myristamine oxide and its most important characteristics is its ability to dissolve and spread very fast in the genital mucosa (39, 147). *In vitro* studies showed that C31G has a broad-spectrum activity against bacteria and viruses including HIV (32, 78). This compound also has sperimicidal activities that may limit its use in communities that stand against contraception. However, C31G was tested in two phase III clinical trials in Ghana and Nigeria that had to be abandoned because of lower than expected rate of HIV seroincidence in the selected populations (39). Nonoxynol-9 (N-9) is another well known surfactant that has been studied as a HIV-1

microbicide candidate. Although N-9 demonstrated strong anti-HIV-1 activities *in vitro*, this compound was originally manufactured for use as a spermicide or contraceptive (53). Two phase III trials were conducted with N-9 in Africa. One amongst sex workers in Cameroon and showed no difference in the rate of HIV infection between the study group and the placebo group (127). However, this study also found that there was an association between the use of N-9 and the higher incidence of genital ulcers among trial participants. The other clinical trial tested the use of 52.5 mg of gel-formulated N-9 that had to be applied before and after sexual intercourse (147). Upon unblinding the trial it was found that the N-9 gel increased the risk of contracting HIV infection by 50% and this risk was even higher in women who reported a reduced use of condom. The increased risk of HIV transmission associated with N-9 is likely to be related to the high incidence of superficial de-epithelialisation, changes in the genital microflora as well as the high rate of petechial haemorrhage and erythemia associated with this formulation.

Sodium lauryl sulphate (Invisible Condom, Univesité Laval, Quebec, Canada) is a non-specific inhibitor of HIV-1 and other pathogens that cause sexually transmitted infections (STI) (39, 118). This compound is liquid at room temperature and converts into a gel that coats the vaginal tract and protects against HIV-1 at body temperature. Sodium lauryl sulphate has been shown to be safe in two phase I trials and in a rabbit model. However, the result of a phase II trial, conducted in Cameroon, is still pending.

1.5.4. Polyanionic polymers

Polyanionic polymers interact with the positively charged V3 loop of HIV-1 gp120 (46, 47, 147) and have been shown to be active against both X4 and R5 viruses. In

addition to the positive charge of the V3 loop, the charge mediated binding of polyanionic polymers is enhanced by the conformational changes that unmask charged regions of gp120 during the virus entry into target cells (47).

Naphthalene sulfonate (PRO2000; Indevus Pharmaceuticals, Lexington, MA, USA) and the seaweed derived sulphated polysaccharide carrageenan (Carraguard/515, Population Council, New York, NY, USA) are the most studied polyanionic polymers (39, 147). PRO2000 has been shown to have anti-HIV and anti-HSV activities (75); and in a phase I safety trial this compound was shown to be generally well tolerated in humans (149). However, although PRO2000 is able to inhibit SIV transmission in rhesus macaques it failed a phase III efficacy trial in South Africa; this microbicide interaction with semen was mentioned among the possible reasons for the failure (124). Carraguard anti-HIV activities was shown in a mouse model (115). This formulation was also tested in phase I trials and proved to be safe in both HIV-positive and HIV-negative men and women (18, 36, 151). However, a phase III clinical trial conducted amongst women in South Africa showed that carraguard is not protective against HIV-1 infection (39). However, it should be noted that poor adherence from trial participants was also suspected to be a possible cause of carraguard lack of efficacy.

Cellulose sulphate (Ushercell, Polydex Pharmaceuticals, Toronto, ON, Canada and Topical Prevention of Conception and Disease [TOPCAD], Chicago, IL, USA) binds to HIV-1 gp120 V3 loop and *in vitro* studies demonstrated this compound efficacy against X4 and R5 viruses (133). Cellulose sulphate safety for use in humans was shown in a phase I clinical trial (39, 89). However, in a phase III trial a slightly higher incidence of HIV-1 infection occurred in the cellulose sulphate group

compared to the placebo group (148). This led to this compound being abandoned as a HIV-1 microbicide candidate.

Cellulose acetate phthalate (CAP) is a polyanionic polymer that has a unique mode of action against HIV-1. This compound acts synergistically with soluble CD4 to inhibit the virus infection of cells. Its binding to gp120 results in the formation of the hybrid six helix conformation, an intermediate in HIV-1 entry mechanism (96, 107). However, although similar to the six-helix bundle formed by Env during the unhindered viral entry, the one induced by CAP is premature and non-functional. The only limitation with the use of CAP as a topical microbicide is against viruses that *in vitro* showed the ability to infect cells independently of the CD4 receptor (147).

Betacyclodextrin (BCD) is a polyanionic polymer that depletes cholesterol from the viral and cellular membrane, therefore, inhibiting the function of their lipid rafts (147). Given that the CD4 receptor and HIV-1 co-receptors are located in these cholesterol containing rafts, an interference with their functionality inhibits the virus entry into cells (87, 122). In addition, the assembly of new viral particles is also dependent on the cell membrane lipid rafts, suggesting that BCD may also inhibit the budding process of HIV-1 (108, 147).

Dendrimers are the newest members of the anionic polymers family. These compounds are made of a core, interior branches and terminal surface groups for selective interaction with specific targets (39). Dendrimers can bind multiple locations on different cells at the same time. SPL7013 (Vivagel, Starpharma Holdings Ltd, Melbourne, Australia) is the first of this class of compounds to be formulated as a microbicide gel. This dendrimer was shown to be protective against the simian/human

immunodeficiency virus (SHIV) chimera in a macaque model (72). A 3% formulation of SPL7013 is in phase I clinical trials in Kenya and USA.

1.5.5. Reverse transcriptase inhibitors

Reverse transcriptase inhibitors (RTI) interfere with the conversion of HIV-1 RNA into DNA by the viral reverse transcriptase (39). This group of drugs comprises nucleotide and nucleoside reverse transcriptase inhibitors (NRTI); these are defective analogs of naturally occurring nucleotides and nucleosides, respectively. However, candidate microbicides also include non-nucleoside reverse transcriptase inhibitors (NNRTI); they are inhibitors of HIV-1 reverse transcriptase that are not analogs of cellular nucleosides or nucleotides (24, 147).

The thiocarboxanilide derivative UC-781 is a NNRTI microbicide candidate that is characterized by a high affinity for HIV-1 reverse transcriptase. It has been shown *in vitro* that cells treated with UC-781 have a long term protection or "memory" against HIV-1 (8, 14, 20). UC-781 showed safety for use in humans in a phase I clinical trial that evaluated a once-daily dosing of this compound for 6 days (39). In addition, this group of microbicides include SJ-3366, produced by Samjin pharmaceutical Inc., a homocyclic pyrimidinedione that has a dual mode of action against HIV-1. It inhibits both HIV-1 entry and reverse transcription (147). Lastly, TMC120 (4-[{4-[(2,4,6-trimethylphenyl)amino]pyrimidin-2-yl}amino] benzene carbonitrile), another NNRTI that showed no toxicity in animal models and showed inhibitory activities against HIV *in vitro* and *in vivo*, is also found among these microbicide candidates (45, 152). There are on going phase I and II clinical trials with TMC120.

Tenofovir is a NRTI that acts by mimicking adenosine nucleotide (40). Once incorporated in the nascent HIV-1 cDNA it prematurely terminates chain elongation. Tenofovir has a cellular half-life of 9 to 50 hours depending on the cell type and it can be effective in non-dividing cells such as macrophages that have limited phosphorylation ability (5, 7, 126). A study by Tsai *et al.* using macaques model showed that pre-exposure and post-exposure prophylaxis with tenofovir protects against intravenous challenge with SIV (146). This report was one of the first to provide evidence on the possibility of using tenofovir to prevent HIV-1 infection in humans. Since then tenofovir has become the leading antiretroviral used in HIV-1 vaginal microbicide research.

Recently, a clinical trial conducted amongst South African women in Kwazulu Natal using a 1% tenofovir gel within 12 hours before and after sex showed protection against heterosexual transmission of HIV-1 (1). More precisely, after 30 months of trial the gel was effective in preventing HIV-1 transmission by 39%. However, the study also revealed that women who had a higher adherence to the gel had a higher protection compared to those with a lower adherence. HIV-1 RNA genotyping of women who got infected during the trial did not reveal the presence of tenofovir resistance mutations which suggested that the use of tenofovir as a vaginal gel may not result in HIV-1 resistance (1). However, it is not yet known if using the gel over a longer period would lead to resistance mutations.

1.5.6. Proteins

Amongst proteins proposed as HIV-1 microbicide candidates are lectins. These compounds inhibit HIV-1 infection of cells by binding to chains of carbohydrates on the gp41-gp120 complex (21). The specificity of lectins for viral glycoproteins, their

relatively small size and potent anti-HIV-1 activities give them advantage over other potential Microbicides. Lastly, CCR5 inhibitors and fusion inhibitors that bind to gp41 to inhibit the fusion of the viral envelope with the cell membrane are also among HIV-1 microbicide candidate proteins (39).

1.5.6.1. Griffithsin

Griffithsin (GRFT) is a lectin isolated from the red alga *Griffithsia* species (103). This lectin is a 121 amino acid protein with a molecular weight of ~ 13kDa that have been reported by Emau *et al.* (54) to be able to withstand both acidic and alkaline pH. GRFT has a domain swapped dimer structure in which two β-strands of one monomer combine with 10 β-strands of the other monomer to form a β prism made of three four-stranded sheets (167, 168) (Figure 1.20). Each monomer of GRFT contains three binding sites that have high affinity for mannose residues. Both native and recombinant GRFT have displayed potent antiviral activities against laboratory strains and primary isolates of X4 and R5 viruses (54, 103). A study by O'Keefe *et al.* showed that GRFT produced in the tobacco related plant *Nicotiana benthamiana* have a broad neutralizing activity against different subtypes of HIV-1 (111). This recombinant GRFT was also shown to be non-toxic in a rabbit vaginal irritancy model and in human cervical explants. GRFT inhibits HIV-1 infection by binding to the mannose-rich glycans of the gp41-gp120 glycoprotein complex. Although this compound is not HIV-1-specific, it targets mannose-rich arrays that are present on all HIV-1 envelope glycoproteins. Since such arrays are uncommon in mammalian cells, GRFT is not likely to be toxic to human cells even at relatively high concentrations (6, 9).

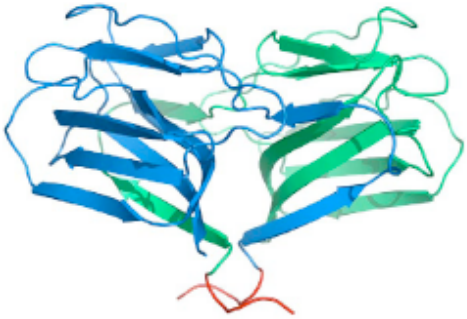


Figure 1.20: A ribbon model of the GRFT dimer. The two monomers making the dimer are shown in blue and green. The N-terminal extension resulting from the cloning procedure is shown in red. This Figure was taken from Ziolkowska *et al.* (167).

1.5.6.2. Cyanovirin-N

Cyanovirin-N (CV-N) is a lectin of 101 amino acids and a molecular weight of ~ 11 kDa. This compound was isolated from the blue green alga *Nostoc Ellipsosporum* and shown to exist in solution as a compact, quasi-symmetric two-domain monomer (Figure 1.21a and b). However, CV-N has the crystal structure of a domain swapped dimmer (22, 156, 168).

a) b)

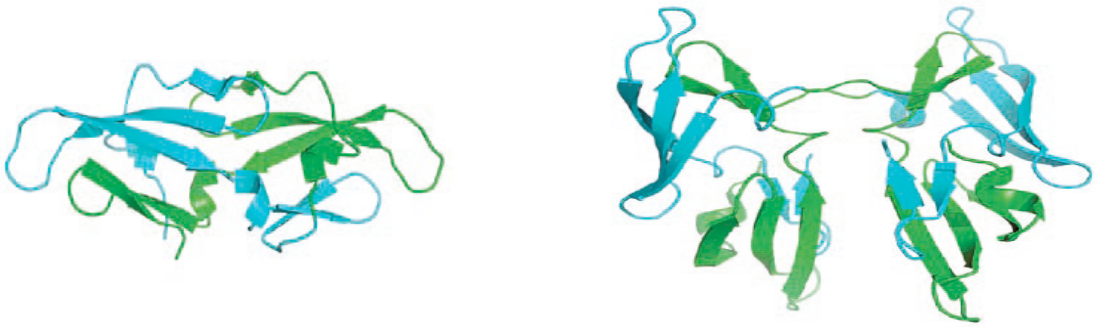


Figure 1.21: Ribbon models of CV-N. (a) The two domains of monomeric CV-N are showed in cyan and green. (b) Molecular structure of dimerized CV-N. This Figure is an adaptation from Ziolkowska and Wlodawer (168).

Each monomer of CV-N has two binding sites for mannose residues (168). Both recombinant and native CV-N have shown potent and broad anti-viral activities

against a range of viruses including HIV-1 and HIV-2; and like GRFT, CV-N neutralizes HIV by binding to mannose-rich glycans of gp41 and gp120 (19, 21, 22, 55, 110, 123). In addition, Balzarini *et al.* showed the ability of CV-N to inhibit the DC-SIGN mediated HIV-1 subtype B transfer from dendritic cells to $CD4^+$ T cells (10). However, HIV-1 was reported to develop resistance to CV-N by mutating glycans at positions 230, 332, 339, 386, 392 and 448 (11). This was supported by Hu *et al.* (2007) who also reported that glycan deletion is the pathway used by HIV-1 to develop resistance to CV-N (71). Although CV-N has not yet been tested in a human clinical trial it is noteworthy that this compound was shown to be effective in protecting pigtailed macaques after vaginal and rectal challenges with high dose of SHIV 86.9P (144, 145).

1.5.6.3. Scytovirin

Scytovirin (SVN) is a lectin isolated from the *cyanobacterium scytonema varium* and this compound is made of 95 amino acids with a molecular weight of ~ 9.7 kDa (17, 158). The amino acids sequence of SVN is contained in a single chain with extensive internal sequence duplication that divides the molecule into two functional domains. The scytovirin domain 1 (SD1) and domain 2 (SD2) are 75% identical and are linked together by 5 inter-domain disulfide bonds (95, 168). SVN exists exclusively as a monomer that has two binding sites for mannose residues, one in each domain (Figure 1.22) (104). Like GRFT and CV-N, SVN inhibits HIV-1 interaction with cellular receptors by binding to mannose-rich residues on its envelope. Both the native and recombinant SVN were shown to be strongly inhibitory to laboratory strains and primary isolates of HIV-1; and Xiong *et al.* showed that while SD1 has an anti-viral activity equaling that of the entire SVN molecule SD2 is significantly less

inhibitory to the virus (17, 158). However, unlike GRFT and CV-N, SVN has been studied exclusively *in vitro*.

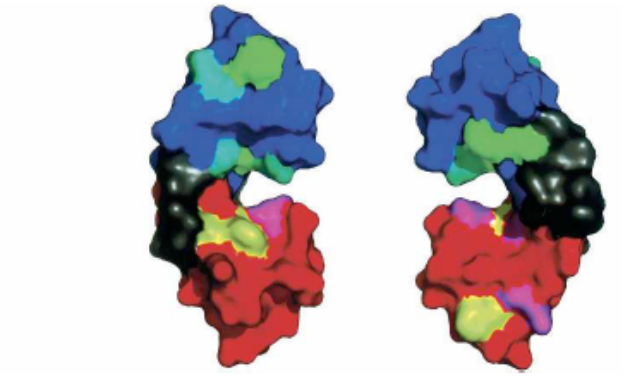


Figure 1.22: SVN amino sequence and structure. The Figure shows two different views (180º rotation) of a SVN molecule. Yellow and magenta indicate the carbohydrate binding residues of SD1 while green and cyan show the carbohydrate binding residues of SD2. This Figure was taken from Moulaei *et al.* (104).

1.5.6.4. Other HIV-1 microbicide candidate proteins

PSC-RANTES potently binds the CCR5 co-receptor to inhibit its interaction with HIV-1. This protein showed inhibitory activities against all subtypes of HIV-1 *in vitro* (39, 143); and *in vivo* a high dose of PSC-RANTES was found to be protective against SHIV SF162 in macaques (84). CMPD167 is another CCR5 inhibitor that also showed a high protective activity against SHIV in a macaques model (153). Additionally, it has been shown that the combination of CMPD167 and BMS-378806 that binds gp120 and C52L that binds gp41 results in a 100% protection of macaques from SHIV challenge. No irritation or toxicity of the genital tract was associated with the use of these compounds. Lastly, PRO140, a CCR5 inhibitor, PRO542, a tetravalent soluble CD4 construct, T20 and T1249 that bind gp41; thrombospondin type I (TSP), a plasma protein that blocks HIV-1 entry; and the pokeweed antiviral

protein (PAP) that inhibits HIV-1 replication are also among these candidate microbicides (147).

1.6. Microbicides: modes of delivery

1.6.1. Gels

Many delivery systems are being studied for dispensing HIV-1 microbicides into the female genital tract (106). However, among them gels are the most commonly used as so far most clinical trials have been conducted with gel formulated microbicides. Although they can sometimes be uncomfortable when applied gels have the advantage of being easy to use (106). Often gel formulated microbicides are designed for application before sexual intercourse; however, in some cases, such as in the tenofovir trial, they have also been applied after sexual intercourse (1).

1.6.2. Vaginal rings

Vaginal rings as a mode of delivery of HIV-1 microbicides involve the insertion of a ring containing an anti-HIV-1 compound into the female genital tract for a long-term and continuous release of the anti-viral drug to provide an environment hostile to the virus (128). Vaginal rings are made of polymers such as poly(dimethylsiloxane), silicon, ethylene vinyl acetate or styrene butadiene (106). One of the key benefits of vaginal rings is that they provide an alternative to users' sustained adherence. TMC120 or Dapiravine is one of HIV-1 potential microbicides being studied for delivery through a vaginal ring (106, 128).

1.6.3. Tablets and suppositories

Tablets and suppositories are designed to melt in the genital tract once applied thereby releasing the microbicidal agent (106). With these systems the microbicide can be delivered over several hours. Tablets are often formulated with mucoadhesive polymers and this increases their retention time in the genital tract (4). However, some tablets also use silicon matrices. Polystyrene sulfonate and praneem, containing purified extracts of *azadirachta indica*, are amongst HIV-1 microbicide candidates studied for delivery as vaginal tablets (128). A major advantage of using tablets and suppositories for the delivery of HIV-1 microbicides is that they are already in use as delivery systems for various intra-vaginal drugs. For example, cervical ripening prior to child birth is achieved by the delivery of a suppository into the genital tract (106).

1.6.4. Nanoparticles

Nanoparticles are also being researched as potential vehicles for HIV-1 microbicides (106). This system involves binding nanoparticles of a noble-metal such as silver with an anti-HIV-1 protein for delivery into the genital tract. Nanoparticles offer the advantage of being able to protect the anti-viral agent from degradation and also of facilitating its penetration into tissues containing HIV-1 target cells (128). Currently PSC-RANTES is being investigated for delivery via nanoparticles (68). A study using human ectocervical tissues showed that PSC-RANTES encapsulated into nanoparticles has a 4.8 times greater uptake than the nonencapsulated one (128).

1.7. Antibodies against HIV-1

1.7.1. Introduction

Like with other viruses, an HIV-1 infection induces the synthesis of antibodies in the infected individual. Some of these antibodies are able to prevent virus infection of cells *in vitro* and are called neutralizing antibodies; others, however, are not able to neutralize the virus and are called non-neutralizing antibodies (12). The main difference between neutralizing and non-neutralizing antibodies is that the former bind the trimeric form of gp41-gp120 while the latter only recognise the monomeric form (101, 121). In addition, some HIV-1 infected individuals develop broadly neutralizing antibodies which are able to neutralize heterologous viruses including those of other subtypes (61, 94, 102, 138). From these individuals monoclonal antibobidies (mAbs) have been isolated and just like the plasma from which they have been derived these antibodies have demonstrated potent and broad antiviral activities *in vitro* (12, 16, 23, 29). It is important to note that some of these broadly neutralizing antibodies are being studied as potential HIV-1 microbicides (9, 83).

1.7.2. The 2G12 antibody

The anti-HIV-1 monoclonal antibody 2G12 is broadly neutralizing and targets carbohydrates on gp120 (21, 30, 31). The Fab region of 2G12 has a domain swapped dimer structure resulting from the exchange of domains between the two arms of the antibody heavy chains (Figure 1.23) (30). This monoclonal antibody directly binds glycans at positions 332, 339 and 392 (31), while glycans at positions 295, 386 and 448 indirectly influence the conformation of its epitope (130, 131). Mutations from asparagine (N) to alanine (A) at the 295, 332, 339, 386, and 392 glycosylation sites have been shown to significantly reduce 2G12 affinity for HIV-1 gp120 (131).

Moreover, subtype C viruses are commonly resistant to 2G12 neutralization. This has been partly attributed to their frequent lack of the 295 glycosylation site (see Figure 1.10) as the re-introduction of this glycan only partially restores their sensitivity to 2G12 (16, 35, 60).

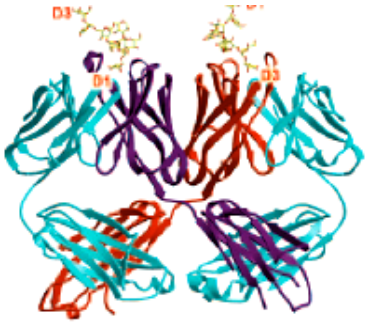


Figure 1.23: A ribbon model of the 2G12 antibody Fab region. The 2G12 Fab dimer is shown in complex with mannose$_8$; in cyan are the light chains while the heavy chains are shown in purple and red (30).

1.7.3. The b12 antibody

The monoclonal antibody b12 is a HIV-1 broadly neutralizing antibody that binds to a conserved region of gp120 overlapping the CD4bs (163). The presence of the mannose-rich glycan at position 386 has been shown to play an important role in this interaction. Eighty two percent of the b12 epitope is located in the outer domain of gp120 (163) that it contacts via its heavy chain complementarity-determining regions (CDR) (Figure 1.24). Most importantly, tryptophan 100 of CDR H3 intercalates between arginine 419 and N-glycosylated asparagine (asn) 386 of gp120 (http://www.nature.com doi: 10.1038/nature05580). The involvement of asn 386 in b12 binding to gp120 was also shown by Zwick *et al.* (169) as mutation to alanine of b12 tyrosine 53, a residue that intercalates between threonine 373 and asn 386 of gp120, resulted in the reduction of b12 binding to the glycoprotein. However, three other studies reported that the presence of the glycan at position 386 renders HIV-1 resistant to b12. More precisely Duenas-Decamp *et al.* showed that asn 386 together with arginine 373 decreases HIV-1 sensitivity to b12 (50) and this was supported by

Gray *et al*. and Sanders *et al*. in studies showing the removal of the 386 glycan significantly increased the sensitivity of the virus to this antibody (60, 129).

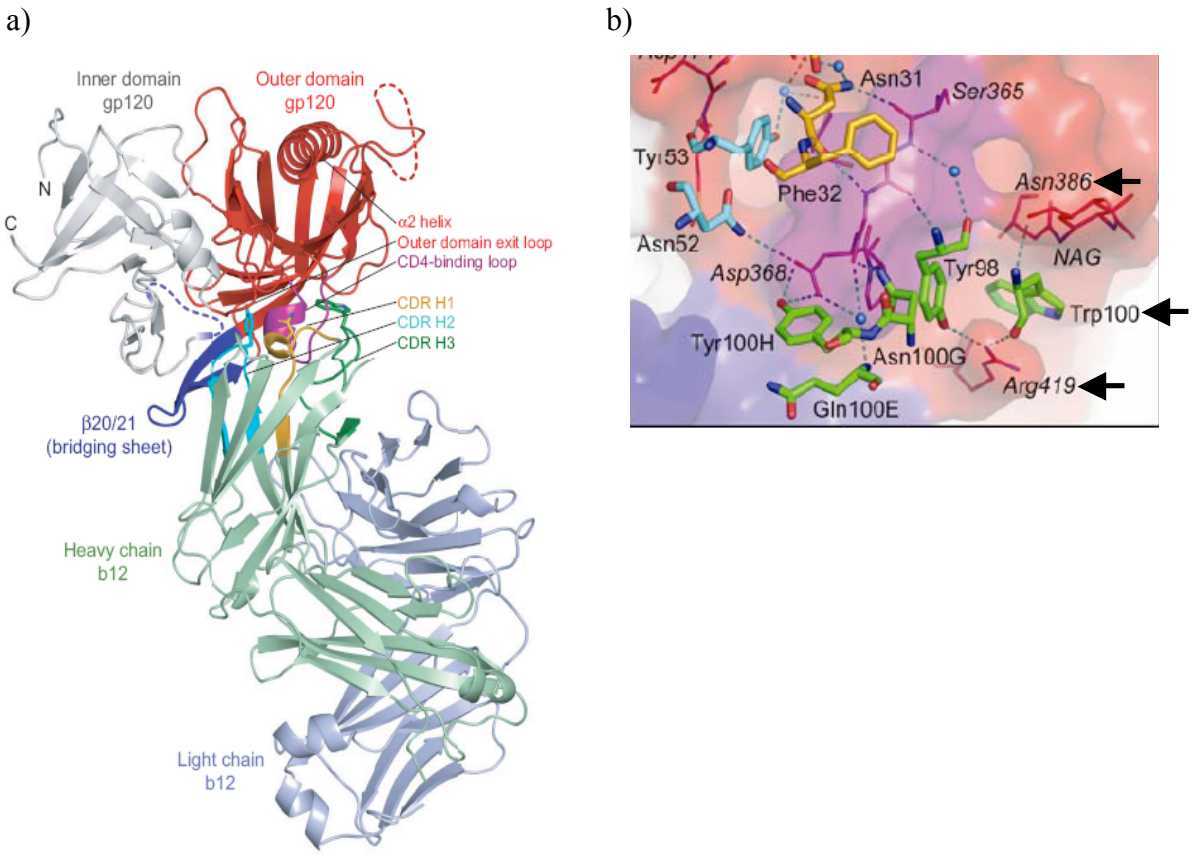


Figure 1.24: The structure of b12 in complex with gp120. (a) A ribbon model of b12 bound to gp120. CDR H 1, 2 and 3 that make contact with the outer domain of gp120 are shown in orange, cyan and dark green, respectively. (b) A closer look at the interaction between tryptophan 100 of CDR H3 and gp120 asparigine 386 and arginine 419. These three amino acids are indicated by arrows. Amino acids in gp120 and b12 are labelled in italic and normal script, respectively. This Figure was adapted from Zhou *et al.* (163).

1.7.4. Other HIV-1 broadly neutralizing antibodies

VRC01 is the broadest HIV-1 neutralizing antibody isolated to date as it neutralizes over 90% of circulating isolates (162). Like b12 VRC01 binds to the CD4bs; however, unlike many CD4bs antibodies its binding to this site avoids conformational masking (79) and the glycan that shield the CD4bs. VRC01 broad neutralization breadth is partly due to the extensive affinity maturation it undergoes and the fact that

87% of its interaction with gp120 is made with the conformationally invariant outer domain (162). Although, 13% of VRC01 contact with gp120 involve the bridging sheet and the conformationally flexible inner domain this interaction has a minor influence on the antibody binding to HIV-1.

Like VRC01, PG9 and PG16 are potent and very broad HIV-1 neutralizing antibodies (154). More precisely, they neutralize 70-80% of circulating HIV-1 isolates (113). PG9 and PG16 are somatically related and are derived from the same combination of heavy and light chain. In addition, these antibodies target a quaternary epitope on gp41-gp120 trimer involving the V2 and V3 loops. Two of the main characteristics associated with PG9 and PG16 broad neutralizing activities are the extensive affinity maturation they undergo and the unusually long CDR H3 they possess (113).

The monoclonal antibodies 2F5 and 4E10 are HIV-1 broadly neutralizing antibodies that target the membrane-proximal external region (MPER) of gp41 (33, 49, 66, 112). The core of the 2F5 epitope is made of the sequence ELDKWA while the sequence NWFDIT forms the core of 4E10 epitope. The binding of 2F5 and 4E10 to HIV-1 can be divided into two steps that are: attachment to the virion associated lipids and binding to the MPER epitope that becomes exposed during the virus-cell fusion (94). Together with 2G12, b12, VRC01, PG9, PG16, 2F5 and 4E10 are the broadest and most potent HIV-1 antibodies isolated to date. Figure 1.25 summarizes the location of these seven antibodies epitopes on trimeric gp41-gp120.

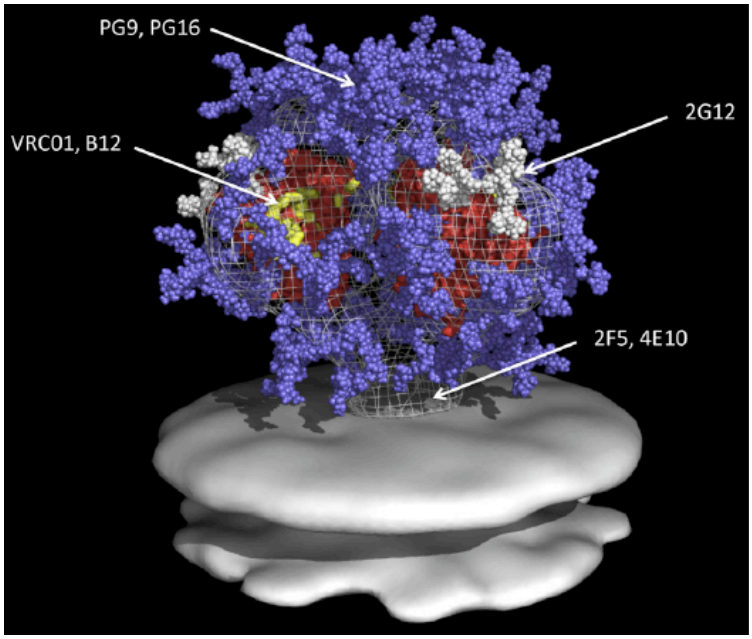


Figure 1.25: Summary of 2G12, b12, VRC01, PG9, PG16, 2F5 and 4E10 epitopes on gp41-gp120 trimer. The locations of the seven antibodies epitopes are indicated by arrows. In addition, the Figure shows the core gp120 in red, glycans constituting the 2G12 epitope are shown in white while the rest of gp160 glycans are shown in blue, and VRC01 and b12 epitopes are shown in yellow. This Figure was taken from McElrath and Haynes (94).

Although not as broad and potent as the antibodies mentioned above, the V3 loop specific antibody F425-B4e8 has been shown to have cross-neutralizing activities against HIV-1 subtype B, C and D isolates (15). F425-B4e8 epitope is made of residues surrounding the sequence GPGRA that constitutes the apex of the V3 loop in subtype B viruses and Isoleucine 309, arginine 315 and phenylalanine 317 play a critical role in this epitope (15). However, much of F425-B4e8/V3 interaction involve arginine 315 since this residue mutation to alanine or glutamine markedly reduces the antibody affinity for gp120 (114).

The monoclonal antibody 17b neutralizes HIV-1 by binding to the CD4 induced (CD4i) epitope that is formed following CD4 binding to gp120 (142). Most importantly, 17b prevents HIV-1 entry into cells by inhibiting steps that are

subsequent to CD4-gp120 interaction. Furthermore, this antibody binding to gp120 was shown to be enhanced in the presence of sCD4 and *in vitro* 17b showed broad neutralizing activities against HIV-1 subtypes (142). Lastly, in a study by Decker *et al.* 17b demonstrated potent inhibitory activities against HIV-2 in the presence of sCD4 (42).

1.8. Study aims

The main objective of this study was to investigate the ability of GRFT, CV-N and SVN to inhibit HIV-1 subtype C infection of cells and to study the binding sites of these lectins on gp120. This was achieved by dividing the study in four parts:

1. Since HIV-1 subtype B has been shown to be sensitive to GRFT, CV-N and SVN the first part of the project involved testing the sensitivity of subtype C viruses to these lectins. This section also examined glycans on HIV-1, including those in the 2G12 epitope, that are involved in GRFT, CV-N and SVN binding to the virus.
2. While studying the involvement of the 2G12 epitope in GRFT, CV-N and SVN binding to HIV-1 we observed that GRFT enhanced the binding of the mAb b12 to HIV-1. We, therefore, investigated the ability of GRFT to expose the CD4bs, thereby, making it more accessible to CD4bs specific antibodies. In this section we also studied whether GRFT synergized with these antibodies to neutralize HIV-1.
3. The DC-SIGN receptor plays an important role during the sexual transmission of HIV-1; it mediates the transfer of the virus to its target cells. Therefore, in this part of the project we investigated GRFT, CV-N, and SVN inhibition of

HIV-1 binding to the DC-SIGN receptor as well as their inhibition of the DC-SIGN receptor-mediated transfer of the virus to target cells.

4. Because HIV-1 subtype B has been shown to develop resistance to CV-N we investigated the ability of the subtype C virus to develop resistance to GRFT, CV-N and SVN. We also analyzed glycans on the viral envelope that are involved in this resistance.

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CHAPTER TWO

MANNOSE-RICH GLYCOSYLATION PATTERNS ON HIV-1 SUBTYPE C GP120 AND SENSITIVITY TO THE LECTINS, GRIFFITHSIN, CYANOVIRIN-N AND SCYTOVIRIN

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Mannose-rich glycosylation patterns on HIV-1 subtype C gp120 and sensitivity to the lectins, Griffithsin, Cyanovirin-N and Scytovirin

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ABSTRACT

Griffithsin (GRFT), Cyanovirin-N (CV-N) and Scytovirin (SVN) are lectins that inhibit HIV-1 infection by binding to multiple mannose-rich glycans on the HIV-1 envelope glycoproteins (Env). Here we show that these lectins neutralize subtype C primary virus isolates in addition to Env-pseudotyped viruses obtained from plasma and cervical vaginal lavages. Among 15 subtype C pseudoviruses, the median IC_{50} values were 0.4, 1.8 and 20.1 nM for GRFT, CV-N and SVN, respectively, similar to what was found for subtype B and A. Analysis of Env sequences suggested that concomitant lack of glycans at positions 234 and 295 resulted in natural resistance to these compounds, which was confirmed by site-directed mutagenesis. Furthermore, the binding sites for these lectins overlapped that of the 2G12 monoclonal antibody epitope, which is generally absent on subtype C Env. This data support further research on these lectins as potential microbicides in the context of HIV-1 subtype C infection.

Introduction

Griffithsin (GRFT) is a lectin isolated from the red algae *Griffithsia* sp. found in the coastal waters off New Zealand (Mori et al., 2005). It is a 121 amino acid dimeric protein with a domain-swapped structure (Ziolkowska et al., 2006). This lectin neutralizes HIV-1 by binding to mannose-rich glycans found on the envelope glycoproteins. Both native and recombinant GRFT display potent antiviral activities against laboratory-adapted strains and primary isolates of M- and T-tropic HIV-1. This compound was also shown to be active against a broad range of HIV-1 including 4 subtype C viruses (O'Keefe et al., 2009). A second lectin, Cyanovirin-N (CV-N) isolated much earlier from the blue green algae *Nostoc ellipsosporum* (Boyd et al., 1997) is a 101 amino acid protein that has been shown to exist either as a quasi-symmetric two-domain monomer or as a domain-swapped dimer (Barrientos et al., 2002; Botos and Wlodawer, 2005). Like GRFT, CV-N binds to mannose-rich glycans and both native and recombinant CV-N have shown potent anti-HIV-1 activity *in vitro* (Bolmstedt et al., 2001; Esser et al., 1999). A third protein, Scytovirin (SVN) is a 95 amino acid lectin isolated from the cyanobacterium *Scytonema varium* (Bokesch et al., 2003). SVN is expressed as a single amino acid chain with extensive internal sequence duplication. This protein is found exclusively as a monomer and has been shown to neutralize both laboratory-adapted strains and primary isolates of HIV-1 by interacting with mannose-rich glycans on the viral envelope (Moulaei et al., 2007; Xiong et al., 2006; Ziolkowska and Wlodawer, 2006).

As a result of their ability to block HIV-1 entry *in vitro*, GRFT, CV-N and SVN have been proposed as potential microbicides to prevent the sexual transmission of HIV-1. Although these compounds are not HIV-specific, they target high-mannose arrays that are present on all HIV envelope glycoproteins. Since such arrays are uncommon in mammalian cells, these compounds are not likely to be toxic to human cells *in vivo* even at relatively high concentrations (Balzarini, 2005; Balzarini and Van Damme, 2007). Furthermore, a recombinant GRFT produced in the tobacco-like plant *Nicotiana benthamiana* was shown to be non-toxic in a rabbit vaginal irritancy model and in human cervical explants (O'Keefe et al., 2009). Although GRFT, CV-N and SVN have not yet been tested in human clinical trials it is noteworthy that CV-N was shown to be effective in protecting pigtailed macaques after vaginal and rectal challenges with high dose of SHIV 86.9P (Tsai et al., 2004, 2003).

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HIV-1 can develop resistance to CV-N by partial deglycosylation, specifically deleting glycans at positions 230, 289, 295, 332, 339, 386, 392 and 448 in HIV-IIIB (Balzarini et al., 2006; Hu et al., 2007). This suggests that resistance to CV-N may be hard to generate as it will require multiple mutations in the viral genome although additional studies using primary viruses are needed. Some of these glycans also form part of the epitope for the broadly neutralizing monoclonal antibody (mAb) 2G12. This antibody directly binds glycans at positions 332, 339 and 392 (Calarese et al., 2003), while glycans at positions 295, 386 and 448 though not directly involved in this epitope, may influence its conformation (Sanders et al., 2002; Scanlan et al., 2002). Subtype C viruses are commonly resistant to 2G12 neutralization. This has been attributed to their frequent lack of the 295 glycosylation site although re-introduction of this glycan only partially restores sensitivity to this mAb (Binley et al., 2004; Chen et al., 2005; Gray et al., 2007b). Although it is known that GRFT and SVN bind to mannose-rich glycans found on HIV-1 envelope, the specific glycans involved in this binding have not yet been identified.

Despite subtype C viruses being responsible for over 50% of global infections (http://www.unaids.org) many of the antiviral studies with GRFT, CV-N and SVN have been done on subtype B viruses. Significant differences exist in the pattern of glycosylation between subtype B and C envelope glycoproteins (Zhang et al., 2004). For example, about 80% of subtype B envelopes are glycosylated at position 295 while only ~20% of subtype C viruses are glycosylated at the same position. The opposite pattern is observed at position 230 where over 70% of subtype C envelopes are glycosylated compared to only 20% of subtype B. It is known that differences between subtypes can affect their susceptibility to neutralizing agents as for the 2G12 mAb described above (Binley et al., 2004; Gray et al., 2006). Therefore, we aimed to examine the sensitivity of a large collection of subtype C viruses to GRFT, CV-N and SVN and compare these to subtypes B and A strains. Furthermore, we examined the role of the 2G12 epitope in these lectin's binding sites.

Results

GRFT, CV-N and SVN neutralize HIV-1 subtype C isolates from blood and cervico-vaginal lavages

To determine the ability of GRFT, CV-N and SVN to inhibit HIV-1 subtype C infection of peripheral blood mononuclear cells (PBMC), we performed neutralization assays using blood-derived subtype C primary isolates Du151, COT9 and Du179. Viral infection was measured by p24 ELISA after 4 days of culture. The concentration of lectin that inhibited 80% of virus infection (IC_{80}) is reported. All three lectins neutralized these viruses in a dose-dependent manner (Fig. 1). The IC_{80} values were similar for all viruses with GRFT and CV-N being more potent than SVN. The mean IC_{80} values for all 3 viruses were 42.7 ± 4.4, 77.0 ± 18.2 and 368.4 ± 22.7 nM for GRFT, CV-N and SVN, respectively.

Next we tested the ability of GRFT, CV-N and SVN to inhibit 50% (IC_{50}) HIV-1 infection in the TZM-bl cell-line based neutralization assay (Montefiori, 2004). For this we used 11 subtype C Env-pseudotyped viruses cloned from plasma of adult patients with acute or early HIV infection and from pediatric patients during chronic infection (see Materials and methods for details). For comparison we included 6 viruses from the subtype B panel described by Li et al. (Li et al., 2005) and 5 subtype A viruses from acutely infected individuals (Long et al., 2002; Neilson et al., 1999). All viruses used the CCR5 coreceptor. GRFT and CV-N potently neutralized most subtype C viruses with a median IC_{50} of 0.4 and 1.2 nM, respectively, (Fig. 2) which were not significantly different. SVN was the least effective with a median IC_{50} of 20.1 nM. The single-cycle pseudovirus assay required considerably lower concentrations of lectin to inhibit viral infection compared to the PBMC assay even when comparing IC_{80} values (not shown). Nevertheless, the trends were the same in both assays as was noted for Du151.2 and COT9.6 pseudoviruses compared to their matched primary viruses in PBMC (Table 1 and Fig. 1). There were no significant differences in mean IC_{50} values between subtypes C, B and A viruses for all 3 lectins ($p>0.05$) (Table 1). However, 2 subtype C viruses (CAP206.08J and CAP63.A9J) and one subtype B virus (CAAN5342.A2) showed unusually high IC_{50} values suggesting natural resistance to these compounds (Fig. 2). We also found a significant positive correlation ($p<0.05$) between GRFT and CV-N sensitivity for subtype C, and between CV-N and SVN for both subtype C and B Env-pseudotyped viruses (data not shown).

We also tested the sensitivity of four subtype C Env isolated from cervical–vaginal lavages (CVL) of HIV-1 positive women during acute infection (Bronwen Lambson, unpublished data). These viruses also showed varying sensitivities to the lectins with one (CAP177.cvl96) showing a high level of resistance to both GRFT and CV-N (Fig. 3). For CAP63.cvl6GA a matched blood sample was also available and the CVL and plasma derived viruses showed similar sensitivities to the three lectins (Table 1). In general, viruses from CVL were inhibited by GRFT, CV-N and SVN with IC_{50} in the nanomolar range similar to those derived from plasma.

Correlation between HIV-1 mannose-rich glycosylation patterns and sensitivity to GRFT, CV-N and SVN

Given that these lectins bind mannose-rich glycans, we analyzed the predicted glycosylation patterns of all the viruses used in our neutralization assays to determine if there was any correlation with their sensitivity to GRFT, CV-N and SVN. This analysis included all 11 predicted mannose-rich sites on HIV-1 gp120 some of which are involved in mAb 2G12 binding (labeled with asterisks in Table 1). Significant differences were noted between subtypes as previously reported (Zhang et al., 2004). In this study, 13 of the 15 subtype C viruses lacked the 295 glycan compared to more than half of the subtype B and A viruses where this glycan was intact. On average, viruses from subtypes C and B lacked 2 of the 11 predicted glycans although there was a range with some viruses lacking as many as 5 sites. The subtype A viruses had a larger number of missing sites and they all lacked the 230 and 289 glycans. Only one virus was predicted to have all 11 glycans, a subtype C virus (CAP270.cvl44) isolated from CVL.

The five viruses (four subtype C, including two from CVL, and one subtype B) that were the least sensitive to GRFT lacked both the 234 and 295 glycosylation sites. Two of these five viruses (CAP206.08J and CAAN5342.A2) were also the least sensitive to CV-N and SVN and a third (CAP177.cvl96) also required above average levels of CV-N for inhibition. We concluded that the absence of both the 234 and 295 glycosylation sites was responsible for this decreased sensitivity to these lectins. However, the lack of either of these two glycans in isolation did not affect sensitivity. Thus WITO4160.33 and Q842.d12 which lacked the 234 glycan but had the 295 glycan showed similar sensitivity to viruses with both glycans. Similarly viruses that lacked the 295 glycan but retained the glycan at position 234 (most subtype C viruses) were not unusually resistant. Overall there was no correlation between the number of missing glycans and resistance to these lectins. The fewer mannose-rich glycosylation sites on subtype A Envs did not reduce their sensitivity.

The 234 and 295 glycosylation sites are involved in GRFT, CV-N and SVN neutralization of HIV-1

In order to examine the contribution of the glycan at position 295 to lectin neutralization, we introduced this glycosylation site in 3 viruses, Du151.2, COT9.6 and COT6.15, that already had the 234 glycan (see Table 1). For all three viruses the introduction of the 295 glycosylation site further increased their sensitivity to GRFT by 7 to 50

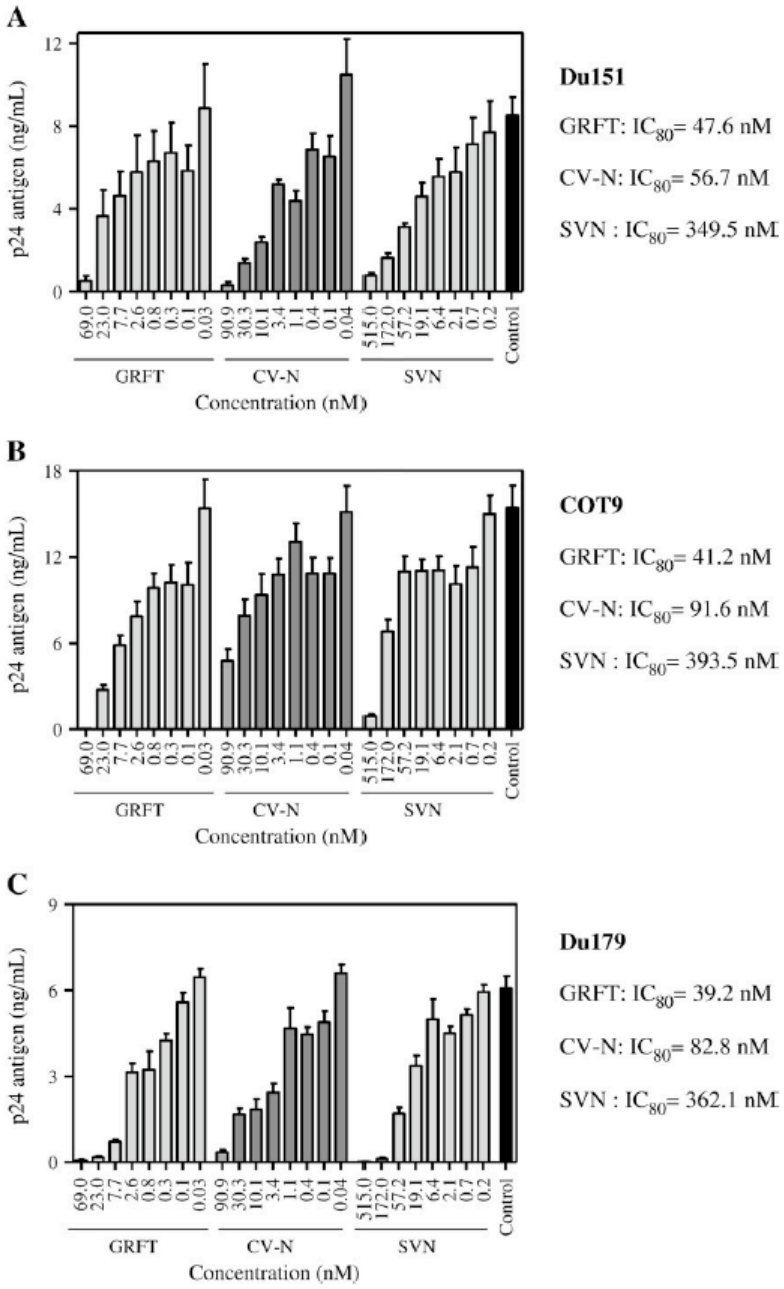

Fig. 1. GRFT, CV-N, and SVN inhibit HIV-1 subtype C infection of PBMC. Three primary isolates, Du151 (A), COT9 (B) and Du179 (C) were treated with increasing concentrations of GRFT, CV-N and SVN before infection of PBMC. Data are shown as the average plus standard deviations of three independent experiments. Untreated virus is shown in black (positive control). The IC_{80} values of the lectins are indicated next to each graph.

fold (Table 2). However, the addition of the 295 glycan had no effect on their sensitivity to CV-N and SVN suggesting that this glycan is essential for GRFT binding only. As GRFT is more promiscuous in its binding to high-mannose oligosaccharides than either SVN or CV-N (which only bind to oligomannose-8 or -9) this could also indicate that the glycan at position 295 is a smaller oligosaccharide such as oligomannose-5 or -7.

We also introduced both the 234 and 295 glycosylation sites into 3 viruses, CAP206.08J, CAP63.A9J and CAAN5342.A2 that lacked both of these sites and were the most resistant to the lectins, to determine if this would increase their sensitivity. Reconstruction of the 234 glycosylation site in both CAP206.08J and CAP63.A9J reduced virus infectivity of TZM-bl cells by ~2-fold. We were unable to determine whether this was due to a decrease in the production of viral particles in 293T cells or a defect in the virus's capacity to infect cells. However, the introduction of 295 and to a lesser extent 234, increased the sensitivity of all three viruses to GRFT (Fig. 4A). Furthermore, when these two glycans were introduced simultaneously there was an additional increase in sensitivity. The introduction of the 234 and 295 glycosylation sites also had a similar effect on CV-N sensitivity, with the exception of CAP63.A9J where addition of the 234 glycosylation site had no effect (Fig. 4B). For SVN, the 234 glycosylation site had a more significant impact on sensitivity than the 295 although for CAP63.A9J addition of a glycan at position 295 actually increased

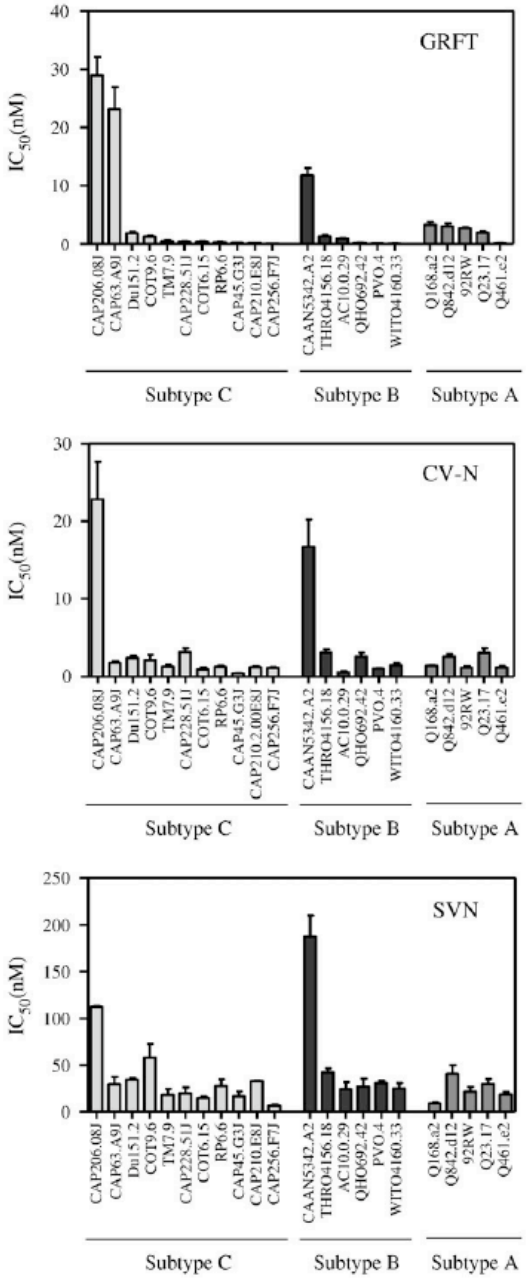

Fig. 2. GRFT, CV-N, and SVN inhibit HIV-1 infection in the TZM-bl assay. Neutralization of HIV-1 subtype C, B and A pseudoviruses by GRFT (A), CV-N (B), and SVN (C). HIV-1 subtype B and A pseudoviruses were used for comparison to subtype C. Each virus was tested at least three times. Pseudoviruses are ranked by GRFT sensitivity.

sensitivity above the wild-type (Fig. 4C). Collectively, these data suggested that the 234 and 295 glycosylation sites are involved in lectin binding to the HIV-1 envelope glycoproteins and subsequent virus neutralization, although for CV-N and SVN their involvement may be strain-dependent.

GRFT, CV-N and SVN binding sites overlap the 2G12 epitope

Since five of the 11 mannose-rich sites on gp120 form the 2G12 mAb epitope, we investigated whether lectin binding interfered with access to this epitope. We used a competition ELISA to measure the amount of p24 antigen captured onto 2G12-coated plates after pre-incubation of the virus with GRFT, CV-N or SVN. The 2G12 mAb was competed against itself as a positive control. We tested the subtype B virus QH0692.42 which is sensitive to 2G12 with an IC_{50} of 2.8 μg/ml (Li et al., 2005). Subtype C viruses are generally insensitive to 2G12 and so we used modified subtype C gp160 where the 2G12 epitope had been partially reconstituted (Gray et al., 2007b). Thus, for COT6.15 the addition of glycans at positions 295 and 448 (COT6-V295N/S448N) increased sensitivity from IC_{50} >100 μg/ml to 64.5 μg/ml. There was also increased binding of the 2G12 mAb to this mutant (Gray et al., 2007b). For COT9.6 the addition of glycans at positions 295 and 442 (COT9-V295N/K442N) increased the sensitivity to 2G12 from an IC_{50} of 68.7 μg/ml to 33.9 μg/ml.

Pre-incubation of the 3 viruses with each lectin significantly reduced the amount of virus captured onto 2G12-coated plates suggesting competition between these compounds (Fig. 5). Similar to what was seen in the neutralization assays; GRFT and CV-N were more potent than SVN at inhibiting virus capture. The effect was dose-dependent although higher amounts of lectin were required to inhibit virus capture compared to neutralization. However, for all 3 viruses, virus capture by 2G12 was inhibited at equivalent lectin concentrations despite the 10–20 fold difference in sensitivities to 2G12 neutralization. Together with the mutagenesis results (Table 2) these data suggested an overlap between the 2G12 epitope and GRFT, CV-N and SVN binding sites.

Discussion

In this study we have shown that HIV-1 subtype C viruses, including those from the vaginal tract are sensitive to the mannose-binding lectins, GRFT, CV-N and SVN. Despite subtype-specific differences in mannosylation patterns, their sensitivity was similar to what has been previously reported for subtype B viruses (Bokesch et al., 2003; Boyd et al., 1997; Mori et al., 2005). GRFT and CV-N were more potent than SVN which required higher concentrations to prevent HIV-1 entry. We identified glycans at positions 234 and 295, in addition to the 2G12 mAb epitope, as playing an important role in the binding of these lectins. Our data support the continued development of GRFT, CV-N and SVN as potential microbicides since these compounds are likely to be effective against HIV-1 subtype C mucosal transmissions.

Inhibition of HIV-1 subtype C infection by GRFT, CV-N and SVN in both primary cells and a cell line were similar although higher concentrations were required for PBMC neutralization. The increased sensitivity in TZM-bl cells compared to PBMC is consistent with studies of antibody neutralization, where single-cycle pseudovirus assays are generally more sensitive than PBMC infectivity assays (Fenyo et al., 2009). The longer duration of culture and multiple replication cycles in the PBMC assay probably account for this. Unlike Huskens et al., we did not find any enhanced infectivity of HIV-1 in PBMC treated with CV-N, indicating that CV-N was not mitogenic in our hands (Huskens et al., 2008). The greater neutralization potency of GRFT and CV-N compared to SVN may be due to the fact that a GRFT dimer has six mannose-binding sites and a CV-N dimer has four binding sites. In contrast, the SVN monomer has only two mannose residue binding sites (Moulaei et al., 2007; Ziolkowska et al., 2006; Ziolkowska and Wlodawer, 2006). The correlation between IC_{50} values of the compounds within certain subtypes suggested that these lectins share some binding sites, a finding which we confirmed for a few loci. Although, we have shown efficacy of these compounds against subtype C viruses in primary PBMC, it will be important to test the anti-HIV activity of GRFT, CV-N and SVN using dendritic cells and macrophages as these cells play an important role during the sexual transmission of HIV (Lederman et al., 2006; Pohlmann et al., 2001).

Subtype C viruses isolated from both adult and pediatric HIV infections as well as those from acute and chronic infection were sensitive to these lectins. Our data are in line with previously reported

Table 1
Pattern of predicted mannose-rich glycans on gp120 of HIV-1 and sensitivity to GRFT, CV-N, and SVN.

HIV-1 envelope pseudoviruses	Predicted N-linked mannose-rich glycosylation sites[a]											IC_{50} (nM)[b]		
	230	234	241	262	289	295[c]	332[c]	339[c]	386[c]	392[c]	448[c]	GRFT	CV-N	SVN
Subtype C (n=15)														
CAP177.cvl96		x			x	x	x			x		55.9±5.0	7.5±1.1	21.8±5.4
CAP206.08J		x			x	x				x	x	30.9±6.2	18.0±1.2	112.8±1.1
CAP63.A9J		x				x						23.2±6.5	1.8±0.3	29.8±13.3
CAP63.cvl6GA		x				x						25.7±2.6	3.1±0.1	43.8±8.5
CAP261.cvl93						x			x			4.2±0.4	1.8±0.6	6.2±0.9
Du151.2						x		x		x		1.5±0.8	2.0±1.0	27.5±9.6
COT9.6						x						1.2±0.2	3.9±2.7	57.6±26.9
COT6.15						x					x	0.4±0.2	0.9±0.5	10.8±6.9
CAP228.51J						x				x		0.4±0.1	3.1±0.9	20.1±11.1
CAP270.cvl44												0.3±0.1	0.8±0.07	11.7±4.8
RP6.6						x		x				0.2±0.1	1.1±0.1	11.3±3.3
TM7.9						x				x		0.2±0.1	1.1±0.4	12.1±1.6
CAP45.G3J						x	x			x	x	0.2±0.1	0.4±0.1	16.9±8.8
CAP210.E8J						x						0.2±0.0	1.2±0.2	33.1±1.1
CAP256.F7J	x							x				0.1±0.0	1.1±0.1	6.7±2.3
Median												*0.4*	*1.8*	*20.1*
Subtype B (n=6)														
CAAN5342.A		x				x		x				11.7±2.3	16.7±6.0	187.6±38.6
THRO4156.18					x		x					1.2±0.3	3.0±0.8	42.6±6.9
AC10.0.29	x					x			x			0.9±0.2	0.3±0.1	24.5±12.9
QHO692.42					x					x		0.2±0.1	2.5±0.8	27.3±14.1
PVO.4	x									x		0.1±0.0	1.0±0.1	25.2±14.0
WITO4160.33		x			x							0.05±0.0	1.5±0.5	24.8±10.1
Median												*0.6*	*2.0*	*26.2*
Subtype A (n=5)														
Q168.a2	x				x		x	x		x		3.3±0.9	1.4±0.1	9.4±2.3
Q842.d12	x	x			x		x					3.0±0.9	2.5±0.6	40.9±15.8
Q23.17	x				x	x						1.9±0.3	3.0±1.1	29.8±9.0
92RW009	x				x			x	x			1.8±0.4	1.2±0.3	21.8±8.9
Q461.e2	x				x	x	x			x		0.12±0.06	1.2±0.3	18.9±4.2
Median												*1.9*	*1.4*	*21.8*

[a] Mannose-rich glycosylations were identified from the amino acid sequence of each envelope clone (Kwong et al., 1998; Leonard et al., 1990). Missing mannose-rich glycosylation sites are marked by x. Viruses are ranked according to GRFT sensitivity.
[b] The IC_{50} is the concentration of GRFT, CV-N, and SVN that reduced HIV-1 infection by 50%. Mean ± SD of 3 independent experiments is shown.
[c] Glycans involved in binding the monoclonal antibody 2G12.

inhibitory concentrations for subtype B viruses and confirm that these compounds are active against multiple subtypes (Bokesch et al., 2003; Boyd et al., 1997; Mori et al., 2005; O'Keefe et al., 2009). Given that CV-N and GRFT are being proposed as microbicides, we also considered it important to test them against viruses found in the vaginal tract. It has been reported that virus compartmentalization can occur during HIV infection raising the possibility of differential sensitivity of viruses from CVL compared to blood (Kemal et al., 2003). However, our data showed that CAP63.A9J from plasma and CAP63.cvl6GA from CVL of the same HIV-positive woman had similar sensitivity to all three lectins. We attributed this to the fact that these two viruses have minimal genetic differences and the same mannose-rich glycosylation patterns (Table 1). Also these viruses are from early infection and, therefore, it may be too early for compartmentalization to develop. Nevertheless, further studies with more pairs of plasma and CVL viruses, from the same individual with a demonstrated virus compartmentalization, are needed to determine if viruses from CVL are as sensitive as those present in blood.

Despite differences in mannose-rich glycosylation patterns, subtype C, B and A viruses (Zhang et al., 2004) showed similar sensitivities to GRFT, CV-N, and SVN. This result was unexpected given that these compounds bind high-mannose residues. Analysis of the glycosylation patterns suggested that absence of glycans at positions 234 and 295 was associated with natural resistance to GRFT and in some cases to CV-N and SVN. Since subtype C viruses generally lack 295N it was surprising that these viruses were as sensitive to the lectins as subtype B and A viruses that generally retained this site. However, a concomitant lack of the 234 glycosylation site was also required to reduce sensitivity and since this glycan is present on ~80% of subtype C viruses, this may account for the comparable sensitivity with subtype B. The important role of these glycans in conferring sensitivity to these lectins was confirmed by site-directed mutagenesis. Interestingly, when the 295 or 234 glycosylation sites were individually reconstituted they had a significant impact in some viruses. This suggests that in natural Env there may be considerable redundancy or overlap in the glycans that are bound by these compounds. We did not find a correlation between the number of predicted mannose-rich glycans and sensitivity to the lectins, unlike an earlier study on CV-N in subtype B viruses (Balzarini et al., 2006). For example, CAP270.cvl44 which had all predicted 11 mannose-rich glycans was less sensitive to GRFT than viruses that had fewer predicted glycans. In fact our data suggested that the location of the glycan was more important than the number of glycans. Thus, in addition to mannose-rich glycans there may be other factors in the viral envelope that determine the sensitivity to GRFT, CV-N and SVN. These factors may include the Env structure, that can result in glycans that occupy the same position being exposed differently, and the fact that not all potential sites are glycosylated (Go et al., 2009).

Three studies examining CV-N escape pathways using laboratory-adapted HIV-1 subtype B viruses selected *in vitro* showed that deletion of glycans caused resistance to CV-N (Balzarini et al., 2006; Hu et al., 2007; Witvrouw et al., 2005). The large number of glycans involved suggested that these compounds have a high genetic barrier to resistance. In this study, we showed that for subtype C primary

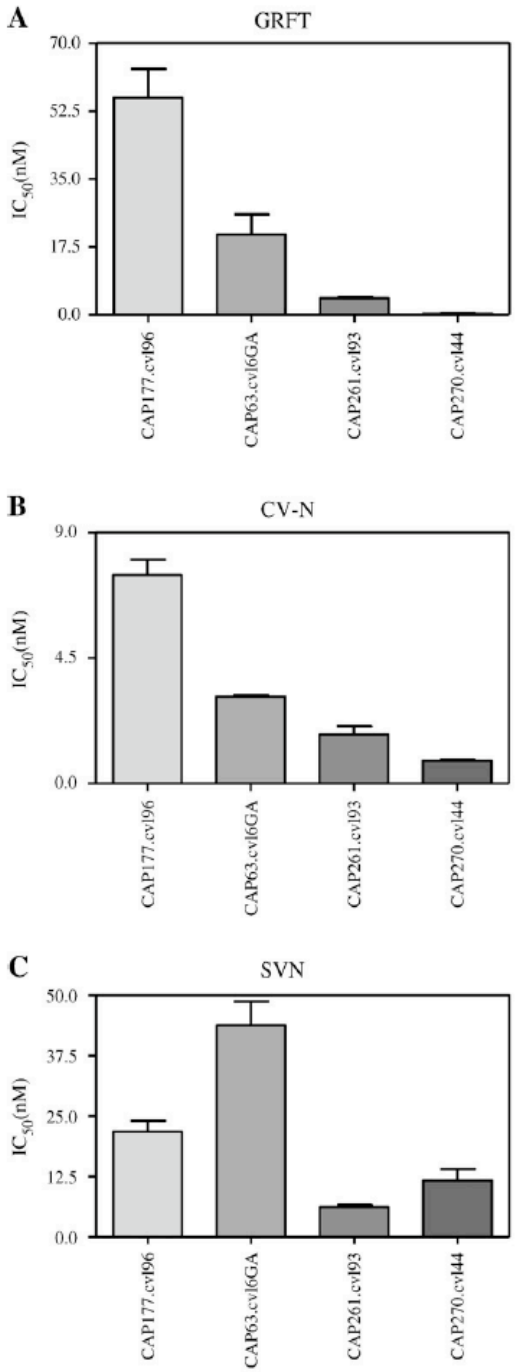

Fig. 3. GRFT, CV-N, and SVN inhibit HIV-1 isolates from CVL. Sensitivity of 4 HIV-1 subtype C pseudoviruses containing functional envelope genes amplified from cervicovaginal lavages against GRFT (A), CV-N (B) and SVN (C) are shown.

viruses that lack the 295 glycan, the absence of a single glycan at position 234 could in some cases confer natural resistance to these lectins. However, it is important to bear in mind that generating resistant variants *in vitro* is likely to be different to identifying loci associated with natural resistance as we have done here. Furthermore, naturally-occurring resistant viruses may have compensatory mutations that are isolate-specific or not commonly present. Indeed, glycan

Table 2
Effect of the 295 glycan on sensitivity to GRFT, CV-N and SVN.

Envelope		GRFT	CV-N	SVN
		IC_{50} (nM)[a]		
Du151.2	Wild-type	1.5±0.8	2.0±1.0	27.5±9.6
	V295N	**0.03±0.02**	1.2±0.4	32.5±4.9
COT9.6	Wild-type	1.2±0.2	3.9±2.7	57.6±26.9
	V295N	**0.06±0.04**	4.4±3.9	63.0±35.9
COT6.15	Wild-type	0.4±0.2	0.9±0.5	10.8±6.8
	V295N	**0.06±0.0**	1.1±0.6	16.1±9.2

Cases where the mutant IC_{50} was more than 5-fold lower than the wild-type virus IC_{50} are bolded.

[a] IC_{50} is the concentration of GRFT, CV-N, and SVN that reduces viral infection by 50%.

234 may play an important functional role given its relative conservation in subtype C, and this is first study to report its involvement in CV-N resistance in both subtypes C and B. Further studies are needed to clearly elucidate the position and number of glycans involved in CV-N resistance in primary viruses. It will also be important to investigate the ability of HIV-1 to develop resistance to GRFT and SVN, which may help to identify other GRFT and SVN binding sites on the virus.

The ability of GRFT, CV-N and SVN to compete against the 2G12 MAb for binding to HIV-1 implicates the 2G12 epitope in the binding of these compounds to HIV-1. These data are in agreement with previous reports that glycans in the 2G12 epitope are involved in CV-N and GRFT binding to HIV-1 gp120 (Esser et al., 1999; Mori et al., 2005). However, our study is the first to demonstrate the involvement of the 2G12 epitope in SVN binding to HIV-1. Nevertheless, the involvement of this epitope in the activity of these lectins to subtype C viruses is unclear as these viruses are generally insensitive to the 2G12 mAb (Chen et al., 2005; Gray et al., 2007b; Sanders et al., 2002). The sensitivity of subtype C viruses to GRFT, CV-N and SVN is likely due to the fact that these lectins bind many more sites than those involved in the 2G12 epitope.

GRFT, CV-N and SVN are being actively pursued as microbicides (Bokesch et al., 2003; O'Keefe et al., 2009; Shattock and Moore, 2003; Xiong et al., 2006). The search for HIV microbicides is driven by the challenges encountered in developing an effective HIV vaccine (Johnston and Fauci, 2007) and the fact that the majority of HIV infections around the world are sexually transmitted (Stein, 2003). Potential microbicides include non-specific inhibitors such as buffering agents that inactivate viruses by maintaining the acidic pH in the vaginal tract and moderately specific inhibitors such as anionic polymers that neutralize the virus by binding to its positively charged envelope glycoproteins (Cutler and Justman, 2008). Anti-retroviral agents that target the reverse transcriptase enzyme are being actively researched and herald a new phase in prevention strategies. GRFT, CV-N and SVN, however, hold an advantage over the above-mentioned compounds by selectively targeting the virus to inhibit its entry into susceptible cells thereby preventing integration. These compounds are small proteins that can be readily and cheaply produced. Furthermore, commensal lactobacilli can be engineered to produce these compounds which could be used to colonize vaginal mucosa and create an environment hostile to HIV-1 (Pusch et al., 2005). Since these compounds are not being developed as therapeutics, their exclusive use as prevention agents would avert the complications that may arise with the use of ARV as microbicides. In conclusion, our data support the continued development of GRFT, CV-N and SVN as microbicides against HIV-1 subtype C mucosal transmissions.

Materials and methods

Viruses, gp160 envelope (Env) clones, MAbs, cell lines and lectins

HIV-1 subtype C isolates Du151, and Du179 were isolated in South Africa from individuals infected with HIV-1 subtype C (van Harmelen

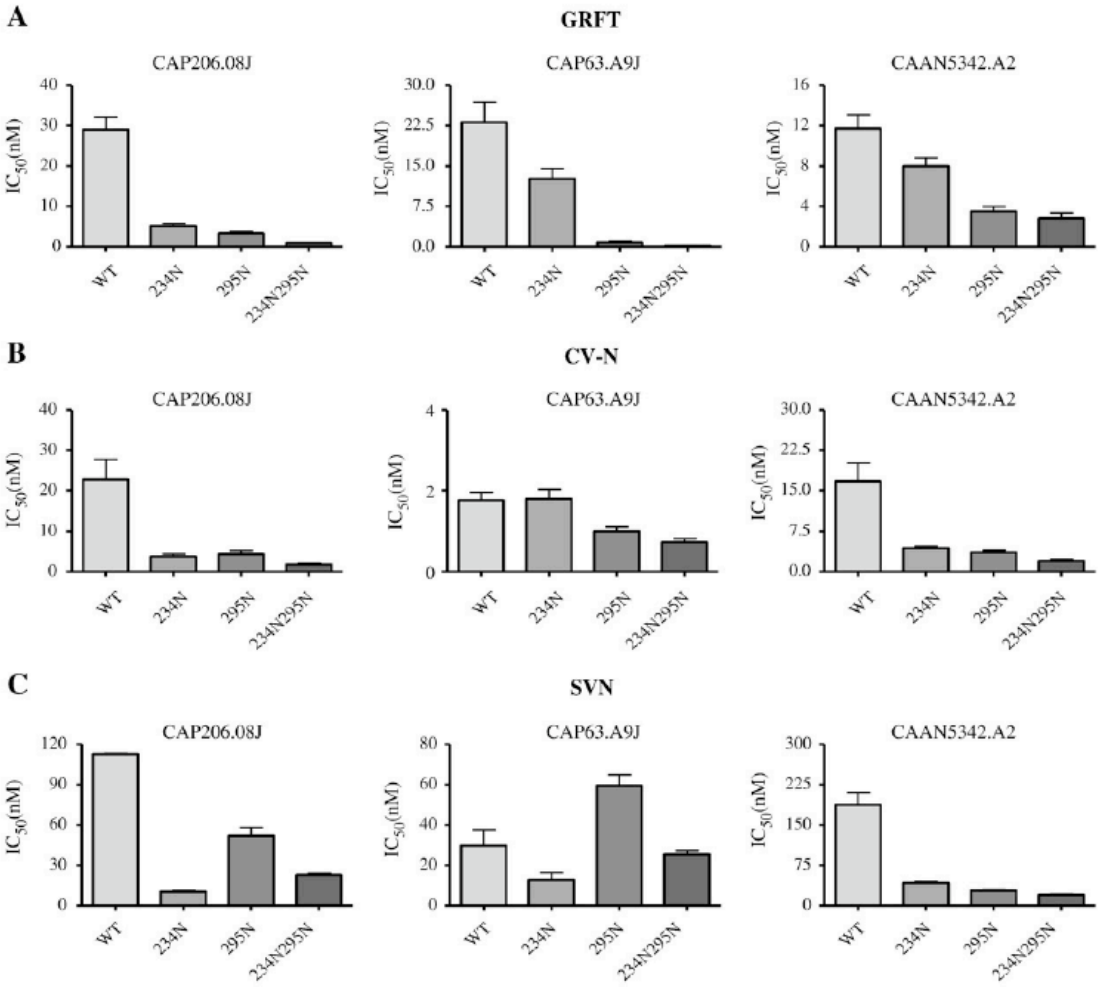

Fig. 4. Glycans at positions 234 and 295 increase HIV-1 sensitivity to GRFT, CV-N and SVN. Glycosylation sites at 234 and/or 295 were introduced in CAP206.08J, CAP63.A9J and CAAN5342.A2 by site-directed mutagenesis. Mutant viruses were tested in neutralization assays against GRFT (A), CV-N (B) and SVN (C) in TZM-bl cells. Each virus was tested at least three times.

et al., 2001). COT9 is a South African pediatric isolate (Choge et al., 2006). HIV-1 subtype C Env clones Du151.2, CAP45.G3J, CAP210.E8J, CAP206.08J, CAP63.A9J, CAP228.51J and CAP256.F7J were amplified from South African individuals at the acute or early stage of HIV infection (Gray et al., 2007a; Li et al., 2006). HIV-1 subtype B Env, CAAN5342.A2, WITO4160.33, THRO4156.18, AC10.0.29, QH0692.42 and PVO.4 were amplified from acutely infected individuals from the U.S.A, Trinidad and Tobago and Italy (Li et al., 2005). Subtype C Env COT9.6, COT6.15, RP6.6 and TM7.9 were derived from South African pediatric HIV-1 isolates. CAP63.cvl6GA, CAP177.cvl96, CAP261.cvl93 and CAP270.cvl44 were amplified from cervico-vaginal lavages (CVL) obtained during the acute phase of infection from HIV-positive women in the CAPRISA cohort in South Africa. The pSG3Δ*env* plasmid was obtained from Beatrice Hahn. The mAb 2G12 was obtained from the NIH Reference and Reagent Program and the IAVI Neutralizing Antibody Consortium. The TZM-bl cell line was obtained from the NIH Reference and Reagent Program (Cat No 8129) and the 293T cell line was obtained from the American Type Culture Collection. Both cell lines were cultured in DMEM containing 10% fetal bovine serum (FBS). Cell monolayers were disrupted at confluence by treatment with 0.25% trypsin in 1 mM EDTA. Recombinant GRFT, CV-N, and SVN purified from *E. coli* were provided by Dr. Barry O'Keefe from NCI.

HIV-1 neutralization assay in peripheral blood mononuclear cells

The neutralization assay in PBMC was carried out as described by Bures et al. (Bures et al., 2000). Briefly, a three-fold dilution series of GRFT, CV-N, and SVN in 40 μL of RPMI 1640 containing 20% FBS and interleukin-2 (growth medium) was prepared in triplicate in a U-bottom 96-well plate. Five hundred $TCID_{50}$ of HIV-1 primary isolate in 15 μL of growth medium was added to each well and the plate was incubated at 37 °C for 1 h. Then 100 μL of 5×10^6 cells/mL phytohemagglutin/interleukin-2 stimulated PBMC (PHA-PBMCs) was added to each well. The following day cells were washed 3 times with RPMI 1640 with 20% FBS and resuspended in 155 μL of fresh growth medium. The culture supernatant was collected twice daily and replaced with an equal amount of fresh growth medium. For each harvest the p24 antigen concentration in the virus control wells was measured by ELISA using the Vironostika HIV-1 Antigen Microelisa System (Biomerieux, Boseind, the Netherlands), according to the manufacturer's instructions. The inhibitory activity of the lectins was measured at the time-point that corresponded to the early part of the linear growth period of the virus control (Zhou and Montefiori, 1997). The IC_{80} were calculated by plotting the lectin concentration vs. the percentage inhibition in a linear regression using GraphPad Prism 4.0.

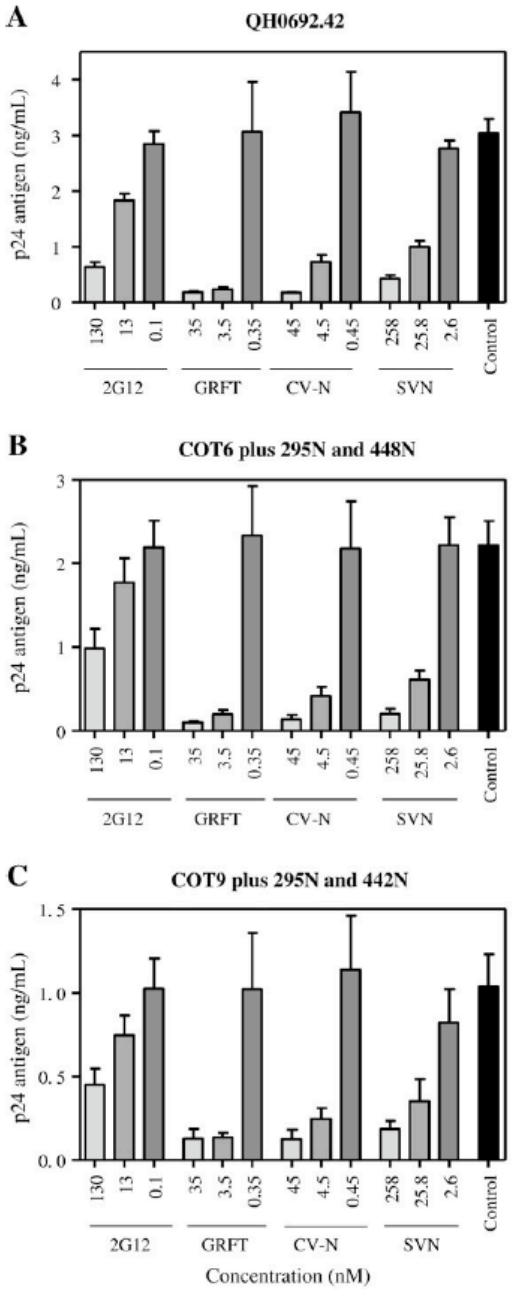

Fig. 5. GRFT, CV-N, and SVN compete with the 2G12 mAb for binding to HIV-1. Pseudoviruses QH0692.42 (A), COT6.15-V295N/S448N (B) and COT9.6-V295N/K442N (C), the latter two with a reconstituted 2G12 epitope, were incubated with GRFT, CV-N and SVN prior to capture with the 2G12 mAb. 2G12 was competed against itself as the experimental control. Experiments were done at least 3 times.

Generation of Env-pseudotyped virus stock

HIV-1 pseudoviruses were generated by co-transfection of the Env and pSG3Δ*env* plasmids (Wei et al., 2003) into 293T cells using the Fugene transfection reagent (Roche Applied Science, Indianapolis, IN). The $TCID_{50}$ of each virus stock was quantified by infecting TZM-bl cells with serial 5-fold dilutions of the supernatant in quadruplicate in the presence of DEAE dextran (37.5 μg/mL) (Sigma-Aldrich, St. Louis, MO). The Bright Glo™ Reagent (Promega, Madison, WI) was used to measure infection after 48 h of tissue culture, according to the manufacturer's instructions. Luminescence was measured in a Wallac 1420 Victor Multilabel Counter (Perkin-Elmer, Norwalk, CT). The $TCID_{50}$ was calculated as described elsewhere (Johnson and Byington, 1990).

Single-cycle neutralization assay (TZM-bl assay)

The pseudovirus neutralization assay was performed as described elsewhere (Montefiori, 2004). Briefly, three-fold dilution series of each lectin in 100 μL of DMEM with 10% FBS (growth medium) were prepared in a 96-well plate in duplicate. Two hundred $TCID_{50}$ of pseudovirus in 50 μL of growth medium was added and the mixture was incubated for 1 h at 37 °C. Then 100 μL of TZM-bl cells at a concentration of 1×10^5 cells/mL containing 37.5 μg/mL of DEAE dextran was added to each well and cultured at 37 °C for 48 h. Infection was evaluated by measuring the activity of the firefly luciferase. Titers were calculated as the inhibitory concentration that causes 50% reduction (IC_{50}) of relative light unit (RLU) compared to the virus control (wells with no inhibitor) after the subtraction of the background (wells without both the virus and the inhibitor).

HIV-1 virion capture assay

High binding 96-well plates (Corning Incorporated Corning, New York, U.S.A) were coated overnight with 1 μg/well of the mAb 2G12 in $NaHCO_3$ (pH 8.5). The coated plates were washed three times with phosphate buffered saline (PBS) and blocked with 3% bovine serum albumin (BSA) in PBS at 37 °C for 2 h. Pseudoviruses were pre-incubated for an hour with different concentrations of 2G12, GRFT, CV-N, or SVN. After discarding the blocking solution the virus was added to the plate and left at 37 °C for 2 h. The plate was then washed three times with PBS and captured viruses were lysed with 150 μL of 0.5% Triton X-100. The amount of p24 was measured using the Vironostika HIV-1 Antigen Microelisa System (Biomerieux, Boseind, The Netherlands), according to the manufacturer's instructions.

Site-directed mutagenesis

Putative glycosylation sites were introduced in HIV-1 gp120 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA). The presence of the mutation was confirmed by sequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystem, Foster City, CA) and resolved on the ABI 3100 automated genetic analyzer.

Statistical analysis

The Mann–Whitney *t*-test was used to compare the median IC_{50} of each lectin for the neutralization of subtype C, B and A viruses and the Wilcoxon matched pairs test was used to compare the median IC_{50} of GRFT and CV-N for the neutralization of subtype C. GraphPad Prism 4.0 was used for the Spearman's nonparametric rank test. *p*-values of ≤ 0.05 were considered statistically significant.

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CHAPTER THREE

BINDING OF THE MANNOSE-SPECIFIC LECTIN, GRIFFITHSIN, TO HIV-1 GP120 EXPOSES THE CD4-BINDING SITE

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Binding of the Mannose-Specific Lectin, Griffithsin, to HIV-1 gp120 Exposes the CD4-Binding Site▽

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The glycans on HIV-1 gp120 play an important role in shielding neutralization-sensitive epitopes from antibody recognition. They also serve as targets for lectins that bind mannose-rich glycans. In this study, we investigated the interaction of the lectin griffithsin (GRFT) with HIV-1 gp120 and its effects on exposure of the CD4-binding site (CD4bs). We found that GRFT enhanced the binding of HIV-1 to plates coated with anti-CD4bs antibodies b12 and b6 or the CD4 receptor mimetic CD4-IgG2. The average enhancement of b12 or b6 binding was higher for subtype B viruses than for subtype C, while for CD4-IgG2, it was similar for both subtypes, although lower than observed with antibodies. This GRFT-mediated enhancement of HIV-1 binding to b12 was reflected in synergistic neutralization for 2 of the 4 viruses tested. The glycan at position 386, which shields the CD4bs, was involved in both GRFT-mediated enhancement of binding and neutralization synergism between GRFT and b12. Although GRFT enhanced CD4bs exposure, it simultaneously inhibited ligand binding to the coreceptor binding site, suggesting that GRFT-dependent enhancement and neutralization utilize independent mechanisms. This study shows for the first time that GRFT interaction with gp120 exposes the CD4bs through binding the glycan at position 386, which may have implications for how to access this conserved site.

HIV-1 gp120 is heavily glycosylated, and N-linked glycans account for ~50% of its molecular mass (30, 32). Three types of glycans are found on gp120, namely, high-mannose glycans composed of 7 to 9 terminal mannose residues, complex glycans containing terminal sialic acid residues, and hybrid glycans, which are a mixture of both (9, 20, 29, 62). There are approximately 11 glycosylation sites on monomeric gp120 that are occupied by either mannose-rich or hybrid glycans, while the remaining sites bear complex glycans (32). However, recently Doores et al. reported that 98% of glycans on native HIV-1 envelope (Env) are barely processed beyond $Man_5GlcNAc_2$, i.e., glycans containing five mannose residues (16). Glycosylation patterns between HIV-1 subtype B and C envelopes have also been reported to differ in number and frequency (60). In addition to their role in promoting the proper folding of gp120 and mediating its interaction with cellular receptors, glycans protect HIV-1 from antibody neutralization by masking sensitive epitopes on the envelope (19, 34, 35, 37–39, 54, 55).

The CD4-binding site (CD4bs) on gp120 is highly conserved among HIV-1 subtypes and is a target for antibodies (29, 58). Among HIV-1 antibodies that target the CD4bs is the broadly neutralizing monoclonal antibody b12. The epitope of this antibody is located primarily in the neutralizing face of gp120, and 82% of its binding site is in the outer domain of the viral envelope (61). However, the high-mannose glycan at position 386 located inside the CD4bs shields this site from antibodies, as its removal has been shown to increase HIV-1 sensitivity to b12 (24, 48, 52, 66). In addition, the CD4bs is a target of nonneutralizing antibodies, such as b6. However, unlike b12, which binds both monomeric and trimeric gp120, b6 binds only to the monomeric form of the glycoprotein (48).

High-mannose glycans on gp120 are also targets of glycan-specific agents, such as lectins. Several lectins have been identified in recent years that potently block the infectivity of viruses, such as HIV and influenza virus (6, 41, 46). One of the most potent of these is griffithsin (GRFT). GRFT is a 121-amino-acid and ~13-kDa-molecular-mass lectin that was originally isolated from the red alga *Griffithsia* sp. (41). GRFT exists exclusively as a dimer and has a domain-swapped structure in which two β-strands of one monomer combine with 10 β-strands of the other monomer to form a β prism of three four-stranded sheets (63, 64). Each GRFT monomer contains three binding sites that have high affinity for mannose residues. Both native and recombinant GRFT display potent antiviral activities against primary HIV-1 isolates by binding to high-mannose glycans on the viral envelope spike (41, 47). We previously showed that the 234 and 295 glycosylation sites play an important role in GRFT neutralization of HIV-1 (1).

Since GRFT binds high-mannose oligosaccharides, including the one at position 386 that conceals the b12 epitope, we wished to explore whether this lectin affected exposure of the

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CD4bs. We examined binding using both a virus capture assay and neutralization. We found that GRFT enhanced HIV-1 binding of b12 and the nonneutralizing CD4bs monoclonal antibody (MAb) b6, as well as CD4-IgG2, which was used here as a surrogate for the CD4 receptor molecule. Importantly, GRFT and b12 synergized to render some HIV-1 isolates more sensitive to neutralization. The glycan at position 386 on gp120 was found to play a role in both enhancement and synergy, suggesting that GRFT could be used to increase exposure of the CD4bs of HIV-1.

MATERIALS AND METHODS

Viruses and reagents. HIV-1 subtype B envelope clones QH0692.42 and PVO.4, amplified from acutely infected individuals (33), were obtained from the NIH Reference and Reagent Program. The cloned subtype C envelopes COT9.6 and COT6.15 were derived from chronic pediatric infections (21), while Du151.2 and CAP239.G3J were amplified from acutely infected patients (23, 56). Infectious primary viruses were isolated from subtype B (DS12)- and subtype C (Du151 and CM9)-infected adults (12, 13), while RP1 was isolated from a chronic pediatric patient (21). The HIV-2 envelope clone 7312A was provided by George Shaw, and the pSG3Δ*env* plasmid was obtained from Beatrice Hahn. The MAbs IgG1b12 (b12) and IgG1b6 (b6) were kindly provided by Dennis Burton. MAbs 4E10, F240, and 17b were obtained from the NIH Reference and Reagent Program. The subtype C-specific anti-V3 MAb 3468L was isolated from an HIV-positive patient (22). The anti-CCR5 inhibitor PRO140, the CD4 receptor surrogate CD4-IgG2, and soluble CD4 (sCD4) were generously provided by Progenics Pharmaceuticals, Inc. (Tarrytown, NY). Recombinant GRFT and CV-N were purified from *Escherichia coli* at the National Cancer Institute, Frederick, MD (6, 41).

Generation of *env*-pseudotyped viruses. HIV-1 pseudoviruses were generated by cotransfection of the gp41-gp120 (Env) and pSG3Δ*env* plasmids (55) into 293T cells using the Fugene transfection reagent (Roche Applied Science, Indianapolis, IN). The 50% tissue culture infective dose ($TCID_{50}$) of each virus stock was determined by infecting TZM-bl cells with serial 5-fold dilutions of the supernatant in quadruplicate in the presence of DEAE dextran (37.5 μg/ml) (Sigma-Aldrich, St. Louis, MO). The Bright Glo Reagent (Promega, Madison, WI) was used to measure infection after 48 h of culture, according to the manufacturer's instructions. Luminescence was measured in a Wallac 1420 Victor Multilabel Counter (Perkin-Elmer, Norwalk, CT). The $TCID_{50}$ values were calculated as described elsewhere (27).

Site-directed mutagenesis. N-linked glycosylation site signal sequences were introduced into HIV-1 gp120 by QuikChange Site Directed Mutagenesis (Stratagene, LaJolla, CA). The presence of the mutation was confirmed by sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and resolved on the ABI 3100 automated genetic analyzer.

Single-cycle neutralization assay (TZM-bl assay). Pseudovirus neutralization assays were performed as described elsewhere (40). Briefly, a 3-fold dilution series of GRFT in 100 μl of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (growth medium) was prepared in a 96-well plate in duplicate. Two hundred $TCID_{50}$ of pseudovirus in 50 μl of growth medium was added, and the mixture was incubated for 1 h at 37°C. Then, 100 μl of TZM-bl cells at a concentration of 1×10^5 cells/ml containing 37.5 μg/ml of DEAE dextran was added to each well and cultured at 37°C for 48 h. Infection was evaluated by measuring the activity of the firefly luciferase. Titers were calculated as the inhibitory concentration that causes a 50% reduction (IC_{50}) in relative light units (RLU) compared to the virus control (wells with no inhibitor) after the subtraction of the background (wells without both the virus and the inhibitor).

To measure synergism, HIV-1 was incubated with a 3-fold dilution series of GRFT and b12 in 100 μl of DMEM-10% FBS. The lectin and the MAb were diluted alone and in combination. This was followed by the addition of the virus to TZM-bl cells. The IC_{50} and IC_{80} of GRFT and b12 were determined in the wells containing each compound alone and in the wells containing a mixture of both compounds. Synergism between GRFT and HIV-positive plasma or serum was measured as for b12, except that the 50% infective dose (ID_{50}) and ID_{80} were calculated. Since PRO140 is a CCR5 inhibitor, TZM-bl cells were first incubated with a dilution series of the compound prior to the addition of the virus with or without GRFT (in a dilution series) to allow for PRO140 binding to the coreceptor. The IC_{50} and IC_{80} of the lectin and PRO140 when used alone and in combination were calculated. Synergism was determined by calculating the combination index (CI) using both the IC_{50} and the IC_{80} (11, 59). A CI of 0.3 to 0.7 was deemed indicative of synergism, 0.7 to 0.85 of moderate synergism, 0.85 to 0.9 of slight synergism, and 0.9 to 1.1 of an additive effect, as previously defined (11, 67).

GRFT inhibition of HIV-1 infection of U87-CCR5 and U87-CXCR4. U87-CCR5 or U87-CXCR4 at a concentration of 2×10^4 cells/well were cultured for 24 h in a flat-bottom plate. This was followed by the addition of HIV-1 primary isolates that were preincubated for 1 h with a 3-fold dilution series of GRFT in 150 μl of DMEM with 10% FBS. The following day, the cells were washed three times and cultured for 10 days. The percent inhibition of infection was determined by comparing the p24 concentration of GRFT-containing wells to the control wells. The IC_{50} of GRFT inhibition of HIV-1 was calculated by plotting the lectin concentration against the percent inhibition in a linear regression using GraphPad Prism 4.0.

HIV-1 virion capture assay. A high-binding 96-well plate (Corning Inc., Corning, NY) was coated overnight with 100 μl/well of a 10-μg/ml solution of b12, b6, F240, 4E10, 3468L, or CD4-IgG2 in $NaHCO_3$ (pH 8.5). The coated plate was washed three times with phosphate-buffered saline (PBS) and blocked with 3% bovine serum albumin (BSA) in PBS at 37°C for 2 h. HIV-1 was incubated for an hour with GRFT, CV-N, CD4-IgG2, or the MAbs, and the virus was then added to the plate and left at 37°C for 2 h. The plate was washed three times with PBS, captured virus was lysed with 150 μl of 0.5% Triton X-100, and the p24 concentration was measured by enzyme-linked immunosorbent assay (ELISA). Control wells contained HIV-1 captured in the absence of the lectins, MAbs, or CD4-IgG2.

The assay was modified to assess competition between GRFT and 17b for binding to the CD4-induced epitope (CD4i) using the HIV-2 virus 7312A in the presence of sCD4 (15). After 1 h incubation with different concentrations of the lectin, the virus was incubated with 25 μg/ml of sCD4 for another 1 h before addition to a 17b-coated plate for 2 h. After the plate was washed three times with PBS, the amount of captured p24 was quantified as explained above. In another version of the same experiment, the virus was incubated first with 25 μg/ml of sCD4 and then with different concentrations of the lectin before addition to the 17b-coated plate for 2 h. For GRFT competition with 17b, using a plate coated with CD4-IgG2, the virus was first incubated with the CD4-IgG2-coated plate for 1 h. This was followed by the sequential addition of 25 μg/ml of sCD4 (to expose the CD4i on Env spikes that did not bind to the surface-bound CD4-IgG2); then by 30, 6, or 1.2 μg/ml of 17b; and thereafter by 30 μg/ml of GRFT, each for 1 h. The plate was washed with PBS three times between incubations. Rabbit anti-GRFT polyclonal antibodies and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody were used to measure the amount of GRFT bound to the virus using an optical density of 450 nm.

RESULTS

HIV-1 binding to b12 and b6 MAbs was enhanced in the presence of GRFT. We previously showed that GRFT competes with the glycan-specific MAb 2G12 for binding to HIV-1 (1). During this investigation, we observed that GRFT enhanced the binding of another anti-HIV MAb, b12, which targets the CD4bs and is broadly neutralizing (4, 5). In order to further explore this observation, we made use of a virus capture assay (VCA) that is highly reproducible and commonly used (8, 10, 45, 50). Most importantly, VCA offers a simple way of evaluating HIV-1 interaction with both neutralizing and nonneutralizing antibodies, as both capture viruses (10), although some gp41 antibodies have been reported to be captured only weakly (31). However, this assay has the disadvantage of being unable to provide information about the neutralization capacity of the antibody studied, so in this study, we performed both VCA and neutralization in parallel. We used two subtype B (QH0692.42 and PVO.4) and four subtype C (COT6.15, COT9.6, Du151.2, and CAP239.G3J) pseudoviruses, which were preincubated with GRFT and then added to b12-coated plates. Captured viruses were lysed, and the amount of p24 antigen was measured relative to wells with no lectin. As shown in Fig. 1A, preincubation with GRFT signif-

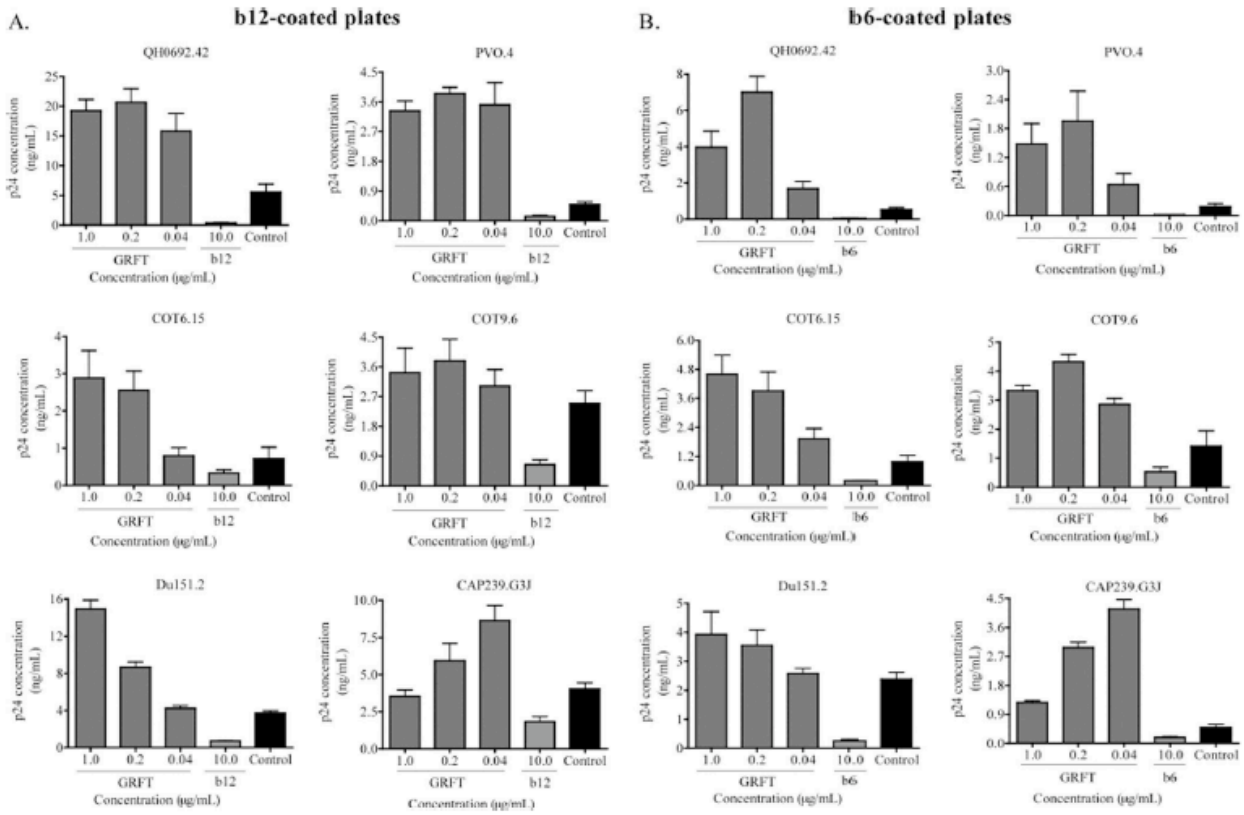

FIG. 1. HIV-1 capture by MAbs b12 and b6 was enhanced in the presence of GRFT. HIV-1 *env*-pseudotyped viruses from subtype B (QH0692.42 and PVO.4) and subtype C (COT6.15, COT9.6, Du151.2, and CAP239.G3J) were incubated with three different concentrations of GRFT before being added to plates coated with either b12 (A) or b6 (B). The amount of captured virus was assessed by p24 ELISA. Each MAb was also competed against itself as a positive control. The controls (black bars) show the amounts of virus captured on b12- or b6-coated plates in the absence of GRFT. The bars represent the means and standard deviations (SD) of three different experiments.

icantly enhanced virus capture by the b12 MAb above control b12-coated wells with no GRFT. The greatest effect was observed with the 2 subtype B viruses: for PVO.4, it was 8-fold above the control, and for QH0692.42, it reached 4-fold. The effect was slightly less pronounced for the four subtype C viruses: for Du151.2 and COT6.15, binding increased by 4-fold, while for CAP239.G3J, binding increased by 2-fold, and for COT9.6, the increase was 1.5-fold above the control.

In order to determine if this GRFT enhancement of binding was specific to the b12 MAb, we tested another CD4bs MAb called b6, which is nonneutralizing despite its epitope overlapping with that of b12 (48, 51). GRFT enhanced the binding of all six viruses to b6, and the effect was even more striking than with b12 (Fig. 1B). Again, the subtype B viruses showed higher levels of binding than the subtype C viruses. The subtype B virus QH0692.42 exceeded the control by 13-fold, while among subtype C viruses, CAP239.G3J was the highest, reaching 6.2 times the control. For both b12 and b6, most viruses reached maximum enhancement between 0.2 and 1 μg/ml of GRFT, suggesting saturation of CD4bs exposure with little additional effect at higher concentrations. In most cases, a reduction in binding was observed for both b12 and b6 at 0.04 μg/ml, indicating a dose-response effect. CAP239.G3J reached maximal enhancement at the lowest GRFT concentration tested, perhaps because of its unusual sensitivity to GRFT compared to other subtype C viruses (see Table 5). Both b12 and b6 competed against themselves, as expected (Fig. 1A and B).

Since the CD4bs overlaps both the b12 and b6 epitopes, we also studied whether GRFT increased CD4 receptor binding to HIV-1 gp120. To achieve this, we carried out a capture assay using the chimeric molecule CD4-IgG2 and the viruses QH0692.42 and COT6.15, which showed the highest enhancement of b12 and b6 binding. As shown in Fig. 2, the presence of GRFT modestly enhanced capture with CD4-IgG2, and this enhancement reached ~2 times the control for both viruses.

Enhancement of b12 and b6 binding was specific to GRFT and does not involve HIV-1 virion cross-linking. In order to explore the specificity of the observed enhanced exposure of the CD4bs by GRFT, we turned to CV-N, another mannose-specific lectin with strong anti-HIV neutralizing activity (64). QH0692.42 and COT6.15 were captured with either b12 or b6 in the presence of CV-N. Contrary to the observations with GRFT, CV-N blocked virus capture by both antibodies (Fig. 3A to D), showing that the ability of GRFT to enhance the binding of b12 and b6 to HIV-1 was specific to this lectin.

Next, we determined whether the GRFT-mediated enhance-

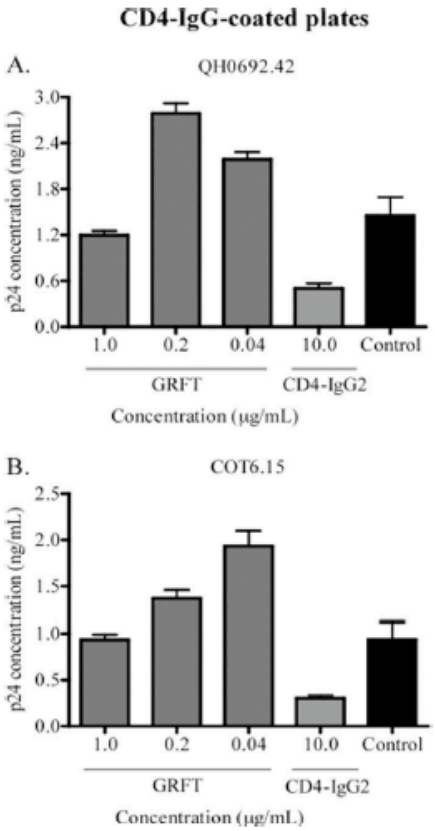

FIG. 2. CD4-IgG2 capture of HIV-1 was enhanced by GRFT. HIV-1 subtype B pseudovirus QH0692.42 (A) and the subtype C pseudovirus COT6.15 (B) were incubated with different concentrations of GRFT and added to a plate coated with CD4-IgG2. The amount of captured virus was assessed by p24 ELISA relative to an untreated control with no GRFT (black bars). CD4-IgG2 was also competed against itself as a positive control. The bars represent the means and SD of three different experiments.

ment of b12 and b6 binding was the result of cross-linking of viral particles via the mannose residues. If so, similar enhancement should be observed if b12 or b6 were substituted for another antibody whose epitope does not overlap the CD4bs. We selected the MAbs F240 and 4E10, which bind gp41 at the immunodominant epitope and the membrane-proximal external region (MPER), respectively (7, 36), and 3468L, a MAb that binds the V3 loop (22), as the substitute antibodies. An ELISA plate was coated with them, and the pseudoviruses QH0692.42, PVO.4, and Du151.2, which had been preincubated with different concentrations of GRFT, were added. We observed that unlike b12 and b6, GRFT had no effect on virus binding to F240- and 4E10-coated plates and that p24 levels were equivalent to those of control wells without GRFT (Fig. 4A and B show data for 1 representative virus). There was also no enhanced binding of viruses to the anti-V3 MAb-coated plate, but unexpectedly, GRFT competed with 3468L for binding to these viruses (Fig. 4C). Nevertheless, these data indicated that the GRFT-mediated enhancement of binding to gp120 is specific to CD4bs antibodies and does not involve HIV-1 virion cross-linking by the lectin.

The glycan at position 386 was involved in GRFT-mediated enhancement of HIV-1 binding to b12 and b6. In order to determine if the number or position of mannose-rich glycans influenced the GRFT-mediated enhancement of b12 and b6 binding, we compared the glycosylation sites of all the viruses used in this study (Table 1). We observed that, except for COT9.6 and Du151.2, which lacked one and three glycans, respectively, all viruses lacked two glycosylation sites, suggesting that the number of glycans was not responsible for the variance in enhancement observed with these viruses. Three of the 4 subtype C viruses lacked the glycan at position 295, which is typical of viruses from this subtype (60). All six viruses, however, had intact glycosylation sites at positions 241, 262, 332, and 386.

We and others have previously shown that removal of the glycan at position 386 in HIV-1 gp120 increases the virus sensitivity to b12 neutralization (17, 24, 52). We therefore investigated whether this glycan might be involved in the GRFT-mediated enhancement of b12 and b6 binding to HIV-1. We deleted this site in PVO.4 and QH0692.42, and in both cases, the deletion resulted in a roughly 2-fold decrease in GRFT-mediated enhancement of binding to b12 and b6 (Table 2), suggesting that the 386 glycosylation site was involved in the GRFT-mediated effect.

Since subtype C viruses showed lower levels of GRFT-mediated enhancement of b12 and b6 binding than subtype B viruses, we reconstituted all the mannose-rich glycans on COT6.15 and COT9.6 to determine if this increased the levels of binding. Addition of the 295N and 448N glycans to the COT6.15 envelope resulted in ~2-fold-greater enhancement of binding to b6, while for COT9.6, addition of 295N increased both b6 and b12 binding compared to the corresponding wild-type viruses. Confirming what was seen for the 2 subtype B viruses, deletion of the 386 glycosylation site in these reconstituted subtype C viruses resulted in a similar decrease in the enhancement of b12 binding (Table 2).

GRFT synergized with b12 to inhibit HIV-1 infection. To determine whether GRFT enhancement of b12 binding to HIV-1 translated into a synergistic interaction between the two compounds, we infected TZM-bl cells in the presence of GRFT and b12, individually and in combination. PVO.4 and CAP239.G3J were not neutralized by b12, and the presence of the MAb did not affect their sensitivity to GRFT, so synergy could not be measured. Viral infection was quantified after 48 h, and synergism between the antibody and the lectin at IC_{50} and IC_{80} was measured by calculating the CI (59). Both GRFT and b12 when tested individually neutralized all four pseudoviruses (Table 3). We observed synergism between b12 and GRFT for the neutralization of QH0692.42 and COT6.15, but not for COT9.6 or Du151.2. The CI values for QH0692.42 (at a GRFT/b12 molar ratio of 1.2 to 1) were 0.6 at IC_{50} and IC_{80}, and for COT6.15 (at a GRFT/b12 molar ratio of 1 to 16.5), it was 0.7 at IC_{50}. We were not able to calculate the CI value at IC_{80} for COT6.15, as the highest inhibition of the virus with b12 was 65% (data not shown). Lastly, we tested for synergism between GRFT and b6 for the neutralization of the six viruses mentioned above, and we observed no synergism (data not shown). In addition, the presence of b6 did not affect the sensitivity of these viruses to the lectin (data not shown).

Since we showed that the glycan at position 386 was involved

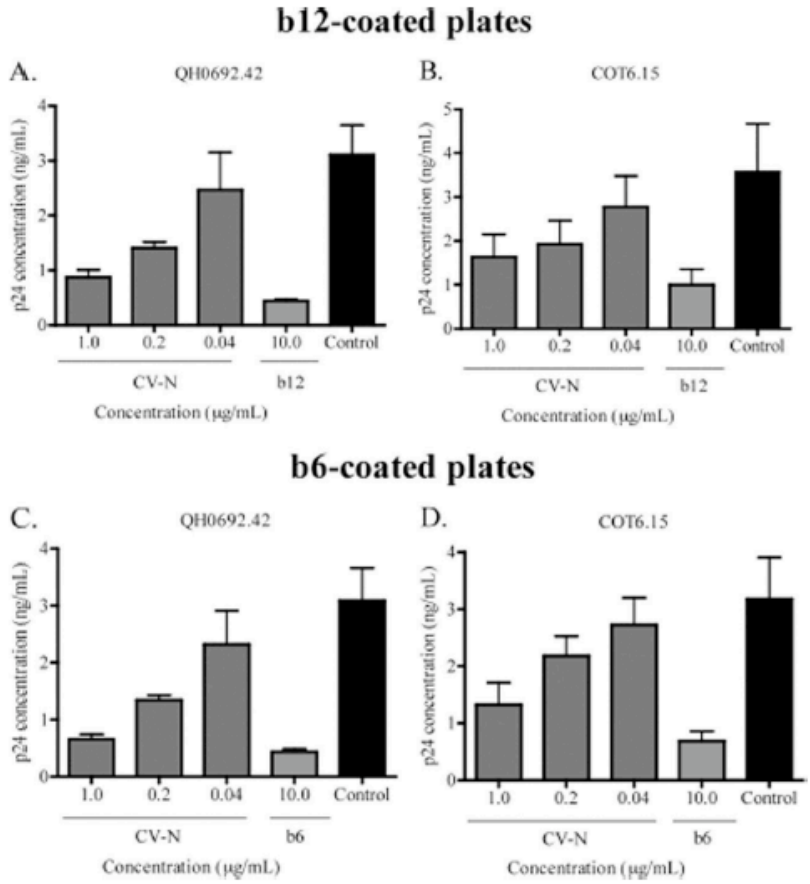

FIG. 3. CV-N competed with b12 and b6 for binding to HIV-1. The subtype B pseudovirus QH0692.42 and the subtype C pseudovirus COT6.15 were incubated with different concentrations of CV-N and then added to plates coated with either b12 (A and B) or b6 (C and D). The amount of captured virus was assessed by p24 ELISA relative to an untreated control with no CV-N (black bars). MAbs were also competed against themselves as positive controls. The bars represent the means and SD of three different experiments.

in the GRFT-mediated enhancement of binding, we investigated its effect on the observed synergy. For this experiment, we used QH0692.42 and the COT6.15 virus reconstituted with the 295N and 448N glycans, which showed the same level of synergy as the parental virus (Table 3). The N386Q mutant in this reconstituted virus was, however, considerably more sensitive to b12, as previously reported (Table 3) (24). For both QH0692-N386Q and COT6-V295N/S448N/N386Q, there was a loss of synergism between GRFT and b12 at IC_{50}, implicating the 386 glycan in the synergistic interaction between GRFT and b12 for neutralization of the viruses tested.

To determine which compound benefited the most in this synergistic interaction, we analyzed the shift in the inhibition curves for GRFT and b12, when used alone or in combination. As shown in Fig. 5A and B, the combination of GRFT and b12 shifted the inhibition curves to the left relative to the curves for the single compounds. This suggested that QH0692.42 and COT6-V295N/S448N were more sensitive to each compound when used in combination. These observed shifts, while not statistically significant, were consistent. For b12, the IC_{50}s were 13-fold lower in the presence of GRFT, while for GRFT, they were 2-fold lower, suggesting that b12 benefited the most from this combination. The deletion of the 386 glycan in both viruses resulted in a loss of synergy. This was shown by overlapping of the curves for GRFT when used either singly or in combination with b12 (Fig. 5C and D). In agreement with what we observed previously, the effect of the removal of the 386 glycan on GRFT neutralization curves was consistent but minor and not statistically significant. The curves for b12 were also affected; thus, for QH0692-N386Q, there was a 63% reduction in the shift between the single and combination curves for b12, while for COT6-V295N/S448N/N386Q, there was a 40% reduction despite the increased sensitivity of the virus to b12. These data confirm the involvement of the 386 glycan in GRFT and b12 synergistic interaction.

Synergy between GRFT and anti-CD4bs plasma antibodies. Since plasma from some HIV-infected individuals contains antibodies that target the CD4bs (25), we investigated whether GRFT affects ID_{50} titers for the neutralization of HIV-1. We used HIV-positive plasma from BB10 that was shown to contain antibodies to the CD4bs and compared it to BB34, which contained anti-MPER antibodies (25). TZM-bl cells were infected with HIV-1 in the presence of GRFT with or without plasma starting at a 1:20 dilution. Only the CI at ID_{50} was measured, as the highest neutralization with both plasmas was below 80%. GRFT and BB10 acted synergistically to neutralize

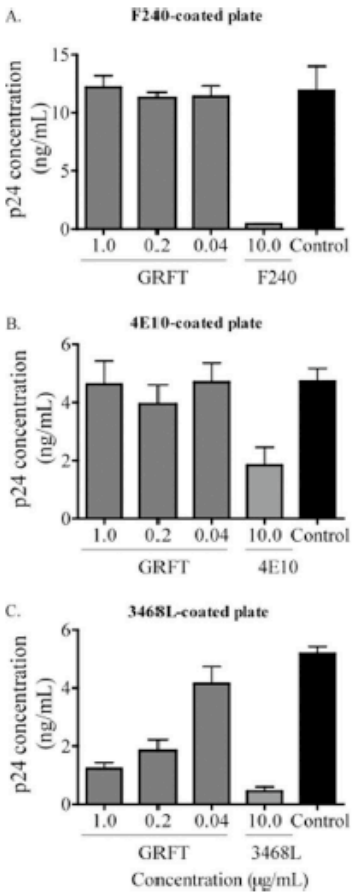

FIG. 4. No enhancement of HIV-1 binding to F240-, 4E10-, and 3468L-coated plates by GRFT. The subtype B virus QH0692.42 was incubated with different concentrations of GRFT and then added to plates coated with the MAbs F240 (A), 4E10 (B), and 3468L (C). The amount of captured virus was assessed by p24 ELISA relative to an untreated control with no GRFT (black bars). Each antibody was also competed against itself as a positive control. The bars represent the means and SD of three different experiments.

all 4 viruses tested, with CI values ranging from 0.2 to 0.7. In contrast, GRFT and BB34 synergized to neutralize only CAP239.G3J, with a CI of 0.4, while there was antagonism for QH0692.42 (Table 4).

CAP239.G3J showed synergy with both heterologous plasmas, so we examined GRFT in combination with the autologous serum. However, we observed no synergism between the lectin and the serum (data not shown), consistent with the observation that it did not contain anti-CD4bs antibodies (John R. Mascola, unpublished data). In conclusion, these data show that GRFT can increase HIV-1 sensitivity to plasma containing CD4bs antibodies.

GRFT inhibited sCD4-induced 17b binding and blocked infection via both CCR5 and CXCR4. Given that GRFT inhibits HIV infection by binding glycans and blocking receptor engagement, we asked how the lectin might promote virus binding to the CD4 receptor while inhibiting viral infection. We hypothesized that GRFT inhibits HIV infection by blocking steps that follow the CD4 receptor binding event, such as the coreceptor binding step. To investigate this possibility, we used the HIV-2 pseudovirus 7312A, which, following incubation with soluble CD4, becomes sensitive to the 17b MAb that binds to CD4i epitopes (15). First, we established that 7312A sensitivity to GRFT was similar to that of HIV-1 by carrying out a neutralization assay in TZM-bl cells. GRFT inhibited the virus with an IC_{50} of 4 nM, which is comparable to what we obtained with HIV-1 (1). We incubated 7312A with GRFT and then with sCD4 prior to capture with 17b. We also reversed the order, i.e., we incubated the virus with sCD4 first before incubation with GRFT prior to capture on the ELISA plate. As shown in Fig. 6A and B, GRFT reduced the amount of virus captured with 17b in the presence of sCD4 irrespective of when sCD4 was added, suggesting that GRFT inhibits the interaction of 17b with the coreceptor binding site.

To determine if 17b interfered with GRFT binding, we added 7312A to an ELISA plate coated with CD4-IgG2 and then sequentially added sCD4, 17b, and GRFT to the plate before adding rabbit anti-GRFT polyclonal antibodies. Figure 6C shows that the increase in the concentration of 17b had no effect on GRFT binding to the virus. Similarly, GRFT binding to an ELISA plate coated with monomeric gp120 did not reduce 17b binding (data not shown), confirming that GRFT does not interfere with 17b binding. Taken together, these data indicate that GRFT and 17b do not occlude each other's binding sites, i.e., there is no steric obstruction between the two compounds.

Given the possibility that GRFT interferes with coreceptor binding to HIV-1, we analyzed whether lectin inhibition of the virus was coreceptor specific. U87 cells expressing either the CCR5 or CXCR4 coreceptor were infected with the dual-

TABLE 1. Mannose-rich glycosylation pattern and GRFT-mediated enhancement of HIV-1 binding to b12 and b6

HIV-1 envelope pseudovirus (subtype)[a]	Predicted N-linked mannose-rich glycosylation site[b]											Fold increase with GRFT at 0.2 μg/ml	
	230	234	241	262	289	295	332	339	386	392	448	b12-coated plate	b6-coated plate
PVO.4 (B)	x									x		8.2 ± 2.1	11.1 ± 0.9
QH0692.42 (B)					x					x		4.1 ± 1.5	13.5 ± 1.7
COT6.15 (C)						x					x	4.5 ± 1.9	4.5 ± 1.8
Du151.2 (C)						x		x		x		2.3 ± 0.04	1.5 ± 0.2
CAP239.G3J (C)		x			x							1.5 ± 0.4	6.2 ± 1.2
COT9.6 (C)						x						1.5 ± 0.06	3.9 ± 2.1

[a] Viruses are ranked by subtype and according to their enhancement by GRFT.
[b] Mannose-rich glycosylations were identified from the amino acid sequence of each envelope clone (32). Missing sites are marked by x.

TABLE 2. Effects of glycan mutations on GRFT-mediated enhancement of binding to b12 and b6

HIV-1 envelope pseudovirus	Predicted N-linked mannose-rich glycosylation sites[a]											Fold increase with GRFT at 0.2 μg/ml	
	230	234	241	262	289	295	332	339	386	392	448	b12-coated plate	b6-coated plate
PVO.4	x									x		8.2 ± 2.1	11.1 ± 0.9
PVO-N386Q	x								x	x		4.8 ± 2.0	4.9 ± 1.9
QH0692.42					x					x		4.1 ± 1.5	13.5 ± 1.7
QH0692-N386Q					x				x	x		2.8 ± 0.3	6.5 ± 2.7
COT6.15						x					x	4.5 ± 1.9	4.5 ± 1.8
COT6-V295N/S448N												3.8 ± 0.02	8.7 ± 1.8
COT6-V295N/S448N/N386Q									x			1.7 ± 0.4	7.2 ± 2.8
COT9.6						x						1.5 ± 0.06	3.9 ± 2.1
COT9-V295N												2.8 ± 0.3	7.7 ± 3.2
COT9-V295N/N386Q									x			1.3 ± 0.2	1.7 ± 0.2

[a] Mannose-rich glycosylations were identified from the amino acid sequence of each envelope clone (32). Missing mannose-rich glycosylation sites are marked by x.

tropic infectious HIV-1 Du151, DS12, CM9, and RP1 in the presence of GRFT. As shown in Fig. 7, GRFT inhibited infection in both cell lines with similar IC_{50}s regardless of which coreceptor the virus used more efficiently.

Since different classes of entry inhibitors have been shown to act synergistically to inhibit HIV-1 infection (43, 44, 53, 65), we next determined whether the anti-CCR5 antibody PRO140 synergized with GRFT to inhibit HIV-1 infection. Six pseudoviruses were tested at a GRFT and PRO140 molar ratio of 1 to 50. As shown in Table 5, four viruses showed moderate synergy between GRFT and PRO140 of 0.6 to 0.7 at IC_{80}. This suggested that while GRFT and the anti-CCR5 MAb PRO140 can synergize to inhibit HIV-1 infection, this effect requires high concentrations of both compounds. In addition, since we previously showed that GRFT enhanced QH0692.42 and COT6.15 binding to CD4IgG2 (Fig. 2), we investigated whether the lectin synergized with sCD4 to neutralize these viruses. We found that there was synergism between GRFT and sCD4 for the neutralization of QH0692.42 with CIs of 0.7 and 0.6 at IC_{50} and IC_{80}, respectively.

DISCUSSION

In this study, we have shown that GRFT enhanced HIV-1 binding to the CD4bs MAbs b12 and b6 and the CD4 surrogate CD4-IgG2. The observed enhancement was specific to GRFT, as no effect was seen with CV-N, another mannose-binding lectin. The enhancement of binding with GRFT was not due to lectin cross-linking of viruses, as HIV-1 binding to gp41- and V3-specific MAbs was not affected. This enhanced binding resulted in moderate synergistic neutralization between b12 and GRFT for some viruses. The high-mannose glycan at position 386 on gp120 was implicated in both the enhancement and synergism between GRFT and b12, probably because the glycan shields the CD4bs (18). Lastly, in this study, we also showed that GRFT inhibits CCR5- and CXCR4-mediated HIV-1 infection with similar potencies and that the lectin can synergize with plasma from HIV-positive individuals and with the anti-CCR5 inhibitor PRO140 to neutralize HIV-1.

GRFT enhancement of HIV-1 binding was higher for subtype B than for subtype C viruses. Glycosylation differences in the envelopes of the two subtypes may explain this (60), although a previous study suggested that these differences had no impact on neutralization by GRFT (1). Another possible reason is that subtype B viruses show higher affinity for b12 than subtype C. Indeed, subtype B viruses are neutralized by b12 and b6 more frequently and more potently than subtype C viruses (5). However, this is unlikely to be the only reason, as PVO.4, which is resistant to b12, showed the highest levels of

TABLE 3. Synergy between GRFT and b12 for neutralization of HIV-1

HIV-1 envelope pseudovirus	Single				Combined				CI[a]	
	GRFT		b12		GRFT		b12			
	IC_{50}[b] (μg/ml)	IC_{80}[c] (μg/ml)	IC_{50} (μg/ml)	IC_{80} (μg/ml)	IC_{50} (μg/ml)	IC_{80} (μg/ml)	IC_{50} (μg/ml)	IC_{80} (μg/ml)	IC_{50} (μg/ml)	IC_{80} (μg/ml)
QH0692.42	0.002	0.008	1.2	2.8	0.001	0.005	0.09	0.05	**0.6**	**0.6**
QH0692-N386Q	0.004	0.03	0.6	2.0	0.004	0.02	0.003	0.2	1.0	0.8
COT6.15	0.06	0.2	33.6	ND[d]	0.03	0.1	5.6	18.6	**0.7**	ND
COT6-V295N/S448N	0.003	0.008	44.1	ND	0.002	0.006	0.02	0.9	**0.7**	ND
COT6-V295N/S448N/N386Q	0.001	0.004	1.1	3.8	0.002	0.3	0.02	11.0	2.0	1.0
Du151.2	0.03	0.1	0.3	0.7	0.03	0.1	0.3	0.9	1.3	2.2
COT9.6	0.1	0.4	1.7	9.0	0.06	0.3	2.2	11.0	1.9	2.0

[a] A CI of 0.3 to 0.7 indicates synergism, 0.7 to 0.85 indicates moderate synergism, 0.85 to 0.9 indicates slight synergism, and 0.9 to 1.1 indicates an additive effect. Cases where synergism occurred are in boldface.
[b] The IC_{50} is the concentration needed to reduce HIV-1 infection by 50%.
[c] The IC_{80} is the concentration needed to reduce HIV-1 infection by 80%.
[d] ND, not determined.

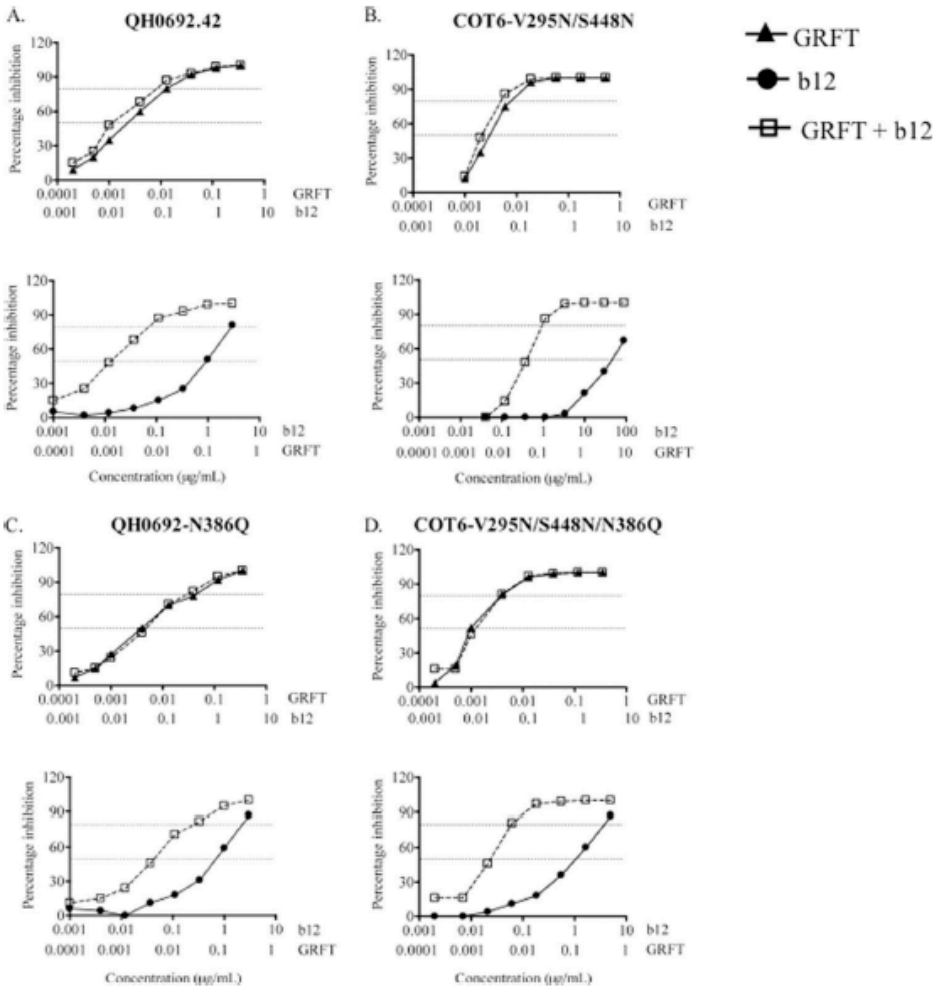

FIG. 5. Effects of the 386 glycosylation site on the HIV-1 inhibition curves of GRFT and b12. (A and B) HIV-1 QH0692.42 (A) and COT6-V295N/S448N (B) were neutralized in TZM-bl cells with GRFT and b12, in combination and alone. The shift in the inhibition curves of the two compounds combined relative to each compound alone are shown. (C and D) The same experiment as in panels A and B but using QH0692-N386Q and COT6-V295N/S448N/N386Q, respectively. The dashed lines indicate 50 and 80% inhibition. The graphs are representative of three different experiments.

b12 binding in the presence of GRFT. Clearly, a larger number of viruses from both subtypes should be tested to determine whether substantial differences in GRFT-mediated enhancement of b12 and b6 binding exist.

In general, b6 binding was higher than b12 binding, suggesting that the b6 epitope may be more affected by the GRFT-mediated exposure of the CD4bs. GRFT also increased binding of CD4-IgG2, although the enhancement was lower than for the anti-CD4bs antibodies of the 2 viruses tested. This may be due to the fact that the CD4 receptor binds further away

TABLE 4. Synergy between GRFT and HIV-1-positive plasma for neutralization of HIV-1

HIV-1 pseudovirus envelope	BB10					BB34				
	Single		Combined		CI[a]	Single		Combined		CI[a]
	GRFT IC_{50} (nM)	BB10 ID_{50}	GRFT IC_{50} (nM)	BB10 ID_{50}		GRFT IC_{50} (nM)	BB34 ID_{50}	GRFT IC_{50} (nM)	BB34 ID_{50}	
CAP239.G3J	0.3	0.009	0.06	0.0005	**0.2**	0.3	0.03	0.1	0.0008	**0.4**
COT6.15	1.4	0.03	0.7	0.005	**0.7**	1.4	0.0007	1.4	0.0007	1.1
COT9.6	0.7	0.01	0.3	0.003	**0.7**	0.6	0.03	0.3	0.02	1.2
QH0692.42	0.07	0.04	0.05	0.0003	**0.7**	0.1	0.009	0.2	0.002	2.2

[a] A CI of 0.3 to 0.7 indicates synergism, 0.7 to 0.85 indicates moderate synergism, 0.85 to 0.9 indicates slight synergism, and 0.9 to 1.1 indicates an additive effect. Cases where synergism occurred are in boldface.

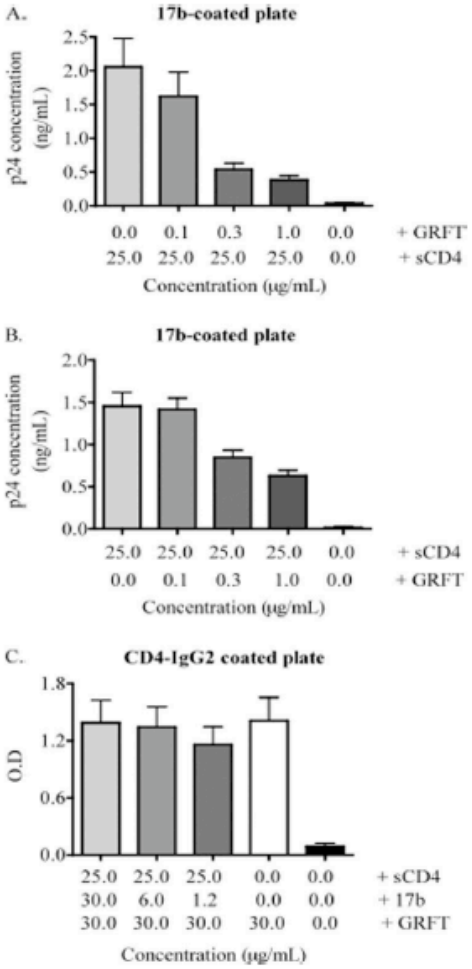

FIG. 6. GRFT inhibition of 17b MAb binding to the CD4i epitope. (A) The HIV-2 pseudovirus 7312A was first incubated with different concentrations of GRFT and then with 25 μg/ml sCD4 prior to capture on a 17b-coated plate. The well containing the immobilized 17b only (0 μg/ml of GRFT and sCD4) is the experimental control well. The amount of captured virus was measured by p24 ELISA. The bars represent the means and SD of three different experiments. (B) Same experiment as in panel A, except that 7312A was first incubated with 25 μg/ml of sCD4 before incubation with different concentrations of GRFT. (C) The HIV-2 pseudovirus 7312A was captured with CD4-IgG2 and then incubated with sCD4. This was followed by sequential addition of 17b, GRFT, and anti-GRFT antibody to the captured virus. The white bar (the well containing the virus and GRFT only) is the positive control, while the black bar (the well containing the virus only) is the negative control. The amount of GRFT bound to the virus was measured by measuring the optical density (O.D.) at 450 nM after the addition of horseradish peroxidase and the substrate. The bars represent the means and SD of three different experiments.

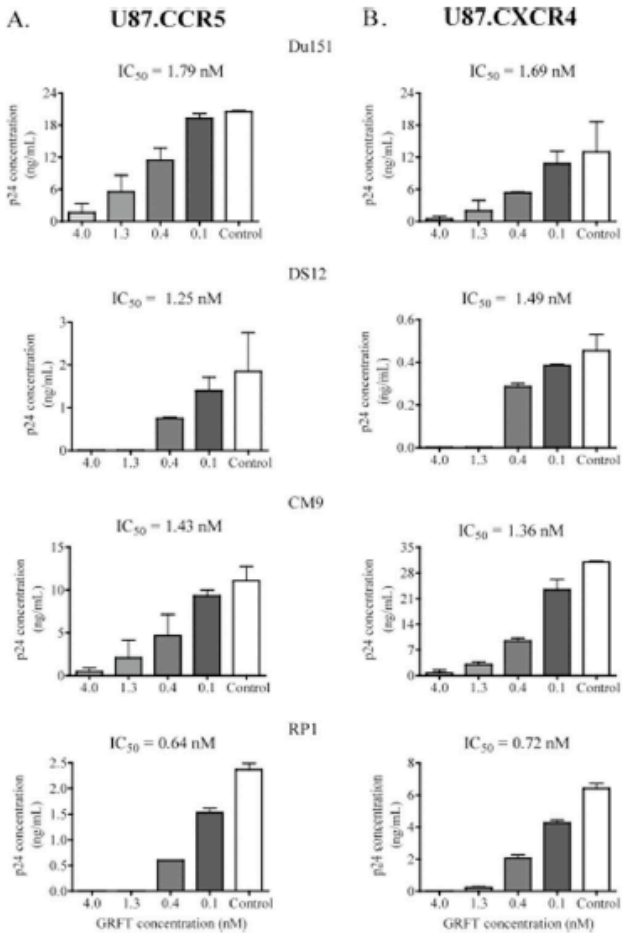

FIG. 7. GRFT inhibited HIV-1 infection in U87-CCR5 and U87-CXCR4 cells. HIV-1 subtype C Du151, DS12, CM9, and RP1 were treated with different concentrations of GRFT before infection of U87-CCR5 (A) and U87-CXCR4 (B) cells. The data are shown as the averages plus standard deviations of three independent experiments. Untreated virus is shown in white (positive control). The IC_{50}s of GRFT are indicated above each graph.

from glycan 386 or because it is simply less affected by GRFT binding to gp120. However, the use of antibodies and the CD4 mimetic provides compelling evidence that GRFT exposes the entire CD4bs and not just the binding sites of the antibodies.

In 2 of the 4 viruses tested, GRFT-mediated enhanced binding to b12 resulted in synergistic neutralization between GRFT and b12. The individual virus sensitivity to GRFT and b12 neutralization, as well as the level of GRFT-mediated enhancement of binding to b12, is likely to be an important determining factor for neutralization synergy to occur. Thus, QH0692.42 and COT6.15, the 2 viruses in which synergy was observed, showed the highest levels of enhancement, and both were sensitive to GRFT and b12 neutralization. However, for COT6.15, the ratio of b12 to GRFT used to achieve synergism was very high, probably because high levels of b12 were required for COT6.15 neutralization. The lack of synergy for COT9.6 may be explained by the comparatively low levels of enhancement of b12 binding in the presence of GRFT. The Du151.2 virus did not show synergism despite being sensitive to b12 and GRFT, and its enhancement was similar to that of COT6.15, suggesting that other factors were involved; for example, this virus was the only one that lacked the 339 glycan. Two viruses were not sensitive to b12, so synergy could not be assessed; PVO.4 resistance was probably due to trimer shielding, as the virus had all the required b12 contact residues, while in CAP239.G3J, resistance to b12 was due to sequence varia-

TABLE 5. Synergy between GRFT and PRO140 for neutralization of HIV-1

HIV-1 envelope pseudovirus	Single				Combined				CI[a]	
	GRFT		PRO140		GRFT		PRO140		IC_{50}	IC_{80}
	IC_{50} (nM)	IC_{80} (nM)	IC_{50}	IC_{80}	IC_{50}	IC_{80}	IC_{50}	IC_{80}		
PVO.4	0.1	0.1	9.4	33.5	0.1	0.1	2.3	5.1	1.2	1.2
QH0692.42	0.1	0.3	79.2	263.9	0.1	0.2	6.3	19.3	1.1	**0.7**
COT6.15	0.8	2.6	21.2	128.6	0.3	0.6	13.1	46.4	1.0	**0.6**
Du151.2	1.5	2.3	16.0	41.5	0.3	0.6	13.4	30.5	1.0	1.0
CAP239.G3J	0.2	0.9	36.2	165.8	0.2	0.4	6.5	22.1	1.2	**0.6**
COT9.6	2.7	6.3	31.0	147.2	0.5	1.6	23.8	53.0	1.0	**0.6**

[a] A CI of 0.3 to 0.7 indicates synergism, 0.7 to 0.85 indicates moderate synergism, 0.85 to 0.9 indicates slight synergism, and 0.9 to 1.1 indicates an additive effect. Cases where synergism occurred are in boldface.

tion at these sites (61). Although no synergism was noted between GRFT and b6, as expected given that it is a nonneutralizing antibody, the lectin acted synergistically with sCD4 to neutralize QH0692.42. This suggested that, like the enhancement of binding, GRFT-mediated synergy involved more than just the b12 binding site. Furthermore, synergy between GRFT and HIV-positive plasma BB10, which contains anti-CD4bs antibodies (25), for all the viruses tested is consistent with GRFT exposure of the CD4bs. However, the lack of synergistic interaction between the lectin and BB34 for 3 of these viruses is in agreement with the fact that the plasma contains antibodies that target the MPER (25). This supports the data in Fig. 4 that show GRFT does not enhance HIV-1 binding to 4E10.

We also observed synergism at IC_{80} between GRFT and the CCR5 inhibitor PRO140 for the neutralization of HIV-1 in 4 of the 6 viruses tested. However, the mechanism of this interaction is most probably unrelated to that of GRFT/b12, since the PRO140 MAb binds CCR5 on the cell surface, thereby blocking coreceptor interactions, while the b12 MAb binds gp120, blocking the initial association with CD4. Synergy between PRO140 and other CCR5 inhibitors has been noted previously with CI values between 0.38 and 0.61 (43). This is similar to what is reported in this study, which for GRFT in combination with PRO140, b12, or sCD4 was between 0.6 and 0.7, while for BB10 plasma it was 0.2 to 0.7. Comparison to CIs that have been published for other HIV-1 inhibitors, including a fusion inhibitor, such as AMD3100 and T20 (0.03) and PRO542 and T20 (0.14), suggests that the synergistic interactions we observed with GRFT were moderate (44, 53).

The high-mannose glycan at position 386 is located within the CD4bs (29, 32, 48), and deletion of this glycan has been shown to increase HIV-1 neutralization sensitivity to b12 (24, 52). It is possible that the mechanism of GRFT-mediated enhancement of b12 binding and synergy is the result of GRFT binding to 386 and shifting the glycan to increase accessibility to the CD4bs. Removal of the glycan resulted in a decrease in b12 binding and also decreased synergy between GRFT and b12. Deletion of the 386 glycan probably impacted GRFT binding to gp120, as was suggested by QH0692.42 (Table 3). Duenas-Decamp et al. (17) showed that the 386 glycosylation site requires arginine at position 373 to modulate HIV-1 resistance to b12. However, given that none of the viruses we tested had arginine 373, this residue is most probably not important for the GRFT-mediated enhancement of HIV-1 binding to b12.

CV-N, like GRFT, binds high-mannose residues, and the glycan at position 386 has also been implicated in its binding site (3). However, the lectin did not show the same effects as GRFT, suggesting that GRFT interacts with high-mannose glycans on gp120 somewhat differently than CV-N. Perhaps the arrangement of the 6 glycan binding sites on GRFT may allow greater cross-linking of glycans on gp120 than the 4 binding sites of CV-N. Recent structural studies on monomeric GRFT have indicated that such intraspike cross-linking is important for the antiviral activity of GRFT (42). This being said, it is clear that the GRFT enhancement is not due to cross-linking between viral particles, as binding to the gp41 and anti-V3 MAbs was not enhanced. The competition between GRFT and 17b for binding to HIV-2 7312A in the presence of sCD4 suggested that GRFT may inhibit HIV-1 binding to the coreceptor after CD4 receptor engagement (Fig. 6A and B). GRFT is likely to achieve this regardless of whether it binds before or after the CD4 receptor binding to gp120, although the effect is more pronounced when GRFT exposure precedes CD4 binding. However, the lack of steric obstruction between 17b and GRFT suggested that GRFT interference with the coreceptor binding site may not involve direct steric hindrance. We speculate that dimeric GRFT binding to multiple glycans on gp120 interferes with the structural rearrangement induced by CD4 binding necessary to form the CD4i epitope. Lastly, the similarity in the potencies of GRFT neutralization of CCR5- and CXCR4-mediated HIV-1 infection is likely to be related to the fact that the lectin inhibits the virus by targeting the viral envelope and not cellular receptors.

The CD4-binding site is a very important target for HIV-1 neutralizing antibodies, since the site is conserved among different HIV-1 subtypes (14, 29, 61). This is supported by the fact that b12, one of the few HIV-1 broadly neutralizing antibodies, targets this epitope (26). More recently, highly potent anti-CD4bs MAbs, VRC01, VRC02, and VRC03, which neutralize a much broader spectrum of isolates from diverse genetic subtypes, have been isolated (57). The vital role played by the CD4 receptor in the HIV-1 life cycle (14, 28) suggests that antibodies to this site could block replication *in vivo*, as has been shown in macaque studies (49). Given the unique mode of action of lectins and the fact that most are not toxic *in vitro* or in animal models, it has been suggested that these compounds represent a novel approach for intravenous targeting of HIV (2). As has been shown, the ability of GRFT to expose the CD4bs may offer a way of making HIV-1 more susceptible to

neutralization by anti-CD4bs antibodies, which are found in some HIV-positive patients (25). The study reported here increases our understanding of the interaction of GRFT with HIV-1 gp120 and also suggests a new way of increasing the exposure of the CD4bs.

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CHAPTER FOUR

THE C-TYPE LECTINS GRIFFITHSIN, CYANOVIRIN-N AND SCYTOVIRIN INHIBIT HIV-1 INTERACTION WITH THE DC-SIGN RECEPTOR

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The lectins griffithsin, cyanovirin-N and scytovirin inhibit HIV-1 binding to the DC-SIGN receptor and transfer to CD4$^+$ cells

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ABSTRACT

It is generally believed that during the sexual transmission of HIV-1, the glycan-specific DC-SIGN receptor binds the virus and mediates its transfer to CD4$^+$ cells. The lectins griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN) inhibit HIV-1 infection by binding to mannose-rich glycans on gp120. We measured the ability of these lectins to inhibit both the HIV-1 binding to DC-SIGN and the DC-SIGN-mediated HIV-1 infection of CD4$^+$ cells. While GRFT, CV-N and SVN were moderately inhibitory to DC-SIGN binding, they potently inhibited DC-SIGN-transfer of HIV-1. The introduction of the 234 glycosylation site abolished HIV-1 sensitivity to lectin inhibition of binding to DC-SIGN and virus transfer to susceptible cells. However, the addition of the 295 glycosylation site increased the inhibition of transfer. Our data suggest that GRFT, CV-N and SVN can block two important stages of the sexual transmission of HIV-1, DC-SIGN binding and transfer, supporting their further development as microbicides.

Introduction

HIV-1 is mainly transmitted through sexual intercourse that accounts for ~80% of infections around the world (http://www.unaids.org). In females, transmission begins when viral particles released into the vaginal tract cross the epithelial cell lining and infect target cells (Shattock and Moore, 2003). Some of these viruses bind to intraepithelial or submucosal dendritic cells (DC) via the interaction of mannose-rich glycans on the HIV-1 envelope with carbohydrate binding receptors such as DC-SIGN, DC immune receptor (DCIR) and mannose receptors (Hong et al., 2002; Lambert et al., 2008; Li et al., 2010; Liu et al., 2004; Piguet and Sattentau, 2004; Pohlmann et al., 2001). Similarly, in men, the foreskin of the penis contains DC that express the DC-SIGN receptor and are believed to play a role in female to male transmission (Fischetti et al., 2009; Hussain and Lehner, 1995; McCoombe and Short, 2006; Patterson et al., 2002; Soilleux and Coleman, 2004). The DC-SIGN receptor is also expressed on rectal mucosa mononuclear cells and may mediate infection, as these cells have been shown to transfer HIV-1 to CD4$^+$ T cells in vitro via this receptor (Gurney et al., 2005).

DC are antigen-presenting cells that become activated upon interaction with an invading pathogen (Piguet and Sattentau, 2004). Following this they migrate to the lymph nodes to stimulate naïve T-helper cells. HIV-1 interaction with the DC-SIGN receptor exploits this process by enabling the virus to reach the lymph nodes and infect CD4$^+$ T cells (Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2001). Previous studies suggested that HIV-1 binding to this receptor can result in its internalization by DC, the so called Trojan Horse model of *trans*-infection (Piguet and Sattentau, 2004; Pohlmann et al., 2001). However, more recent studies dispute this and propose that surface-bound viral particles mediate DC transfer of HIV-1 to susceptible cells (Cavrois et al., 2008; Yu et al., 2008). Nonetheless, in addition to HIV-1 infection in *trans* (virus transfer to target cells), it has been shown that DC-SIGN can also promote the infection in *cis* (infection of the cell expressing the receptor) of immature DC and macrophages (Burleigh et al., 2006; Pohlmann et al., 2001).

Like the DC-SIGN receptor, carbohydrate binding agents or lectins, bind to mannose-rich glycans found on HIV-1 envelope (Bokesch et al., 2003; Boyd et al., 1997; Leonard et al., 1990; Mori et al., 2005; Ziolkowska et al., 2006). Griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN) were isolated from the red algae *Griffithsia sp*, the cyanobacteria *Nostoc ellipsosporum* and *Scytonema varium*, respectively. While CV-N is found in both monomeric and dimeric forms, SVN exists exclusively as a monomer and GRFT as a dimer (Barrientos et al., 2002; Botos and Wlodawer, 2005; Moulaei et al., 2007; Ziolkowska and Wlodawer, 2006; Ziolkowska et al., 2006). Both the native and recombinant forms of these lectins have demonstrated potent and broad anti-viral activity against laboratory

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adapted strains and primary isolates of HIV-1 (Alexandre et al., 2010; Bolmstedt et al., 2001; Esser et al., 1999; O'Keefe et al., 2009; Xiong et al., 2006). Since these compounds are inhibitors of HIV-1 entry, they are being actively pursued as potential microbicides for the prevention of HIV-1 transmission (Balzarini and Van Damme, 2007; Bokesch et al., 2003; O'Keefe et al., 2009).

Previously we showed that GRFT, CV-N and SVN potently inhibit infection of TZM-bl cells by cell-free viral particles (Alexandre et al., 2010, 2011). Studies by others have shown that CV-N can inhibit the DC-SIGN mediated HIV-1 transfer to a cell line expressing the CD4 receptor (Balzarini et al., 2007). In this study, we investigated the ability of GRFT and SVN, in addition to CV-N, to inhibit both HIV-1 binding to the DC-SIGN receptor and the DC-SIGN-mediated transfer to target cells. We found that these lectins are efficient inhibitors of the DC-SIGN-mediated transfer of HIV-1 to both $CD4^+$ TZM-bl cells and PBMC. As such, these lectins may be useful in blocking early events in HIV-1 transmission in mucosal tissues.

Results

GRFT, CV-N and SVN inhibit HIV-1 binding to the DC-SIGN receptor

The lectins GRFT, CV-N and SVN interact with mannose-rich glycans on the HIV-1 envelope to inhibit the infection of susceptible cells (Bokesch et al., 2003; Boyd et al., 1997; Mori et al., 2005; Ziolkowska and Wlodawer, 2006). Since the DC-SIGN receptor also

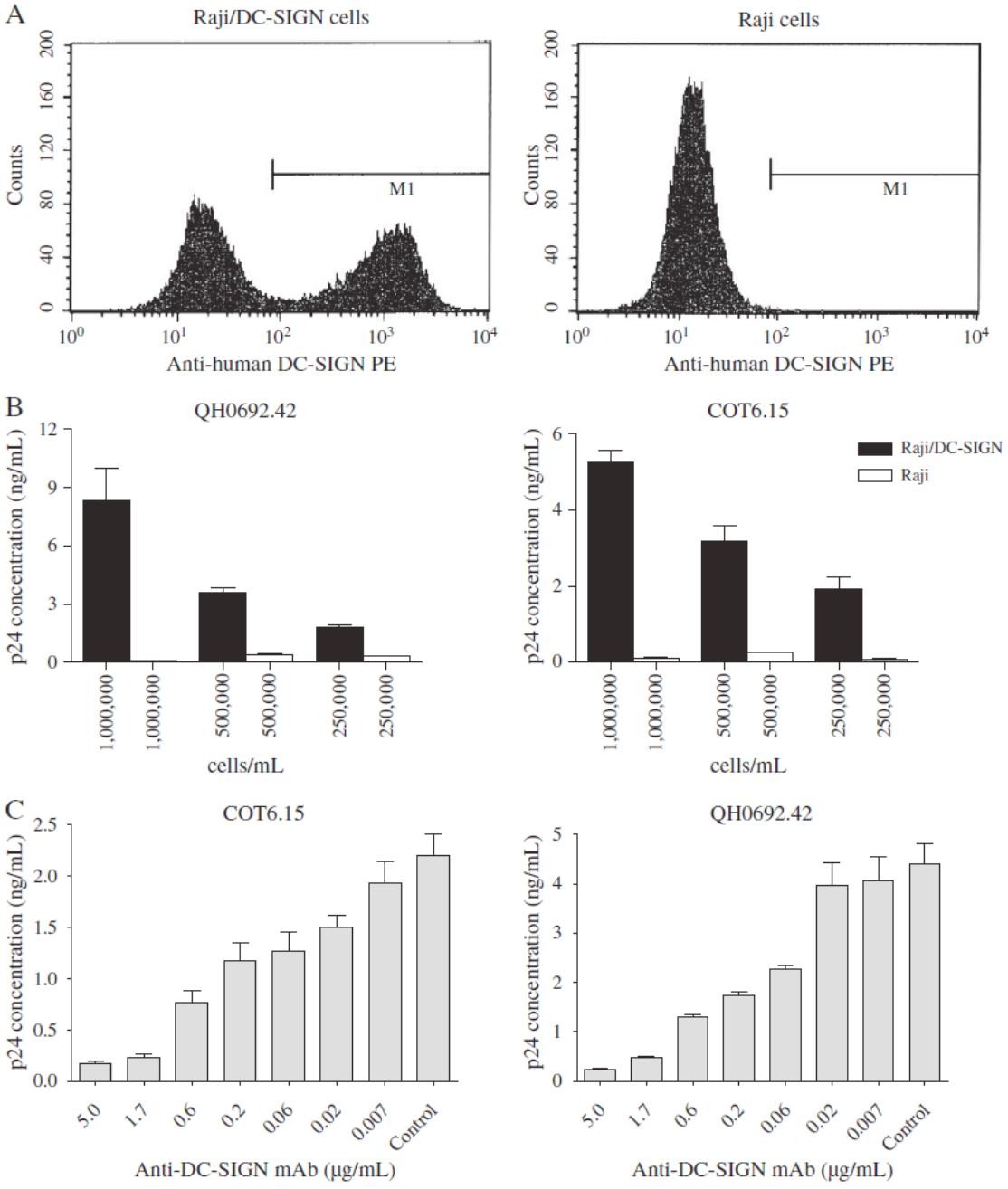

Fig. 1. DC-SIGN receptor expression on Raji/DC-SIGN cells. (A) Raji/DC-SIGN and Raji cells were stained with phycoerythrin (PE)-labeled mouse anti-human CD209 (DC-SIGN) and analyzed by flow cytometry. DC-SIGN expression is shown beneath the bar labeled M1. (B) HIV-1 subtype B, QH0692.42 and subtype C, COT6.15 were captured by Raji/DC-SIGN and Raji cells at 3 different cell concentrations. The amount of captured virus was measured by p24 ELISA. (C) Inhibition of HIV-1 binding to Raji/DC-SIGN cells by mouse anti-human CD209 antibody. The amount of captured virus was measured by p24 ELISA.

binds mannose-rich glycans on the viral envelope (Piquet and Sattenteau, 2004; Pohlmann et al., 2001), we investigated the ability of GRFT, CV-N and SVN to inhibit HIV-1 binding to the DC-SIGN receptor. For this we used Raji/DC-SIGN cells, a Burkitt's lymphoma cell line that has been engineered to express the DC-SIGN receptor (Wu et al., 2004). Using a DC-SIGN specific antibody, we found that ~55% of Raji/DC-SIGN cells expressed this receptor as measured by flow cytometry while control Raji cells did not (Fig. 1A). We first evaluated the ability of HIV-1 to bind to the DC-SIGN receptor by incubating the virus with Raji/DC-SIGN cells and measuring the amount of cell-bound p24 after repeated washing. Using the subtype B QH0692.42 and the subtype C COT6.15, we found that both pseudoviruses bound to Raji/DC-SIGN cells while their binding to control Raji cells was negligible (Fig. 1B). In addition, the binding to Raji/DC-SIGN was dependent on the cell concentration used, with 10^6 cells/mL showing the highest binding. However, for subsequent experiments we chose to use 500,000 cells/mL since the differential binding was in a workable range and minimized the number of cells required. To discount the possibility that the virus interacted with Raji/DC-SIGN cells via a mechanism other than binding to the DC-SIGN receptor, we incubated these cells with mouse anti-human DC-SIGN antibody prior to the addition of the virus. As shown in Fig. 1C, the antibody inhibited the binding of two HIV-1 isolates to Raji/DC-SIGN cells confirming the involvement of the DC-SIGN receptor in this interaction.

We then tested GRFT, CV-N and SVN inhibition of HIV-1 binding to the DC-SIGN receptor by incubating the virus with the lectins prior to capture with Raji/DC-SIGN cells. Unbound viruses were removed by repeated washing and the amount of captured virus was measured by p24 ELISA. Five subtype C (COT6.15, Du151.2, Du156.12, COT9.6 and CAP63.A9J) and three subtype B (QH0692.42, PVO.4 and CAAN5342.A2) pseudoviruses were used. Fig. 2 shows the dose response graphs for two representative viruses that displayed lectin-mediated inhibition of binding to DC-SIGN. All three compounds inhibited in a dose-dependent manner with SVN showing the weakest effect. Table 1 shows the data for all eight viruses where the percentage inhibition was calculated based on the amount of p24 antigen at the highest concentration of the lectin relative to the control. This ranged from 10 to 90% and was highest for CV-N. There was no correlation between the number and pattern of mannose-rich glycosylation sites on gp120 and sensitivity to GRFT, CV-N and SVN (Table 1). For example, COT6.15 (subtype C) and PVO.4 (subtype B) that both lacked two mannose-rich glycans had very different sensitivities to these lectins. Although there was a trend towards better inhibition of subtype B compared to subtype C viruses for GRFT ($p=0.072$) and CV-N ($p=0.054$), this was only significant for SVN ($p=0.015$). The number and position of complex glycans (i.e., not mannose-rich) also did not affect the sensitivity as expected given that complex glycans do not bind these lectins (data not shown).

Effects of the 234 and 295 glycosylation sites on lectin inhibition of HIV-1 binding to the DC-SIGN receptor

Previously, while investigating the association between mannose-rich glycosylation patterns and HIV-1 sensitivity to GRFT, CV-N and SVN, we observed that glycans at positions 234 and 295 in gp120 increased HIV-1 neutralization sensitivity in TZM-bl cells to all three lectins (Alexandre et al., 2010). Therefore, we investigated whether the addition of these sites also affected the inhibition of HIV-1 binding to the DC-SIGN-receptor. CAAN5342.A2 and CAP63.A9J viruses together with their 234N and 295N mutants were incubated with the lectins prior to capture with Raji/DC-SIGN cells. Contrary to what we expected, the addition of the 234 glycosylation site almost completely abolished lectin inhibition of CAAN5342.A2 and CAP63.A9J while the introduction of the 295 glycosylation site had no effect (Fig. 3). Interestingly, when comparing the amount of p24 antigen captured by Raji/DC-SIGN cells in the absence of lectin

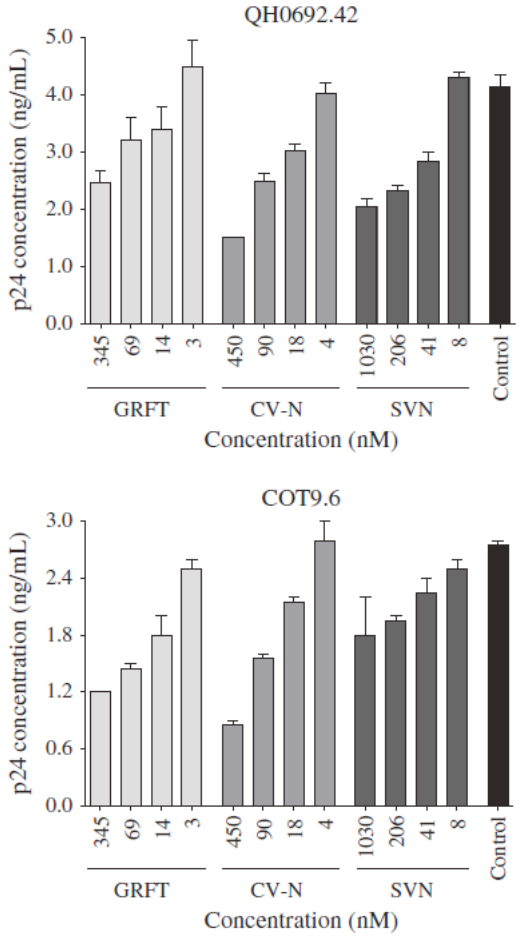

Fig. 2. Preincubation of HIV-1 with GRFT, CV-N and SVN inhibited binding to the DC-SIGN receptor. HIV-1 was incubated with GRFT, CV-N and SVN prior to capture on Raji/DC-SIGN cells. Bound virus was lysed and the amount of captured p24 was measured by ELISA. Bars represent mean ± SD of three different experiments. Untreated controls are shown in black.

(control wells shown as black bars in Fig. 3), the addition of the 234 glycosylation site decreased CAAN5342.A2 binding to the DC-SIGN receptor by ~50% while it increased CAP63.A9J binding by ~100%. To further examine this, we also tested COT6.15 which naturally expresses 234N. The deletion of 234N in COT6.15 resulted in a 62% decrease in DC-SIGN binding (the wild-type virus bound 2.1 ng/mL p24 compared to 0.8 ng/mL bound by the COT6-N234Q mutant). Taken together, our data suggest the glycan at position 234 affects the binding of HIV-1 to DC-SIGN and also ablates the lectin's ability to inhibit this interaction.

Inhibition of the DC-SIGN-mediated HIV-1 transfer with GRFT, CV-N and SVN

Since the DC-SIGN receptor is purported to play a critical role in transferring the virus to susceptible cells during the sexual transmission of HIV-1 (Piguet and Sattentau, 2004; Pohlmann et al., 2001), we tested the ability of GRFT, CV-N and SVN to inhibit DC-SIGN-mediated transfer of HIV-1 to $CD4^+$ TZM-bl cells. We proceeded by using two methods to mimic the possibility that when used as a microbicide, the lectin may interact with the virus prior to or after binding to the DC-SIGN receptor. In the first method, termed the post-DC-SIGN binding method, the virus was preincubated with Raji/DC-SIGN cells followed by the addition of the lectin. The lectins were used at concentrations previously found to be inhibitory to HIV and non-toxic to host cells (Alexandre et al., 2010). SVN was the least potent requiring higher concentrations while GRFT, being the most potent, was

Table 1
Inhibition of HIV-1 binding to the DC-SIGN receptor expressed on Raji cells.

HIV-1 envelope pseudovirus	[a] Predicted N-linked mannose-rich glycosylation sites											[b] Percentage inhibition		
	230	234	241	262	289	295	332	339	386	392	448	[c] GRFT	CV-N	SVN
Subtype C														
COT9.6		■				■						56.4 ± 1.0	69.1 ± 1.8	34.8 ± 18.9
CAP63.A9J		■				■						37.9 ± 3.2	68.4 ± 2.9	39.1 ± 11.9
Du156.12	■								■	■		27.7 ± 5.9	34.1 ± 2.0	19.7 ± 0.9
Du151.2						■		■		■		16.5 ± 7.6	46.6 ± 0.4	28.5 ± 6.0
COT6.15						■					■	9.8 ± 3.7	46.7 ± 22.5	43.9 ± 3.2
Median												27.7	46.7	34.8
Subtype B														
CAAN5342.A2		■				■		■				66.8 ± 0.6	89.7 ± 0.3	50.3 ± 13.0
PVO.4	■									■		64.3 ± 23.2	82.3 ± 20.6	57.9 ± 26.4
QH0692.42					■							40.1 ± 11.1	63.7 ± 2.6	50.8 ± 11.7
Median												64.3	82.3	50.8

[a]Mannose-rich glycosylation sites were identified from the amino acid sequence of each envelope clone (related to HxB2) based on a study using monomeric gp120 (Leonard et al., 1990). Absent glycans are indicated by black boxes.
[b]The percentage inhibition was tested at 345 nM, 450 nM and 1030 nM of GRFT, CV-N and SVN, respectively.
[c]Viruses are ranked according to their sensitivity to GRFT.

used at lower concentrations. Virus-bound Raji/DC-SIGN cells were then co-cultured with TZM-bl cells and the inhibition of transfer was determined after 48 h. As shown in Table 2, GRFT, CV-N and SVN inhibited HIV-1 transfer with IC_{50} in the low nanomolar range. Similar to our earlier findings in the TZM-bl neutralization assay (Alexandre et al., 2010), GRFT was the most potent of the three compounds followed by CV-N and SVN. It is likely that the inhibition of HIV-1 transfer observed here is related to the neutralization potency of the lectin. In addition and also in agreement with our previous study (Alexandre et al., 2010), we observed no correlation between the number of mannose-rich glycans on gp120 and sensitivity to GRFT, CV-N and SVN (see Table 1 for glycan patterns). Despite the differences in glycosylation patterns (Zhang et al., 2004), HIV-1 subtype B and C showed similar sensitivity to lectin inhibition of DC-SIGN-mediated transfer (Table 2).

We also investigated whether GRFT, CV-N and SVN synergized to inhibit the DC-SIGN mediated HIV-1 transfer. Synergism between the three lectins was measured by inhibiting the virus transfer with each lectin alone and in combination and by measuring the combination index (CI). We used two subtype C, COT9.6 and CAP63.A9J, and two subtype B, CAAN5342.A2 and QH0692.42, in this experiment. As shown in Table 3, there was no synergism between GRFT, CV-N and SVN. On the contrary, the three compounds acted antagonistically when used in combination, with CI values of >1.1 probably due to the fact that they share common binding sites on the viral envelope (Alexandre et al., 2010).

In the second method, termed the pre-DC-SIGN binding method, the virus was first incubated with the lectin before sequentially adding Raji/DC-SIGN cells and TZM-bl cells. Although, GRFT, CV-N and SVN inhibited the DC-SIGN-mediated transfer, the sensitivity of the virus to the three compounds was markedly reduced (Table 2). Similar to the post-DC-SIGN binding method, GRFT was the most potent and there was no difference between subtypes. Dose–response curves for two representative viruses in both formats are shown in Fig. 4. Note that the concentrations used in the pre-DC-SIGN method were 5–10 fold higher since the lectins are less potent in this format. We also tested for synergistic interaction between the three lectins when combined using the pre-DC-SIGN binding method and similar to the post-DC-SIGN binding method there was antagonism (Table 3).

We then asked the question of whether the combination of both the post and pre-DC-SIGN binding methods would increase GRFT, CV-N and SVN inhibition of HIV-1 transfer via the DC-SIGN receptor. This was investigated by first incubating the virus with the lectins followed by the addition of Raji/DC-SIGN cells. After washing off unbound viruses, Raji/DC-SIGN cell bound viruses were again incubated with the lectins before the addition of TZM-bl cells. For this study we used COT9.6, CAP63.A9J, CAAN5342.A2 and QH0692.42. Compared to each method alone, the combination of the pre and post-DC-SGN binding methods resulted in an increased inhibition of transfer for COT9.6 and QH0692.42, while the effect was less clear for CAP63.A9J with CV-N and CAAN5342.A2 with all three lectins (Fig. 5). Lastly, similar to the post and pre-DC-SIGN binding methods, we observed antagonism when all three lectins were tested together (Table 3).

Since PBMC are more relevant than TZM-bl cells as HIV-1 targets, we also tested the inhibition of virus transfer to these primary cells. To achieve this we used infectious replicating isolates SW7 and

Table 2
Inhibition of HIV-1 transfer to TZM-bl cells via the DC-SIGN receptor.

HIV-1 envelope pseudovirus	[a]Post-DC-SIGN binding method			[b]Pre-DC-SIGN binding method		
	GRFT	CV-N	SVN	GRFT	CV-N	SVN
Subtype C						
COT9.6	4.8 ± 0.5	31.5 ± 7.3	70.6 ± 14.6	19.4 ± 4.2	154.1 ± 25.6	230.5 ± 9.8
CAP63.A9J	35.0 ± 5.4	24.0 ± 5.6	441.3 ± 62.9	38.7 ± 5.2	33.7 ± 3.7	590.0 ± 82.4
Du156.12	6.4 ± 0.4	69.2 ± 8.3	161.9 ± 11.6	28.8 ± 8.3	136.8 ± 73.3	284.0 ± 65.8
Du151.2	4.8 ± 0.3	30.8 ± 3.2	82.8 ± 8.7	11.4 ± 1.0	131.5 ± 46.0	386.5 ± 71.3
COT6.15	5.0 ± 0.1	31.2 ± 0.6	75.9 ± 0.8	18.6 ± 4.5	151.3 ± 22.7	149.7 ± 36.3
Median	5.0	31.2	82.8	19.4	136.8	284.0
Subtype B						
CAAN5342.A2	5.1 ± 0.1	18.8 ± 1.7	115.0 ± 2.4	7.1 ± 0.5	24.2 ± 2.1	153.0 ± 9.7
PVO.4	4.4 ± 0.2	35.7 ± 2.1	82.4 ± 3.0	17.6 ± 2.7	162.8 ± 1.1	308.6 ± 16.2
QH0692.42	5.3 ± 0.1	36.9 ± 1.4	77.3 ± 5.7	21.7 ± 8.3	135.4 ± 18.3	438.4 ± 58.8
Median	5.1	35.7	82.4	17.6	135.4	308.6

[a,b]Results are shown as IC_{50} (nM) which is the concentration needed to inhibit HIV-1 transfer by 50%.

Table 3
Antagonism between GRFT, CV-N and SVN for the inhibition of DC-SIGN-mediated HIV-1 transfer.

		COT9.6			CAP63.A9J			CAAN5342.A2			QH0692.42		
		Post	Pre	Pre + Post	Post	Pre	Pre + Post	Post	Pre	Pre + Post	Post	Pre	Pre + Post
Single lectin	GRFT	11.3 ± 2.5	37.6 ± 4.3	3.5 ± 1.3	71.9 ± 5.0	33.7 ± 7.2	15.6 ± 8.8	9.9 ± 1.0	8.4 ± 0.6	9.0 ± 0.5	9.7 ± 2.4	19.5 ± 9.2	1.4 ± 0.3
	CV-N	46.9 ± 10.1	201.8 ± 38.2	22.6 ± 4.7	48.8 ± 7.9	141.7 ± 22.3	80.6 ± 42.7	43.4 ± 4.9	30.1 ± 8.7	45.1 ± 7.5	35.1 ± 5.5	180.3 ± 24.0	10.0 ± 5.2
	SVN	118.2 ± 12.4	396.0 ± 72.6	67.4 ± 5.7	357.9 ± 49.8	311.3 ± 32.8	185.1 ± 129.1	98.6 ± 7.4	152.0 ± 11.5	103.8 ± 14.7	84.8 ± 20.7	769.1 ± 67.8	67.4 ± 7.4
Combined lectin	GRFT	7.5 ± 1.9	15.7 ± 2.2	4.0 ± 0.3	11.1 ± 1.9	29.7 ± 4.8	15.8 ± 10.4	5.8 ± 1.3	7.3 ± 1.3	7.4 ± 1.4	10.4 ± 2.7	18.4 ± 3.4	1.3 ± 0.3
	CV-N	48.0 ± 12.6	203.6 ± 42.2	25.6 ± 1.9	59.4 ± 6.2	185.6 ± 19.8	66.8 ± 21.1	49.4 ± 7.8	47.3 ± 8.6	46.4 ± 7.6	26.0 ± 5.4	117.0 ± 19.7	0.3 ± 0.1
	SVN	110.8 ± 20.7	112.8 ± 15.3	58.1 ± 4.5	318.6 ± 36.1	189.7 ± 54.9	231.0 ± 160.4	94.8 ± 7.0	89.3 ± 5.1	105.5 ± 17.0	55.8 ± 9.0	128.7 ± 39.4	7.1 ± 0.8
CI		2.6	1.7	3.1	2.3	2.8	3.1	2.7	3.0	2.9	2.5	1.8	1.1

Data are shown as IC_{50} which is the concentration needed to reduce HIV-1 transfer by 50%.
The CI is the Combination Index: 0.3 to 0.7 indicates synergism, 0.7 to 0.85 indicates moderate synergism, 0.85 to 0.9 indicates slight synergism, 0.9 to 1.1 indicates an additive effect and >1.1 indicates antagonism.

Du179 (subtype C) and QH0515 (subtype B). Viruses were tested in both the post and pre-DC-SIGN binding methods and similar to what we observed in TZM-bl cells, GRFT, CV-N and SVN were inhibitory to HIV-1 transfer with GRFT being the most potent (Figs. 6A and B). Furthermore, similar to TZM-bl, viruses were more sensitive to the lectins in the post-DC-SIGN binding method compared to the pre-DC-SIGN binding method. We also tested the combination of the pre and post-DC-SIGN binding methods against SW7 and QH0515 and observed that there was a moderate increase in lectin inhibition of HIV-1 transfer (Fig. 7). In conclusion, our data show that GRFT, CV-N and SVN inhibit the DC-SIGN-mediated HIV-1 infection of PBMC, suggesting that these compounds may be able to inhibit the virus transfer to primary $CD4^+$ T cells in vivo.

Effects of the 234 and 295 glycosylation sites on lectin inhibition of DC-SIGN-mediated HIV-1 infection of TZM-bl cells

We next investigated whether the inhibition of the DC-SIGN mediated HIV-1 transfer was also affected by the 234 and 295 glycosylation sites. To test this, CAAN5342.A2, CAP63.A9J and their respective mutants were incubated with Raji/DC-SIGN cells prior to the sequential addition of the lectin and TZM-bl cells (post-DC-SIGN binding method). Similar to what we observed for the inhibition of binding, the addition of the 234 glycosylation site totally abolished the sensitivity to GRFT, CV-N and SVN (Table 4). However, examination of the virus control (without lectin) showed that the 234N CAAN5342.A2 virus was 30-fold less efficiently transferred (RLU of 4946 compared to 157,591 for the wild-type virus), while CAP63.A9J-234 N was 3-fold more efficiently transferred compared to the wild-type virus (RLU of 16,498 and 5637). The introduction of the 295 glycosylation site considerably increased CAP63.A9J and CAAN5342.A2 sensitivity to the lectins. Similar data were obtained using the pre-DC-SIGN binding method (Table 4). Note that the 295N mutation had no effect on the efficiency of virus transfer. When put together, these data suggest that the 295 glycan may play a role in GRFT, CV-N and SVN inhibition of HIV-1 transfer via the DC-SIGN receptor while the 234 glycan may interfere with this inhibition.

Discussion

In this study we showed that GRFT, CV-N and SVN inhibit both HIV-1 binding to the DC-SIGN receptor and the DC-SIGN mediated transfer of the virus to target cells. However, the inhibition of binding to DC-SIGN was moderate compared to the inhibition of transfer. In addition, HIV-1 was more sensitive to the inhibition of transfer when it bound the lectins after binding to the DC-SIGN receptor. These effects were modulated by glycan changes at positions 234 and 295 in gp120. Despite documented differences in glycosylation patterns, subtype B and subtype C viruses overall did not show significantly different sensitivities to GRFT, CV-N and SVN inhibition of binding and transfer via DC-SIGN. Given the central role of DC-SIGN in HIV transmission, further investigation of these compounds as potential microbicides to prevent the sexual transmission of HIV-1 is warranted.

Lectin inhibition of HIV-1 binding to the DC-SIGN receptor was partial even at concentrations that were more than 3000-fold higher than required for neutralization (Alexandre et al., 2010). This is likely attributed to the promiscuity of the DC-SIGN receptor which can in addition to mannose-rich also bind complex glycans (Hong et al., 2007; Liao et al., 2011; Lue et al., 2002). Thus while the lectins block virus binding via mannose-rich glycans they would have no effect on virus binding to DC-SIGN via other glycans. It is interesting that CV-N was the most potent of the 3 lectins in inhibiting the binding to DC-SIGN, suggesting that CV-N shares more binding sites with DC-SIGN than GRFT or SVN. These data are in agreement with studies

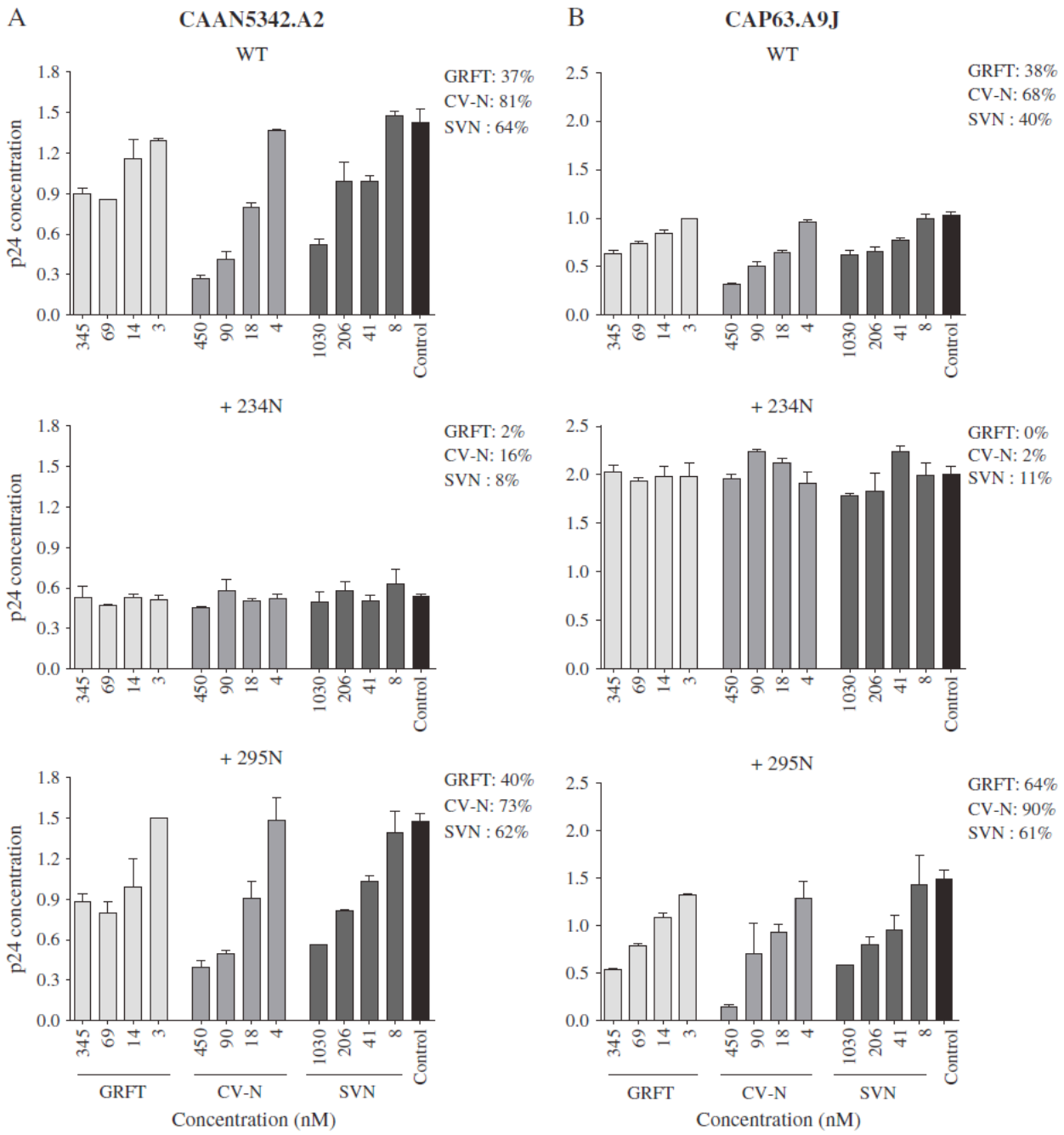

Fig. 3. Effects of 234 and 295 glycans on GRFT, CV-N and SVN inhibition of HIV-1 binding to the DC-SIGN receptor. (A) CAAN5342.A2 and (B) CAP63.A9J mutants lacking 234N and 295N were captured with Raji/DC-SIGN cells in the presence of GRFT, CV-N and SVN. The amount of virus was measured by p24 ELISA and expressed in ng/mL. Bars represent mean ± SD of three different experiments. Untreated controls are shown in black. The highest percentage inhibition obtained with each lectin is show next to the graphs.

by Spear who reported that CV-N partially inhibited HIV-1 binding to DC-SIGN and Banerjee who reported that GRFT incompletely inhibited monomeric gp120 binding to the DC-SIGN receptor, even at concentrations as high as 10 μM (Banerjee et al., in press; Spear et al., 2003). A study by Hong showed CV-N did not inhibit HIV-1 JR-CSF gp120 binding to the DC-SIGN receptor even at 1 mM (Hong et al., 2002), but given the partial nature of lectin inhibition of binding to DC-SIGN and the range of sensitivities seen with different viruses (Table 1), this is perhaps not too surprising. The degree of inhibition appeared not to be related to the number of mannose-rich or complex glycans. However, it may be possible that the absence or presence of single or combinations of mannose-rich glycans is involved in this differential sensitivity of lectin inhibition of binding to DC-SIGN. This is suggested by our previous studies showing that the position of the missing mannose-rich glycans on HIV-1 envelope was more important than the number in determining sensitivity to GRFT, CV-N and SVN (Alexandre et al., 2010). While the mannose-rich glycosylation patterns differ between subtypes B and C (Kwong et al., 1998; Zhang et al., 2004), this did not have a major impact on lectin sensitivity. Furthermore, recent data from Go and coworkers showing that the glycosylation patterns of transmitted founder viruses from subtypes B and C are similar (Go et al., 2011). Although this is a single study of one virus from each subtype, it nevertheless suggests that a GRFT, CV-N or SVN-based microbicide would be universally effective.

Our data are consistent with that of Balzarini and colleagues who showed that CV-N inhibited the DC-SIGN-mediated transfer of HIV-1 to $CD4^+$ cells (Balzarini et al., 2007). In addition to CV-N, we show

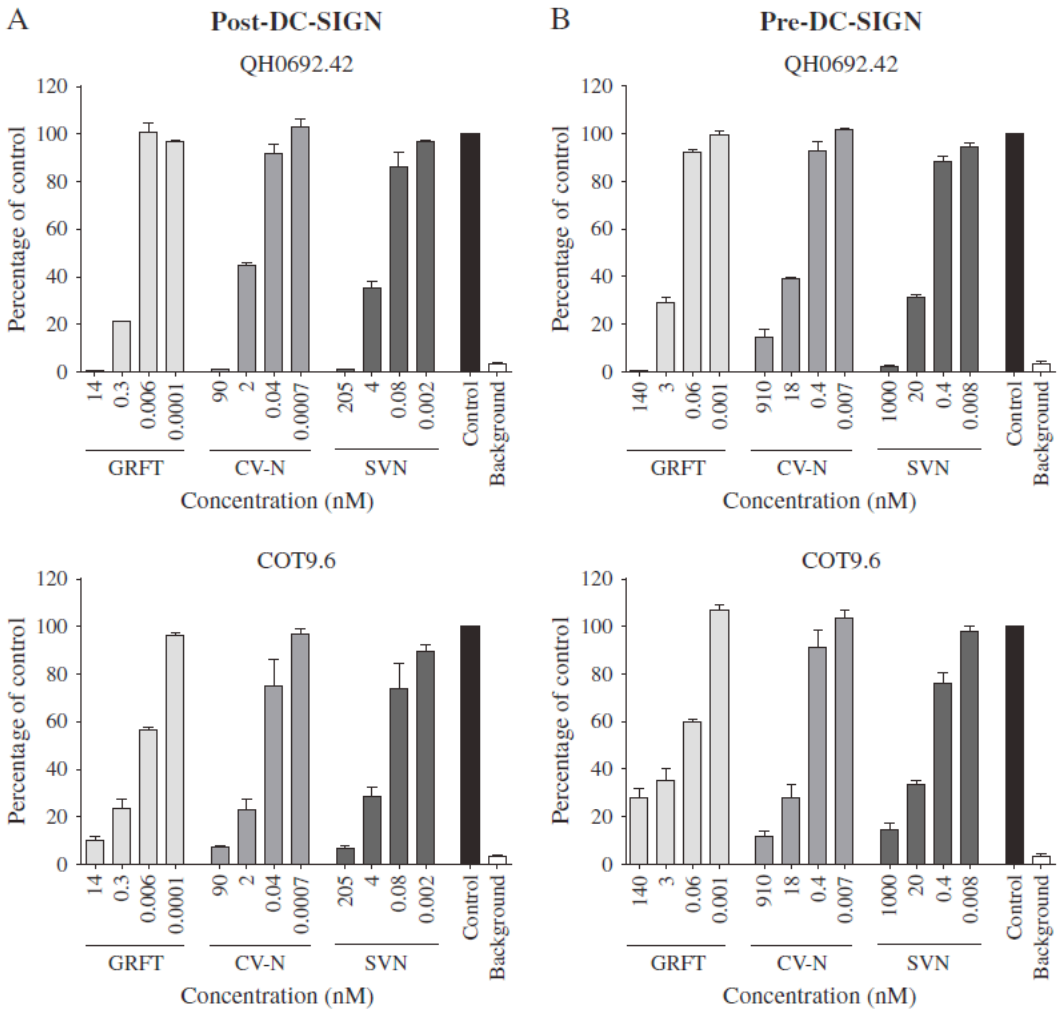

Fig. 4. Inhibition of DC-SIGN mediated HIV-1 transfer by post and pre-DC-SIGN binding methods. (A) In the post-DC-SIGN binding method, HIV-1 subtype B QH0692.42 and subtype C COT9.6 were incubated with Raji/DC-SIGN cells prior to the addition of GRFT, CV-N and SVN and co-culture with TZM-bl cells. (B) In the pre-DC-SIGN binding method, the virus was first incubated with GRFT, CV-N and SVN prior to the addition of Raji/DC-SIGN cells and co-culture with TZM-bl cells. The lectins were used at higher concentrations (5 to 10 fold more) in the pre-DC-SIGN binding format. The inhibition of transfer for both methods was determined by measuring the RLU. Bars represent mean ± SD of three different experiments. Untreated controls are shown in black.

here that GRFT and SVN are also inhibitory for HIV-1 transfer with GRFT being the most potent. The DC-SIGN-mediated HIV-1 transfer to target cells can be visualized as a two step process: step 1 is the binding of the virus to the DC-SIGN receptor and step 2 is transfer to target cells for infection. Thus, the partial sensitivity of HIV-1 to GRFT, CV-N and SVN inhibition of binding to the DC-SIGN receptor and its higher sensitivity to the lectin inhibition of the DC-SIGN-mediated transfer suggests that these compounds are more active during the second step of the process. This is consistent with the fact that these three lectins are strong inhibitors of HIV-1 infection of cells (Alexandre et al., 2010; Bokesch et al., 2003; Boyd et al., 1997; Mori et al., 2005; O'Keefe et al., 2009). Indeed, the post-DC-SIGN method measures inhibition of the specific interaction with CD4, as opposed to the rather promiscuous interaction with DC-SIGN via multiple glycans measured in the pre-DC method. Another possible explanation for the higher potency of the post-DC-SIGN method is that HIV-1 binding to DC-SIGN could increase the exposure of mannose-rich glycans on the viral envelope allowing them to bind more of the inhibitory lectin. However, it is clear that *in vivo* the inhibition of transfer by the post-DC-SIGN binding method will require that the lectins cross the cervico-vaginal mucosa to reach the sub-epithelium where the virus interacts with DCs and $CD4^+$ T cells (Lederman et al., 2006; Shattock and Moore, 2003). Since micro-abrasions of the vaginal mucosa are very common during sexual intercourse this may offer a way by which these compounds can breach the mucosal barrier (Norvell et al., 1984). The combination of the pre and post-DC-SIGN binding methods resulted in increased sensitivity to GRFT, CV-N and SVN for some of the viruses tested. A possible explanation for this is that this combination increased the likelihood of lectins occupying all or most of their binding sites on the virus, thereby, enhancing their potency. The antagonism observed with the combination of the three compounds was somewhat expected since these lectins binding sites overlap on the viral envelope (Alexandre et al., 2010). The finding that these lectins also inhibited DC-mediated transfer in PBMC in the pre- and post-DC-SIGN format, suggests that they will also be effective in inhibiting transfer to $CD4^+$ T cells resident in the cervico-vaginal mucosa (Lederman et al., 2006; Shattock and Moore, 2003).

The introduction of the 234 glycosylation site abolished GRFT, CV-N and SVN inhibition of HIV-1 binding to the DC-SIGN receptor and transfer to target cells. Previously we showed that the 234 glycan rendered viruses more sensitive to lectin-mediated neutralization presumably because they bound more lectin. The increased binding to the DC-SIGN receptor for 2 of 3 viruses in which the 234 glycan was present suggested that this glycan may be involved in DC-SIGN binding, similar to complex glycans at positions 158, 276 and 355 and the mannose-rich glycan at position 386 (Hong et al., 2007; Liao et al., 2011; Lue et al., 2002). The loss of lectin inhibition in the

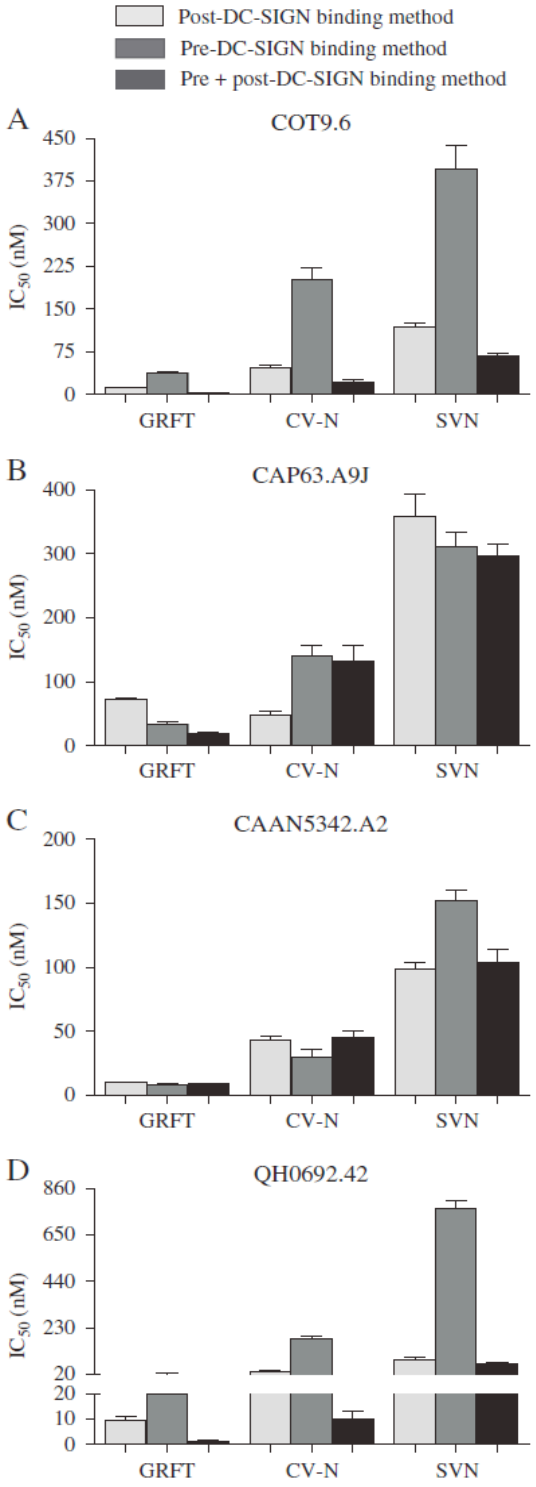

Fig. 5. Comparison of IC_{50} values between the post, pre and the combined method using TZM-bl cells. GRFT, CV-N and SVN inhibition of HIV-1 (A) COT9.6, (B) CAP63.A9J, (C) CAAN5342.A2 and (D) QH0692.42 transfer to TZM-bl cells using the three formats. The values shown are average of three different experiments.

presence of 234N could, therefore, be the result of increased interaction of HIV-1 with DC-SIGN that supersedes the sensitivity to the lectins. This is suggested by CAP63.A9J and COT6.15 which showed increased binding to DC-SIGN when 234N was present and a decrease when this glycan was deleted. The loss of inhibition of binding to DC-SIGN in the presence of the 234N was also observed for CAAN5342.A2 although the decrease in DC-SIGN binding in the presence of this glycan was more difficult to understand. Similarly, the 234N mutants differed in their ability to be transferred by DC-SIGN. It is important to note that CAAN5342.A2 differed from CAP63.A9J in that it also lacked the 339 glycan. It is possible that this extra deglycosylation may be involved in the differences observed between these two viruses. The importance of the 234 glycosylation site is suggested by the fact that it is conserved in about 80% of subtype B and C viruses (Zhang et al., 2004), possibly because it participates in interactions with receptors such as DC-SIGN. However, we did not observe a correlation between naturally occurring 234N and binding to DC-SIGN, for example PVO.4 which has 234N bound less well to the DC-SIGN receptor compared to CAP63.A9J and CAAN5342.A2 (data not shown), suggesting this interaction may be contextual.

Similar to 234N we also showed previously that the addition of the 295 glycosylation site increased HIV-1 sensitivity to GRFT, CV-N and SVN (Alexandre et al., 2010). However, we saw no effect of 295N on DC-SIGN binding while there was increased HIV-1 sensitivity to GRFT, CV-N and SVN inhibition of the DC-SIGN-mediated transfer in the presence of 295N. It is, therefore, possible that the effect of the 295 glycosylation site observed here is related to its effect on GRFT, CV-N and SVN neutralization of HIV-1 only.

The difficulties encountered in developing an effective HIV-1 vaccine (Johnston and Fauci, 2007; McElrath and Haynes, 2010) and the fact that the majority of HIV-1 infections are sexually transmitted (http://www.unaids.org) (Stein, 2003) makes the development of microbicides that prevent the sexual transmission of the virus crucial. GRFT, CV-N and SVN block HIV entry into cells and are being actively pursued as potential HIV-1 microbicides (Bokesch et al., 2003; O'Keefe et al., 2009; Shattock and Moore, 2003; Xiong et al., 2006). These compounds have shown little to no toxicity of mammalian cells in vitro while CV-N tested in a gel formulation as a microbicide was able to prevent the vaginal and rectal transmission of SHIV89.6P in macaques (Kouokam et al., 2011; O'Keefe et al., 2009; Tsai et al., 2003, 2004). In addition, a study conducted in mice showed that CV-N secreted by engineered vaginal *lactobacillus* did not induce a mucosal immune response (Liu et al., 2006). Except for tenofovir gel, all microbicide candidates that have been tested in human clinical trials have demonstrated little or no efficacy and some have proven to be harmful (Abdool Karim et al., 2010; Cutler and Justman, 2008; Ramjee et al., 2008). The advantage of GRFT, CV-N and SVN is that they selectively target mannose-rich glycan arrays on HIV-1 that are not found on mammalian cells (Balzarini, 2005). These lectins also have the benefit over ARV, such as tenofovir, since they are not being used for routine HIV-1 treatment. Lastly, GRFT has been shown to synergize with tenofovir, maraviroc and enfuvirtide against HIV-1 (Ferir et al., 2011), opening up the possibility of combining lectins with other compounds in a single formulation. In conclusion, our data suggest that GRFT, CV-N and SVN could play an important role in preventing the sexual transmission of HIV-1 by inhibiting the interaction of the virus with the DC-SIGN receptor in addition to the CD4 receptor. This supports further investigations on the use of these lectins for HIV-1 prevention.

Materials and methods

Viruses, cell lines and lectins

SW7 and Du179 are HIV-1 subtype C infectious viruses isolated from an AIDS and an acutely infected patient, respectively, while QH0515 is an infectious subtype B from an acutely infected individual (Cilliers et al., 2003; Li et al., 2005; van Harmelen et al., 2001). The subtype C envelope clones, COT9.6 and COT6.15 used to generate pseudoviruses were derived from pediatric isolates (Choge et al., 2006) while Du151.2, Du156.12 and CAP63.A9J were isolated during the acute phase of the infection (Gray et al., 2007; Li et al., 2006).

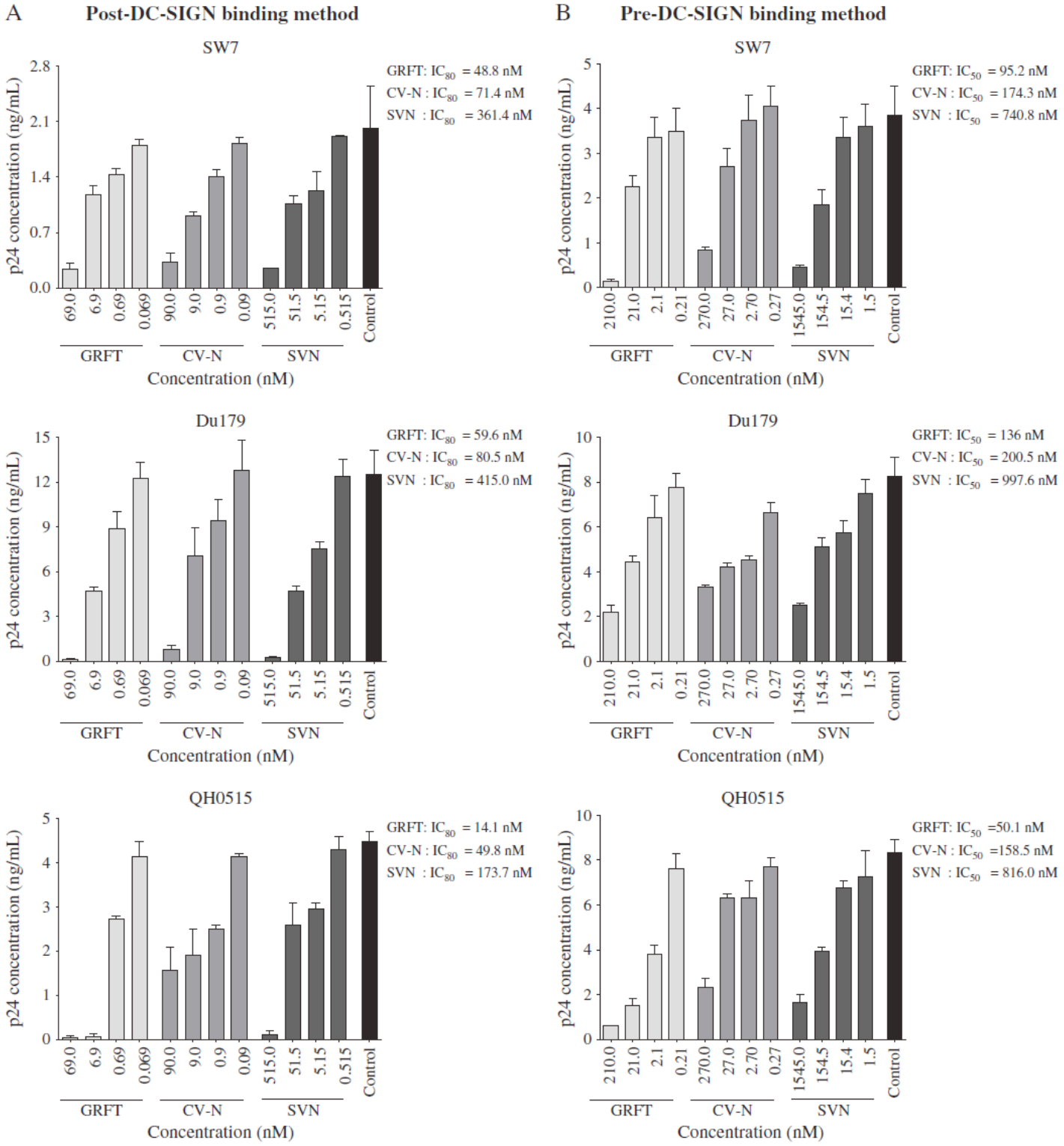

Fig. 6. DC-SIGN-mediated HIV-1 transfer to PBMC is inhibited by GRFT, CV-N and SVN. HIV-1 subtype C infectious viruses SW7 and Du179 and subtype B QH0515 transfer to PBMC was inhibited with GRFT, CV-N and SVN using (A) the post and (B) pre-DC-SIGN binding methods. The inhibition of transfer was measured by p24 ELISA. Bars represent mean ± SD of three different experiments. Untreated controls are shown in black. Levels of p24 antigen in the absence of virus or lectins were below detection (not shown).

HIV-1 subtype B envelopes CAAN5342.A2, QH0692.42 and PVO.4 were from the clade B reference panel (Li et al., 2005). The 234 N and 295 N mutants of CAAN5342.A2 and CAP63.A9J were generated by site directed mutagenesis using the QuikChange Site Directed Mutagenesis Kit (Stratagene, LaJolla, CA) (Alexandre et al., 2010). The pSG3Δ*env* plasmid was obtained from Dr. Beatrice Hahn. HIV-1 pseudoviruses were generated by co-transfection of the Env and pSG3Δ*env* plasmids (Wei et al., 2003) into 293 T cells using the Fugene transfection reagent (Roche Applied Science, Indianapolis, IN). Raji cells (Cat No. 9944), a Burkitt's lymphoma cell line (Wu et al., 2004), and Raji/DC-SIGN cells that express the DC-SIGN receptor (Cat No. 9945) were provided by the NIH Reference and Reagent Program and were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS). The TZM-bl cell line was obtained from the NIH

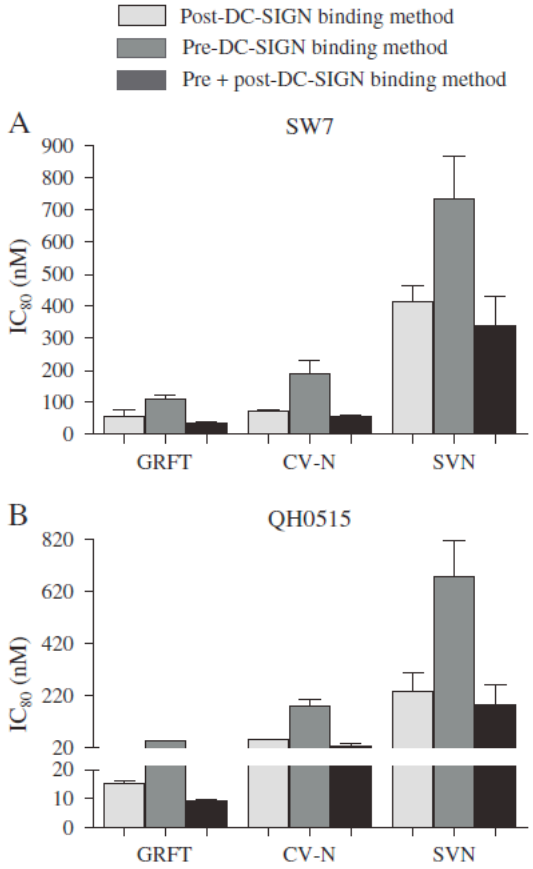

Fig. 7. Comparison of IC_{80} values between the post, pre and combined methods in PBMC. SW7 (A) and QH0515 (B) transfer to PBMC was inhibited with GRFT, CV-N and SVN using the post, pre and combined methods. The values shown are average of three different experiments.

Reference and Reagent Program (Cat No. 8129) while the 293 T cell line came from the American Type Culture Collection. Both cell lines were cultured in DMEM containing 10% fetal bovine serum (FBS). Cell monolayers were disrupted at confluence by treatment with 0.25% trypsin in 1 mM EDTA. Recombinant GRFT, CV-N and SVN were purified from *E. coli* at the National Cancer Institute, MA, USA (Bokesch et al., 2003; Boyd et al., 1997; Mori et al., 2005).

Expression of the DC-SIGN receptor on Raji/DC-SIGN cells

One million Raji/DC-SIGN or Raji cells were centrifuged at 2000 rpm for 5 min, resuspended in 100 μL of RPMI 1640 containing 10% FBS and stained with 10 μL of phycoerythrin (PE)-labeled mouse anti-human CD209 (DC-SIGN) (BD, San Jose, U.S.A) for 1 h at 4 °C. Cells were then washed by adding 3 mL of phosphate buffered saline (PBS) containing 5% fetal bovine serum (FBS) and centrifuged at 2000 rpm for 5 min. The supernatant was then removed and 300 μL of 1% formaldehyde in PBS (fixer) was added. After 10 min incubation at 4 °C, cells were analyzed by flow cytometry for the expression of the DC-SIGN receptor.

DC-SIGN receptor binding assay

HIV-1 pseudoviruses in 20% FBS DMEM were centrifuged for 25 min at 4500 rpm and 4 °C using the 100,000 MWCO PES vivaspin 20 column (Sartorius Stedim biotech, Aubagne, France), to remove free p24 from the viral samples. This was followed by the determination of the p24 concentration of the supernatant using the Vironostika HIV-1 Antigen Microelisa System (Biomerieux, Boseind, the Netherlands), according to the manufacturer's instructions. HIV-1 binding to the DC-SIGN receptor was measured by adding 5 ng of p24 to 1.0×10^5, 0.5×10^5 and 0.25×10^5 Raji/DC-SIGN cells in 100 μL of RPMI 1640 containing 10% FBS, in a U bottom 96 well plate. The virus and the cells were incubated at 37 °C for 2 h and then washed three times with RPMI 1640 by centrifuging at 2000 rpm for 5 min. Cell bound viral particles were then lysed with 150 μL of 0.5% Triton-X 100 and the amount of captured p24 was measured by ELISA.

The interaction between HIV-1 and the DC-SIGN receptor on Raji/DC-SIGN cells was confirmed by incubating 10^5 cells with a serial dilution of mouse anti-human CD209 antibody (BD Biosciences, San Jose, California) for 1 h. The cells were then washed three times with RPMI 1640, by centrifuging at 2000 rpm for 5 min, and incubated with 5 ng of p24 for 2 h at 37 °C. After the incubation they were again washed three times and bound viruses were lysed with 150 μL of 1% empigen. The amount of captured p24 was measured by ELISA.

GRFT, CV-N and SVN inhibition of HIV-1 binding to the DC-SIGN receptor was measured by preparing a five-fold dilution series of these lectins in 100 μL of RPMI 1640 containing 10% FBS, in a U bottom 96-well plate. This was followed by the addition of 50 μL of 5 ng/mL of HIV-1 pseudovirus-containing supernatant and 1 h incubation at 37 °C. Subsequently 100 μL of 0.5×10^5 Raji/DC-SIGN cells/mL was added and incubated at 37 °C for 2 h. The plate was then washed three times with RPMI 1640 by centrifuging at 2000 rpm for 5 min and virus bound to Raji/DC-SIGN cells was lysed with 150 μL of 0.5% Triton-X 100. The amount of captured p24 was measured by ELISA in the presence and absence of lectins.

DC-SIGN-mediated transfer of HIV-1 infection

The DC-SIGN-mediated HIV-1 transfer to TZM-bl cells was carried out by incubating 7.5×10^4 Raji/DC-SIGN cells/well with HIV-1 pseudovirus in a U bottom 96 well plate at 37 °C for 1 h. Cells were then washed three times with RPMI 1640 by centrifuging at 2000 rpm for 5 min to remove unbound viruses and resuspended in 150 μL of 10% FBS DMEM (growth medium). This was followed by the transfer of 100 μL to the corresponding wells of a flat bottom plate. Subsequently, 3×10^4 TZM-bl cells/100 μL of growth medium/well were added

Table 4
Effect of HIV-1 glycosylation on DC-SIGN transfer.

Envelope	Genotype	Post-DC-SIGN binding method[a] IC_{50} (nM)			Pre-DC-SIGN binding method IC_{50} (nM)		
		GRFT	CV-N	SVN	GRFT	CV-N	SVN
CAAN5342.A2	[b]WT	5.1±0.1	18.8±1.7	115.0±2.4	7.1±0.4	24.2±2.1	153.0±9.7
	234N	>50	>50	>500	>50	>50	>500
	295N	1.5±0.2	7.3±0.1	66.8±3.0	3.5±0.3	13.4±0.3	140.6±9.0
CAP63.A9J	WT	35.0±5.4	24.0±5.6	411.3±62.9	38.7±5.2	33.7±3.7	590.0±82.4
	234N	>50	>50	>500	>50	>50	>500
	295N	7.9±0.2	9.2±0.1	228.3±3.0	11.5±0.7	18.2±1.0	357.1±47.3

[a] The concentration needed to inhibit HIV-1 transfer by 50%.
[b] Wild type.

to the plate that was then placed at 37 °C for 48 h. The Bright Glo™ Reagent (Promega, Madison, WI) was used to measure infection after 48 h of culture, according to the manufacturer's instructions. Luminescence was measured in a Wallac 1420 Victor Multilabel Counter (Perkin-Elmer, Norwalk, CT).

GRFT, CV-N and SVN inhibition of DC-SIGN-mediated HIV-1 infection was measured by using two methods. In the first method (post-DC-SIGN binding method) Raji/DC-SIGN cells were incubated with the virus for an hour and after the removal of unbound viruses a five-fold dilution series of GRFT, CV-N and SVN in 150 μL of growth medium was added. This was followed by a 1 hour incubation at 37 °C and transfer of 100 μL from each well to the corresponding well of a flat bottom 96 well plate. Subsequently, 3×10^4 TZM-bl cells/100 μL of growth medium/well were added to the plate that was then placed at 37 °C for 48 h; HIV-1 infection was measured as described above. Raji cells co-cultured with TZM-bl cells, in the absence of the virus, were used here as background controls. In the second method (the pre-DC-SIGN binding method), HIV-1 pseudovirus was first incubated for 1 h at 37 °C with serially diluted GRFT, CV-N and SVN in 150 μL of growth medium before the subsequent addition of Raji/DC-SIGN cells and TZM-bl cells. When the pre and post-DC-SIGN binding methods were combined, the virus was incubated with the lectins for an hour before addition of Raji/DC-SIGN cells for 2 h. This was followed by washing and a second incubation with the lectins. The cells were then co-cultured with TZM-bl cells for 48 h. To determine synergism between GRFT, CV-N and SVN the inhibition of transfer was tested for each lectin alone and in combination. Synergism, additive effect or antagonism was determined by calculating the combination index (CI) using IC_{50} (Chou and Talalay, 1984; Xu et al., 2001). A CI of 0.3 to 0.7 was deemed indicative of synergism, 0.7 to 0.85 of moderate synergism, 0.85 to 0.9 of slight synergism, 0.9 to 1.1 of an additive effect and >1.1 of antagonism as previously defined (Chou and Talalay, 1984; Zwick et al., 2001).

For the inhibition of HIV-1 transfer to phytohemagglutin (PHA) / interleukin-2 stimulated peripheral blood mononuclear cells (PBMC), the post and pre-DC-SIGN binding method described above were used with some modifications. With the post-DC-SGN binding method, Raji/DC-SIGN were first allowed to bind to infectious HIV-1 subtype C primary isolates, before incubation with serially diluted GRFT, CV-N and SVN. Subsequently virus-bound Raji/DC-SIGN cells were co-cultured with PHA-activated PBMC (5×10^5 cells/well). The cells were cultured in RPMI 1640 containing 20% FBS and interleukin-2. PBMC cultured with Raji cells in the absence of the virus and lectins were used as negative controls. After 24 h Raji/DC-SIGN cells and PBMC were washed three times by centrifuging at 1200 rpm for 5 min. All the washing steps were performed with 20% FBS RPMI 1640. Thereon the culture supernatant was collected twice daily and replaced with an equal amount of fresh growth medium. For each harvest the p24 antigen concentration in the virus control wells was measured. The inhibitory activity of the lectins was measured at the time-point that corresponded to the early part of the linear growth period of the virus control (Zhou and Montefiori, 1997). The IC_{80} were calculated by plotting the lectin concentration vs. the percentage inhibition in a linear regression using GraphPad Prism 4.0. With the pre-DC-SIGN binding method, HIV-1 was first incubated for 1 h at 37 °C with serially diluted GRFT, CV-N and SVN before the subsequent addition of Raji/DC-SIGN cells and PBMC. In the combined method the virus was incubated with the lectins prior and after incubation with Raji/DC-SIGN. Virus bound Raji/DC-SIGN cells were then co-cultured with PBMC and HIV-1 infection was determined as explained above.

Acknowledgments

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CHAPTER FIVE

THE MECHANISMS OF HIV-1 SUBTYPE C RESISTANCE TO GRFT, CV-N AND SVN

Mechanisms of HIV-1 subtype C resistance to GRFT, CV-N and SVN

Running Title: HIV-1 resistance to lectins.

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ABSTRACT

Glycans on the HIV-1 trimer protect neutralization sensitive epitopes and contribute to the structural integrity of the envelope. Those on gp120, particularly the mannose-rich glycans are targets for lectins that can inhibit virus entry into cells. We examined the ability of HIV-1 subtype C to develop resistance to the inhibitory lectins, griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN) which bind multiple mannose-rich glycans on gp120. HIV-1 strains Du179, Du151, Du422 and COT9 cultured under escalating concentrations of the three lectins became increasingly resistant to these compounds tolerating 2 to 12 times their 50% inhibitory concentrations. Envelope sequence analysis of lectin-selected viruses showed that most had deletions of 1 to 5 mannose-rich glycans on gp120. Glycosylation sites at positions 230, 234, 241, 289 located in the C2 region and 339, 392 and 448 in the C3-C4 region were affected. Differences in the glycan deletion patterns in response to the three lectins were noted, although sites 230, 392 and 448 were common. Furthermore, deletions and insertions of up 4 amino acids near mannose-rich glycosylation sites in the variable region 4 (V4) of gp120 were also observed. These data suggest that gp120 deglycosylation and rearrangement of glycans are mechanisms of HIV-1 subtype C escape from GRFT, CV-N and SVN. They also suggest that the C2, C3 and V4 regions are important for lectin binding to the viral envelope.

INTRODUCTION

The surface of the HIV-1 envelope is populated with glycans that play an important role in protecting neutralization sensitive epitopes (21, 26, 27, 31, 43, 44, 49), promoting gp120 structural integrity and mediating interaction with cellular receptors (20, 28-30, 32). The majority of glycans on the HIV-1 envelope trimer are mannose-rich comprising 7 to 9 terminal mannose residues (9, 18) although the precise number and location remains elusive. Complex glycans with terminal sialic acid residues are likely also present (27). Mannose-rich glycans are targets for lectins or carbohydrate binding agents (CBAs) such as griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN) isolated from naturally occurring algae (8, 10, 36-38, 50, 51). These lectins showed potent and broad anti-HIV-1 activities *in vitro* and are, therefore, being investigated for use in HIV-1 prevention, mostly in the form of microbicides (3, 39, 41, 42).

Since the neutralization activity of lectins involves interaction with glycans, one potential mechanism of HIV-1 escape from these compounds is deglycosylation. Indeed studies on HIV-1 subtype B have shown deletion of mannose-rich glycans is a mechanism of resistance to CV-N (6, 23). More specifically, Balzarini and colleagues showed that loss of mannose-rich glycans at positions 230, 289, 295, 332, 339, 386, 392 and 448 was associated with resistance in HIV-1_{IIIB} and HIV-$1_{NL\text{-}4.3}$ (6). Similarly, Hu *et al.* reported the deletion of six of these glycans including those at positions 230 and 386 also on HIV-1_{IIIB} cultured under escalating concentrations of CV-N (23). In addition, HIV-1 resistance to the lectins *Galanthus nivalis* agglutinin and *Hippeastrum* hybrid agglutinin, was reported to occur via partial envelope deglycosylation (4, 5). Furthermore, HIV-1 resistance to the broadly neutralizing antibody 2G12, that targets glycans on gp120, involves the deletion of mannose-rich glycans and this is supported by the fact that most subtype C viruses are resistant to this antibody due to their lack of the 295 glycosylation site (7, 13, 22, 33). Thus, partial deglycosylation is an escape mechanism frequently used by HIV-1.

The glycosylation pattern on the HIV-1 subtype C envelope differs from subtype B (47), and the ability of these viruses to develop resistance to lectins is unknown. In the current study we describe the mechanism of resistance to CV-N among four subtype C primary viruses and show that it was similar to subtype B, involving the deletion of

mannose-rich glycans on the envelope. Some of these glycans were the same as those already identified for subtype B. However, we also observed the involvement of the 234 glycan, not previously shown, and amino acids deletion in the V4 region. In addition, HIV-1 escape from GRFT and SVN followed a similar pathway to CV-N. Thus, changes of glycosylated and non-glycosylated amino acid sequences accompanying resistance to these three lectins suggest multiple mechanisms of escape.

MATERIALS AND METHODS

Viruses, cell lines and lectins: Infectious HIV-1 subtype C, R5-using virus Du151 and Du422 were isolated from acute infections while the R5X4 Du179 was isolated from a chronic infection in South Africa (45). COT9 is a R5 isolate from a chronically infected pediatric patient (14). The pSG3Δ*env* plasmid was provided by Dr. Beatrice Hahn. The TZM-bl cell line was from the NIH Reference and Reagent Program (Cat No 8129) while the 293T cell line was obtained from the American Type Culture Collection. These two cell lines were cultured in DMEM containing 10% fetal bovine serum (FBS). Cell monolayers were disrupted at confluence by treatment with 0.25% trypsin in 1 mM EDTA. Recombinant GRFT, CV-N and SVN were purified from *E. coli* at the National Cancer Institute, MA, USA (8, 10, 36).

Selection of GRFT, CV-N and SVN resistant viruses: One thousand $TCID_{50}$ of each HIV-1 subtype C infectious isolate were grown under escalating concentrations of GRFT, CV-N and SVN. Viruses were cultured in 2 mL of 4 x 10^6 peripheral blood mononuclear cells (PBMC), depleted of $CD8^+$ T cells by means of RosetteSep CD8 depletion cocktail (StemCell Technologies, Vancouver, Canada). The starting concentrations of the lectins were the IC_{50} (50% inhibitory concentration) for each virus. Cultures without lectins were included as experimental controls. All cultures were maintained in RPMI 1640 containing 20% FBS and IL2 (0.05 μg/mL). Viruses were passaged every 7 days by transferring 500 μL of the previous culture into fresh CD8 depleted PBMC. The concentration of GRFT, CV-N and SVN was increased whenever the p24 antigen level in the lectin containing cultures was similar or higher than the control cultures. When these levels were lower the lectin concentration was reduced. After every passage 500 μL aliquots of culture supernatants were stored at -70 ºC for genotyping and neutralization assays.

HIV-1 neutralization assay in peripheral blood mononuclear cells: The neutralization assay in PBMC was carried out as described by Bures *et al.* (11). Briefly, a three-fold dilution series of GRFT, CV-N, and SVN in 40 μL of RPMI 1640 containing 20% FBS and IL2 (growth medium) was prepared in triplicate in a U-bottom 96-well plate. Five hundred $TCID_{50}$ of the HIV-1 isolate in 15 μL of growth medium was added to each well

and the plate was incubated at 37ºC for 1 hour. This was followed by the addition of 5 x 10^5 cells/well/100 µL of phytohemagglutin/IL-2 stimulated PBMC (PHA-PBMCs). After an overnight incubation, cells were washed 3 times with RPMI 1640 containing 20% FBS and resuspended in 155 µL of fresh growth medium. The culture supernatant was collected twice daily and replaced with an equal amount of fresh growth medium. The p24 antigen concentration in the virus control wells was measured by ELISA using the Vironostika HIV-1 Antigen Microelisa System (Biomerieux, Boseind, the Netherlands). Levels of p24 in the lectin cultures were measured at the time-point corresponding to the early part of the linear growth period of the virus control (48). The 80% inhibitory concentrations (IC_{80}) were calculated by plotting the lectin concentration versus the percentage inhibition in a linear regression using GraphPad Prism 4.0.

HIV-1 envelope amplification and sequencing: HIV-1 RNA was extracted from stored aliquots of infectious viruses grown under increasing concentrations of GRFT, CV-N and SVN. RNA was reverse transcribed to cDNA using the Superscript III Reverse Transcriptase according to the manufacturer's instructions (Invitrogen, CA). For both the single genome amplification (SGA) and the total population amplification, envelope gene PCR was carried out as described by Salazar-Gonzalez *et al.* (40). The PCR products were gel purified using the Qiagen Gel Purification Kit according to the manufacturer's instruction (Hilden, Germany), sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and resolved on an automated genetic analyzer. Changes in the sequenced envelopes were identified using Sequencher v.4.5 (Genecodes, Ann Arbor, MI), Clustal X (ver. 1.83) and Bioedit (ver. 5.0.9).

Generation of mutants Env-pseudotyped virus stock: Glycosylation sites and amino acid deletions and insertions associated with GRFT, CV-N and SVN resistance were introduced in HIV-1 envelope clones using the QuikChange Site Directed Mutagenesis Kit (Stratagene, LaJolla, CA). The presence of the mutations was confirmed by sequencing as described above. HIV-1 pseudoviruses were generated by co-transfection of the Env and pSG3Δ*env* plasmids (44) into 293T cells using the Fugene transfection

reagent (Roche Applied Science, Indianapolis, IN). This was followed by the quantification of the $TCID_{50}$ of each virus stock by infecting TZM-bl cells with serial 5-fold dilutions of the supernatant in quadruplicate in the presence of DEAE dextran (37.5 μg/mL) (Sigma-Aldrich, St. Louis, MO). After 48 hours of culture, HIV-1 infection was measured using the Bright Glo™ Reagent (Promega, Madison, WI), according to the manufacturer's instructions. Luminescence was quantified in a Wallac 1420 Victor Multilabel Counter (Perkin-Elmer, Norwalk, CT) and the $TCID_{50}$ was calculated as described elsewhere (25).

Single cycle neutralization assay (TZM-bl assay): The pseudovirus neutralization assay was carried out as described previously (35). Briefly, three-fold dilution series of GRFT, CV-N and SVN in 100 μL of DMEM with 10% FBS (growth medium) were prepared in a 96-well plate in duplicate. This was followed by the addition of 200 $TCID_{50}$ of pseudovirus in 50 μL of growth medium and the mixture was incubated for 1 hour at 37ºC. Then 100 μL of TZM-bl cells at a concentration of 1 x 10^5 cells/mL in 10% FBS DMEM containing 37.5 μg/mL of DEAE dextran was added to each well and the plate was placed at 37ºC for 48 hours. HIV-1 infection was evaluated by measuring the activity of firefly luciferase. Titers were calculated as the inhibitory concentration that causes 50% reduction (IC_{50}) of relative light unit (RLU) compared to the virus control (wells with no inhibitor) after the subtraction of the background (wells without both the virus and the inhibitor).

RESULTS

Replication of HIV-1 subtype C isolates in the presence of sub-inhibitory concentrations GRFT, CV-N and SVN

HIV-1 subtype B has previously been shown to develop resistance to CV-N after repeated passages in the presence of escalating concentrations of this compound (4, 5, 23). Since the glycosylation patterns of HIV-1 subtype C envelope differs (47), we determined the ability of viruses of this subtype to develop resistance to CV-N and two other lectins, GRFT and SVN. Four primary isolates were cultured in CD8 depleted PBMC in the presence of increasing lectin concentrations for 11 to 22 weeks, starting with the concentration equal to the IC_{50} for each compound (Table 1). Viral growth was measured weekly by p24 antigen ELISA which showed variation during the course of the experiment and on some occasions was markedly reduced in the lectin-containing cultures when compared to the control (culture containing no lectin) (Figure 1). When this happened the lectin concentrations were reduced accordingly in order to facilitate ongoing replication.

The Du179 isolate developed the greatest loss of sensitivity to all three compounds, tolerating at least 10 times the starting concentration of each lectin (Table 1). Du151, Du422 and COT9 grew at 3 times the starting concentrations of GRFT and SVN and 5 times the starting concentration of CV-N (Table 1). Altogether, these data showed that the continuous growth of HIV-1 subtype C under lectin selective pressure resulted in increased resistance to these compounds.

Lectin-selected isolates showed decreased sensitivity and cross-resistance

We next determined whether viruses cultured in the presence of GRFT, CV-N and SVN showed reduced sensitivity to these compounds in the PBMC neutralization assay, using an 80% neutralization (IC_{80}) cut-off (12, 19). Du179 selected by the 3 lectins (Du179/GRFT.R, Du179/CV-N.R and Du179/SVN.R) showed a >5 fold increase in IC_{80} compared to the control viruses, passaged in the absence of the lectin (Figure 2A). For Du151 and Du422 there was an increase in IC_{80} that ranged from 2 to 4 fold for all 3 lectins (Figure 2B and C), while for COT9, the GRFT resistant virus showed a ~2 fold increase in IC_{80} (Figure 2D). However, there was no change in IC_{80} for COT9 cultured

under CV-N and SVN, despite their ability to grow under increased concentrations of these lectins.

We next investigated whether viruses that were resistant to each compound also showed cross-resistance to the other two lectins. We used Du179 for this study as this virus developed the greatest resistance to the lectins. Du179/GRFT.R showed a ~3 fold increase in resistance to CV-N and SVN neutralization (Figure 3). A similar increase was observed for Du179/CV-N.R against GRFT and SVN. Lastly, Du179/SVN.R developed strong resistance to GRFT with its IC_{80} increasing ~7 fold. However, this virus resistance to CV-N increased only ~3 fold. These data suggest that resistance to one lectin confers cross-resistance to the other two probably as a result of the overlapping glycans binding sites on gp120 (1).

Resistance was associated with amino acid changes and deletions at and around mannose-rich glycosylation sites

In HIV-1 subtype B, resistance to CV-N occurred through the deletion of mannose-rich glycosylation sites (4, 5, 23). In order to assess whether this was also the case in subtype C, we compared the full envelope sequences of the total viral population of both wild type (viruses passaged in the absence of the lectin) and their corresponding resistant samples. Unpassaged Du179, Du151 and COT9 each had 10 intact mannose-rich glycosylation sites while Du422 had nine. All four viruses lacked the 295 glycosylation site as is common among subtype C viruses (26, 47). Of the 12 selected viruses (four for each lectin), nine had deletions of glycans on gp120 with no changes in gp41 glycosylation patterns. Out of the 11 glycosylation sites on gp120 that have been confirmed to contain mannose-rich glycans (27), seven were involved in resistance to GRFT, CV-N and SVN (Figure 4). Deletions of the 230, 392 and 448 glycans were observed among viruses selected by all three lectins with the 448 glycan observed in 6 out of the 12 selected viruses. Except for position 289, deletions at the seven sites occurred in response to more than one lectin, in agreement with the overlap of their binding sites.

Examination of glycans changes to individual lectins showed that the greatest number of deleted glycans was conferred by GRFT selection (Table 2). The loss of the

glycan at position 339 occurred in three out of four GRFT resistant viruses (Table 2 and Figure 4), with those at position 230 and 234 occurring in two. In GRFT resistant Du179 there was also the deletion of the 442 glycan, predicted to be complex in studies conducted with monomeric gp120 (26, 27). The loss of sensitivity to GRFT in Du422 restored the glycan at position 386 that was absent in the wild type virus (Table 2). With CV-N resistant viruses, the loss of the 448 glycan was the most common, occurring in half of these viruses (Table 2). However, we did not observe a change in COT9 and Du422 sequences that accompanied their reduced sensitivity to CV-N. Three out four SVN resistant viruses had the 448 deglycosylation while two out four had the 339 deletion (Table 2). The same as with GRFT, SVN resistance restored the 386 glycan in Du422. Lastly, similar to CV-N, COT9 resistance to SVN was not associated with an apparent change in glycan.

In addition to gp120 deglycosylation, we observed deletions and insertions of amino acid sequences near mannose-rich glycosylation sites located in the fourth variable (V4) region. GRFT resistance was associated with the deletion of four amino acids at position 400-403 and 396-399 in Du179 and COT9, respectively (Figure 5A and D). However, in Du422 resistance to GRFT resulted in the insertion of five amino acids at position 398-402 (Figure 5C). Similarly, CV-N resistance led to the deletion of four amino acids in Du179 at position 392-395 (Figure 5A). In Du422, SVN resistance resulted in the insertion of five amino acids at position 398-402, this was similar to GRFT (Figure 5C). We observed no deletions or insertions of amino acids in Du151 under the selective pressure of any of the three lectins. In conclusion our data show that HIV-1 subtype C developed resistance to GRFT, CV-N and SVN by deleting amino acids that may result in shifting the position of neighboring glycans, this in addition to directly deleting these glycans.

Single genome amplification of GRFT, CV-N and SVN resistant viruses

Since the loss of multiple glycans observed by total viral population sequencing could be the result of a mixture of viruses carrying fewer deglycosylations, we performed single genome amplifications (SGA) of gp160. We selected Du179 for this experiment since it

was the most deglycosylated of all viruses studied. Using SGA we isolated seven clones from GRFT and CV-N selected viruses and six clones from the SVN selected virus.

All but one GRFT clone had five deglycosylation involving the 230, 234, 339, 442 and 448 sites. The remaining clone had the deletion of the 393 glycosylation site in addition to these sites making its deglycosylation pattern similar to the total population sequence. Furthermore, every GRFT clone had the deletion of four amino acids in V4 mainly at position 400 to 403. For CV-N, most clones had more glycans deletions than the total population sequence with the majority having four to five deglycosylation compared to only three in this sequence. Also the 234 and 442 deletions observed in most CV-N clones were absent in the total population sequence. However, similar to this sequence, all clones had the deletion of four amino acids in V4. With SVN, in most cases, both the number and deglycosylation pattern of isolated clones were similar to the total population sequence although unlike it, one had the 442 deletion while two carried deletions of amino acids in V4. Lastly, almost all GRFT, CV-N and SVN clones had the sequence GPGK at the tip of the V3 loop changed to GPGQ that is common in viruses using the CCR5 co-receptor (17). Taken together, these data show that populations of GRFT, CV-N and SVN resistant viruses comprise differentially deglycosylated clones.

Confirmation of resistance conferring mutations by site-directed mutagenesis

To confirm the association between glycans and V4 amino acid deletions observed in GRFT, CV-N and SVN resistant viruses (shown in Table 2 and Figure 5) and increased resistance to the lectins, we introduced these deletions into corresponding wild type cloned envelopes by site-directed mutagenesis. Sensitivity to the three lectins between mutant viruses and their wild type counterparts was compared in a TZM-bl cell neutralization assay. The introduction of glycans and V4 amino acid deletions associated with GRFT and SVN resistance in the four cloned envelopes caused a decrease in HIV-1 sensitivity to the lectins judging from the increased IC_{50} of the mutant viruses (Table 3). There was a ~3 fold decrease in GRFT sensitivity while the decrease in sensitivity to SVN ranged from 5 to 16 fold. When we introduced glycans and amino acids deletions associated with CV-N resistance in Du179.14 and Du151.2 we obtained pseudoviruses

that were unable to infect TZM-cells. Nevertheless, these data suggest that the changes we observed in HIV-1 gp120 sequence were indeed resistance-conferring mutations.

Sensitivity to HIV-positive plasma of GRFT and SVN resistant viruses

HIV-1 resistance to CV-N has been shown to be associated with increased sensitivity to HIV-positive sera due to loss of glycans (23). Therefore, we investigated whether GRFT and SVN resistant viruses, generated by site directed mutagenesis, had increased sensitivity to six HIV-positive plasma samples from chronically infected subjects using the neutralization assay in TZM-bl cells. Du179-GRFT.R_M and COT9-GRFT.R_M were as sensitive as the wild type viruses to most of the samples tested (Table 4). However, Du151-GRFT.R_M mostly showed a decrease in sensitivity, a trend that was partly followed by Du422-GRFT.R_M (Table 4). There was no change in sensitivity to five of the six plasma samples for Du179-SVN.R_M and Du422-SVN.R_M while Du151-SVN.R_M and COT9-SVN.R_M showed an increase in sensitivity in most cases. In all, our data suggest that HIV-1 escape from GRFT and SVN can in some cases affect sensitivity to plasma antibodies from HIV-1 positive individuals.

DISCUSSION

In this study we demonstrated that the continuous growth of HIV-1 subtype C isolates under escalating concentrations of GRFT, CV-N and SVN resulted in reduced sensitivity to these lectins. This was associated with the deletion of 1 to 5 mannose-rich glycans on gp120 and in some cases insertions or deletions of amino acids sequences near mannose-rich glycosylation sites. We confirmed these associations by site-directed mutagenesis of envelope clones. This study is the first to report the mechanism of HIV-1 subtype C resistance to GRFT, CV-N and SVN, contributing to a better understanding of the gp120 binding sites of these compounds.

The association between mannose-rich glycans deletions on subtype C viruses and increased resistance to GRFT, CV-N and SVN is consistent with reports showing that these deletions mediate HIV-1 subtype B resistance to CV-N (6, 23, 46). However, it should be noted that these studies used laboratory adapted strains while our study was based on circulating primary isolates. The deleted glycans were located in the C2, C3, V4 and C4 regions of gp120 and suggest these regions are involved in the lectins binding to the viral envelope. As shown in Figure 7 the structural arrangement of gp120 exposes the C2, C3, V4 and C4 and, therefore, renders them available to interact with the lectins. V4 in particular may be of prime importance to GRFT, CV-N and SVN binding to gp120 given that in addition to the removal of glycans there were significant deletions and insertions of amino acids in this region in many resistant viruses. These changes in V4 amino acid sequence were near mannose-rich glycosylation sites, thus, probably affecting glycans exposure. This finding is in agreement with Witvrouw and colleagues who showed that HIV-1 cultured in increasing concentration of CV-N had amino acids deletions in V4 (46). Similar to V4, the C2 region is likely to play an important role in the interaction of the lectins with HIV-1 since, compared to other regions, it contains the highest density of mannose-rich glycans and also had the highest level of deglycosylation in our study (Figures 5 and 7). The symmetric arrangement of mannose binding sites on GRFT and CV-N (50, 51) and the observation that some deleted glycans were in close proximity of each other (Figure 7) suggests that these lectins may cross-link glycans on the viral envelope and this is consistent with what has been previously shown for GRFT (38). In addition, the spatial arrangement of the 339 and 392 glycans, both deleted under

GRFT and SVN selective pressure, suggests that this cross-linking may involve glycans on different regions of gp120.

The observation that in all cases resistance to the lectins was accompanied by the deletion of mannose-rich glycans exclusively located in gp120 suggests that glycans in gp41 are not involved in GRFT, CV-N and SVN interaction with HIV-1, in agreement with Hu and colleagues (23). In some Du179 resistant viruses we observed the deletion of the 442 glycosylation site which does not contain a mannose-rich glycan, as shown on monomeric gp120 (27). However, recent studies indicated that, contrary to monomeric gp120, glycans on gp120 trimer are more resistant to mannose-trimming that generates complex glycans (9, 18). Thus, it can be speculated that on the later the 442 site contains a mannose-rich glycan. The observation of the 448 deletion in 50% of selected viruses suggests this glycan plays an important role in GRFT, CV-N and SVN binding to gp120. This agrees with Huang and colleagues who showed that the 448 glycan was critical for GRFT interaction with gp120 (24). The involvement of the 230, 392 and 448 glycans in resistance to the three lectins is in agreement with a previous study that showed resistance to CV-N involved the loss of these three glycans on HIV-1 subtype B viruses (6). Furthermore, the lack of deglycosylation at position 262 is consistent with previous subtype B studies (6, 23). However, it is likely that in some cases there are subtype-specific pathways to resistance. For example, the 289 glycan was lost in subtype B viruses that developed resistance to CV-N (23) while none of the CV-N resistant viruses studied here showed this loss. Previously we showed the concomitant absence of the 234 and 295 glycosylation sites renders HIV-1 naturally resistant to GRFT, CV-N and SVN (1). In the current study we observed the deletion of the 234 glycosylation site in resistant viruses of the three lectins suggesting this glycan can participate in both natural and *in vitro* induced resistance to these compounds.

Our data did not show an association between the number of deleted mannose-rich glycans and the concentration of GRFT, CV-N and SVN the viruses tolerated. For example the highest concentrations of GRFT that Du151 and Du422 withstood were similar although these viruses lost four and one mannose-rich glycan, respectively. The same observation was made when comparing the SVN resistant Du151 and Du422. This

supports our study that suggested that GRFT, CV-N and SVN sensitivity is more affected by the position of deleted mannose-rich glycans than by their number (1).

In this study we showed that a population of GRFT, CV-N and SVN resistant viruses can consist of individual strains that have varying patterns of glycan loss. This was not apparent when we analyzed the envelope sequences of the bulk population of Du179, Du151, Du422 and COT9 resistant viruses. However, we were able to observe this by SGA of GRFT, CV-N and SVN resistant Du179. To a certain extent this mixed population of differentially deglycosylated strains should have been expected since, as already stated, we previously showed that the number of deleted mannose-rich glycans is less important than their position (1). Due to this, viruses that have different levels of deglycosylation will tend to coexist in a given resistant population as long as they have glycan losses that render them resistant to the lectins. It can as well be speculated that the presence of strains with different levels of glycan loss is the result of differences in their mechanisms of escape.

Compared to the other viruses we used, Du179 developed the highest resistance to the lectins. This difference is probably not caused by the number or pattern of mannose-rich glycosylation sites on these viruses since like COT9 and Du151, Du179 lacked only the 295 glycosylation site. It is also unlikely that the initial sensitivity of these viruses to GRFT, CV-N and SVN played a role in the extent to which they developed resistance to the lectins because in most cases they had similar sensitivity to these compounds. However, Du179 was the only dual-tropic virus tested here (16, 45). Thus, testing a larger number of dual tropic viruses and comparing them to non-dual tropic viruses may reveal to which extent the R5X4 property affects the rate at which HIV-1 develops resistance to GRFT, CV-N and SVN. Here it is interesting to note that we previously showed GRFT inhibits HIV-1 infection of cells by interfering with the co-receptor binding to the virus (2). Therefore, it may be possible, at least in the case of GRFT, that the dual tropic property might have played a role in Du179 higher escape rate. Note as well that all but one Du179 clone (Figure 6) had the GPGQ sequence at the tip of the V3 loop that is characteristic of R5 viruses (15, 17), suggesting that Du179 may have given prominence to the use of the CCR5 co-receptor under the lectins selective pressure.

The level of resistance to the lectins observed in our study was not as high as previously shown (6, 46). For example, Balzarini and colleagues, growing HIV-1 under escalating concentration of CV-N in CEM cells obtained viruses that could tolerate up to 20 times the starting concentration of this lectin (6) while our viruses tolerated only 2 to 12 times the starting concentrations of GRFT, CV-N or SVN. The reason for this partial resistance may be due to our use of primary cells that probably did not support HIV-1 replication the same as it might be the case in the cell line used by these investigators. Also our viruses were circulating isolates which may have different replication capacity compared to molecular clones used in previous studies (6, 23, 46).

GRFT, CV-N and SVN are among leading CBAs that are being studied for use in HIV-1 prevention. However, until now much of what we know about HIV-1 resistance to these lectins is the result of studies conducted with subtype B viruses that compared to subtype C have a different glycosylation pattern (47). Thus the current study makes an important contribution to our understanding of the mechanism of resistance to GRFT, CV-N and SVN in HIV-1 subtype C viruses, the main cause of HIV infections around the world. In addition, the extensive loss of glycans and amino acid sequence changes associated with resistance to the three lectins may indicate the high genetic barrier the virus needs to overcome in order to become resistant to these compounds. This is supported by the observation that our viruses' resistance to the lectins was relatively low. However, since partial resistance to these lectins is more likely to happen than complete resistance, it is important to understand its mechanism. Taken together, our study supports further research in the use of these compounds to prevent the spread of HIV-1.

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LEGENDS

Figure 1. *In vitro* generation of GRFT, CV-N and SVN resistant viruses. HIV-1 subtype C Du179 (A), Du151 (B), Du422 (C) and COT9 (D) were cultured under escalating concentration of GRFT, CV-N and SVN. The concentration of each lectin was gradually increased or reduced depending on whether the viral growth in the culture was the same, higher or lower than the control culture (containing no lectin) as determined by p24 ELISA. The axis on the left and right of each graph indicate the p24 and lectin concentration, respectively. The arrows show the lectin concentration at the time the virus was isolated.

Figure 2. GRFT, CV-N and SVN escape viruses showed a decreased sensitivity to the lectins in a PBMC neutralization assay. HIV-1 subtype C Du179 (A), Du151 (B), Du422 (C) and COT9 (D) resistant to GRFT, CV-N and SVN were tested against the three lectins in a PBMC neutralization assay. The neutralization of HIV-1 infection was measured by p24 ELISA and the IC_{80} of the resistant virus and the corresponding wild type were determined by linear regression.

Figure 3. GRFT, CV-N and SVN selected viruses from Du179 show cross resistance in a PBMC neutralization assay. GRFT (A), CV-N (B), and SVN (C) resistant Du179 were tested against GRFT, CV-N and SVN. HIV-1 neutralization was measured by p24 ELISA and the IC_{80} of the resistant virus (grey) and the corresponding wild type (white bar) were determined by linear regression.

Figure 4. Mannose-rich glycosylation sites deleted in GRFT, CV-N and SVN resistant viruses. The X-axis shows the positions of mannose-rich glycans deleted in the four isolates under lectin selective pressure. The positions of glycans are numbered according to the HxB2 virus (27) and were identified by sequence analysis. The Y-axis shows the number of resistant viruses of the four isolates that had the deletion.

Figure 5. Mannose-rich glycans deglycosylation and amino acid deletions and insertions in HIV-1 resistant viruses. The regions and positions of the changes observed in Du179 (A), Du151 (B), Du422 (C) and COT9 (D) are shown. The sequence of the deleted and inserted amino acids (aa) are also shown. The arrows indicate the location of the deletion or insertion in each virus. Symbols show glycans that were deleted in GRFT (*), CV-N (○) and SVN (Δ) resistant viruses. The red color indicates that the glycan was deleted in resistant viruses of all three lectins while orange means it was added due to lectin selection.

Figure 6. Amino acid sequence of isolated GRFT, CV-N and SVN resistant Du179 clones. GRFT, CV-N and SVN resistant Du179 clones gp120 sequence isolated by single genome amplification. The grey shading shows the presence of a potential mannose-rich glycosylation site, yellow shading indicates the site deletion while the red box shows the tip of the V3 loop (34). Note that the glycan at position 393 is labelled as 392 in the text for comparison with other viruses.

Figure 7. Positions of mannose-rich glycosylation sites associated with resistance. Mannose-rich glycans on gp120 (Protein Data Bank ID 1GC1) deleted in GRFT, CV-N and SVN resistant viruses. Regions of gp120 associated with deleted glycans (shown as spheres) are color coded. The picture was created and visualized using PyMOL (DeLano Scientific LLC, South San Francisco, CA, USA, http://www.pymol.org).

Table 1: IC_{50} values of GRFT, CV-N and SVN for the neutralization of HIV-1

Virus	Pre-selection [a] IC_{50} (nM)			Fold increase after selection		
	GRFT	CV-N	SVN	GRFT	CV-N	SVN
Du179	37.8	102.2	134.1	10	10	12
Du151	40.3	41.2	128.5	3	5	3
Du422	39.5	82.1	215.6	3	5	2
COT9	85.8	77.1	449.5	3	4	2

[a] 50% inhibitory concentration.

Table 2: Changes in gp120 mannose-rich glycosylation patterns associated with resistance to GRFT, CV-N and SVN

Lectin	Virus	[a] Predicted mannose-rich glycosylation sites										
		230	234	241	262	289	295	332	339	386	[b] 392	448
GRFT	Du179											
	Du151											
	Du422											
	COT9											
CV-N	Du179											
	Du151											
	Du422											
	COT9											
SVN	Du179											
	Du151											
	Du422											
	COT9											

[a] Mannose-rich glycosylation sites were identified from the amino acid sequence of each envelope clone (related to HxB2) based on a study using monomeric gp120 (24).

[b] Note that for Du179 and Du151 the 392 glycan is shifted to position 393 but it is placed at position 392 for simplicity.

Red colored boxes indicate glycosylation sites that were deleted under GRFT, CV-N or SVN selective pressure.
Green colored boxes indicate glycosylation sites that were added under GRFT, CV-N or SVN selective pressure.
Grey colored boxes indicate sites that were unchanged.
Blank boxes indicate sites that were absent in the wild-type virus.

Table 3: Change in IC_{50} of mutant viruses compared to the corresponding wild type

	[a] IC_{50} (nM)	
Pseudovirus	**GRFT**	**SVN**
Du179.14 (WT)	3.6 ± 0.6	9.8 ± 1.6
Du179/GRFT.R_M (N230T/T236M/N339D/N393S/N448K/400-403 aa deletion)	13.7± 0.6 [b](4)	
Du179/SVN.R_M (N230D/N339K/N393D/N448I)		46.0 ± 1.6 (5)
Du151.2 (WT)	3.3 ± 0.2	14.0 ± 0.7
Du151/GRFT.R_M (N234S/S243G/S291Y)	10.5 ± 0.4 (3)	
Du151/SVN.R_M (N241K/N448D)		106.4 ± 39.2 (8)
Du422.1 (WT)	0.8 ± 0.3	7.4 ± 1.2
Du422/GRFT.R_M (N230T/D386N/ 398-402 aa insertion)	3.4 ± 0.8 (4)	
Du422/SVN.R_M (N339K/N448T/398-402 aa insertion)		117.5 ± 46.4 (16)
COT9.6 (WT)	2.7 ± 0.9	21.6 ± 7.5
COT9/GRFT.R_M (T341I/396-399 aa deletion)	9.8 ± 3.7 (3)	
[c] COT9/SVN.R_M (E440K/V454L/Y456F)		30.8 ± 7.7

[a] The concentration needed to inhibit HIV-1 infection by 50%.

[b] Change in IC_{50} in fold of the WT.

The M in the subscript indicates that resistance was generated by mutagenesis.

The aa changes that were introduced by mutagenesis are shown between brakets next to each virus.

[c] COT9/SVN.R_M did not have glycans deletion, the changes made are those closest to glycosylation sites.

Table 4: Sensitivity of GRFT, CV-N and SVN resistant viruses to HIV-positive plasma

Plasma	Du179			Du151			Du422			COT9		
	WT	GRFT.R	SVN.R	WT	GRFT.R	SVN.R	WT	GRFT.R	SVN.R	WT	GRFT.R	SVN.R
3B8	334	780	1,830	199	503	423	106	182	40	105	372	1,056
3B12	2,895	3,460	5,216	1,814	545	4,861	2,602	631	154	1,896	2,162	6,254
3B28	873	483	348	272	73	4,262	232	254	193	125	315	1,307
3B55	4,354	2,373	2,670	328	95	3,293	223	189	193	381	755	4,551
3B70	15,812	20,621	18,261	6,321	1,289	21,542	3,764	479	1,594	2,454	3,851	10,061
3B106	692	520	230	201	3,901	2,412	106	1,161	160	147	131	315

Red and green boxes indicate at least a 3 fold increase and decrease, respectively, in sensitivity to the plasma compared to the wild type virus while grey boxes show no change in sensitivity.

Data shown in the Table are reciprocal dilutions of plasma used to neutralize HIV-1 by 50%.

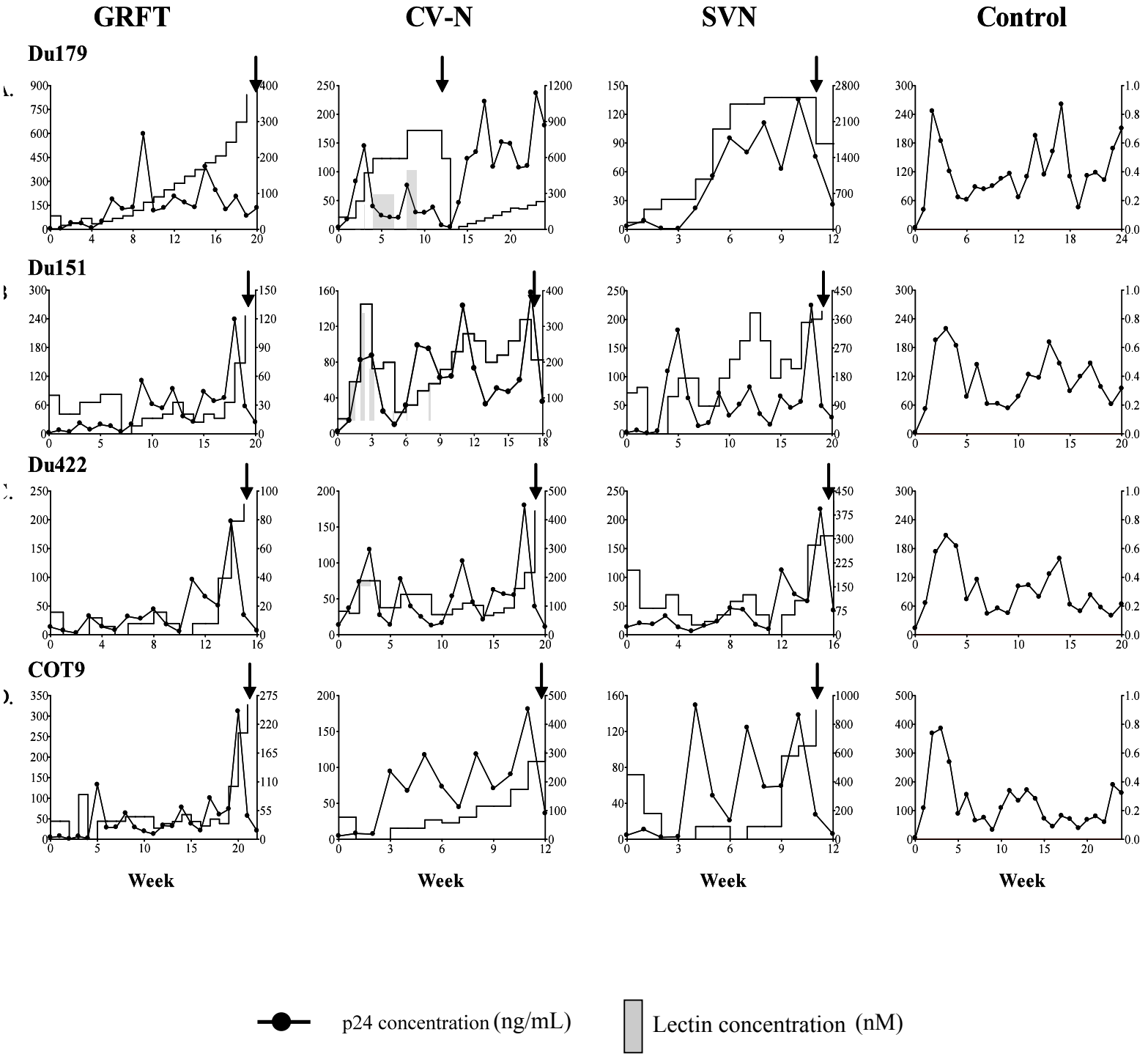

GRFT
CV-N
SVN
Control
Du179
Du151
Du422
COT9
Week
Week
Week
Week
p24 concentration (ng/mL)
Lectin concentration (nM)

Figure 1.

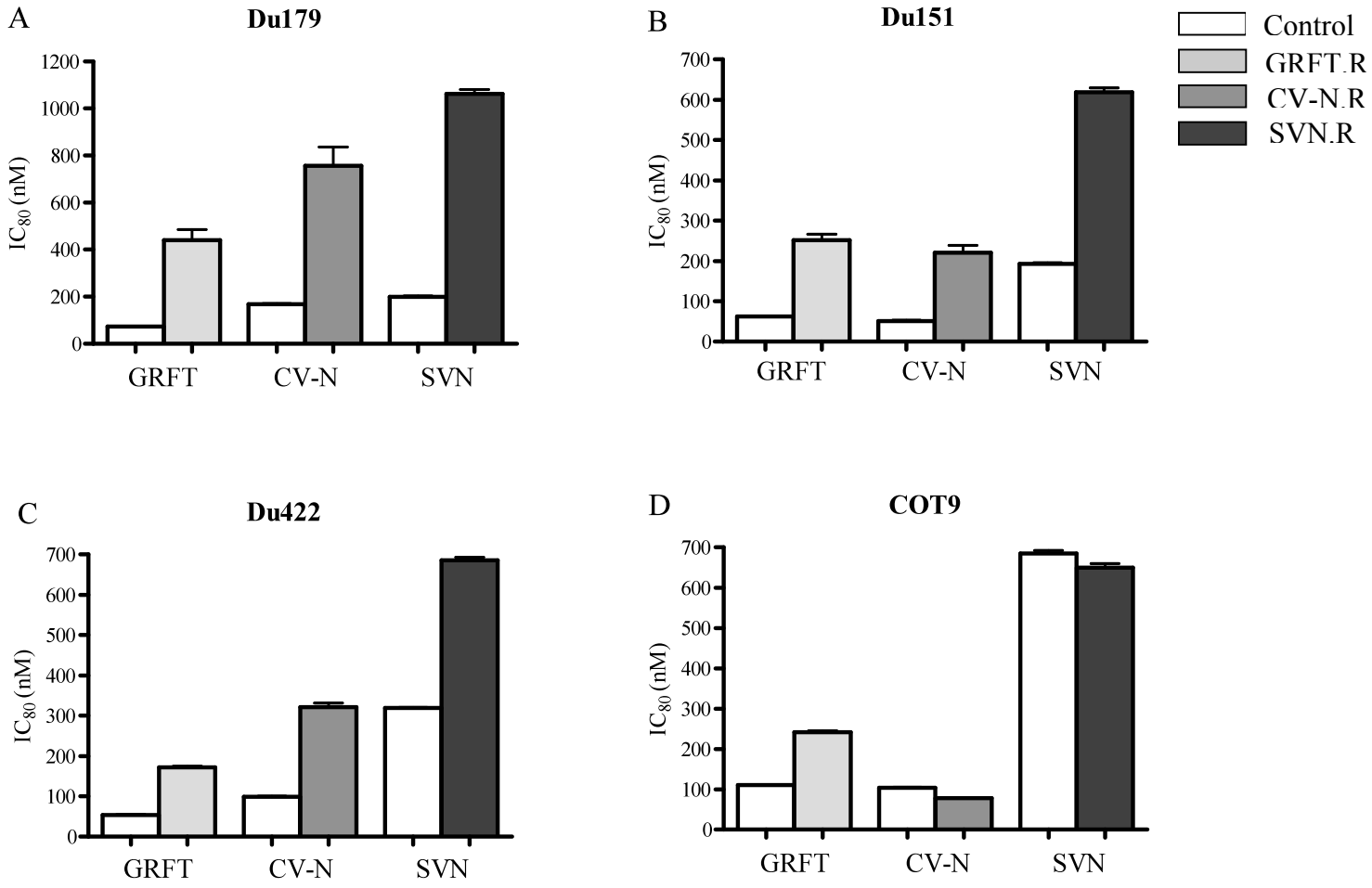

A
Du179
1200
1000
800
600
400
200
0
IC80 (nM)
GRFT
CV-N
SVN
B
Du151
700
600
500
400
300
200
100
0
IC80 (nM)
GRFT
CV-N
SVN
Control
GRFT.R
CV-N.R
SVN.R
C
Du422
700
600
500
400
300
200
100
0
IC80 (nM)
GRFT
CV-N
SVN
D
COT9
700
600
500
400
300
200
100
0
IC80 (nM)
GRFT
CV-N
SVN

Figure 2.

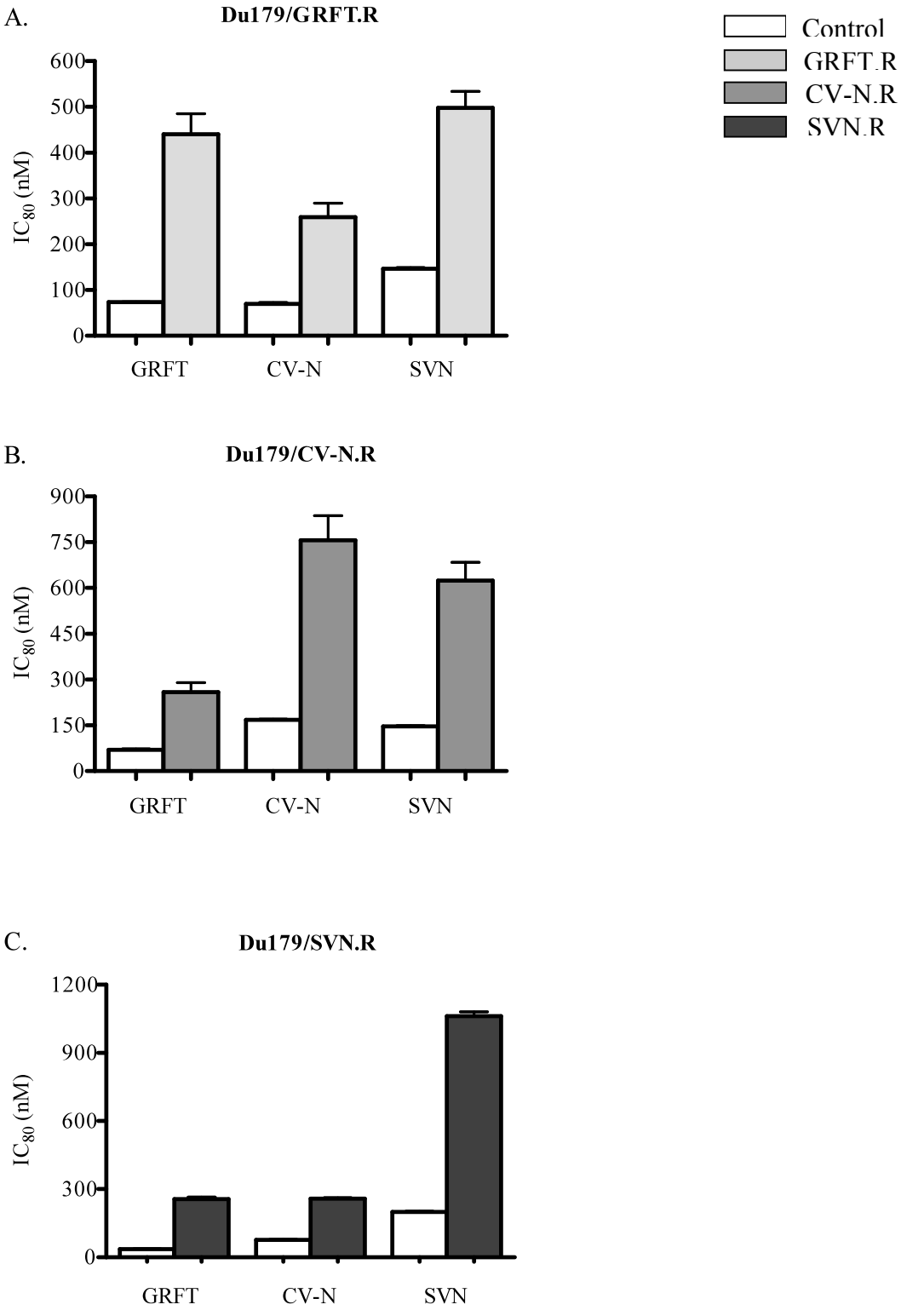

A.
Du179/GRFT.R
Control
GRFT.R
CV-N.R
SVN.R
IC80 (nM)
600
500
400
300
200
100
0
GRFT
CV-N
SVN
B.
Du179/CV-N.R
IC80 (nM)
900
750
600
450
300
150
0
GRFT
CV-N
SVN
C.
Du179/SVN.R
IC80 (nM)
1200
900
600
300
0
GRFT
CV-N
SVN

Figure 3.

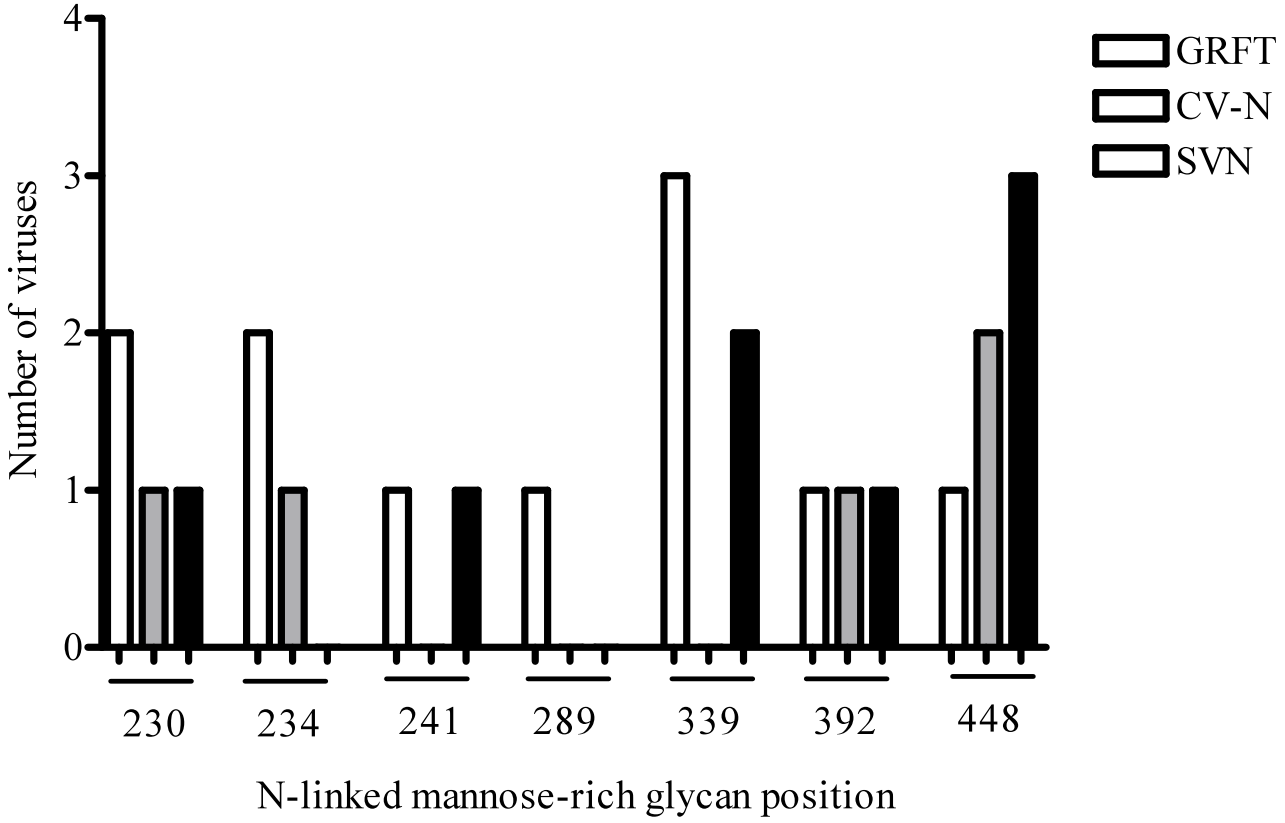
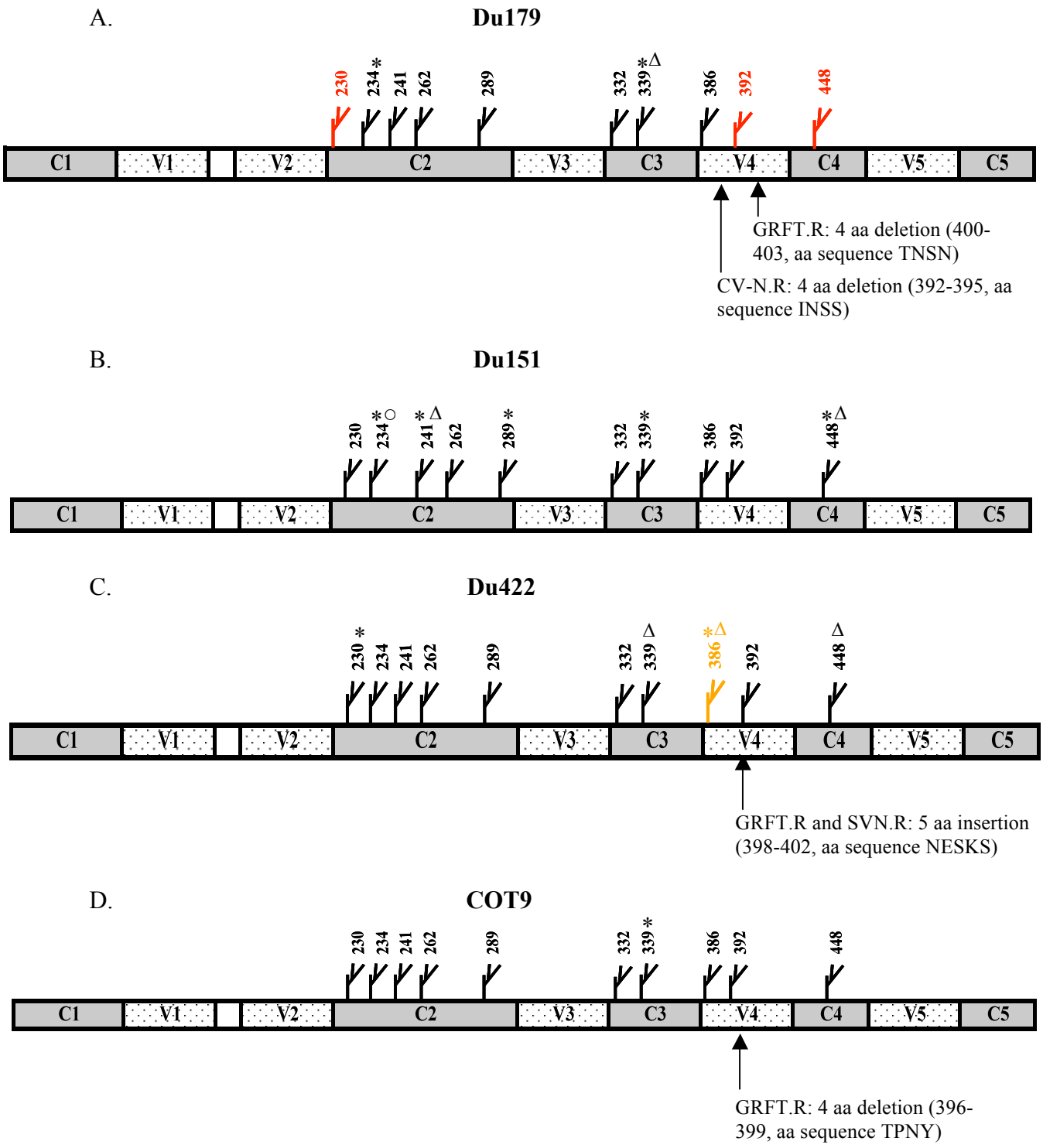

4
3
2
1
0
Number of viruses
GRFT
CV-N
SVN
230
234
241
289
339
392
448
N-linked mannose-rich glycan position

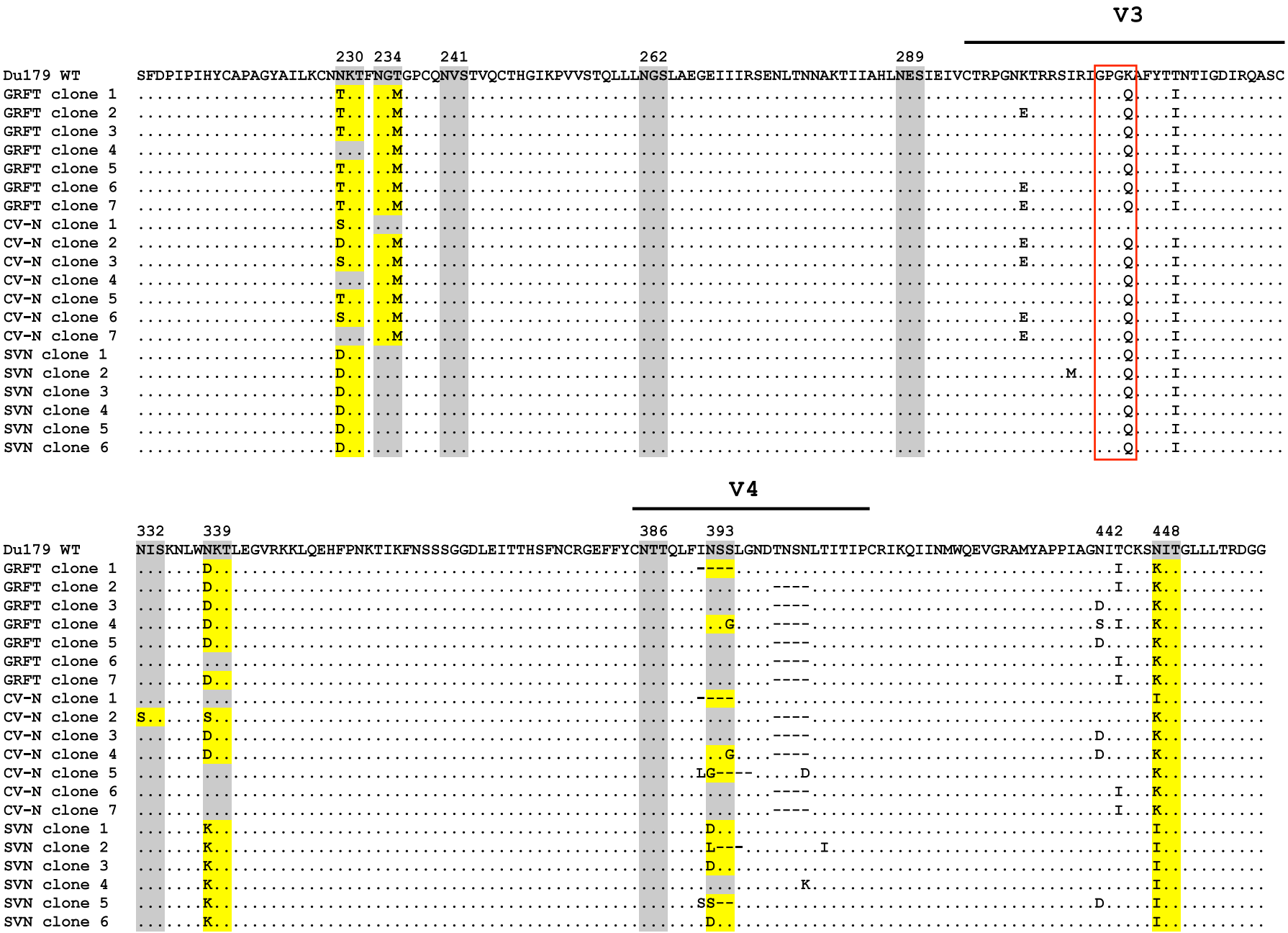
Figure 4.



(*) Glycans changed in GRFT resistant virus, (○) removed in CV-N resistant virus and (Δ) changed in SVN resistant virus. In red are glycans that were deleted in resistant viruses of all three lectins. In orange is the glycan that was added under lectin selective pressure.

Figure 5

Figure


V3
V4
Du179 WT
GRFT clone 1
GRFT clone 2
GRFT clone 3
GRFT clone 4
GRFT clone 5
GRFT clone 6
GRFT clone 7
CV-N clone 1
CV-N clone 2
CV-N clone 3
CV-N clone 4
CV-N clone 5
CV-N clone 6
CV-N clone 7
SVN clone 1
SVN clone 2
SVN clone 3
SVN clone 4
SVN clone 5
SVN clone 6

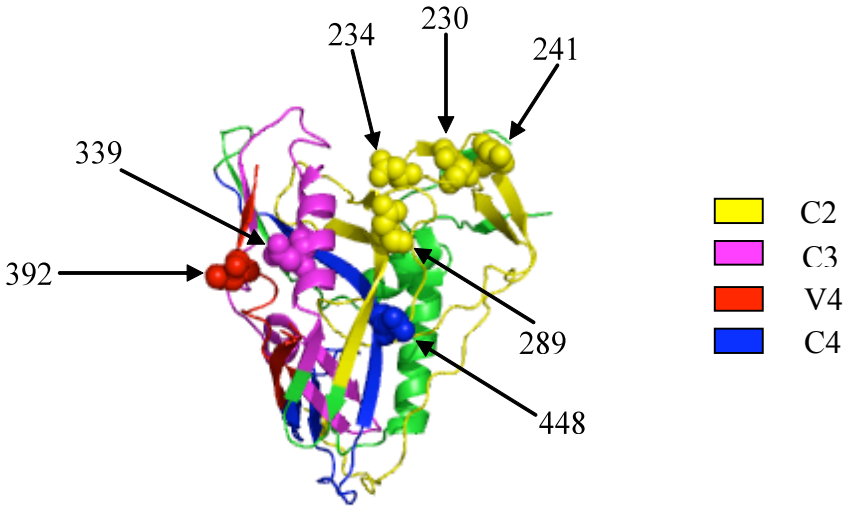


Figure 7.

CHAPTER SIX
SUMMARY AND CONCLUSIONS

Given the difficulties of finding an effective HIV-1 vaccine, microbicides offer a suitable alternative for infection prevention. The case for the use of microbicides against HIV-1 has been strengthened by the result of the CAPRISA 004 trial that showed for the first time that vaginally applied gels containing tenofovir can prevent up to 39% of infections (1). However, the potential complications that may arise by utilizing antiretrovirals as microbicides, such as the emergence of drug resistant viruses, requires that new molecules with different inhibitory mechanisms be investigated. These molecules include the lectins griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN) that bind mannose-rich glycans on gp120 (5, 32, 52). These compounds have been studied mainly against subtype B viruses although subtype C are the major cause of infections around the world. Therefore, this study involved an investigation into the ability of GRFT, CV-N and SVN to inhibit HIV-1 subtype C infection focusing on the array of mannose-rich glycans on the envelope glycoproteins. Since HIV-1 interaction with the DC-SIGN receptor on dendritic cells (DC) is very important for sexual transmission, I also examined GRFT, CV-N and SVN inhibition of HIV-1 binding to DC-SIGN. Lastly, given that HIV-1 has a propensity to evade antiviral compounds, the mechanism involved in its resistance to GRFT, CV-N and SVN was also studied.

In Chapter Two, I show that despite their significant differences in mannose-rich glycosylation patterns, HIV-1 subtype C sensitivity to GRFT, CV-N and SVN is similar to subtype B (48). This study suggested that these lectins binding sites on HIV-1 are flexible. The importance of the 234 and 295 glycans in GRFT, CV-N and SVN binding to HIV-1 was also shown in this study. Nevertheless, it will be important to further investigate the role of specific glycans in the binding of these compounds. Such a study

will help determine the susceptibility of different circulating subtype C viruses to GRFT, CV-N and SVN. Lastly, given the high diversity of HIV-1 viruses (8), it is advisable to test these compounds against other subtypes such as D and F. The fact that these lectins inhibited subtypes C, B and A with comparable potency suggests that they may also be effective against other subtypes.

Given that GRFT, CV-N and SVN are potential microbicides that will be applied in the female genital tract or in the rectum, for men who have sex with men, a study comparing the sensitivity of matched blood / genital or rectal canal viruses will be important. In Chapter Two, I showed that CAP63.A9J from blood and a matched envelope clone from CVL have similar sensitivity to GRFT, CV-N and SVN. However, further investigations with additional viruses from blood and genital or rectal canal needs to be conducted since compartmentalization has been shown to affect glycosylation patterns (22). Furthermore, semen contains cell associated and cell free viruses and the shedding of HIV-1 in this body fluid is a critical factor during the male to female transmission of the virus (9). Therefore, an important factor that should be considered in future research is that microbicides will mainly be used by HIV negative women and so the prime targets will be viruses that originate from semen of infected men. Thus, an investigation of the potency of candidate microbicides such as GRFT, CV-N and SVN against semen derived viruses would be important.

In Chapter Three, I show that GRFT binding to gp120 exposes the CD4bs, therefore, making it more accessible to antibodies that target this epitope. This work has implications for HIV-1 vaccine research, given the importance of the CD4bs in the virus

life cycle and the fact that this site is highly conserved across HIV-1 subtypes (12, 23, 47, 50). An important suggestion from this study is that GRFT, or compounds that mimic its action, could be used in combination with vaccines that elicit antibodies to the CD4bs. Similarly, there is a possibility of combining this lectin with CD4bs antibodies during passive immunization to prevent HIV-1 infection. In addition, GRFT and other mannose-binding lectins have shown no toxicity to mammalian cells and Balzarini suggested that they or their smaller derivates could be used *in vivo* against HIV-1 (3). Thus, HIV-1 infected individuals, many of whom have antibodies to the CD4bs (18), may be treated with GRFT to increase the virus vulnerability to these endogenous antibodies. This idea is supported by the fact that GRFT synergized with BB10 (Table 4 of Chapter Three), an HIV-positive plasma that contains CD4bs antibodies, to neutralize HIV-1.

The inability of CV-N, which like GRFT has a symmetrical arrangement of carbohydrate-binding sites (52), to enhance HIV-1 binding to the CD4bs suggests that GRFT's ability to expose this epitope has nothing to do with this geometric arrangement. Furthermore, GRFT belongs to the family of ß-prism-I lectins made of jacalin-related proteins (39). This family includes lectins such as jacalin (2), artocarpin (21) and heltuba (6). Thus, although GRFT shares less than 30% sequence identity with these other ß-prism-I family members (51), it will be interesting to investigate whether they too share this ability to expose the CD4bs. Such a study will help in our understanding of GRFT's ability to expose this conserved epitope.

GRFT and b12 enhanced each others neutralization potency against HIV-1. A similar observation was made between GRFT and PRO140. These synergistic interactions have important implications for the use of this lectin as an HIV-1

microbicide since they suggest the possibility of using combinations of entry inhibitors in a single microbicide formulation. They also suggest that this lectin should be tested with other CD4bs antibodies such as VRC01, VRC02 and VRC03 as well as with other compounds that target HIV-1 co-receptors such as AMD3100 and maraviroc to determine whether there is synergism (14, 15, 40, 49). In brief, given the observations made in Chapter Three, it could be concluded that GRFT through its ability to bind glycans has a unique ability to synergize with compounds that target different sites on the virus or on susceptible cells and this warrants further investigations.

Lectin receptors play the important role of mediating HIV-1 transfer to cells that are susceptible to infection at the sites of mucosal transmission (13, 41) and help disseminate the virus to the lymph nodes (35). The DC-SIGN receptor expressed on dendritic cells is one of the most important lectin receptors used by HIV-1 (36). Chapter Four shows that GRFT, CV-N and SVN are able to inhibit HIV-1 subtype B and C binding to DC-SIGN as well as the DC-SIGN-mediated transfer to target cells. The importance of this work stems from the fact that these three compounds are candidate microbicides and their primary site of application is the female genital tract that is rich with cells expressing the DC-SIGN receptor. Compared to the inhibition of transfer, GRFT, CV-N and SVN inhibition of HIV-1 binding to the DC-SIGN receptor was moderate. Thus, we postulate that the strong inhibition of HIV-1 transfer by these lectins is due to their potent inhibitory activities against the virus infection of cells, shown in Chapter Two. However, the relationship between lectin neutralization of free viral particles and their inhibition of DC-SIGN-mediated transfer is not a straight forward one

i.e. the ability of GRFT, CV-N and SVN to neutralize a given HIV-1 particle does not imply that they should also be able to inhibit DC-SIGN-mediated transfer. A plausible example is provided by CAP63.A9J and CAAN5342.A2 and their 234N mutants. In Chapter Two, the addition of the 234 glycosylation site markedly increased CAP63.A9J and CAAN5342.A2 neutralization sensitivity to GRFT, CV-N and SVN while in Chapter Four this mutation completely abolished lectin inhibition of the DC-SIGN-mediated transfer of these viruses. In Chapter Four, I also show that, at least in some viruses, the addition of the 234 glycosylation site increased HIV-1 interaction with the DC-SIGN receptor. This may explain why the presence of 234N affected lectin inhibition of HIV-1 binding to DC-SIGN and transfer to target cells. Since the 234 glycan is conserved in ~ 80% of subtype C viruses (48), its role in HIV-1 interaction with the DC-SIGN receptor needs further investigation.

Like the DC-SIGN receptor, the mannose and the dendritic cell immune receptor (DCIR) are lectin receptors and have been shown to mediate HIV-1 infection of cells (10, 24). More precisely, the mannose receptor was reported by Liu and colleagues to mediate HIV-1 infection *in cis* of astrocytes while the DCIR was shown by Lambert *et al.* to promote the infection *in trans* of $CD4^+$ T cells (24, 29). Since, cells that express these receptors are also found in the cervico-vaginal tract (9, 25, 41), a study of the ability of GRFT, CV-N and SVN to inhibit the mannose receptor and the DCIR binding and transfer of HIV-1 to susceptible cells is needed. If these compounds are found to inhibit these interactions it would strengthen their case for further development as microbicides.

Previously, HIV-1 subtype B was shown to develop resistance to CV-N and other mannose binding lectins *in vitro* and the main mechanism of this resistance was the deletion of mannose-rich glycans (4, 20). Similarly, in Chapter Five, I show that subtype C viruses can become resistance to GRFT, CV-N and SVN via deletions of mannose-rich glycans. However, the fact that only Du179, out of the four viruses tested, tolerated concentrations of these lectins that were above five times their IC_{50} suggested that the generation of subtype C viruses that are resistant to these compounds may be difficult. This is supported by the observation that GRFT, CV-N and SVN resistant Du179 could still be inhibited with nanomolar concentrations of the lectins. The fact that glycans on HIV-1 envelope play important functional and structural roles (17, 26, 28-31, 44, 45) may explain why they cannot be readily deleted. This ultimately means that GRFT, CV-N and SVN can be effective as HIV-1 subtype C microbicides since it may be difficult for the virus to escape their inhibitory activity.

An important study that can be added to the one conducted in Chapter Five is the investigation of whether the deletion of mannose-rich glycans to escape GRFT, CV-N and SVN affects HIV-1 interaction with the DC-SIGN receptor. Given the promiscuity of this receptor in interacting with these glycans (19, 27, 31), one could speculate that loosing one or two mannose-rich glycans on the viral envelope may not affect DC-SIGN binding to HIV-1. However, the deletion of four or five glycans, as was observed with Du179 and Du151, has the potential to affect this binding. This study can also be performed with other lectin receptors such as the mannose receptor and DCIR.

Inevitably, there will be the question of what makes GRFT, CV-N and SVN better HIV-1 microbicide candidates than compounds that are already being researched for the same purpose; in particular tenofovir, the first microbicide candidate to show efficacy against HIV-1 in a human trial. As mentioned at the beginning of this Chapter, the main advantage GRFT, CV-N and SVN have over microbicide candidates such as tenofovir is that they are not used to treat HIV-1 infection and this removes the possibility that their topical use in infected individuals will generate drug resistant strains. Another advantage that lectins have over tenofovir is that they could also provide protection to a man engaging in sexual intercourse with an infected woman since the presence of these compounds in the vaginal tract could prevent infection of the male partner. Compared to other envelope inhibitors, especially those already in clinical trials (11, 37, 38), GRFT, CV-N and SVN are more potent with IC_{50} in the low nanomolar range across HIV-1 subtypes (3, 5, 7, 34). CV-N was shown to protect macaques against SHIV89.6P while GRFT inhibited HIV-1 infection of human cervical explants (34, 42, 43). However, in humans, these lectins efficacy is likely to be affected by adherence. As shown in the CAPRISA 004 trial, poor adherence can lower the efficacy of microbicides against HIV-1 infection (1). A possible solution to this is the use of vaginal rings containing the lectin, since once incorporated into the cervico-vaginal tract the ring will continuously release the microbicide over an extended period of time without the user's intervention (33). Lastly, since GRFT, CV-N and SVN binding sites overlap on HIV-1 envelope, their combination in a single microbicide formulation is likely to result in competition for binding to the virus, thus, reducing their efficacy. The ideal way of using these lectins in

combination is, therefore, by using them with compounds that do not share the same binding sites on the HIV-1 envelope.

The developing world, especially Sub-Saharan Africa, is the center of the global HIV-1 epidemic (http://www.unaids.org). Women in Sub-Saharan Africa are the most affected by the virus and as already mentioned in Chapter One, this is due to their economic dependence on men, domestic violence, high incidence of rape and their inability to negotiate safe sex practices with their male partners (16). Thus, the availability of a female controlled microbicides is likely to result in a marked decrease in the rate HIV-1 transmission among women in this region and will lead to a significant decrease in the global epidemic. Note that a recent study found that an 80% use of the tenofovir gel could avert 2.33 million new infections and save 1.3 million lives in the next 20 years (46). Even a 25% adherence to this gel has the potential to save 0.29 million lives, this study also found. Unlike in Sub-Saharan, in the developed world the sexual transmission of HIV-1 is mostly between men (http://www.unaids.org). However, since microbicides are also designed for application in the rectum they are likely to decrease the rate of infections in this part of the world as well.

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CHAPTER SEVEN
APPENDICE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Kabamba Alexandre

CLEARANCE CERTIFICATE	M090601
PROJECT	Sensitivity of HIV-1 Subtype C Viruses to Griffithsin, Cyanovirin-N and Scytovirin: Potential HIV-1 Microbicied
INVESTIGATORS	Mr Kabamba Alexandre.
DEPARTMENT	AIDS Unit
DATE CONSIDERED	09.06.26
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 09.06.26 CHAIRPERSON .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof L Morris

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...
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