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Immune recognition and early virus-cell interactions: Glycobiological aspects on HIV-1 gp120

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II. Hansen J-ES, Jansson B, Gram GJ, Clausen H, Nielsen JO and Olofsson S. Sensitivity of HIV-1 to neutralization by antibodies against O-linked carbohydrate epitopes despite deletion of O-glycosylation signals in the V3 loop. *Arch Virol* (1996), 141(2): 291-300

III. Schønning K, Joost M, Gram GJ, Machuca R, Nielsen C, Nielsen JO, Hansen J-ES. Chemokine receptor polymorphism and autologous neutralizing antibody response in long-term HIV-1 infection. *J Acquir Immune Defic Syndr Hum Retrovirol* (1998), 18(3):195-202.

IV. Gram GJ, Bolmstedt A, Schønning K, Biller M, Hansen J-ES, Olofsson S. Orientation-specific anti-gp120 antibody reactivity in sera from HIV infected individuals determined by a new N-glycanase protection assay. *Submitted for publication.*

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Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) are the etiological agents of AIDS in humans. The viral envelope glycoproteins gp120 and gp41 mediate the early virus-cell interaction of HIV with the target cells. The envelope glycoprotein is organised into trimeric complexes on the virion surface and the heavily glycosylated gp120 contains both N- and O-linked glycans. The glycosylation is necessary for generation of correct conformation but it is also an important component to decrease the susceptibility of the virus to immune responses of the host. These and other factors are likely to influence the progression of the natural infection in HIV infected individuals. Several aspects of N-linked glycans and O-linked glycans of gp120 were investigated.

We characterised two N-glycan in the V1-loop and V3-loop of gp120 and found both N-glycans to be of the complex type. Further, these N-glycans was found to influence the sensitivity of the virus to neutralising antibodies. We deleted all potential O-glycan sites in the V3-loop of gp120, to pin-point the unknown location of broadly O-linked neutralisation epitopes of gp120. However, the location of these neutralisation epitopes must be outside of the V3-loop. Using a novel deglycosylation assay, we identified N-glycans located in the vicinity of the CD4 binding site and epitopes on gp120 recognised by antibodies from HIV infected individuals. However, these recognition sites were found to be only partly overlapping. These antibody responses are likely to influence the progression of infection in HIV infected individuals, but the presence of autologous neutralising antibodies was not a requirement in persons displaying a slow progressing HIV infection. However, a non-syncytium viral phenotype in conjunction with a mutant allele carrying a 32 base pair deletion in the CCR5 co-receptor gene, was found to be predictive for slow progressing infection.

Keywords: Human immunodeficiency virus type 1 (HIV-1), glycoprotein, gp120, glycosylation.

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List of publications

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Table and figure numbers

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Table 2: Page 10, bottom.

Figure 1: Page 10.

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Abbreviations

Ab	Antibody
AIDS	Acquired immune deficiency syndrome
CCR5	β-chemokine receptor - coreceptor for HIV
CD4	Cluster of differentiation antigen 4; cell membrane molecule; receptor for HIV
CD4bs	CD4 binding site - the region of gp120 interacting with CD4
CXCR4	β-chemokine receptor - coreceptor for HIV
D7324	Antibody against the C5 region of gp120
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
gp	Glycoprotein
HIV	Human immunodeficiency virus
HIV Ig	Immunoglobulins from HIV infected individuals
HTLV	Human T-cell leukemia virus.
HAART	Highly active antiretroviral therapy
Ig	Immunoglobulins
2G12	Human anti-gp120 MAb directed against a discontinous epitope of $gp120^{195}$
LTNP	Long-term nonprogressors
MAb	Monoclonal antibody
NEA9205	Antibody against the tip of the V3 loop of gp120
NSI	Non-syncytium inducing
P4/D10	Antibody against the V3 loop of $gp120^{1}$
PCR	Polymerase chain reaction
RIPA	Radio-immuno-precipitation-assay
RNA	Ribonucleic acid
RPI	Rapid progressing infection
sCD4	Recombinant soluble form of the CD4 molecule
SI	Syncytium inducing
SIV	Simian immunodeficiency virus
SPI	Slow progressing infection
Th epitope	T-helper cell epitope
Tn	O-linked carbohydrate neo-antigen, GalNac-Ser/Thr

Brief introduction & Aims

The human immunodeficiency virus (HIV), is the etiological agent of acquired immunodeficiency syndrome (AIDS). The virus expresses on the surface the viral glycoprotein, gp120 that is important in the early virus-cell interactions for infection of target cells. The extensive glycosylated gp120 molecule is modified by both N-linked and O-linked glycans that have structural as well as functional roles. Some of the O-linked glycans can serve as neutralising structures, but their location on gp120 still remains unknown. On the other hand, the more bulky N-linked glycans can modulate the accessibility and recognition of antibodies to gp120. Available data suggests that N-glycosylation significantly contributes to the viral escape from the immune responses in HIV infected individuals. However, other factors are likely to influence the progression of the HIV infection.

The aims of this dissertation were:

1. To characterise the role of a defined N-linked glycan strategically located in the triple-nested S-S bridged V1 region of gp120, and to structurally classify this particular glycan.

2. To determine whether the broadly reactive carbohydrate gp120 neutralisation targets, consisting of short O-glycan neoantigens, are localised in the V3 region.

3. To establish a method for determining N-linked glycan accessibility after antibody binding to gp120 and appreciate the density of glycans in the vicinity of neutralising epitopes and the CD4-binding domain.

4. To apply this assay and a crossed neutralisation assay, combining consecutive pairs of sera and HIV-1 isolates from patients for analysis of disease progression in HIV-infected individuals.

General background

Retroviruses

Retroviruses are RNA viruses, characterised by their ability to create a DNA copy of their viral RNA genome, which later becomes integrated in the host cell chromosomes. Retroviruses have probably existed for millions of years, but it was not until the late 1960's that retroviruses were identified as a special family of RNA viruses through discovery of the reverse transcriptase in Murine leukemia virus and Rous sarcoma virus[10;193]. David Baltimore and Howard Temin received the Nobel for this discovery in 1975. In 1980, the first human pathogenic retrovirus, Human T-cell leukemia virus, was identified[159;160] and only a few years later the human immunodeficiency virus type 1 (HIV-1) was identified by French and American scientists[11;60;101;197].

Retroviruses have been identified for all vertebrate species and have been categorised based on their morphology in electron microscopy[16;63]:
A-type: 'Intracisternal particles'. Non-enveloped, immature particles only seen inside cells, believed to result from endogenous retrovirus-like genetic elements.
B-type: Enveloped, extracellular virus particles with a condensed, acentric core and prominent envelope spikes, e.g. MMTV.
C-type: As B-type, but with a central core and barely visible spikes - e.g. most mammalian and avian retroviruses (MLV, ALV, HTLV, HIV).
D-type: Usually the largest virus particle (to 120 nm) and spikes less prominent, e.g. MPMV.
An improved understanding of the genetic relationships between retroviruses resulted in a classification into several orders, suborders, families and genera ([79] and ICTV web: www.ncbi.nml.nih.gov/ICTV/) (Table 1).

Class Order Suborder Family Genera	Type Species	
Retroelementopsida		
Retrovirales		
Orthoretroviridae		
Retroviridae		
Alpharetrovirus	*Avian leukosis virus*	
Betaretrovirus	*Mouse mammary tumor virus*	
Gammaretrovirus	*Murine leukemia virus*	
Deltaretrovirus	*Bovine leukemia virus*	
Epsiloretrovirus	*Walley dermal sarcoma virus*	
Lentivirus	*Human immunodeficiency virus 1*	Bovine immunodeficiency virus (BIV) Equine infectious anemia virus (EIAV) Feline immunodeficiency virus (FIV) Caprine arthritis encephalitis virus (CAEV) Visna/maedi virus Human immunodeficiency virus 1 (HIV-1) Human immunodeficiency virus 2 (HIV-2) Simian immunodeficiency virus (SIV)
Spumavirus	*Human spumavirus*	

Table** Fejl! Ukendt argument for parameter.. **Classification and genera of vertebrate retroviruses. Members of the lentivirus genus is shown. Modified from Hull, 1999 [79].

Retroviruses share a common basic genetic composition of gag (structural), pol (enzymatic) and env (envelope) genes flanked by two long terminal repeats (LTR) (Fig. 1).

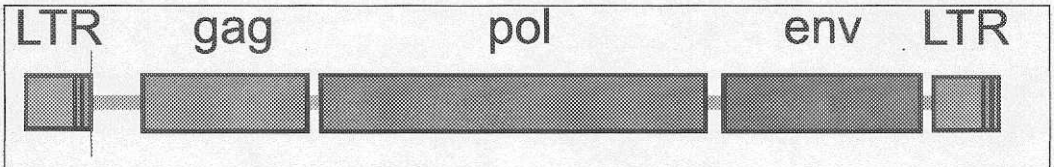

Figure Fejl! Ukendt argument for parameter.. **Basic composition of the retroviral genome. The gag, pol, and enc genes in the provirus is flanked by two long terminal repeats, LTR. Modified from Wain-Hobson, S. et al., 1985 [197]**

The retroviral gene products have common functions and locations in the virus particle (Table 2 and Figure 2).

Gene	Name	Protein	Function
gag	**MA**	matrix	matrix protein; lines envelope
gag	**CA**	capsid	capsid protein; protects the core; most abundant protein in virus particle
gag	**NC**	nucleocapsid.	nucleocapsid protein; protects the genome; forms the core
pol	**PR**	Protease	Essential for gag protein cleavage during maturation of the viral particle
pol	**RT**	Reverse transcriptase	Reverse transcribes the RNA genome; has RNAseH activity
pol	**IN**	Integrase	Needed for integration of the provirus
env	**SU**	surface glycoprotein	The outer envelope glycoprotein; major virus antigen
env	**TM**	transmembrane protein	The inner component of the mature envelope glycoprotein

Table Fejl! Ukendt argument for parameter.. **Names and functions of retroviral gene products. Modified from Freed, 1998 [57].**

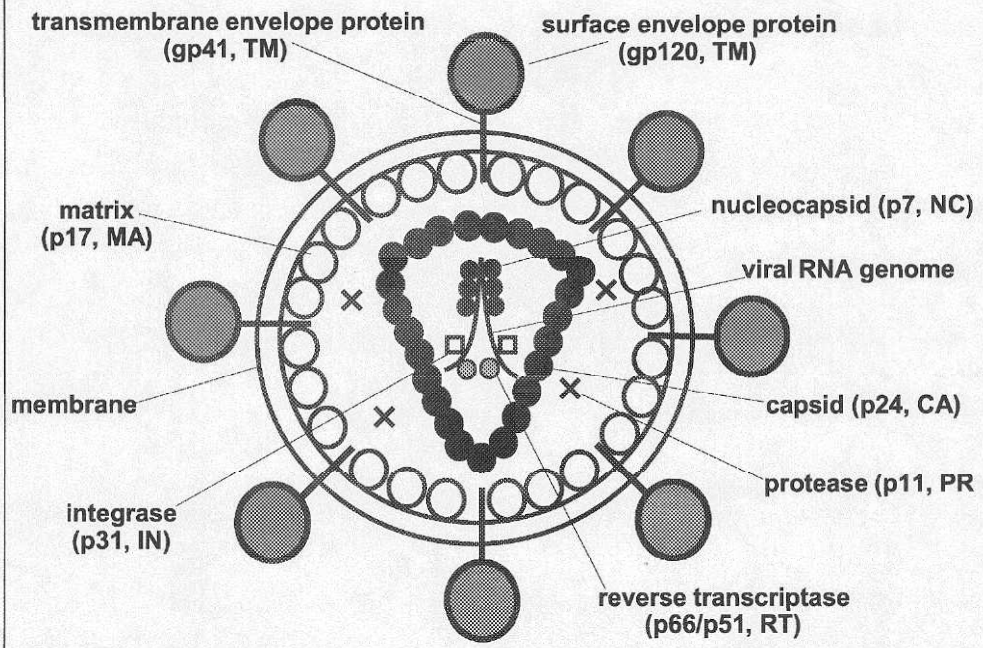

Figure Fejl! Ukendt argument for parameter.. **Composition of the retroviral particle. The viral proteins are highly organised in the viral particle. The HIV-1 particle is shown. Modified from Freed, 1998** [57].

HIV-1 and HIV-2

A few years after the identification of HIV a homologous, but distinct, type of HIV was identified in Africa[34;92]. This HIV virus was termed HIV type 2 (HIV-2). HIV-1 appear to be the result of multiple zoonotic transmissions of humans from SIV infected non-human primate chimpanzees, whereas HIV-2 appear to be originating from non-human sooty mangabeys. The zoonotic event believes to have occurred around 1931[61;62;89;213]. This estimate coincides with the French colonisation of equatorial Africa, that may have increased the risk factors for zoonotic transmission of HIV-1[32].

HIV-1 and HIV-2 are classified as slowly pathogenic viruses, lentiviruses. This genus includes other mammalian retroviruses, such as EIAV (Equines, horses), CAEV (caprines, goats), Visna/maedi virus (ovines, sheeps), BIV (bovines, cattle), FIV (felines; cats) SIV (simian, monkeys)[63;141] (Table 1).

AIDS epidemic

The acquired immunodeficiency syndrome (AIDS) was first described in the beginning of 1980's where immunodeficiency-associated conditions, such as *Pneumocystis carinii* pneumonia, Kaposis sarcoma, *candidia albicans*, and chronic herpes simplex lesions, were found in male homosexuals and intravenous drug abusers[64;80;117;184]. In the following years, the number of AIDS cases increased rapidly and now it was also found among haemophiliacs, blood transfusions recipients and sex partners of AIDS patients too. In 1983/1984 the HIV virus was isolated from AIDS patients, and the etiological agent of AIDS was identified.

By the end of 1999, the total number of HIV-infected individuals was estimated to be about 34.2 million. During the year 1999, more than 5.4 million had been infected with HIV and approximately 2.8 million had died of AIDS. Accumulated, 18.8 million people have died of HIV/AIDS in the 20th century and over 53 million people have been infected with HIV (www.unaids.org).

Clinical course and parameters of HIV-1 infection

The clinical course of HIV infection begins with exposure to HIV. Humans are exposed to and infected with HIV via different routes (in alphabetical order): artificial insemination, birth, blood contaminated syringes, blood transfusions (blood derivatives in medicine), breast milk, intimate sexual contact, *in utero*, transplantation. Intimate sexual contact is the primary route of HIV infection.
The natural HIV infection is chronic and will eventually lead to death of the infected individual. The clinical course of HIV infection is divided into several phases; Infection, seroconversion, asymptomatic, symptomatic and AIDS (Figure 3).

The duration of these phases may vary considerably from patient to patient, but despite intense investigation the underlying factors for this remain unclear[29;150]. Fortunately, in the last couple of years, new chemotherapeutic approaches (combinational therapy) have extended the asymptomatic phase for many HIV

infected individuals[149]. However, currently there is no cure to eradicate HIV in the infected individual. Once the virus is transmitted, the virus will replicate in the CD4 positive cells in the host, producing vast amounts of circulating virus particles (Figure 3 - Plasma RNA).

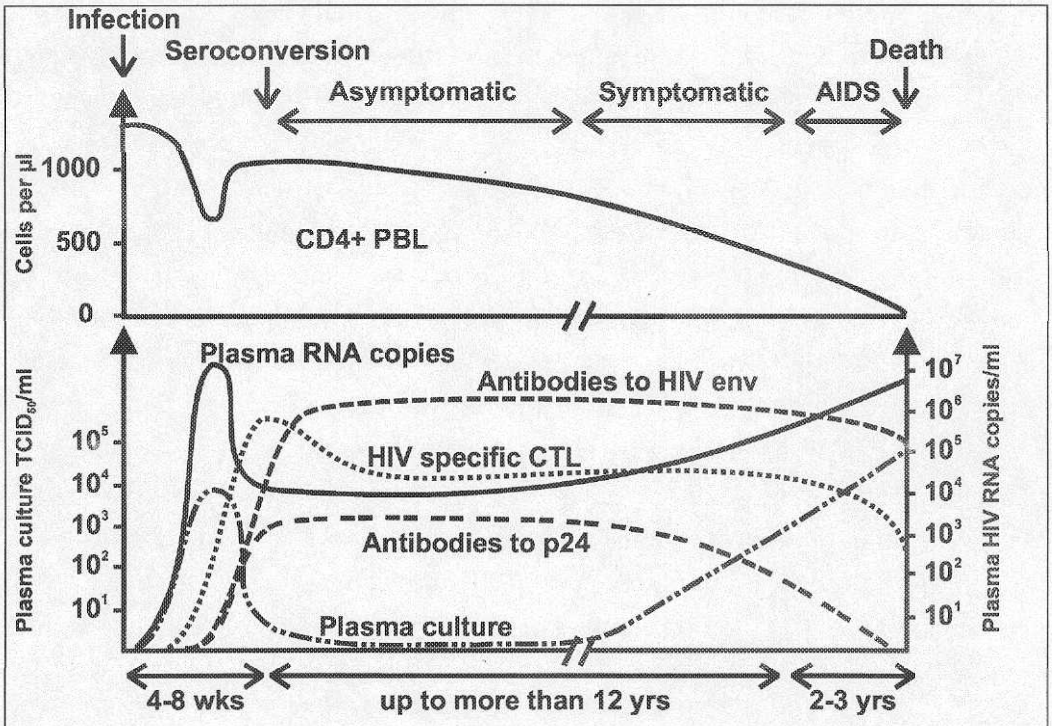

Figure Fejl! Ukendt argument for parameter.. **Immunological and clinical variables during the course of HIV infection. Modified from Profy et al., 1990** [164]

Shortly after infection, specific immune responses against HIV emerge[23;91;128;130] (Figure 3 - HIV specific CTL and Antibodies to p24 and HIV env). The specific reactivities of CTL and antibodies somewhat controls the viral replication for a period of years[130], but the CTL and neutralising antibodies are believed to be responsible for the constant generation of escape mutants in vivo[6].

In the later course of the asymptomatic phase, the concentration of circulating CD4+ T-lymphocytes cells may gradually decline to a critically low level, where the immune response no longer can control the replication of the virus or ordinary non-pathogenic infections. The progression of this immunodeficiency phase eventually leads to uncontrollable immunodeficiency disease and ultimately to death of the HIV infected individual.

Treatment of HIV

The first anti-retroviral drugs used for treatment of HIV infected individuals were inhibitors of the HIV reverse transcriptase (RT) enzyme. These drugs were nucleoside analogues that were aimed at inhibiting the reverse transcription step in the life cycle of HIV and prevent the subsequent integration of the viral genome. Treatment with these nucleoside analogue RT-inhibitors generated escape-mutants quickly and was found to have an insignificant influence on the clinical course of the disease. However, administration of a nucleoside analogue RT-inhibitor for a brief period around the time of delivery can significantly reduce the perinatal HIV transmission from mothers to their new-born babies from around 25% to 8%[36]. Pregnant HIV infected individuals are now like other HIV infected individuals offered an improved anti-viral drug regimen, which reduce the HIV transmission rate at delivery to practically zero[121]. Treatment of HIV and AIDS has undergone considerable change in the last five years. Protease inhibitors and non–nucleoside-analogue RT-inhibitors profoundly suppress viral replication and even increase the CD4 cell counts when used in combination with more ordinary anti-viral drug[149]. This combination drug regimen is called highly active anti-retroviral therapy (HAART). Despite the great success of the HAART treatment, adverse effects and resistant viruses to the drugs have begun to appear in the patients on HAART[115]. These problems with HAART underscore the importance of developing a long-term therapy against HIV and AIDS.

Infectious cycle of HIV-1

HIV-1 infects through binding of the virus particles to the CD4+ target cell by interactions of the envelope gp120 with the CD4 molecule and the CCR5 or CXCR4 molecules on the membrane of the target cell (primarily T-lymphocytes)[39;120] (Figure 4). Fusion of the two lipid bilayers is mediated by conformational changes in the HIV-1 envelope gp120/gp41 complex[58;199]. After fusion, the HIV core is released into cytoplasm of the target cell and the RNA genome is fully reversely transcribed to double stranded DNA. This DNA copy

of the viral genome is transported to the nucleus, where the viral integrase mediates integration of the provirus into the host chromosome[196;211]. In contrast to other retroviruses, e.g. MLV, HIV has the ability to infect non-dividing cells[103;104]. The integrated provirus is transcribed into full-length messenger RNA (mRNA) transcripts that are subsequently processed and spliced into smaller mRNA molecules. These spliced transcripts are translated into the viral regulatory proteins, Tat and Rev[38].

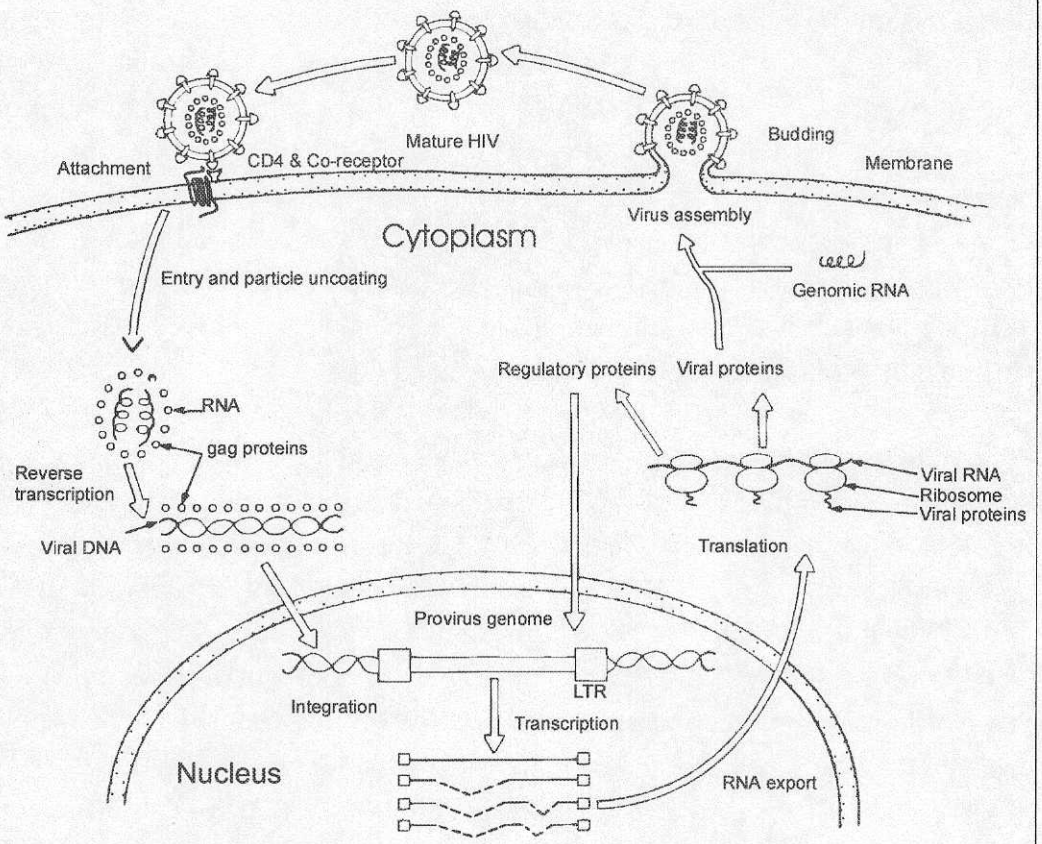

Figure Fejl! Ukendt argument for parameter.. **Replication cycle of HIV-1. See text for details. Modified from Cann & Karn, 1989 [27].**

These two proteins have a significant influence on the levels of transcripts and RNA processing and assist in controlling the concerted appearance of viral elements needed to produce viral particles[37;38]. The viral elements assemble on the inner side of the cell membrane before a premature viral particle buds off. After release of the virus particle from the cell, proteolysis in the particle is associated with condensation of the core which is necessary for viral infectivity,

but not for particle assembly or release[83;155]. The HIV particle is now fully matured and infectious, and a new infectious cycle can begin (Figure 4).

Receptors and co-receptors of HIV

The CD4 molecule was found to function as receptor for HIV infection[39], [82;120]and it was the first retroviral receptor to be identified. The infection process of HIV is independent of pH, in contrast to e.g. the infection processes by MLV and Influenza[163;190]. Isolates of HIV have been classified according to their inversely correlated tropism for either T-lymphocytes (T-tropic, rapid/high) or macrophages (M-tropic, slow/low). This classification was based on the observation that isolates capable of infecting T-cell lines had reduced capacity to replicate in primary macrophages[55]. In addition, HIV isolates were classified as either syncytium inducing (SI) or non-syncytium inducing, according to their ability to induce syncytium formation (multinucleated cells derived from fusion of cells) in T-cells[182].

Based on several observations it was questioned whether the CD4 molecule was neccesary or sufficient for HIV infection e.g. 1) CD4 negative cells, e.g. neuronal cells, could be infected when high doses of viruses were used[105] 2) soluble factors from e.g. CD8+ cells could inhibit the HIV infection of CD4+ cells[198] 3) transfer of human CD4 to mouse cells was not sufficient to confer HIV infection and replication[25;112]. Despite intense efforts to discover a missing co-receptor it was the findings of three members of the chemokine family, namely RANTES, MIP-1α and MIP-1β as potent inhibitors of infection of HIV-1, HIV-2 and SIV[35], that led to the identification of the CXCR4 cell membrane molecule as a co-receptor for HIV infection[53]. Based on these two findings the CCR5 molecule from the same G-protein coupled seven transmembrane (7TM) β-chemokine receptor family was identified as the co-receptor for most HIV-1 strains[41;44]. Additional β-chemokine receptors (CCR2b, CCR3, CCR8, CCR9, CX_3CR1, GPR1, STRL33/Bonzo, GPR15/BOB, APJ, ChemR23, BLTR, US28) have been identified to support HIV or SIV infection, but their biological relevance *in vivo* remains uncertain[119;170;212] (Review[15;109]). Recently, the co-receptor binding site has been mapped on the core-structure of gp120[96;169].

Based on the utilisation of co-receptors CXCR4 or CCR5 a new classification system for HIV-1 have been proposed by Berger et al., 1998[14]. Isolates that use CCR5, but not CXCR4, are termed R5 viruses and isolates that use CXCR4 and not CCR5 are termed X4 viruses. Isolates that use both co-receptors with comparable efficiency are called R5X4 viruses.

Early virus-cell interactions

The interaction between gp120 and CD4 promotes a series of conformational proteins with respect to the target cell membrane. The binding of CD4 to gp120 induces these changes in the conformation of the envelope glycoproteins. Furthermore, several has reported that a few neutralising antibodies, sCD4 and mutations throughout gp120 can induce a similar conformational change described as an "open" or "activated" conformation of gp120. These conformational changes allow high-affinity interaction with co-receptor, CCR5 or CXCR4 and a few antibodies directed against this co-receptor binding-site[191;194]. The CD4-induced translocation of the V1/V2 loops results in the exposure of conserved, discontinuous structures on the HIV-1 gp120 glycoprotein recognised by the 17b and 48d monoclonal antibodies. The 17b and 48d epitopes are proximal to a gp120 region implicated in chemokine receptor binding. A plausible model is that CD4 binding to gp120 repositions the V1/V2 stem, allowing the formation of an antiparallel β-sheet that contributes to the chemokine receptor binding. The V3-loop is necessary for infectivity and small substitutions can abolish infection completely[59]. A recent study demonstrated that substitution of V3-loop sequences, with that of the sequence from a β-chemokine ligand for the HIV co-receptor, preserved infectivity of the mutant virus particles[210]. This indicates that the V3-loop is directly interacting with the co-receptor.

Envelope glycoprotein, gp120

The HIV-1 *env* gene encodes the two envelope glycoproteins, gp120 (surface, SU) and the gp41 (transmembrane, TM), that are associated non-covalently in heterotrimeric complexes in the cell membrane and subsequently in the viral envelope after the viral particle buds off [63;97;199;200]. gp120 are responsible for the

binding and adherence to the target cell, whereas the gp41 are responsible for the subsequent penetration and fusion of the membranes of the cell and the virus[58]. Half the molecular weight of gp120 is derived from the ~500 amino acid residues, whereas the other half is derived from more than the 25 fairly conserved N-linked oligosaccharides located throughout the protein[42;98;100]. This makes gp120 the fourth most glycosylated protein known[139]. gp120 contains a relatively small number (<10) of O-linked glycans[17;68;72;158]. The structure of some of these O-linked glycans are truncated, but still O-glycosylation of gp120 have been shown to significantly contribute to the overall molecular mass[17;72]. The highest diversity in the HIV genome is found in the envelope gene[42]. Variation in gp120 is not, however, dispersed throughout the entire envelope gene, but localised to several regions of lower variation (constant regions) or higher variation (variable regions)[42;127;188].

Biosynthesis of gp120

The viral envelope glycoproteins gp41 and gp120 are generated by proteolytic cleavage of a single precursor protein, gp160. During synthesis, the protein is N-glycosylated in the endoplasmic reticulum, where folding and oligomerisation into trimers occurs[49;97]. At this stage, the oligomeric precursor protein gp160 has already acquired the ability to bind CD4[50]. In the Golgi apparatus three important events takes place, 1) the gp160 is endoproteolytically cleaved into gp41 and gp120 2) the gp120 molecules are modified by addition of O-linked glycans and 3) a trimming and addition of carbohydrates on both N- and O-linked glycans[17;68;90;118;157;189;201]. After trimming of glycans on the envelope products, the envelope complex is transported to the cell membrane for subsequent association with viral particles budding off the cell membrane.

Structure and function of gp120

The envelope glycoprotein gp120 was identified as a molecule that induced antibodies in AIDS patients[3]. Sequence homology analysis of many HIV-1 isolates identified variable regions (V1, V2, V3, V4, V5) regions and constant regions (C1, C2, C3, C4, C5, C6)[127;188]. However, all regions are not equally important for viral functions. The V4 and V5-loop seems to be indispensable,

whereas the V1-loop, V1/V2-loop and the V3-loop is dispensable for replication, although associated with a dramatically reduction in replication[28;187;205].

The extensive glycosylation of gp120 has for many years made crystalisation of gp120 impossible[138]. Finally, a structure of gp120 was deduced from a crystal of gp120[96;203]. The crystal was made with a deglycosylated core fragment of gp120 lacking the V1, V2 V3 loop. The core gp120 was complexed with a CD4 fragment and a Fab antibody specific for the CCR5 binding site[96;203]. The gp120 core fragment has preserved the ability to bind CD4 and CCR5 and displays an overall conserved antigenic profile[19;140;203]. The structure of monomeric gp120 appears to be composed of an inner-domain and an outer-domain, connected by a four-stranded bridging β-sheet[96;203;204] (Figure 5).

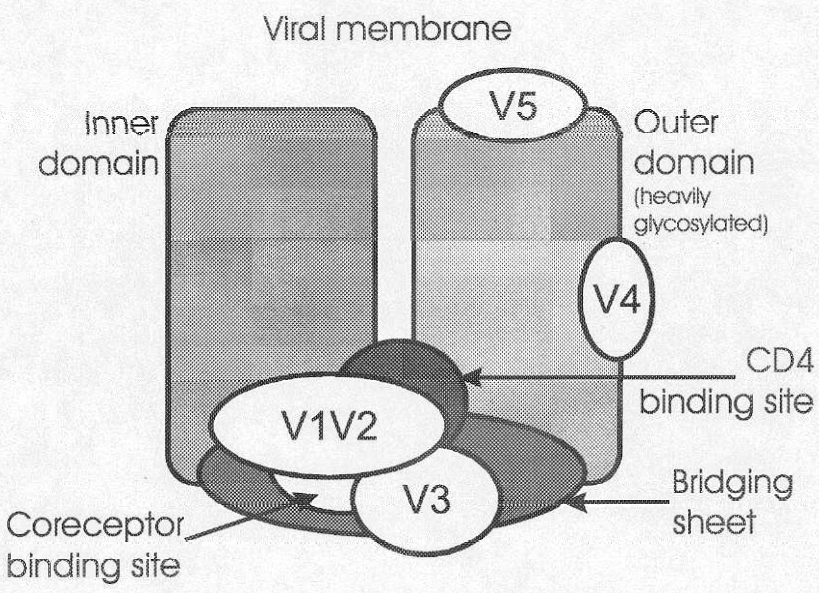

Figure Fejl! Ukendt argument for parameter.. **Structural model of gp120. The model is based on the crystal structure of the gp120 core complexed with a CD4 fragment and a Fab fragment specific for the CCR5 binding site [96]. Modified from Burton & Parren, 2000 [26].**

The inner-domain seems to be engaged in association with gp41 and the C- and N-terminus of gp120, whereas the outer domain is mostly exposed on the surface of the trimer. The interface between the outer and inner domains of gp120 are devoid of glycosylation and forms the binding sites for CD4 and the

co-receptor[96;203] (Figure 5). Much of the contact between gp120 and CD4 seems to come from interactions with Cα-atoms of the amino acids of gp120. Since antibodies usually interact with the side chain component of amino acids, this allows gp120 to alter amino acids in the CD4 binding site without sacrificing the ability to bind CD4[202]. Previously, gp120 was thought to consist of two faces only[133;134]. Elucidation of the structure has revealed a previously unknown face - the silent face. This face is situated on the outer-domain of gp120 but protected by N-linked glycosylation towards recognition by antibodies[96;202].

A structural comparison of the neutralisation sensitive HXBc2 with the neutralisation resistant isolate YU2 have revealed a remarkably similarity of the core gp120 from these isolates[95]. This study suggested that neutralisation resistance is specified by the quaternary interactions of gp120 involving the V1/V2, V3 and V4 loops.

Biological functions of carbohydrates on gp120

The huge carbohydrate moiety of gp120 is an important component of the HIV-1 envelope glycoprotein in several respects. The glycan structures of gp120 are products of the host chromosome coding, specified by the consecutive actions of transferases and glycosidases present in the ER and Golgi. However, the glycan structures are affected by the composition of the microcompartment in the ER and Golgi and e.g. the production rate of the glycoprotein, which may be influenced by the viral infection. Still, all the N-glycosylation sites of gp120 have been found to be utilised[100;209](I, IV). Abnormal glycosylation patterns and aberrant glycan structures have been identified on HIV envelope products not normally found in humans[17;33;68;72;99;158].

The carbohydrate moiety is important for gp120 to attain correct protein conformation and CD4 binding capacity[19;204]. However, once the correct conformation is achieved the carbohydrates of gp120 seem to be dispensable for stabilisation of correct conformation. Deglycosylated gp120 preserves the ability to bind CD4 and deglycosylated virus particles can infect cells, although at much reduced levels[54;96;140;151;203]. However, carbohydrates of gp120 can significantly influence important functions and the structure gp120[2;21;85;86;137].

An important role of the carbohydrate moiety seems to be immunoprotection of gp120[167]. Several [129;153]single N-linked glycans, and even also peripheral glycosylation, have been reported to influence the immune recognition of gp120 [9;21;30;69;177;178;148](I, II).

O-linked glycans of gp120

The exterior envelope glycoproteins from several retroviruses, including HIV-1, have been found to be modified by O-glycosylation[158]. The aberrant short chained O-glycosylation such as the Tn antigen which is abnormally expressed in number of cancers, have been found on the envelopes from HIV-1 and HIV-2[17;33;68;72;158;186]. These O-linked neoantigens serve as broadly neutralisation epitopes and are common on both primary and laboratory adapted strains. The N-linked neo-antigens A_1 and Le^Y have also been identified as neutralising epitopes on HIV[5;68;73]. Pre-existing antibody reactivities towards these neo-antigens has been suggested to be involved in rejection of the initial HIV infection[145].

In contrast to N-linked glycosylation, no exact consensus acceptor sequence has been identified for O-linked glycosylation, which may be explained by the multiple GalNAc transferases identified in the last decade[70;71]. Initial O-linked glycosylation takes place in the cis-Golgi compartment after N-glycosylation, folding and oligomerisation of gp120. Therefore, acceptor sites must be exposed on the surface of oligomer gp120 to be accessible to GalNAc-transferases. Initial O-glycosylation is therefore dependent on both local conformation and overall tertiary/quaternary structure of the glycoprotein. The role of O-linked glycans on gp120 has so far not been identified. However, O-linked glycans have been proposed to alter the recognition by the neutralising antibodies generated in a primate host[30;148].

N-linked glycans of gp120

Individually, every N-linked glycan of gp120 is dispensable for infectivity[98] and multiple N-glycan deletions can be accommodated with only an impairment of infectivity or even without any apparent loss of viral functions[43;69;76;77;108;166]. However, several functions are affected by single or multiple N-glycans; e.g.

binding to CD4 and the co-receptor[12;20;75;147;43;45-47;85-87;110;113](I). Recently, the conserved N-glycan in the V3-loop of a mutant strain of the NDK X4 isolate have been identified as a determining factor for CD4-dependence[46;47] (Hazan, U., personal communication). N-linked glycan have in many cases been shown to modulate the structure of peptides and proteins (Review[143]). Deletion of N-glycans of gp120 can modulate the conformation of gp120 without changing the overall structure [21;85](I, II). Removal of a single N-glycan in the C2-region was found to confer CD4 independence. The authors proposed that this was caused by a structural displacement of the whole V1/V2-region[85].

Analysis of the N-linked glycans of gp120 has identified the presence of both high-mannose, hybrid, complex type glycans[18;100;125;209]. More N-glycan subtypes than addition signals have been identified on gp120, which indicates a micro-heterogeneity in the structure of gp120[125]. Peripheral glycan structures have been shown hide epitopes of gp120[21] and the observed micro-heterogeneity may have both structural and antigenic implications. N-linked glycosylation is thought to be part of HIV's resistance mechanism in order to protect envelope proteins from attack by the host's immune system[13;167;187]. Insertion of N-glycosylation sites has in several cases been identified as an escape mechanism of gp120 to shield underlying or distant epitopes against neutralising antibodies[9;30;108;148;166;167;176-178]. Both O-linked and N-linked carbohydrate structures can be part of an antibody neutralisation epitope[68]. Antibodies to blood group antigens have been found to interfere with viral replication[5;68;73]. However, gp120 specific antibodies that recognise epitopes containing N-linked glycosylation is only rarely found in HIV infected individuals[195].

N-linked glycosylation of gp120 have also been reported to influence the selection of epitopes recognised by T-cells in mice[185]. The reason for this is unknown, but the N-glycans could have altered the processing of the molecule, and hereby the subsequent peptide presentation, as seen for other proteins[114]. A recent DNA immunisation study showed that both the glycosylation and the conformation of the antigen might dictate the selection of T-helper (Th) cell epitopes[192]. All Th-epitopes induced by DNA immunisation were found to be located on the exposed and accessible surface of gp120 when visualised on the crystal structure[192].

The glycosylation of gp120 has been shown to influence sensitivity to neutralising antibodies to the V3-loop and the CD4bs, but also to influence the binding to CD4 and the co-receptor[110;65;69;108;177;178](I). Deletion of the V1/V2-region of gp120 confer increased accessibility to neutralising antibodies, sCD4 and co-receptor[28;84;168;205]. In spite of the multiple functions of the V1/V2 loop it is quite remarkable that viral particles deleted for V1/V2-deleted (ΔV1/V2-gp120) still are infectious[28;205]. This could indicate that the most important function of the V1/V2-region may be to control the interaction with the host cell receptors and to modulate the immune recognition of gp120.

Immune recognition of HIV

The HIV infection induces specific immune responses in the host. After the initial HIV infection, the virus replicates to high levels before specific immune responses appear (see Figure 3). The appearance of HIV-specific CTL's coincides with a decline in viral load, whereas antibodies to env appears subsequently[23;94]. The CTL response is therefore believed to control the initial HIV infection which is supported by CD8-depletion studies[81;130;144;161;175]. The anti-env antibodies produced within the first year of infection are primarily directed against non-neutralising epitopes of gp120[93;128;130]. Autologous neutralising antibodies are found approximately one year after infection whereas heterologous neutralising antibodies are found one year later[128]. The reason for the slow maturation of the autologous and heterologous neutralising antibodies remain unclear. However, most of these matured neutralising antibodies seem to recognise conformational epitopes around the CD4 binding site or epitopes induced after CD4 binding[131].

The specific immune responses stimulate the generation of escape variants[4;6;7;102;126;153]. Escape mutants are generated by single or multiple amino acid substitutions, that often affects the glycosylation sites of gp120[9;51;122;126;177;187]. The persistence of HIV infection is believed to be caused by a constant generation of variants escaping the specific immune responses. Evolvement of virus isolates lacking some N-glycosylation signals, have been associated with prolonged virus propagation *in vitro* or progressed disease and immunosuppression *in vivo*[106]. The variant virus isolates appear to be more

aggressive and to primarily utilising the CXCR4 co-receptor for entry[9;106;182]. These viral characteristics are believed to evolve when the immunocomprimised host no longer can control the virus imunologically.

Antibodies to HIV can protect against infection. Several studies have reported protection of non-human primates against a virus challenge after passive transfer of monoclonal antibodies either alone or in combination with purified antibodies from HIV infected primates[8;183]. These studies encourage the development of a potent gp120 immunogen for an antibody based HIV vaccine.

Methods

Cells and virus

Cells. The human CD4+ lymphocyte cell lines H9[162], C8166[172], MT-2 and MT-4[74;154] were obtained through the AIDS Reagent Project, British Medical Council (MRC) and grown in complete growth medium with antibiotics (I, II, III, IV). The growth medium used for peripheral blood mononuclear cells (PBMC) was supplemented with IL-2 (III).

Virus. The HIV-1 LAI isolate pLAI.2 (formerly pBRU2)[11;31;197] was subjected to site directed mutagenesis to delete consensus sites for N-linked glycans in the V1-loop (I, IV), V3-loop (II, IV) and potential O-linked glycosylation sites in the V3-loop (II). A 2.7 kb BamHI-SalI fragment of pLAI.2 containing most of gp160, was inserted into pUC18 for site directed mutagenesis using mutagenised oligonucleotides. All mutants sequences were verified by DNA sequencing.

Viral stocks were made by transfection of H9 cells with full-length plasmid DNA using a modified DEAE-Dextran transfection procedure[78]. Culture supernatant was harvested after 2-4 weeks of culture, 0.45 μm filtered, aliquoted and stored at -70°C until use. All clonal viral stocks were verified by DNA sequencing as above (I, II, IV). Viral stocks from HIV infected individuals were obtained by cocultivation of OKT4 selected CD4+ cells from patients and healthy donors[180](III).

Virus titration was done as an end-point titration in MT-4 cells or pooled PBMC using quadruplicate or octaplicate cultures and 5-fold viral dilution series. Cell Culture Infective Dose 50% ($CCID_{50}$) was calculated essentially as described by Reed & Muench[165](I, II, III, IV).

Neutralisation assay. Virus supernatant (25 to 100 $CCID_{50}$) and a dilution series of neutralising agent was allowed to interact for 1 hour at RT before addition of permissive MT-4 cells for two hours or PBMC for 16 hours. Unbound virus was removed by extensive wash and cells were resuspended in

appropriate growth medium and quadruplicate cultures were cultured for 4 days (MT-4) or 7 days (PBMC) in a 96-well plate (I, II, III).

Virus phenotype was determined from the ability to form syncytia in the isolation culture of CD4+ cells, in the co-culture for virus-propagation or in co-culture with MT-2 cells[180](III).

CCR5 genotype determinations were done with PCR on genomic extracted DNA using primers spanning the potential 32-bp deletion in the CCR5 gene. The resulting PCR fragments were small in size for easy visual identification of size differences of amplified DNA fragments after agarose gel electrophoresis (III). PCR amplification of the wild type CCR5 gene gave a fragment size of 137 bp.

ELISA

HIV Ag ELISA used purified antibodies from HIV infected individuals (HIV Ig) as a capture antibody[142]. HIV antigens were detected using biotinylated- HIV Ig followed by HPR conjugated avidin. Colorimetric development from the enzymatic conversion of *o*-phenylenediamine (OPD) was measured at 490 nm/630 nm (I, II, III, IV).

gp120 ELISA used the anti-C5-antibody, D7324 (IV), or the anti-V3-antibody, NEA-9205 (II, III) as capture antibody[132;135]. Serum from HIV infected individuals (III), biotinylated HIV Ig (IV) or the anti-V3-antibody NEA-9205 (II, III) were sources of detection antibodies. Alkaline phosphatase-conjugated secondary antibodies were used with the AMPAK amplification kit (DAKO) and measured at 450 nm/630 nm essentially as described[123] (II, III). HPR conjugated avidin and OPD was used with the biotinylated HIV Ig (IV).

Radio immunoprecipitation assay RIPA

^{3}H-GlcNAc labelled gp120 were incubated with mouse anti-V3 antibody NEA9205 (I, II) or the anti-Tn antibody 83D4 (II), and pelleted with a anti-mouse-Ig coupled to proteinA-Sepharose and subjected to SDS-polyacrylamide gelelectrophoresis (SDS-PAGE) for visualisation after exposure to film (I, II).

Deglycosylation assay

Typing of N-glycans (IV) was done with a modified RIPA method; ^{35}S-cysteine/methionine labelled gp120 was caught onto the bottom of a microwell using the anti-V3-antibody NEA-9205 as capture antibody, and subjected to enzymatic degradation using either Endoglycosidase H or N-glycanase/N-glycosidase F. Complexes were solubilised by boiling with SDS and DTT for 30 min., and subjected to SDS-PAGE for visualisation of protein mobility.

Protection of gp120 deglycosylation (IV) was determined with a modified RIPA deglycosylation procedure; ^{3}H-GlcNAc labelled gp120 was caught onto the bottom of a microwell using either the anti-V3-antibody NEA-9205 or the anti-C5 antibody, D7324 as capture antibodies. Wells were preincubated with sCD4, NEA9205, D7324, HIV Ig, or serum from HIV infected individuals, before subjection to N-glycosidase F degradation. Soluble fractions were removed from microwells and measured for the content of radioactivity. Undigested glycans on gp120 still bound onto the microwell were solubilised by boiling with SDS and DTT for 30 min., and measured for the content of radioactivity by liquid scintillation. The two measurements of each well were used to calculate the degree of inhibition of deglycosylation of gp120.

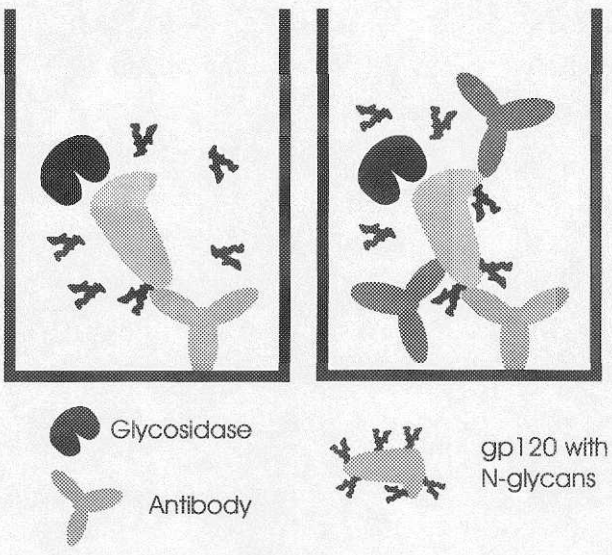

Figure Fejl! Ukendt argument for parameter.. **A schematic representation of the deglycosylation assay for protection of gp120. N-glycanase digestion of N-glycans releases them into the reaction buffer. Left: Unproteced gp120 caught onto the microwell with an antibody. Right: Partially antibody protected gp120 caught onto the microwell by an antibody (IV)**

Summary of results

N-glycans and modulation of antibody sensitivity

Glycosylation of gp120 is necessary to attain functional conformation[138]. In addition to shielding of peptide stretches around N-linked glycans[9;176] the glycosylation of gp120 could also influence the three-dimensional conformation of the molecule, and thus influence the presentation and accessibility of epitopes in a more indirect and spatially discontinuous manner[21;95].

N-glycosylation mutants of gp120 lacking glycosylation sites in the V1-loop, N116Q, (I) or in the V3-loop, A308 (II) were made. Neither of the mutants displayed apparent phenotypic abnormalities and both of the N-glycosylation signals in the V1-loop and V3-loop were shown to be utilised, as identified by an increased electrophoretic mobility of mutant gp120 (I, II). In addition, using a discriminative deglycosylation assay for either high-mannose or complex type N-glycans, both of these N-glycans was found to be of the complex type (IV). However, the neutralisation sensitivity of these N-glycan mutants to anti-V3 antibodies was very different. The A308 mutant were more than 100 times more sensitive to neutralisation by the anti-V3 antibody NEA9205 than the wild type, BRU (II). This topic is contained in the dissertation of Britt Losman[107]. Compared to the wild type, the V1-glycan deletion mutant N116Q was found to be three-fold more resistant to neutralisation by the anti-V3-antibody NEA9205. Additionally, the N116Q mutant was also three-fold more resistant to neutralisation by sCD4, but equal or more sensitive neutralisation by the conformation dependent antibody, 2G12 (I). These results indicated that N-glycans of gp120 could influence the sensitivity to neutralising antibodies. Further, these results indicated that the N-glycan in the V1-loop influenced the conformation of gp120, without changing the overall integrity of the molecule. These results are consistent with the proposed structure of gp120[96;203].

Deletion of potential O-glycans in the V3-loop

Truncated O-linked oligosaccharides constitute broadly neutralising epitopes on gp120[68;72]. These epitopes could be situated in the V3-loop[33], but their exact locations on gp120 remain unknown.

Several mutant HIV-1 infectious clones where some or all potential signals for O-linked glycosylation at Serine or Threonines residues in the V3-loop of HIV-1 gp120 were deleted by Alanine substitution (Figure 7). None of the mutants displayed any apparent phenotypic abnormalities (II). These results indicated that the Alanine substitutions in the V3-loop, including the resulting N-glycan deletion, did not interfere dramatically with viral replication in cell culture (II). Mutant clones were compared with the wild type virus for the sensitivity to neutralisation with antibodies specific for the O-linked oligosaccharides Tn or sialosyl-Tn. If for instance, a Tn epitope was located on one of these sites, deletion of the site would remove a neutralisation epitope and render the virus more resistant to the neutralising by Tn antibody. However, none of the mutants with deletions of O-glycosylation signals in the V3 loop displayed any increased resistance to anti-Tn or anti-sialosyl-Tn antibodies (II). These results indicated that the broadly specific O-linked glycan neutralisation epitopes are located outside the V3 loop of gp120.

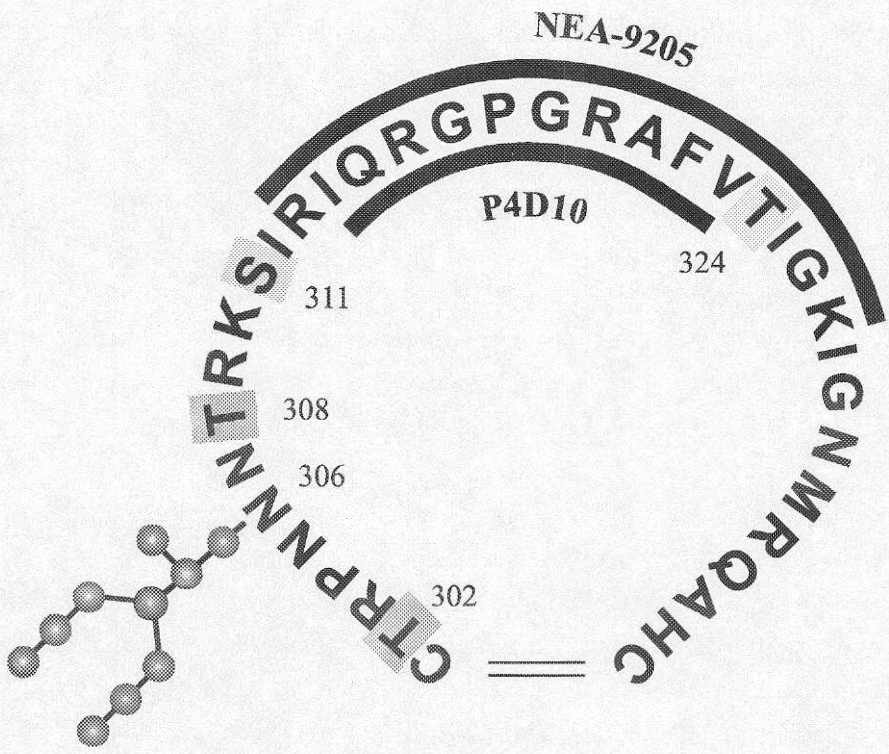

Figure Fejl! Ukendt argument for parameter.. **V3 loop of BRU with Serine/Threonine mutation sites by shading and epitopes for MAbs NEA-9205 and P4D10 indicated by bold lines. The N-linked oligosaccharide at N306 is also indicated. From (II).**

Accessibility to N-glycans of gp120

The intermolecular interactions between antibodies, epitopes and N-linked glycans are complex and poorly understood[145]. Elimination of glycosylation sites prior to antibody binding may not reveal the whole truth and analysis of accessibility of N-linked glycans after binding of ligands to gp120, may disclose important features of N-linked glycan as they appear in the folded and native molecule. We had developed a deglycosylation assay for characterisation of N-glycans types (IV) and developed this assay further to investigate induced changes in the orientation dependent susceptibility of N-linked glycans of gp120 to enzymatic degradation after binding of gp120 ligands (IV).

We achieved orientation-specific exposition of gp120 in ELISA microplates by catching gp120 from ^{3}H-GlcNAc labelled cultures either by D7324 antibody, binding to the C5 domain, or NEA-9205 antibody, binding to the V3 region of gp120[48]. It was unexpected that the heterologous antibody, NEA-9205 was not able to protect gp120 caught by D7324 and vice versa (IV). This demonstrated that antibody binding to accessible gp120 epitopes per se was not sufficient to block adjacent glycans from N-glycanase action. In contrast, sCD4 was able to protect gp120 caught by the anti-C5 antibody from N-glycanase action (IV). This suggested that the CD4-binding site is surrounded by multiple vicinal N-linked glycans. However, pooled HIV IgG was able to better protect gp120 from N-glycanase action when gp120 was caught by the anti-V3-antibody than with the anti-C5 antibody (IV). These results demonstrate that N-linked glycans are situated in the vicinity of epitopes recognised by IgG from HIV infected individuals but the deglycosylation pattern may suggest that these antibodies mainly bind to other faces of gp120 than the CD4-binding one.

The specificity of the antibodies produced in vivo during the natural infection may influence the progression of the disease. In a small pilot study we wanted to explore the orientation dependent susceptibility of gp120 N-linked glycans to enzymatic degradation after binding of sera from HIV infected individuals displaying SPI or RPI (III, IV). Three sera from HIV infected individuals displaying SPI and two sera from individuals with RPI were found to be able to protect gp120 from N-glycanase action (IV). All five HIV+ sera protected gp120 caught with the anti-V3 equally or better than gp120 caught with anti-C5 antibody (IV). This indicated that N-linked glycans are situated in the vicinity of

epitopes recognised by sera from HIV infected individuals with SPI and RPI. No difference between the sera from individuals with slow or rapid progression infection could be discerned here.

Factors influencing disease progression.

The duration of the asymptomatic phase in untreated HIV infection varies considerably among infected individuals and an understanding of the underlying reasons for this variation is likely to provide insights into mechanisms to control of the disease. We approached this by comparing consecutive pairs of sera and HIV-1 isolates from HIV-infected individuals displaying a slow progressing infection (SPI) with individuals displaying a rapid progressing infection (RPI).

We found that neutralising titers of consecutive serum samples against autologous virus isolates were generally higher in persons with SPI than in persons with RPI (III). However, the presence of autologous neutralising activity was not a requirement for SPI. This may indicate a contribution of the cellular immune system in controlling the infection[136].

Virus phenotype was determined both by the ability to induce syncytia in the isolation culture of CD4+ primary cells and by the ability to establish production infection of MT2 cells. We found that 7 of 10 SPI were infected with NSI virus compared to the 4 of 10 in the RPI group (III). In addition, we analysed the chemokine receptor CCR5 gene for the common 32-bp deletion reported to influence viral replication[40;52;173]. The heterozygous individuals were equally distributed in the groups of SPI and RPI (4 of 10), however, a close correlation between presence of the deleted allele, virus phenotype (NSI/SI) and SPI/RPI status was observed (III). Thus, all heterozygous individuals with SPI were infected with NSI virus (4 of 4). In contrast, all heterozygous individuals with RPI were infected with SI virus (4 of 4). This difference is statistically significant ($p=0.029$, Fisher exact test). Thus, in this study, heterozygosity for the deleted CCR5 allele in conjunction with infection with a NSI isolate was predictive for SPI (III).

General discussion

The HIV-1 glycoprotein gp120 is probably the most glycosylated viral protein. Multiple functions are associated with these carbohydrates, but one prominent task from the viral point of view, is to block access for potential neutralising antibodies to otherwise hidden epitopes at the glycoprotein surface. Available data suggest HIV-1 has recruited N-linked glycans for this purpose. On the other hand, the host cell may use carbohydrate units at the viral surface for counteraction of the viral N-linked glycan-dependent escape from neutralising antibodies. This is possible because the strength of the viral strategy to use host cell-specific, i.e. "self" carbohydrate sequences for shielding in a sense also is a weakness, because of the maintained host control of the glycosylation process. Thus, the initiation of the viral transcription program may alter the expression of different host glycosyl-transferase genes, resulting in altered or aberrant glycosylation of host cell as well as viral proteins. These altered carbohydrates may be considered as "non-self" and, therefore, such altered carbohydrate determinants of the viral glycoprotein may be attacked by pre-existing, circulating, and now, neutralising antibodies to various carbohydrate antigens. Here, both these aspects of carbohydrates of viral glycoproteins and antibody neutralisation of HIV-1 are discussed.

The finding that elimination of an N-linked glycan of the V3 region of gp120 resulted in a mutant virus that was more than two orders of magnitude more susceptible to neutralisation by anti-V3 antibodies (II) supported the notion that large N-linked glycans are utilised in a viral strategy to escape potential neutralising antibodies[30;56;108;179]. This was described in the dissertation by Britt Losman[107]. Recent work demonstrates that also glycans situated more distally to the V3 region in the primary protein structure may be engaged in shielding of the V3 region. Thus, Losman and co-workers found that a mutant HIV-1 lacking three N-linked glycans of the V1 region, also was more sensitive to anti-V3 antibodies than wild type virus, whereas glycans of the C2 region seemed to be inert in this context[108]. This probably reflects that the V1 region is adjacent to V3 in the three-dimensional conformation of the gp120 oligomer. However, the

data of the present study (I) showed, that elimination of only one of the three N-linked glycans of the V1 region resulted in a slightly increased resistance to neutralisation by antibodies to V3, which contrast to the findings described above for the mutant virus lacking all 3 glycans. Altogether, these data would suggest that only one or both of the two N-linked glycans N-terminal or C-terminal to the ^{116}N-glycan analysed (position 143c according to the HXB2 isolate[88]) are responsible for shielding of the V3 region. On the other hand it has been shown that deletion of the entire V1-loop did not increase the sensitivity of e.g. V3-loop antibodies[28;205;206]. However, deletion of a whole loop may have other conformational consequences than shielding of the V3-loop.

The great risk, from the viral point of view, using host cell glycans for shielding of B cell epitopes is that the host cell glycosylation machinery may be disturbed by the prominent alteration of host cell metabolism during viral replication. In particular, the implementation of the viral transcriptional program may affect the balance of the often very transiently expressed glycosyl transferases, resulting in carbohydrate neoantigens. Such changes have been described for HIV-infected cells and include expression of histo-blood group substances in normally "silent" T cells, and short O-glycan chains such as Tn and sialosyl-Tn[68;72]. A variety of HIV laboratory strains and patient isolates have been shown to expose these antigens from surface-associated gp120 and these virus strains become readily neutralised by suitable anti-carbohydrate antibodies[68;72]. It is reasonable to assume that naturally occurring antibodies to such unintentional viral carbohydrate antigens, may help to restrict the spread of HIV-1 from e.g. A-positive individuals to B- and 0-positive individuals[5;68]. As the V3-loop constitutes a major neutralisation target, we investigated whether genetic elimination of potential signals in this region could reduce the viral sensitivity to antibodies to O-glycans. However, we found that none of the four potential O-glycosylation sites in the V3-loop (Threonine^{301,308,324} and Serine^{311}) harboured any detectable O-linked oligosaccharide neutralisation target (II). These data suggest that the O-linked glycans, that constitute targets for neutralising antibodies may be situated outside the V3-loop or distributed randomly at Serines and Threonines located on the surface of gp120. Indeed, the results obtained from the N-glycanase protection assay blocking with antibodies to V3

epitopes demonstrated that gp120 contains relatively large patches free from N-linked glycans (IV), which could harbour such O-linked glycans.

The duration of the asymptomatic phase in untreated HIV infection varies considerably among infected individuals and an understanding of the underlying reasons for this variation is likely to provide insights into the pathogenic processes involved in HIV-1 infection and control of the disease. We approached this by studying parameters in HIV-infected individuals who displayed a rapid progressing infection (RPI) and compared them with individuals who displayed a slow progressing infection (SPI). However, this approach is limited by being a comparative study where experimentation is not possible. We found that titers of consecutive serum samples against autologous virus isolates were generally higher in persons with SPI than persons with RPI (III). These results are in agreement with others[29;150;180]. The finding that the presence of autologous neutralising activity was not a requirement for SPI is intriguing. In these HIV infected individuals, factors other than neutralising antibodies must be decisive for the SPI. It is noteworthy that a persistent viral replication can occur for an extended period without the significant generation of autologous neutralising antibodies. The lack of induction of neutralising antibodies may be caused by an immunosuppressed state (for RPI) or insufficiently levels of antigen (for SPI)[152;181]. However, this may indicate a role for cytotoxic T-cells in the control of disease[136].

Glycosylation of gp120 influences the virus phenotype and the interaction with the co-receptor[106;182]. The two important factors, addressed in the present study (III) are likely to have influence on the rate of disease[52;173;182]. Syncytium inducing virus phenotypes appear to be more virulent and emerge late in the course of disease[182]. The mutant allele containing a 32 bp deletion in the CCR5 gene, found in up to 22% of Northern Caucasian population, have been associated with longer AIDS-free survival[52;173]. But, neither phenotype characteristics nor the 32 bp deletion in the CCR5 gene were alone predictive for SPI, as they were represented in both groups (III). The overrepresentation of SI in the RPI group is consistent with the progressed stage of disease seen these individuals[182]. However, NSI phenotype in conjunction with the presence of the 32-bp deletion in the CCR5 gene was predictive for SPI (III). These findings are

consistent with the notion that NSI and SI viruses utilises the CCR5 and the CXCR4 co-receptors, respectively[14], and in accordance with the observation by Michael et al.[124]. The observed correlation between SPI and NSI phenotype in conjunction with heterozygosity for the 32 bp deletion in the CCR5 gene did not include all the individuals displaying SPI. It is reasonable to believe that the underlying reasons for slowly progressing disease of course may be multi-factorial, and other immunological, virological or host factors are likely to be responsible for the slow progression of disease[66;111;150;156;171].

The HIV-1 gp120 is extraordinary highly glycosylated[67] and contains more than 20 sites for N-linked glycosylation, most of which are occupied by any of the two classes of N-linked glycans, complex type on one hand and high mannose or hybrid glycans on the other[18;100;209]. The most important role of these N-glycans seems to be to participate in HIV-1 immune escape mechanisms, by virtue of the capacity of the large N-linked glycans to block access to adjacent peptide epitopes and functional regions[9;65;69;85;146;177]. The glycans of all the glycosylation sites gp120 have been classified as either high mannose or complex type[100]. However, the viral isolate LAI contains one glycosylation site not present in strain BH10 analysed by Leonard et al.[100]. Using our newly developed deglycosylation assay we found that the last uncharacterised N-linked glycan in the V1-loop of the HIV-1 prototype strain LAI in fact is of the complex type which is similar as for the other glycosylation sites of this region[100] (IV). The exposed variable loops of gp120 contains significant sequence variation in response to immune reactivities[7;28;56;95;205]. In addition, N-glycans of the complex type are primarily found in exposed regions of gp120[100;209]. Our characterisation of the N-linked glycan in the V1-loop was based on deletion of the N-glycan in question, which could influence the processing of other N-linked glycans of the molecule. Still, we believe that exposition of the V1-loop to glycanases and glycosidases in the ER and Golgi apparatus is likely to confer the complex nature of this N-glycan.

The interplay between antibodies, epitopes, and N-linked glycans in immune escape mechanisms is complex[145], and the approach of elimination of glycosylation sites prior to antibody binding may not reveal the whole truth.

Thus, analysing the accessibility of N-linked glycans after binding of critical monoclonal antibodies or other important ligands to gp120 may disclose important features of N-linked glycan interference with the presentation of gp120, e.g. to estimate the proportion of physically accessible glycans in the vicinity of important epitopes such as they appear in the folded, native molecule.

We further developed the in-well deglycosylation assay to investigate induced changes in the orientation dependent susceptibility of N-linked glycans of gp120 to enzymatic degradation after binding of monoclonal antibodies (IV). It is interesting that the anti-V3 MAb could not protect gp120 from N-glycanase action and reflects probably that there are too few glycans in the vicinity of the V3 region on monomer gp120 to induce a detectable protection. This is noteworthy considering that gp120 contains more than 20 large N-linked glycans[100] and that the IgG molecule is even more spatious than gp120. Thus, in spite of the large proportion of glycans on gp120, there must be relatively large patches on monomer gp120 not occupied by carbohydrates[96;202;203].

In contrast, the high degree of protection of gp120 caught by an anti-C5 antibody from N-glycanase action by incubation with sCD4, suggested that the CD4-binding site is surrounded by multiple vicinal N-linked glycans. The presence of bulky glycans surrounding the CD4 binding domain could protect the CD4 binding site from antibody recognition. This is in agreement with other reports that demonstrate that the actual CD4 binding site differs from epitopes recognised by CD4 blocking antibodies and consistent with the deduced crystal structure of gp120[96;133;134;203].

Protection of gp120 from N-glycanase action was also observed when IgG antibodies from pooled HIV infected individuals were analysed (IV). However, the most pronounced protection was observed for gp120 caught with the anti-V3 antibody and not with the anti-C5 antibody as seen for sCD4-induced protection. The results demonstrate that N-linked glycans are situated in the vicinity of epitopes recognised by IgG from HIV infected individuals. The deglycosylation pattern suggests that these antibodies mainly bind to other faces of gp120 than the CD4-binding one, which may reflect a viral strategy to escape neutralisation. The specificity of the antibodies produced in vivo during the natural infection has been intensively investigated. Thus, gp120 antibodies produced *in vivo*, are

at least partly, directed to non-neutralising epitopes not present on the oligomer structure, whereas the majority of the broadly neutralising antibodies are directed against the CD4 binding site[24;93;130;131]. However, the fraction of antibodies recognising non-neutralising epitopes present on monomeric gp120, but not oligomeric gp120, still remains unknown, but may constitute as much as half of the circulating anti-gp120 antibodies[24]. Shedded monomer gp120 from infected cells or virions has been proposed to act as a decoy antigen and to stimulate the production of antibodies against non-neutralising epitopes harmless for the virus[24;152]. The results with HIV Ig presented here (IV) may partly reflect this phenomenon.

We explored possible induced changes in the orientation dependent susceptibility of gp120 N-linked glycans to enzymatic degradation after binding of sera from HIV infected individuals displaying SPI or RPI in a small pilot study (IV). Sera from HIV infected individuals displaying SPI (three sera) or RPI (two sera) were tested and found to protect gp120 from N-glycanase action to various extents. All five HIV+ sera protected gp120 caught with the anti-V3 equally or better than gp120 caught with anti-C5 antibody, confirming that N-linked glycans also are situated in the vicinity of epitopes recognised by sera from HIV infected individuals with SPI and RPI. However, the orientation of gp120 appeared to have little influence on the degree of protection seen for all the HIV+ sera. No difference between the sera from individuals with slow or rapid progression infection could be discerned here.

It has become increasingly clear that development of an HIV vaccine should aim at inducing high-affinity neutralising antibodies capable of neutralising several isolates. The established protection of HIV infection after passive transfer of anti-gp120 antibodies to a non-human primate was a hallmark for the development of an antibody based HIV vaccine[116;183]. The induction of these potent anti-gp120 neutralising antibodies seems, however, to be a rare event in humans[195], and current vaccine approaches aim at inducing humoral as well as cellular immune responses[161]. An exiting approach is generation of an immunogen based on stabilised oligomeric structures of gp120[95;97;174;202;207;208], which is likely to induce both humoral and cellular immunity. Immunisation with gp120 carrying specific N-glycan deletions, is likely to unshield epitopes of

gp120 and to facilitate presentation of a more antigenic structure for the stimulation of high-affinity gp120 antibodies and potent HIV specific T-cells[22;136;167;176;185]. Protection from host defences may prove to be the true role of the extensive glycosylation of HIV-1 gp120. One conclusion of the present work is therefore that an optimal vaccine, with the ability to induce neutralising antibodies and not only cell mediated immunity, must present stable oligomeric gp41/gp120 units, where strategical epitope-masking N-linked glycans are eliminated, but where the remaining N-glycans resemble the authentic gp120 glycosylation.

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