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STUDIES ON HIV-2 ANTIBODY MEDIATED NEUTRALISATION, CORECEPTOR USAGE AND *IN VIVO* TROPISM

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ABSTRACT

Human immunodeficiency virus type 2 (HIV-2) is the second virus that causes AIDS in humans. It has a genetic identity with HIV-1 of 40-60% and similar genetic organisation and biological properties, but the two viruses are distinguished certain features. HIV-2's geographical spread is mainly restricted to West Africa, the clinical latency period is significantly longer than for HIV-1, it has a reduced heterosexual and vertical transmission rate and lower levels plasma virus are found in infected individuals. The reasons for these differences are largely unknown. The aim of this thesis was to characterise different biological properties of HIV-2.

Since induction of broadly neutralising antibodies is a desirable feature of a future HIV-2 vaccine, knowledge of neutralising epitopes in the HIV-2 envelope proteins is important. By peptide immunisation of guinea pigs, the central and C-terminal part of the V3 region of HIV-2 gp125 was confirmed to be an important target for neutralising antibodies. However, subtle changes in the sequence and length of peptides resulted in major differences in the ability to elicit HIV-2 neutralising antibodies. The conserved F-315, H-316, W-329 and C-330 amino acid residues were suggested to participate in a conformational neutralising epitope.

Production of recombinant human antibody Fab fragments by combinatorial library/phage display was shown to be a suitable method to obtain anti-HIV-2 antibodies with neutralising capacity. Six Fabs that neutralised the homologous strain SBL6669 were obtained, of which one also neutralised a heterologous virus isolate.

The tropism of HIV is largely determined by the coreceptor usage of the virus. Primary HIV-2 isolates were shown to frequently use CCR5, but were often promiscuous in coreceptor usage. Broadening of coreceptor usage was not associated with disease progression. CXCR4 usage was observed for some isolates recovered from patients with advanced disease, and appeared to correlate with positively charged amino acid residues at positions 314 and/or 313 in the V3 loop. Low level BOB expression in MT-2 cells coupled with promiscuous coreceptor usage among HIV-2 isolates was suggested to account for difficulties in clearly distinguishing distinct phenotypic groups in MT-2 cells. CCR5 or CXCR4 were shown to be required for efficient infection of PBMC by primary HIV-2 isolates *in vitro*. However, inefficient CCR5 and CXCR4 independent infection of PBMC was observed for the majority of isolates tested.

Productive HIV-2 infection in the brain was shown to be restricted to macrophages or microglia. Thus, the broad coreceptor usage and relative CD4 independence of HIV-2 *in vitro* appears to have little influence on the *in vivo* tropism, at least in the brain compartment. Other factors are therefore suggested to account for the higher frequency of encephalopathy observed in HIV-2 than in HIV-1 infection.

Key words: HIV-2, neutralising antibodies, V3, combinatorial library, phage display, Fab fragment, coreceptor, CCR5, CXCR4, HIV encephalitis, macrophages/microglia

To My Family

A. Mörner: Studies on HIV-2 antibody mediated neutralisation, coreceptor usage and *in vivo* tropism. Karolinska Institutet, Stockholm, 2001

LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers and manuscripts, which in the text will be referred to by their roman numerals.

I. Mörner A, Achour A, Norin M, Thorstensson R, Björling E. 1999. Fine characterization of a V3-region neutralizing epitope in human immunodeficiency virus type 2. *Virus Research* 59:49-60.

II. Björling E, von Garrelts E, Mörner A, Ehnlund M, Persson MAA. 1999. Human neutralizing HIV-2 specific Fab molecules generated by phage display. *Journal of General Virology* 80:1987-1993.

III. Mörner A, Björndal Å, Albert J, KewalRamani VN, Littman DR, Inoue R, Thorstensson R, Fenyö EM, Björling E. 1999. Primary human immunodeficiency virus type 2 (HIV-2) isolates, like HIV-1 isolates, frequently use CCR5 but show promiscuity in coreceptor usage. *Journal of Virology* 73:2343-2349.

IV. Mörner A, Björndal Å, Leandersson A-C, Albert J, Björling E, Jansson M. CCR5 or CXCR4 are required for efficient infection of peripheral blood mononuclear cells by promiscuous human immunodeficiency virus type 2 primary isolates. *AIDS Research and Human Retroviruses, in press.*

V. Mörner A, Lucas S, Björling E, Thomas A, McKnight Á. Productive HIV-2 infection in the CNS is restricted to macrophages/microglia. *Manuscript.*

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ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
BPMC	biased probability Monte Carlo
C4	fourth conserved region of gp125
CA	capsid protein (p26)
CCR1 / 2b / 3 / 5	CC chemokine receptor 1 / 2b / 3 / 5
CD4	cluster of differentiation 4
CDR3	complementarity determining region 3
cDNA	complementary DNA
CNS	central nervous system
CTL	cytotoxic T-lymphocytes
CXCR4	CXC chemokine receptor 4
DC	dendritic cell
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
Env	envelope
Fab	recombinant human antibody Fab fragment
Gag	group antigen
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
HAD	HIV associated dementia
HIV-1 / -2	human immunodeficiency virus type 1 / 2
HIVE	HIV encephalitis
IgG, IgM	immunoglobulin of isotype G or M
IFN	interferon
IL	interleukin
LTNP	long-term non-progressor
LTR	long terminal repeat
Mab	monoclonal antibody
MHC	major histocompatibility complex
MIP-1α / ß	macrophage inflammatory protein 1α / ß
MNGC	multinucleated giant cell
mRNA	messenger RNA
Nef	negative regulatory factor
NSI	non-syncytium inducing
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
Pol	polymerase
RANTES	regulated upon activation, normal T-cell expressed and secreted
RNA	ribonucleic acid
RT	reverse transcriptase

SI	syncytium inducing
SIV	simian immunodeficiency virus
SU	surface glycoprotein (gp125)
Tat	transcriptional transactivator
$TCID_{50}$	50% tissue culture infectious dose
TCLA	T-cell line adapted
TM	transmembrane glycoprotein (gp36)
Vpx	viral protein X
V3	third variable region of gp125
7TM	seven transmembrane

Amino acid single letter codes

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophane
Y	Tyrosine

CONTENTS

INTRODUCTION

The AIDS epidemic

In June 1981 came a short report from the United States Centers for Disease Control on five cases of *Pneumocystis carinii* pneumonia in previously healthy men in the Los Angeles area (56). More cases of *Pneumocystis carinii* pneumonia followed over several months, as well as outbreaks of other immune failure related conditions, including disseminated Kaposi´s sarcoma (94). The affected patients were either homosexual men or intravenous drug users and they all had evidence of T-lymphocyte dysfunction. Acquired immunodeficiency syndrome (AIDS) was defined for the first time in 1982, and by the end of the year over 800 cases had been reported. The afflicted populations had expanded to include also Haitian immigrants, hemophiliacs, transfusion recipients, sex partners of risk group members and children born to risk group mothers. In 1983, human immunodeficiency virus (HIV) was discovered to be the causative agent of AIDS, and in the same year a heterosexual AIDS epidemic was revealed in Africa. By 1985, cases of AIDS were found in all regions of the world and the magnitude of the growing epidemic was becoming increasingly clear. In 1991 it was predicted that in sub-Saharan Africa, by the end of the decade, 9 million people would be infected with HIV and 5 million would have died. Today we know that this prediction was a three-fold underestimation, and that the HIV pandemic cannot any longer be regarded as only a health catastrophe. Its disastrous dimensions have made it also into a development crisis and in some parts of the world even a security crisis. During 2000, 5.3 million people were estimated to have become infected, 3 million had died in AIDS and the estimated number of people living with HIV/AIDS was 36.1 million. The total number of AIDS deaths since the beginning of the epidemic was estimated to be 21.8 million (224). Accordingly, the cumulative estimated number of HIV infections in the world was around 58 million.

The discovery of HIV-1 and HIV-2

A transmissible agent, spread trough genital secretions and blood, was believed to be the cause of the disease. In 1983 Luc Montagnier's group at Institute Pasteur in Paris isolated a retrovirus from the lymph node of a patient with lymphadenopathy syndrome (20), which at the time was suspected to be associated with AIDS. The virus was later designated lymphadenopathy-associated virus. It shared some characteristics reported for the human T-cell leukemia virus (HTLV), and was therefore initially believed to belong to that human retrovirus group. However, it was not until April 1984 that the isolation of this virus, now called HTLV-III, from AIDS patients was confirmed, and the evidence for the virus as the

causative agent of AIDS was strengthened (86). The virus was shown to belong to the lentivirus genus of the *Retroviridae* family (92), and was subsequently renamed human immunodeficiency virus (HIV) (53), and later HIV-1.

In 1986, a group of healthy Senegalese was described whose sera demonstrated much stronger antibody responses to the newly discovered simian immunodeficiency virus (SIV), isolated from immunodeficient macaques in 1985 (61), than to HIV-1. This indicated the existence in West Africa of a human virus more closely related to SIV than HIV-1 (Fig. 1), and in the following year this virus, HIV-2, the second AIDS virus, was isolated from two West African AIDS patients (51). The SBL6669 isolate of HIV-2 was isolated in 1987 (7).
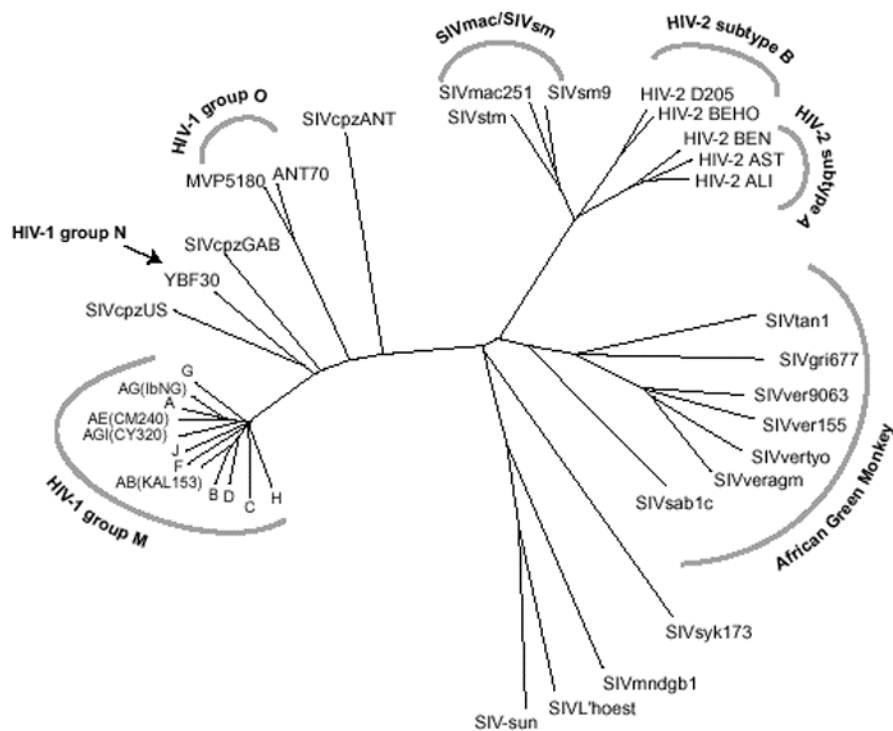

Fig. 1. Phylogenetic tree showing the genetic relationships between primate lentiviruses. The tree was constructed from the complete lentivirus genome alignment. It clearly shows that HIV-2 is closely related to SIV from Sooty mangabeys (SIVsm) and macaques (SIVmac). Adapted from (126).

HIV-2

The disease

The natural history of HIV-2 infection is largely the same as for HIV-1 infection, but shows a prolonged course with a substantially longer clinical latency period (Table 1). The primary infection is associated with a burst of plasma viremia and an acute febrile illness, characterised by non-specific symptomatology and spontaneous resolution (50, 58). The plasma viremia then drops to a relatively low set point, where the control of viral replication has been attributed to several factors, including neutralising antibodies, virus-specific cytotoxic T-lymphocytes (CTL), cytokines, chemokines and availability of HIV coreceptors (reviewed in 78). The levels to which plasma viremia decline and become established are highly predictive of the rate at which an individual will progress to AIDS, where lower levels are associated with prolonged clinical latency (150). Interestingly, the plasma viremia drops to significantly lower levels in HIV-2 than in HIV-1 infection (10, 24, 180, 208). After the drop in plasma viremia, the prolonged asymptomatic period known as clinical latency commences. The clinical latency lasts on average 10-12 years in HIV-1 infection (129, 195), while in HIV-2 it is significantly longer (142) (Table 1). In fact, it is believed that only a small proportion of HIV-2 infected individuals will develop AIDS (181), while the majority may be regarded as long-term non-progressors (LTNP) (134).

Along the course of the disease, the CD4$^+$ T-lymphocytes gradually decrease, and eventually clinical symptoms appear. Apart from the loss of CD4$^+$ T-lymphocytes, the immune system is compromised by hypergammaglobulinemia, impaired function of macrophages, NK cells, T-helper cells, CTL and loss of dendritic cell (DC) numbers and function (130). The clinical manifestations of symptomatic HIV-2 infection are broadly the same as those for HIV-1 (96, 99). These include opportunistic diseases such as bacterial and parasitic infections, tumors and neurologic disease. HIV-2 has been reported to cause encephalopathy more frequently than HIV-1 (139, 153), but Kaposi's sarcoma less frequently (14).

Some HIV-2 infected individuals have been reported to be protected from subsequent HIV-1 infection (221), but other studies have failed to confirm this protective effect (1, 15, 161, 237). In fact, the increasing spread of HIV-1 in HIV-2 endemic areas has led to a growing number of HIV-1 and HIV-2 dually infected individuals (161). Comparison of immunological parameters revealed that dual HIV-1/HIV-2 infection resembles single HIV-1 infection more than infection with HIV-2 (63, 117, 160).

Table 1. Summarised comparison between HIV-1 and HIV-2

	HIV-1	HIV-2	Ref.
Geographical distribution	Global	West Africa	(205)
Modes of transmission	Sexual, blood, vertical		(205)
Sexual spread		Slower	(115)
Mother-to-child transmission	20-25%	<5%	(205)
Age-specific prevalence	Peak 20-40 years	Increases with age	(4, 157, 240)
CD4 decline		Slower	(111)
Time to AIDS	Average 10-12 years	Average >20 years (?)	(142, 205)
Proviral load	No difference		(11)
Plasma viral load		Lower	(10, 24, 180, 208)
Genetic similarity	40-60% homology		(41, 101)

The virus

HIV-2, like HIV-1 and SIV, belongs to the lentivirus genus of the *Retroviridae* family. A schematic structure of the HIV-2 virion is shown in Fig. 2. As mentioned above, HIV-2 is genetically more closely related to certain strains of SIV than to HIV-1 (Fig. 1). On the nucleotide level, the similarity of HIV-2 to SIV from macaques (SIVmac) is around 75% in the *env* gene and 85% in the *gag* gene, while the similarity to HIV-1 is approximately 40% and 60%, respectively (41, 101). The genome organisations of HIV-1, HIV-2 and SIV are similar, but HIV-2 and SIV lack the *vpu* gene, while they carry the *vpx* gene, which does not exist in HIV-1 (Fig. 3). HIV-2 is, like the other primate lentiviruses, characterised by great genetic variation, especially in the *env* gene (82). This diversity is caused by the error prone process of reverse transcription, which in combination with a high viral turnover rapidly gives rise to genetic variants, both within the infected individual and globally. The genetic

variability results in biological diversity, which affects viral properties such as cell tropism and sensitivity to host immune responses, and will be discussed later. By comparison to HIV-1, the genetic diversity of HIV-2 appears to be less extensive and only two subtypes (A and B) have been well characterised. Reports of five other subtypes (C-G) are based on sporadic cases and have not been confirmed by additional samples (87). Subtype A is clearly dominating (205), except in Côte d'Ivoire, where subtype B has been reported do predominate (176).
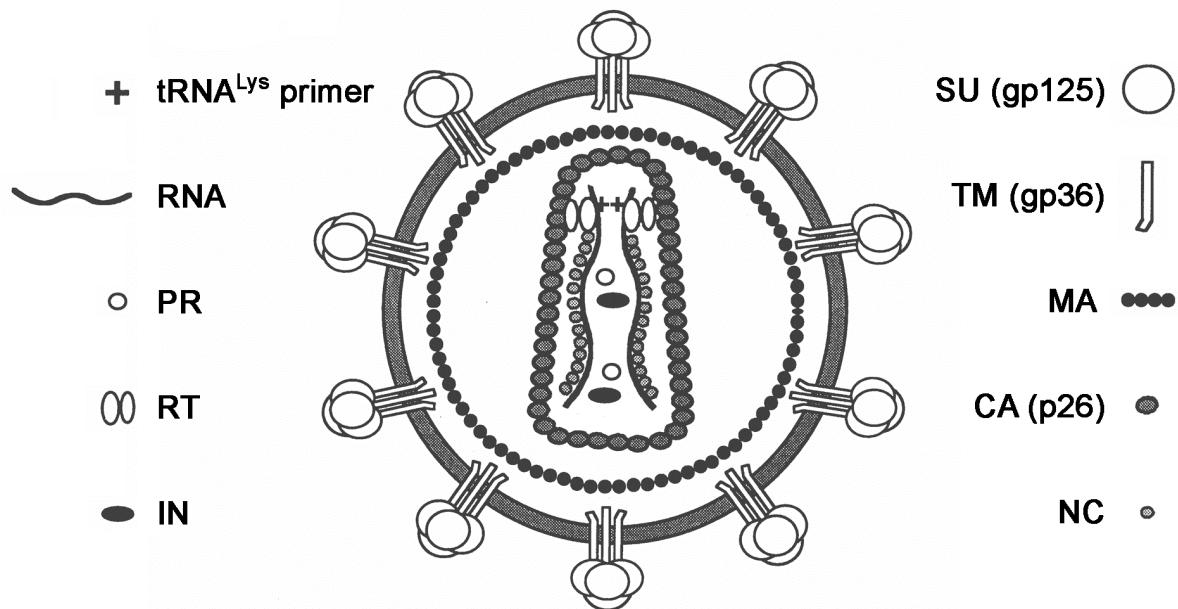
Fig. 2. The HIV-2 virion. Two identical RNA copies, coated with nucleocapsid proteins (NC), are together with the enzymatic proteins protease (PR), reverse transcriptase (RT), and integrase (IN) encapsidated in the viral core, which is composed of the capsid protein (CA or p26). Inside the capsid are also found tRNA^{Lys} primers attached to each RNA copy and the accessory proteins Vpr and Vpx. Vif and Nef may also be included in the virion, while the regulatory proteins Tat and Rev have not been detected in virus particles. The matrix protein (MA) is lining the inside of the viral envelope, which consists of a lipid bilayer of cellular origin with approximately 72 spikes inserted. The spikes are made up of trimers of the non-covalently linked transmembrane (TM or gp36) and surface (SU or gp125) glycoproteins. Modified from (140).

Fig. 3. Genetic map of HIV-2. Modified from (124).

The virus life cycle

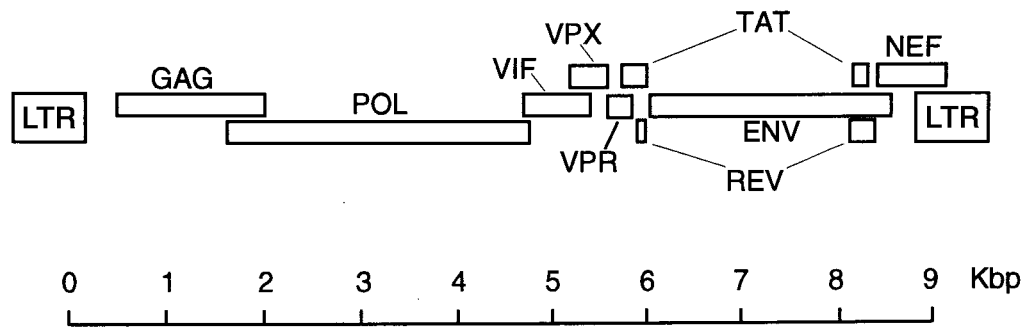The life cycle of HIV-2 has been much less studied than that of HIV-1, therefore, what is described here is largely based on studies of the HIV-1 life cycle (Fig. 4). The life cycle of an infectious primate lentivirus particle begins with the attachment to a permissive cell. The heavily glycosylated surface glycoprotein (SU), which is bound to the membrane anchored transmembrane glycoprotein (TM) on the surface of the virion, binds to the amino-terminal immunoglobulin domain of a CD4 molecule on the cell surface (59, 119). This leads to a conformational change in SU, which allows for the interaction with a secondary receptor, the coreceptor. This receptor is one of several seven transmembrane (7TM) G-protein coupled receptors (GPCR), usually a chemokine receptor. The major coreceptors for HIV-1 are CCR5 (9, 46, 67, 70, 71) and CXCR4 (79, 215). CD4 binding presumably also induces rearrangements in TM, exposing a highly hydrophobic domain at the amino-terminus, which, after SU binding to the coreceptor, inserts into the cell membrane and eventually leads to membrane fusion and virus entry (69).

Once inside the cell, the viral nucleocapsid is partially uncoated, and the RNA genome is reverse transcribed by the virally encoded reverse transcriptase (RT). This leads to the formation of a preintegration complex, which is composed of the double-stranded linear DNA intermediate, the matrix protein, the accessory protein Vpr and specific cellular proteins (152). Unlike many other retroviruses, HIV can infect non-dividing cells, a capacity that depends on active transport of the virus genome into the nucleus of the infected cell. When the virus DNA has entered the nucleus, it is covalently integrated into the host genome, and is at this stage referred to as a provirus.
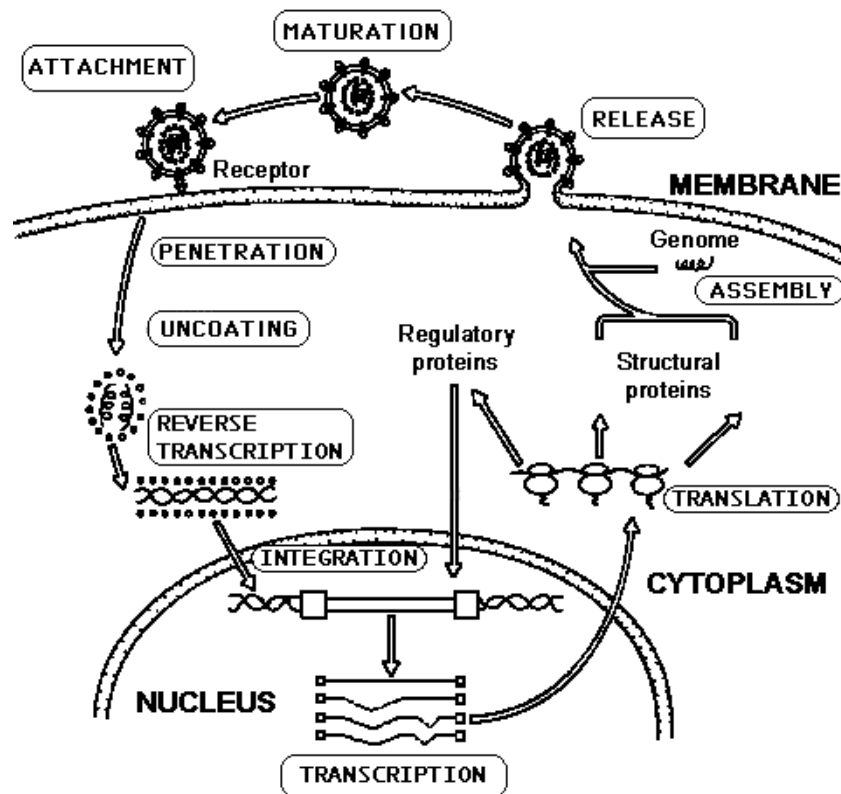
Fig. 4. The primate lentivirus life cycle. Modified from http://www-micro.msb.le.ac.uk/224/Replication.html.

The integrated provirus can either remain silent within the cell, or it can start to be transcribed to produce progeny virions. In the latter case, full-length viral transcipts are expressed from the promoter located in the 5' long terminal repeat (LTR), by the cellular enzyme RNA polymerase II. Some of these transcripts remain unspliced, while others are singly or multiply spliced. Initially, short multiply spliced mRNAs encoding the Tat and the regulator of viral expression Rev regulatory proteins and the accessory protein Nef are exported to be translated in the cytoplasm. Tat is an essential transcriptional activator that binds to the *trans*-activating response element of the beginning RNA transcript. Later, when proteins encoded by unspliced (the Gag and Gag-Pol polyproteins) and singly spliced mRNAs (Env) are needed to assemble infectious virions, Rev functions to mediate the transport of these transcripts to the cytoplasm. The accessory protein Nef, the third early protein, has several functions. Two of them are to promote endosomal degradation of cell-surface CD4 and MHC class I molecules,

both of which may serve as means of avoiding an immune response. *In vivo* Nef increases HIV replication.

The Env precursor polyprotein is synthesised at the endoplasmatic reticulum from a singly spliced mRNA species (reviewed in 147, 242). After folding, oligomerisation, glycosylation and cleavage into its functional units, SU and TM (also referred to as gp120 and gp41 for HIV-1 and gp125 and gp36 for HIV-2) in the endoplasmatic reticulum and the Golgi apparatus, the Env complex is expressed on the surface of infected cell before assembly of progeny virions. The Env-complex expressed on the surface of infected cells and virions is an oligomeric protein, likely a trimer, composed of non-covalently associated heterodimers of SU and the membrane anchored TM.

Unspliced transcripts follow one of two different pathways: they can either become the RNA genome of progeny virions, or they can function as mRNA for the Gag and Gag-Pol polyproteins. The Gag and Gag-Pol precursor polyproteins are incorporated in budding immature virus particles, and are inside the virion proteolytically cleaved by the viral protease to their final products. The major Gag products are: the matrix, capsid protein and nucleocapsid proteins. The capsid protein (commonly referred to as p24 for HIV-1 and p26 for HIV-2) contains some of the most conserved regions within all retroviral Gag proteins and is essential for particle assembly. The Pol portion of the Gag-Pol polyprotein is cleaved into protease, RT and integrase inside the immature virion. Protease is essential in the lentiviral lifecycle since the Gag and Gag-Pol polyproteins must be cleaved by it for mature infectious particles to form. Protease has lately been a prime target for drug design. The role of the second Pol protein, RT, has already been briefly described. It has also been a major target for drug design. The last of the Pol proteins is integrase, whose primary function is to integrate the reverse transcribed viral genome into a host cell chromosome.

Vpu, Vif, Vpr and Vpx are accessory proteins with various functions that are non-essential for virus replication. Of interest, SIV and HIV-2 lack the gene encoding Vpu, which among other functions has been suggested to facilitate Env transport to the cell surface. HIV-1, on the other hand, lacks Vpx, which appears to be necessary for efficient viral replication under certain circumstances (116).

Tropism

The discovery of HIV coreceptors

HIV-1 and HIV-2 were early found to primarily infect cells expressing CD4 (51, 59, 119, 202). Human T-helper lymphocytes, monocytes/macrophages, DCs and microglial cells of the brain express CD4, and consequently represent target cell types *in vivo* (106, 118, 141, 159, 164). However, it was soon discovered that CD4 expression alone was not sufficient for viral entry (141). In 1995, in another field of HIV research, the virus suppressive factors produced by CD8$^+$ T-cells were identified as the ß-chemokines macrophage inflammatory protein-1α (MIP-1α), MIP-1ß and regulated upon activation, normal T-cell expressed and secreted (RANTES). Almost simultaneously, a 7TM GPCR, designated "fusin" (later renamed "CXCR4"), was identified as a cofactor for HIV-1 fusion and entry (79). It was by its amino acid sequence assumed to belong to the family of chemokine receptors, and shortly thereafter a second chemokine receptor, CCR5, was shown also to function as a coreceptor for HIV-1 (9, 46, 67, 70, 71). This coreceptor was the receptor for the aforementioned ß-chemokines MIP-1α, MIP-1ß and RANTES, suggesting that these chemokines inhibit HIV-1 by blocking its entrance to the cell.

Biological phenotypes

Isolates of HIV-1 and HIV-2 have previously been phenotypically divided according to different classification systems. These are shown in Table 2. The classification systems are largely overlapping, so that slow/low viruses are usually NSI and MT-2 negative, while rapid/high viruses are usually SI and MT-2 positive. The M-tropic vs. T-tropic classification differ slightly, since T-tropic viruses often can infect macrophages, albeit with reduced efficiency. The discovery of the HIV coreceptors revealed a strong correlation between coreceptor usage and the division of HIV-1 isolates into the two phenotypic groups. Use of CCR5 rendered a virus to be slow/low, NSI and MT-2 negative, and consequently, if it used CXCR4 it was a rapid/high, SI and MT-2 positive virus (30, 46, 67, 70, 71, 79). Thus, the *in vitro* phenotype of a virus isolate could be explained by two considerations: (i) the virus ability to use either CCR5 or CXCR4 (or both) and (ii) the expression of these coreceptors on different CD4$^+$ target cells. CXCR4 is abundantly expressed by transformed T-cell lines and primary T-lymphocytes, while CCR5 is expressed by macrophages and primary T-lymphocytes.

Table 2. Phenotypical classification systems based on *in vitro* properties of HIV isolates

	Slow/low	Rapid/high	
Slow/low vs. Rapid/high	Slow replication to low titres in PBMC. No replication in transformed T-cell lines or monocytoid cell lines.	Fast replication to high titres in PBMC. Replicate in transformed T-cell lines or monocytoid cell lines.	(81, 251)
	NSI	**SI**	
NSI vs. SI	Do not induce syncytia in PBMC.	Induce syncytia in PBMC.	(218)
	MT-2 -	**MT-2 +**	
MT-2 - vs. MT-2 +	Do not replicate or induce syncytia in the transformed T-cell line MT-2.	Replicate and induce syncytia in the transformed T-cell line MT-2.	(123)
	M-tropic	**T-tropic**	
M-tropic vs. T-tropic	Replicate well in primary macrophages and T-lymphocytes. Do not replicate in established T-cell lines.	Replicate well in primary T-lymphocytes and established T-cell lines. Replicate poorly in primary macrophages.	(44, 75, 90, 230)
	R5	**X4**	
R5 vs. X4	Use CCR5 as coreceptor for cell entry.	Use CXCR4 as coreceptor for cell entry.	(23)

Accordingly, a new classification system, based on coreceptor usage, was introduced (23). It designated viruses that used CCR5 "R5", viruses that used CXCR4 "X4" and viruses able to use both "R5X4". After the discovery of CCR5 and CXCR4, a number of other chemokine receptors and related orphan receptors have been found to function as coreceptors for HIV-1. These include CCR2b, CCR3, CCR8, CCR9, CX_3CR1 (V28), GPR1, BOB (GPR15), Bonzo (STRL33), APJ, RDC1 and US28 (45, 46, 68, 70, 73, 131, 179, 194, 212, 213), however, usually, HIV-1 infection via these coreceptors is inefficient and their use *in vivo* is

questionable. Coreceptor activity has also been reported for more structurally distant receptors such as the leukotriene B_4 receptor and ChemR23 (168, 197).

HIV-2 coreceptor usage

HIV-2 uses, like HIV-1, primarily CCR5 and CXCR4 as coreceptors, and until now, no isolate has been tested that does not use one of them or both (100, 148, 156, 217). However, significant differences exist between HIV-1 and HIV-2 receptor usage. A major difference is that HIV-2, besides CCR5 and CXCR4, can use a broad range of coreceptors readily, whereas HIV-1 usage of alternative coreceptors is usually inefficient. Alternative coreceptors used by HIV-2 are: CCR1, CCR2b, CCR3, CCR4, CCR8, CXCR2, CXCR5, CX_3CR1, GPR1, BOB (GPR15), Bonzo (STRL33), APJ, RDC1 and US28 (34, 68, 74, 100, 114, 135, 148, 156, 167, 179, 186, 194, 212, 213, 217, 250). The relevance of this promiscuity in HIV-2 coreceptor usage is unclear and will be discussed in this thesis.

Mechanism of virus entry

The precise mechanism of HIV-2 cellular entry is not known, but it is likely to follow a model that is widely accepted for HIV-1 (Fig. 5) (69). As for all enveloped viruses, a fusion protein, in this case Env, mediates the fusion of viral and cellular membranes.
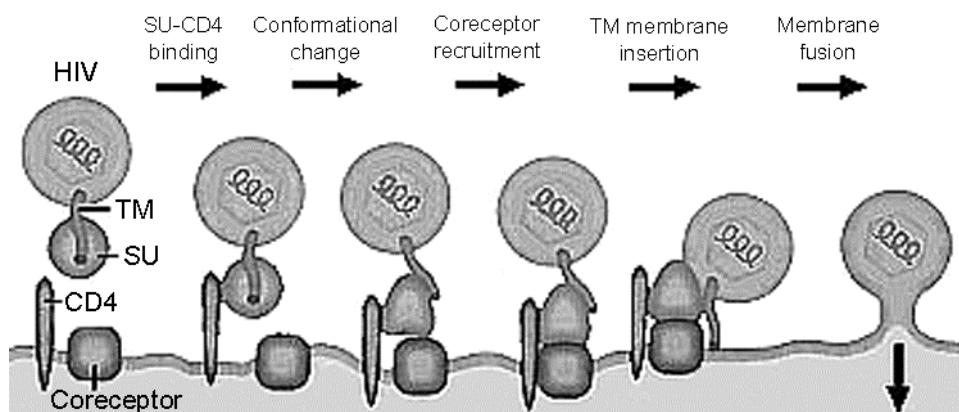
Fig. 5. Proposed model for HIV attachment to and penetration of the host cell membrane. Initial binding to CD4 induces conformational changes in SU that allows interaction with the coreceptor. The fusion process proceeds with TM altering its conformation to a fusogenic state, with its fusion peptide directed towards the cellular membrane. Binding of the coreceptor brings the two membranes closer together and allows the fusion peptide to insert into the target membrane. Coreceptor binding may also aid in the formation of a hairpin-like structure, in which TM "folds back" on it self and forces the membranes to fuse. Modified from http://www-micro.msb.le.ac.uk/335/335Replication.html.

In the current model, CD4 binding induces conformational changes in SU that exposes, creates or stabilises coreceptor-binding determinants. In this way, a conformationally altered SU is allowed to interact with the coreceptor, normally CCR5 or CXCR4. The fusion process then proceeds with the trimeric TM altering its conformation to a fusogenic state, in which a triple-stranded coiled-coil is formed, thereby displacing the N-terminal fusion peptide in direction of the cellular membrane. It is not established whether it is CD4 binding or coreceptor binding that triggers this event. However, binding of the coreceptor brings the two membranes in closer proximity, enabling the fusion peptide to insert into the target membrane and, possibly, aids in the formation of the six-helix bundle, a hairpin-like structure in which TM "folds back" on itself and forces the membranes to fuse. It is likely that this process requires the engagement of several Env trimers with multiple receptor molecules per trimer (69).

Determinants for coreceptor binding

The determinants on SU for coreceptor binding are not well studied in HIV-2. However, the third variable region (V3) has been reported to be important for HIV-2 fusion (84), and the C-terminal half of the V3 loop has been suggested to determine CCR5 or CXCR4 specificity (110). In HIV-1, the V3 loop, a well-known determinant of Env fusogenicity and tropism, has a major role in gp120's activity and specificity for coreceptor binding. In particular, positively charged residues at fixed positions on either side of the conserved tip of the loop determine usage of CXCR4. Although V3 is essential for coreceptor binding, other regions of gp120, including V1, V2 and C4, are also involved. X-ray crystallographic, mutagenic and antigenic studies (127, 188, 241) have led to a model in which the V3 loop and a conserved bridging sheet, composed of the V1/V2 stem and an anti-parallel, four-stranded structure including sequences in the C4 region, interacts with the coreceptor.

HIV-2 CD4 independence

While HIV-1 is highly dependent on CD4 for infection of cells (Fig. 5), T-cell line adapted (TCLA) HIV-2 isolates were shown to use CXCR4 in the absence of CD4 (74, 186). Furthermore, *in vitro* studies using glioma cell lines expressing CCR5 or CXCR4 but not CD4 revealed that also primary HIV-2 isolates have a much lower degree of CD4 dependence (185). The mechanism of HIV-2 CD4 independent infection is not known, but it is possible that the coreceptor binding site on gp125 is already at least partially exposed or formed, allowing interaction between gp125 and the coreceptor without prior binding to CD4. However, most HIV-2 isolates benefit from the presence of CD4, making infection more

efficient and allowing usage of alternative coreceptors. Also, the *in vivo* relevance of this CD4 independence is uncertain as only one HIV-2 strain, the TCLA ROD/B, was capable to infect primary CD4$^-$ astrocytes (185). An hypothesis, which has been deduced from these findings, proposes that the evolutionary predecessors of HIV-1 and HIV-2 strictly used the chemokine receptors for entry and later evolved a CD4 requirement, conferring greater target specificity and protection of the chemokine receptor binding site from the humoral immune system.

Coreceptors and virus transmission

The coreceptor usage has great impact on HIV transmission and disease progression. R5 viruses are the HIV-1 variants that are preferentially transmitted, and they are the ones that are isolated from recently infected individuals (191, 227, 248). The critical role for CCR5 as a mediator of HIV-1 sexual transmission was evidenced by the discovery of a mutant CCR5 allele, designated CCR5Δ32, and its association with HIV-1 resistance (66, 136, 198). CCR5Δ32 has a 32 bp deletion in the region of the open reading frame encoding the second extracellular loop, causing a frame shift and a premature stop codon in the transmembrane domain 5. This truncated protein is not expressed on the cell surface. The highly significant epidemiological data, which were noted in cohorts of individuals exposed both mucosally and parenterally, were supported by *in vitro* experiments, where peripheral blood mononuclear cells (PBMC) from Δ32/Δ32 homozygotes were susceptible to infection with X4 viruses but completely resistant to R5 viruses (136, 183, 198). However, most studies have found no significant effect of the heterozygous genotype on transmission of HIV-1 (66, 109, 249). Exceptional cases, where Δ32 /Δ32 homozygotic individuals have been HIV-1 infected, have all carried virus with a genotype typical for X4 viruses (26, 163, 219). In one case, X4 virus was exclusively and persistently detected (151). These cases demonstrate that CCR5 is not absolutely required for HIV-1 transmission. Another related mechanism of HIV-1 resistance is that of chemokine blockade of transmission. High levels of the ß-chemokines MIP-1α, MIP-1ß and RANTES have been associated with HIV-1 resistance (174, 244). A combination of high ß-chemokine levels and low levels of cell surface CCR5 expression has also been suggested to mediate protection (173).

Infection of the brain

HIV-1, HIV-2 and SIV can all invade the central nervous system (CNS) and cause neurologic disease. HIV associated dementia (HAD) occurs in approximately 15-20% of patients progressing to AIDS (16, 146). Behavioural and personality changes, cognitive defects such

as forgetfulness and loss of concentration, and motor defects such as limb weakness and loss of balance are all symptoms of HAD. Only case reports exist for HIV-2, but the clinical symptoms appear to be similar (22, 72, 107, 121, 137, 190). HIV-1 enters the brain early in the infection (62, 98), however, HAD typically does not present before AIDS (16, 91). It is likely that viral replication in the brain is immunologically suppressed but that control of replication is lost with decline of CD4 counts and development of immune deficiency.

The pathology within the brain associated with HIV encephalitis (HIVE) is characterised by an accumulation of macrophages, multinucleated giant cells (MNGCs) and microglial nodules within the white and subcortical grey matter (97, 209). Astrogliosis and white matter pallor are common findings (35, 48, 145, 158), and synaptic alterations as well as varying degrees of neuronal loss may also be present (77, 239). Reports on *post mortem* findings in brains from HIV-2 AIDS patients are scarce, but diffuse demyelination, perivascular inflammatory infiltration, glial nodules and MNGCs have been described (72, 107).

The cell types primarily found infected in the brain by HIV-1 are microglia and perivascular macrophages (122, 229, 234). Microglial cells are bone marrow derived, have a common lineage to monocytes and are the only resident CD4 expressing cell type of the brain (113, 133). Additionally, microglial cells express CCR3, CCR5 and CXCR4 (102, 128, 200, 226, 243) and therefore meet all the receptor requirements for infection by HIV-1 (and HIV-2). It is therefore not surprising that they are the major targets for HIV-1 infection. It is not known which cells in the CNS are infected by HIV-2.

How HIV infection of the brain results in HAD or encephalitis is not well understood. Although HIVE is associated with infected macrophages/microglia (235), infection of these cell types alone cannot explain pathological dementia and motor/cognitive dysfunction. This would be more easily explained by infection of cells that are directly involved in such processes, such as neurons or astrocytes. Up to 40% of neurons are lost in advanced cases of HAD (77), however, since they are rarely infected *in vivo*, their mechanism of destruction is not known. Still, CD4 negative neurons and microvascular endothelial cells express chemokine receptors (CXCR4 and CCR5) (104) that are used by CD4 independent primary HIV-2 isolates to infect cells *in vitro* (185). Astrocytes are the most abundant cell type in the brain and constitute almost 40% of the total cell population (196). HIV-1 infection of astrocytes *in vivo* in paediatric cases have been reported (reviewed in 32). Abundant expression of Rev and Nef in brain astrocytes has been associated with dementia (184), but

the relevance of astrocytes to HAD is unclear. Unlike macrophages and microglia, the infection of astrocytes is non-productive and requires sensitive *in situ* techniques for detection (33, 120). However, evidence for substantial HIV-1 infection of astrocytes is lacking and proviral load has been strongly correlated to productive infection of macrophages/microglia (238). Astrocytes do not express CD4 and the mechanism of HIV-1 infection is unclear. Chemokine receptor expression in astrocytes is controversial, but CXCR2, CXCR4, CCR1 and CCR5 have been reported to be expressed (104), and astrocytes are therefore potential targets for HIV-2 infection in the brain.

The host defence

The immune responses against intruding microbes can be divided into natural, or innate, and adaptive responses. The natural immune responses act as a first line of defence against intruding microbes, giving the adaptive immunity some time to develop a strong, pathogen specific response. The two types of immune responses are, however, not totally separated from each other; the adaptive immune response is largely dependent on the preceding innate response. The adaptive immunity is made up of cellular and humoral responses. Both are executed by lymphocytes, that upon recognition of foreign antigen are activated, undergo proliferation ("clonal expansion") and differentiate into respective effector cells. The cellular immune functions are carried out by T-lymphocytes, which mature in the thymus, whereas the bone marrow derived B-lymphocytes are responsible antibody production. T-lymphocytes can be divided into two major subsets: $CD8^+$ CTL and $CD4^+$ helper T-cells. $CD8^+$ CTL are responsible for killing infected cells, while $CD4^+$ helper T-cells, by secreting different cytokines, regulate the functions of other cells of the immune system (such as B-cells, $CD8^+$ T-cells and macrophages). Depending on the type of cytokines they produce, T-helper cells can be further divided into type 1 helper (Th1) or type 2 helper (Th2) cells. Th1 cells produce cytokines (such as interferon (IFN)-γ and interleukin (IL)-2) that promote cellular immune responses (for example CTL activity) while Th2 cells mainly secrete cytokines such as IL-4, IL-5 and IL-6, which stimulate the proliferation and differentiation of B-cells into antibody secreting plasma cells (112).

The cell-mediated immune response

The cell-mediated immune response is thought to play a major role in the defence against HIV/AIDS (31, 95). The resolution of the initial viral replication burst coincides with the appearance of virus specific CTL (125, 169), and HIV-1 specific CTL show a tight inverse correlation to viral load (165). There is also evidence for HIV-1 specific CTL and T-helper

responses in LTNP (187) and in exposed uninfected individuals (52, 65). Importantly, T-helper responses are vital for both functional CTL and antibody responses. Indeed, a strong HIV-1 specific T-helper response has been correlated with low plasma viral load (192).

The cell-mediated immune response to HIV-2 is less studied. However, virus specific CTL, predominantly directed against Gag, has been reported to be present in the majority of asymptomatic HIV-2 infected individuals (12, 93), and it has been inversely correlated to proviral load (12). CTL cross-reactive with HIV-1 has been found in the majority of HIV-2 positive subjects (25) as well as in exposed uninfected subjects (193), suggesting a potential mechanism of cross-protection. T-helper responses against HIV-2 Gag and Env in the majority of HIV-2 infected individuals tested have also been reported, as well as cross-reactive responses against HIV-1 in some cases (178).

The humoral immune reponse

The role of antibodies and the control of HIV infection

Neutralising antibodies are a major component of host defence against viral infection, and are particularly important in limiting the spread of cell-free virus. Logically, the appearance of HIV-1 specific IgG is a good correlate of the initial decline in plasma viremia (50, 177), although, virus specific CTL are also strongly correlated with the plasma viremia decline (125, 169). Thus, HIV-1 specific antibodies appear early, usually 3-4 weeks after infection (132). However, although autologous neutralising antibodies (i.e. antibodies able to neutralise virus isolated from the same individual) can appear rapidly (6), most individuals do not develop autologous neutralising antibodies until after the plasma viremia has declined (13, 54, 57). The effectiveness of the early antibodies has therefore been questioned, and the slow development of neutralising antibodies during primary HIV-1 infection could indeed contribute to the viral persistence. Still, non-neutralising antibodies may be an important first response to the virus. For example, HIV-1 specific antibodies that mediate antibody dependent cellular cytotoxicity (ADCC) are induced very early and correlate with the initial drop in plasma viremia (54, 57). Complement-activating antibodies that induce formation of immune complexes may also play a role in the clearance of virus, since complement-containing immune complexes are rapidly eliminated from circulation through the mononuclear phagocytic system (83). The production of virus specific IgM, the most powerful complement activating Ig, often coincides with the initial HIV-1 viremia decline (85).

Neutralising antibodies may also be important in controlling viral replication over time. As mentioned above, the prolonged clinical latency in LTNP is associated with low levels of virus in plasma PBMC and lymph nodes (40, 170). Several studies have shown that the neutralising antibody responses are improved in magnitude and breadth in LTNP. Sera from LTNP show higher neutralising titers against HIV-1 TCLA isolates than sera from progressors (154), and more frequently neutralise heterologous primary isolates (40, 154, 246). Whether the difference in antibody responses in LTNP and progressors is merely a consequence of suppressed immune function in the progressors has been debated. However, Pilgrim *et al.* showed that the breadth of neutralising antibodies, as well as the ability to neutralise autologous virus isolated at the time of serum collection, was improved in sera from LTNP compared to sera from short term non-progressors (defined as individuals with normal CD4$^+$ cell counts who had been infected for 2-7 years) (177). Yet, the question of whether the improved antibody response is a cause or consequence of long-term non-progression remains difficult to answer with certainty.

In most infected individuals, virus neutralising antibodies soon become ineffective due to the emergence of virus variants resistant to neutralisation by contemporary autologous sera (6, 222, 231). However, neutralisation can often be demonstrated with virus isolated 6-12 months prior to serum collection. This selection of virus variants resistant to neutralisation appears to be more frequent in individuals with progressive disease.

Neutralising epitopes can be found in both TM (gp41) and SU (gp120) of HIV-1. In TM, the only confirmed neutralising epitope is defined by the amino acid sequence Glu-Leu-Asp-Lys-Trp-Ala (ELDKWA) in the ectodomain of TM (155), which appears to be exposed in the virion-associated, pre-activation form of the envelope glycoprotein complex. (203). In SU, three neutralising epitope clusters can be recognised (reviewed in 172). Two of these overlap the CD4 and coreceptor binding sites respectively. The third, the V3 loop, is a major target for neutralising antibodies in TCLA viruses; however, the importance of V3 for neutralisation of primary isolates has been questioned (228). This is likely explained by its decreased accessibility in the mature oligomeric Env complex of primary isolates. Consequently, antibody responses elicited by gp120 monomer vaccines are strongly biased towards the V3 loop, neutralise TCLA viruses well but are inefficient against primary isolates. The general mechanism of antibody mediated HIV-1 neutralisation appears to be inhibition of attachment (223, 225).

The humoral immune response to HIV-2

The humoral response against HIV-2 is less well studied than that against HIV-1. The time from infection until appearance of anti-HIV-2 antibodies has only been described in a case report, in which a window period of 35 days was observed (47). Cross-neutralisation between HIV-1 and HIV-2 has been reported in several studies. In some studies a bi-directional cross-neutralisation was observed (38, 39), while others reported only uni-directional (HIV-2 antisera cross-neutralising HIV-1) (236) or sporadic, weak cross-neutralisation (162, 189). In analogy with their closer genetic relationship, stronger cross-neutralising activity was observed between HIV-2 and SIVmac or SIVagm (189). Intriguingly, individuals with HIV-2 infection, in contrast to HIV-1, retain the capability to neutralise autologous virus throughout the infection (29). This is interesting considering the reported correlation between long-term non-progression and prevalence of autologous neutralising antibodies (177) and the prolonged clinical latency in HIV-2 infection (142), suggesting a possible role for neutralising antibodies in the slower disease progression in HIV-2 infection.

The neutralising determinants in HIV-2 are less well characterised than are those in HIV-1. However, the V3-region of HIV-2 Env has been shown to contain important neutralising epitopes (27, 28). V3-specific monoclonal antibodies (Mabs) with neutralising capacity have been produced (28, 144, 149) and peptides have been used for raising neutralising guinea-pig sera (27, 28) and blocking of neutralisation activity of human anti-HIV-2 sera (28). Furthermore, fine mapping has revealed two distinct antigenic sites with conserved motifs within the V3-loop: FHSQ (positions 315-318) and WCR (positions 329-331) (28). These two regions may interact as one discontinuous antigenic site. However, conflicting results have been published, for example, failures to induce neutralising antibodies by peptide immunisation (17, 189) and to inhibit serum neutralisation with peptides (189) have been described. Furthermore, neutralising sites in the second and fourth variable regions of HIV-2 SU and one conserved region in TM have been described (216).

ADCC appears to be more frequent and have a broader specificity in HIV-2 than in HIV-1 (27, 138, 232). As ADCC has been correlated with the decline of initial plasma HIV-1 viremia (21) and long-term non-progression (54), it is possible that it plays a role in the diminished pathogenicity of HIV-2.

AIMS OF THE THESIS

1) To further characterise the neutralising epitopes in the V3 region of HIV-2 gp125.

2) To generate HIV-2 gp125 specific Fab fragments with neutralising capacity, using phage display library technique.

3) To investigate the ability of primary HIV-2 isolates to use different 7TM receptors as coreceptors.

4) To study the coreceptor requirements for HIV-2 infection of PBMC *in vitro.*

5) To investigate the cellular tropism in HIV-2 infection of the CNS.

PATIENT MATERIAL

HIV-2 virus isolates

The HIV-2 isolate SBL6669 used in Papers I and II was recovered from a patient of West African origin, suffering from pulmonary disease and with 110 CD4$^+$ cells/µl (7). The isolate has been propagated extensively in phytohaemagglutinin (PHA) stimulated PBMCs from healthy donors.

In Papers III and IV, eight primary HIV-2 isolates originating from Guinea-Bissau, two from the Ivory Coast (1653 and 1654) and one from an individual of Gambian origin (6669) were studied. Isolates 1816 and 2300 were sequentially obtained samples from one individual, collected with six months interval. The isolates were obtained by cocultivation of PBMCs from HIV-2 infected individuals with PBMCs from healthy blood donors as previously described (8). The biological phenotype of each isolate were previously determined by cocultivation of infected PBMC with CEM, Jurkat-tat and U937 clone 2 cell lines (8). The primary isolates had all been passaged in human PBMC.

Bone marrow lymphocytes

In Paper II, bone marrow lymphocytes for construction of an antibody cDNA library were obtained from a 50-year-old Caucasian male infected by HIV-2 in Africa. At the time of donation, he was asymptomatic, with normal CD4 cell counts and not receiving antiretroviral therapy.

Brain tissue samples

Human brain tissue samples were collected during a large population study in Côte d'Ivoire in 1991 (139). Autopsy brain samples showing HIV associated cerebral pathology were obtained for this study from six HIV-2 and two dually HIV-1/HIV-2 infected patients who had died of HIV/AIDS. The HIV status of patients had been serologically determined by whole virus enzyme-linked immunosorbent assay (ELISA; Genetic Systems, Seattle, Washington, USA) with supplemental testing of repeatedly positive samples using Pepti-LAV 1-2 (Diagnostics Pasteur, Paris, France).

METHODS

This is an overview of the methods used in the studies included in this thesis. More detailed information on experimental procedures can be found in respective paper in the appendix.

Peptide synthesis and immunisations (Paper I)

Forty-four peptides, nine to thirty-seven amino acids long, corresponding to the previously shown neutralising V3 region in gp125 of HIV-2, were synthesised according to the solid-phase multiple peptide method (108). T-Boc chemistry was used, in which the amino group is protected by an acid labile tert-butyloxycarbonyl group, which is removed before each coupling by treatment with triflouroacetic acid. The peptide amino acid sequences were derived from the molecular clone ISY of the HIV-2 isolate SBL6669 (82).

Peptide ELISA (Papers I and II)

Peptide ELISA was performed with each peptide to investigate the reactivity against a panel of human anti-HIV-2 sera, against previously described mouse Mabs (28) and against its homologous hyperimmune guinea pig serum. Samples that displayed values above the mean optical density at 490 nm of negative human sera or preimmunisation guinea pig sera +3 standard deviations were considered to be positive. The guinea pig sera were serially diluted with a dilution factor of ten, and the last positive dilution step was scored as a measure of the potency of each serum to recognise its homologous peptide.

Western blot (Paper I)

Hyperimmune guinea pig sera were tested for reactivity against SBL6669 virion derived gp125 by Western blot analysis, which was performed as previously described (233). Preimmunisation guinea pig sera as well as HIV-2 positive and HIV-2 negative human sera were used as controls.

50% Tissue Culture Infectious Dose (TCID$_{50}$) titrations (Papers I-IV)

TCID$_{50}$ titrations were performed essentially as previously described (29). In brief, frozen supernatants from HIV-2 infected PBMC cultures were thawed and serially diluted in five-fold steps and used to infect 10^5 PHA-stimulated PBMC in five replicate wells in 96-well microtiter plates. Cell culture supernatants were removed and replaced with fresh medium on day 1, 4 (Papers I-IV) and 8 (Paper IV) post infection (p.i.) and the cultures were terminated

on day 7 (Papers I-III) or day 14 (Paper IV) p.i. HIV-2 antigen in supernatants was detected by an in-house HIV-2 capture ELISA (220), and $TCID_{50}$ values were obtained with Reed-Muench calculations.

Neutralisation assay (Papers I and II)

The neutralisation assays in Papers I and II were performed essentially as previously described (28). Fifty $TCID_{50}$ of the HIV-2 isolate SBL6669 was incubated for one hour (Paper I) or two hours (Paper II) with serial dilutions of guinea pig sera (Paper I) or Fab (Paper II). PHA stimulated PBMC were added and the cultures were maintained for one week, with exchange of medium on days one and four p.i. Productive HIV-2 infection was detected by a modified in-house HIV-2 antigen capture ELISA (220). The neutralisation titre was defined as the last dilution step that showed an 80% reduction or more of optical density at 490 nm.

Computer modelling and prediction calculations (Paper I)

Prediction of the three dimensional peptide structures of peptides A43-29 and A68-14 was performed using the Biased Probability Monte Carlo (BPMC) algorithm (2) delivered by ICM Molsoft LLC (3) (http://www.molsoft.com). The resulting models were analyzed and visualized on a Silicon Graphics work station, using the Sybyl 6.3 program package (TRIPOS Associates, 1996) and the Setor program (76).

Generation of recombinant human antibody Fab fragments by phage display (Paper II)

Human heavy (Fd) and light chain cDNAs were PCR-amplified and cloned into the pComb3 vector as previously described (19, 36). Fab expressing phage were recovered from transformed *E. coli* XL-1 Blue cells that had been infected with VCSM13 helper phage. For the selection of HIV-2 gp125 binding clones the phage library was panned against biotinylated affinity purified gp125, and bound phage were rescued with streptavidin coated beads (199). After elution at low pH, phage were amplified by infection of XL1-Blue cells followed by superinfection with helper phage. After the last round of panning, no helper phage was added and phagemid DNA was prepared. The cpIII gene fragment was excised from the phagemid DNA, and soluble Fab fragments were expressed and screened for reactivity against gp125 by ELISA. Selected Fabs were affinity purified and their concentration was determined. The Fabs were analysed for their antigen specificities by ELISA against recombinant gp125 or peptides corresponding to different regions in gp125 and gp36. They were also tested for their neutralising capacity against SBL6669, K135 and

SIVsm. In addition, the complementarity determining region 3 (CDR3) of the heavy chain of the selected Fabs was sequenced to establish their unique identity.

MT-2 assay (Paper III)

MT-2 cells were tested both by cell-free infection and by cocultivation with infected PBMC. Cultures were maintained for at least 21 days, they were split and monitored for HIV-2 antigen production twice a week and visually inspected daily for cytopathic effects. (123)

Determination of coreceptor usage (Papers III and IV)

Two different indicator cell line systems were employed; the human glioma U87.CD4 cell line, stably expressing CD4 and one of the CCR1, CCR2b, CCR3, CCR5 or CXCR4 chemokine receptors (Paper III and IV) (30, 67) and the GHOST(3) cell line expressing CCR5, CXCR4, BOB or Bonzo in conjunction with human CD4 and engineered to express green fluorescent protein (GFP) upon infection with HIV-1, HIV-2 or SIV (Paper III). Infection of the U87.CD4 cells was performed by cocultivation with infected PBMC or by cell-free infection with 1000-2000 $TCID_{50}$ of cell culture supernatant from infected PBMC. The cultures were followed for 10 days, inspection for syncytium formation was performed daily and the cultures were continuously monitored for HIV-2 antigen production in an in-house HIV-2 antigen capture ELISA (220). The GHOST(3) cells were infected only by cell-free virus, essentially as described above, and were in addition monitored by fluorescence microscopy for GFP expression induced by HIV-2 infection.

Detection of BOB mRNA (Paper III)

Detection of BOB mRNA in cell lines was attempted both by Northern blot and by reverse transcriptase-polymerase chain reaction (RT-PCR). Northern blot analysis was carried out with total RNA extracted from each cell line, and probed with full length BOB cDNA labelled with ^{32}P. Equivalent levels of mRNA among the samples were verified by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probing. For RT-PCR, total RNA isolated from each cell line was used for cDNA synthesis with random hexamer primers, which was subsequently used as template for PCR amplification with BOB specific primers. GAPDH specific primers were used as internal controls and parallel cDNA synthesis reactions without the RT enzyme were uniformly negative.

Immunohistochemical detection of productive HIV-2 infection in autopsy brain tissue (Paper V)

Immunohistochemical reactivity of primary anti-HIV-2 or anti-SIV antibodies to HIV-2 infected H9 cells was tested using the DAKO Envision™ System, according to the manufacturers instructions without major modifications. The method involved antigen retrieval by microwaving in 0.01M sodium citrate buffer (pH6.0). Bound primary antibody was detected with a secondary antibody/alkaline phosphatase-conjugated polymer and visualised using 5-Bromo-4-Chloro-3-Indoxyl Phosphate and Nitro Blue Tetrazolium Chloride (BCIP/NBT) (DAKO). The optimal dilution was determined for primary antibodies with strong reactivity. Staining of H9 cells was also performed using DAKO Chem Mate reagents, as described below. Single and double stainings of formalin fixed, wax embedded brain tissue sections were performed using DAKO ChemMate™ reagents and the DAKO TechMate™ automated slide processing system, following the manufacturers instructions. It is a labelled streptavidin-biotin (LSAB) method, which uses horse radish peroxidase (HRP) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) for the first primary antibody and, in double stainings, alkaline phosphatase and Fast Blue for the second primary antibody.

RESULTS AND DISCUSSION

Fine characterisation of a V3-region neutralising epitope in HIV-2 gp125

Although it is debated which relative role the humoral immune response might play in the control of HIV infection, it is conceivable that the induction of broadly neutralising antibodies would be a beneficial property of a future HIV vaccine. An important task in the development of a vaccine capable to induce a potent neutralising antibody response is to thoroughly define the structures to which the neutralising antibodies bind. A major obstacle in the development of an HIV vaccine that induces broadly cross-clade neutralising antibodies is the great variability the Env glycoproteins. It is therefore desirable to identify conserved immunogenic structures within the Env glycoproteins to which neutralising antibodies can bind. It was early found that the V3 loop of HIV-1 represented a linear immunogenic site, to which neutralising antibodies were directed, and it was termed the "principal neutralising determinant". Despite the variability of this region, the tip of the loop represents a relatively conserved sequence. However, later studies reported that primary HIV-1 isolates were generally less sensitive to anti-V3 Mabs than TCLA isolates, and that the exposure of the V3 loop in the trimeric Env complexes on the virion surface of primary isolates was reduced (reviewed in (80)). The V3 loop of HIV-2 has also been identified as an important site for neutralising antibodies (27, 28, 144, 149). In contrast to HIV-1, immune sera from guinea pigs immunised with HIV-2 V3 peptides were capable to neutralise many primary HIV-2 isolates (28). It should be recognised in this context that the V3 loop of HIV-2 is significantly less variable than the V3 loop of HIV-1 (60, 171, 201), and therefore may be less capable to escape from the immune response. It is also possible that the V3 loop of primary HIV-2 isolates is better exposed on the virion surface than its HIV-1 counterpart.

In paper I, the aim was to further characterise the immunogenic sites in the V3-loop of HIV-2 important for eliciting neutralising antibodies, and to study in more detail the importance of different V3 peptide configurations for their ability to elicit anti-HIV-2 neutralising antibodies in a guinea pigs. We synthesised a panel of peptides, ranging from nine- to 27-mer, with small variations in sequence, representing the central and C-terminal part of the V3 region from the HIV-2 isolate SBL6669 molecular clone ISY (aa 311-341) (Fig. 6). All peptides, except one (A68-14) with extensive alanine substitutions, were recognised in ELISA by a panel of 14 human anti-HIV-2 sera. Further, the peptides were tested for reactivity with seven previously described mouse Mabs raised against a mixture of two overlapping peptides, representing aa

311-326 and aa 322-337 of SBL6669-ISY, respectively, (28), all, except Mab 4B9, neutralising against SBL6669. The Mab 3C4 was previously mapped to the FHS (aa 315-317) and WCR (aa 329-331) motifs. The relative inability of 3C4 to recognize the peptides used in this study, while distinctly recognising the two immunisation peptides used for raising the Mabs, further highlights the fine specificity of this Mab.
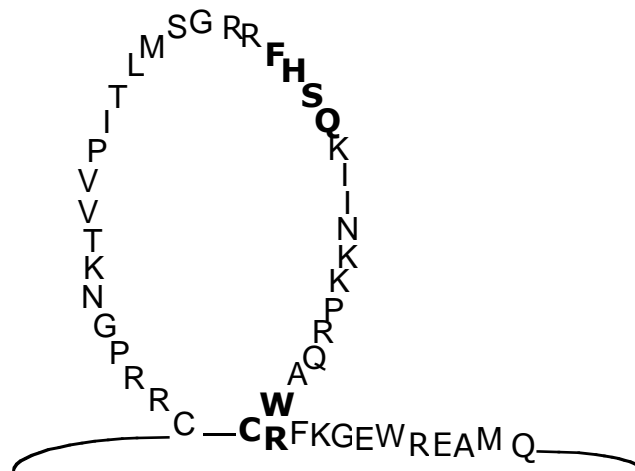

Fig. 6. Schematic representation of the V3 loop of the molecular clone SBL6669-ISY. The two highly conserved motifs FHSQ and WCR, previously shown to be important binding sites for neutralising antibodies (28), are shown in bold.

The hyperimmune sera obtained by immunisation with each peptide were tested for neutralising capacity against the homologous virus isolate SBL6669. Neutralising guinea pig sera, with titres ranging from 40 to 320, were obtained by immunisation with 15-mer or longer peptides of wild type-sequence predominantly, and all neutralising sera reacted with virion derived gp125 in an in-house Western blot. The central part of V3, including the conserved FHSQ motif, appeared to be crucial for induction of neutralising antibodies.

Glycine or alanine substitutions between the two previously identified neutralizing domains FHSQ and WCR abrogated the induction of neutralising antibodies, while a peptide with a deletion of five residues in the same region retained some capacity to induce neutralising antibodies. Attempts to increase the immunogenicity of the peptides, by adding N- and C-terminal lysine "tails" and by the N-terminal addition of a known HIV-1 T-helper epitope (64) or its HIV-2 homologue, were not successful.

In an attempt to analyse the three dimensional structure of the peptides used for immunisation, prediction of the peptide folding was performed using the BPMC computer modeling simulation system. The size of the peptides and the lack of internal disulphide bonds made them difficult to predict. However, we were able to obtain reliable models of two of the peptides, A43-29 (SGRRFHSQKIINKKPRQAWC), representing the unmodified central and C-terminal part the SBL6669-ISY V3 region, and A68-14 (AARRFHSQAAAAARQAWCRA), which covers the same region as A43-29 but is slightly longer and contains alanine substitutions at positions 311-312, 319-325 and 332. A43-29 elicited neutralising antibodies against SBL6669 in guinea pigs, whereas A68-14 did not. Comparison of the predicted three-dimensional structures of the two peptides revealed that in the wild-type peptide, A43-29, the Phe-315, His-316, Trp-329 and Cys-330 were potential participants in a conformational epitope, recognised by neutralising anti-HIV-2 antibodies, while the mutations in A68-14 appeared to cause a conformational alteration of the C-terminal region which resulted in the disintegration of this epitope.

Thus, in Paper I, by immunising guinea pigs with peptides representing the central and C-terminal regions of the HIV-2 V3 loop, we found that even subtle changes in the sequence and length of peptides resulted in major differences in the ability to elicit HIV-2 neutralising antibodies. This is probably explained by the fact that the folding of a peptide into a three dimensional structure is very complex, and therefore, an addition, deletion or substitution of a single residue may radically alter many of its inherent properties, including the ability to be recognised by a specific antibody. Nevertheless, neutralising guinea pig sera were elicited by 17 of the 44 HIV-2 V3 peptides tested in this study, showing the potency of this Env region for induction of neutralising antibodies. The FHSQ motif appeared crucial in eliciting neutralising antibodies, except in one peptide, but appeared to depend on down-stream residues. Computer modelling revealed that the F-315, H-316, W-329 and C-330 likely participate in the formation of a conformational epitope. The ideal peptide for inclusion in a possible future HIV-2 vaccine should therefore be one that spans the central and C-terminal parts of the HIV-2 V3 loop. The central part of the HIV-2 V3 region has also been shown in another study to contain a neutralising epitope (149). However, it remains to be established whether these peptides can give rise to antibodies with broad specificity, capable to neutralise divergent primary HIV-2 isolates, or if they are isolate specific.

Generation of human neutralising HIV-2 gp125 specific Fab fragments by phage display

Although the role of antibody in controlling viral disease is controversial, its potential to prevent infection is well documented. For HIV, studies in animal models have provided evidence that passive transfer of antibody may be protective. Severe combined immunodeficient mice populated with human PBMC (hu-PBL-SCID mice) were protected from infection with a primary HIV-1 isolate after receiving a potent neutralising Mab, even when given several hours after viral challenge (88). In the HIV/SIV chimeric virus macaque model (SHIV), protection against intravenuous as well as intravaginal challenge was afforded by infusion with neutralising anti-HIV antibody (143, 211). Thus, antibody of the right specificity and sufficient concentration seem to be potent in preventing HIV infection, although it remains to be shown in man. Passive immunisation with potently neutralising antibody might therefore be a means of preventing for example vertical transmission and infection after accidental exposure.

In Paper II, we explored the possibility of generating recombinant human antibody Fab fragments (Fabs) with neutralising capacity against HIV-2 through phage display selection from a combinatorial library. The combinatorial library/phage display technique has several advantages; (i) it allows the rapid generation of large numbers of human antibodies (ii) the antibodies (Fab fragments) are produced in *E. coli*, and can readily be sequenced (iii) functional differences can be correlated to the amino acid sequence of the antigen binding domains. It has been successfully used to generate Fab fragments to a broad range of antigens. Of particular interest, the Fab fragment b12 (18), which recognises a conformational epitope that overlaps the CD4 binding site of the HIV-1 envelope, has shown potent and broad neutralising capacity against primary HIV-1 isolates (37).

A cDNA library consisting of the genes for the antibody light chain and first two heavy chain domains was constructed from bone marrow mRNA of an asymptomatic HIV-2 seropositive individual and displayed on the surface of phage. Phage expressing Fabs specific for HIV-2 were selected by "panning" against virion derived gp125 and converted to soluble Fab fragments. The ten best binding Fabs were selected for characterisation of neutralising capacity. Six Fabs, by sequencing of the heavy chain CDR3 regions shown to represent separate clones, neutralised the SBL6669 isolate with varying efficiency. One Fab, 88, showed strong neutralising capacity against SBL6669 (from which the gp125 used for

panning was obtained), but also neutralised the heterologous strain K135. The other five Fabs neutralised SBL6669 in an isolate specific fashion.

The virus isolated from the bone marrow donor was not genetically closely related to SBL6669, showing only 77% sequence identity with SBL6669 in the V3 region, and therefore, our selection method should theoretically favour the isolation of broadly reactive Fabs. Still, cross-neutralisation against the heterologous HIV-2 isolate K135, with 74% V3 sequence identity to SBL6669, was only detected with one clone, Fab 88. It may be that epitopes on gp125 of SBL6669 recognised by our Fabs are not well exposed on other primary HIV-2 isolates, thus explaining the poor cross-neutralisation. A better strategy to obtain broadly neutralising Fabs may be to select against the native polymeric HIV-2 Env expressed on the surface of infected cells.

None of the neutralising Fabs reacted with linear peptides, representing different parts of gp125, though such specificities were present in the donor serum. Instead, these Fabs are likely to interact with conformational epitopes on gp125. Furthermore, their neutralising capacity indicates that they recognise structures exposed in the trimeric Env complexes on the virion surface. They may therefore be useful in structure assessment of immunogens developed for vaccine studies. For use in passive immunisation, they should be expressed as whole IgG, to increase their half-life in the organism.

Primary HIV-2 isolates frequently use CCR5 but show promiscuity in coreceptor usage

Even though HIV-1 and HIV-2 share the same primary receptor, CD4, and have a similar host range, differences exist in their ability to infect certain cells. For example, HIV-2 is able to infect certain human and non-human cells *in vitro* that were non-susceptible to HIV-1 (49). The discovery of HIV coreceptors (9, 46, 67, 70, 71, 79) opened a new possible explanation for these observations.

The development of indicator cell lines expressing CD4 and 7TM receptors opened the way for convenient and reliable determination of the coreceptor usage of individual virus isolates. While the knowledge about HIV-1 coreceptor usage rapidly accumulated, less was reported about the coreceptor usage of HIV-2. HIV-2 envelope glycoproteins had been shown to make CD4 dependent interactions with CCR5, leading to fusion and viral entry (105), while CXCR4, CCR3 and CX_3CR1 (V28) could, with various efficiency, be used by some isolates

in the absence of CD4 (74, 186). Others had reported that TCLA and primary HIV-2 isolates showed a considerable promiscuity in coreceptor usage (34, 100, 103, 148, 217). However, differences in the patterns of coreceptor usage reported existed, and only a limited number of isolates were tested in each study.

In Paper III, we tested the coreceptor usage of eleven primary HIV-2 isolates, previously characterised for biological phenotype (rapid/high or slow/low) and with known V3 sequences. We also tested them for MT-2 tropism. Typical syncytia and HIV-2 antigen production a few days after infection of MT-2 cells were induced only by three isolates; these isolates would therefore be classified as SI. The majority of the remaining isolates showed an atypical cytopathic effect, without evident syncytia, but with single cell killing accompanied by late virus production 10 to 25 day post infection (p.i.) (Fig. 7). Similar observations have earlier been made for HIV-2 (217) and SIV (43). Two of the SI isolates (1010 and 6669) were recovered from AIDS patients, while the third, 2297, originated from a patient with milder disease. Moreover, of the four rapid/high isolates, classified by their ability to infect and replicate in the established CEM and U937-2 cell lines, only two (1010 and 6669) induced typical syncytia in MT-2 cells. Conversely, isolate 2297 did not replicate in CEM or U937-2 cells but replicated and induced syncytia in MT-2 cells. These results indicate that HIV-2, unlike HIV-1, may enter MT-2 cells through more than one pathway.
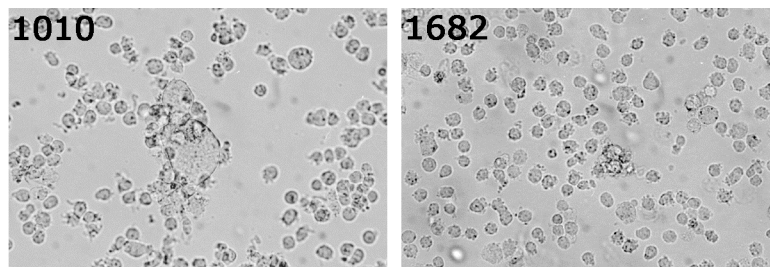

Fig. 7. MT-2 cells infected with the primary HIV-2 isolates 1010 and 1682. 1010 replicated readily and induced typical syncytia, while 1682 replicated slowly and did not induce syncytia.

Next, we tested the coreceptor usage of the same HIV-2 isolates by using the U87 indicator cell lines, stably expressing CD4 and each of the putative coreceptors CCR1, CCR2b, CCR3, CCR5 and CXCR4 (Fig. 8). Infections were carried out both by cocultivation with infected PBMC and with cell-free virus, and syncytium formation and HIV-2 antigen production were monitored. All isolates, except 6669, efficiently induced syncytia and antigen production in

CCR5 expressing cells, whereas only two isolates, the SI isolates 1010 and 6669, used CXCR4. This CXCR4 usage pattern is in agreement with two previous studies (103, 217), but at variance with another (148), in which all HIV-2 isolates were found to use CXCR4. Moreover, the frequent CCR5 usage reported here is supported by two of these studies (103, 148) whereas Sol and coworkers found a relative incapacity by HIV-2 to use CCR5 (217). Whether these differences are due to the collection of HIV-2 isolates used in the different studies or to differences in indicator cell systems remains to be clarified.

The majority of the isolates induced syncytia in CCR1 and CCR3 expressing cells, particularly upon cocultivation with infected PBMC, whereas syncytium formation in CCR2b expressing cells was more rare. None of the isolates replicated in the parental U87.CD4 cells, but 7 of the 11 isolates induced syncytia after cocultivation with infected PBMC. Cocultivation generally induced more efficient syncytium formation than inoculation with cell-free virus, although exceptions were observed. The reason for this is not clear, but may simply reflect a virus dose dependency, assuming that the infected PBMC cultures used for cocultivation produce amounts of virus larger than the amounts used for cell-free infection. Another possible explanation is that cell fusion between infected PBMC and target cells, potentially involving additional cell surface molecules, may be more efficient than only cell-free infection.
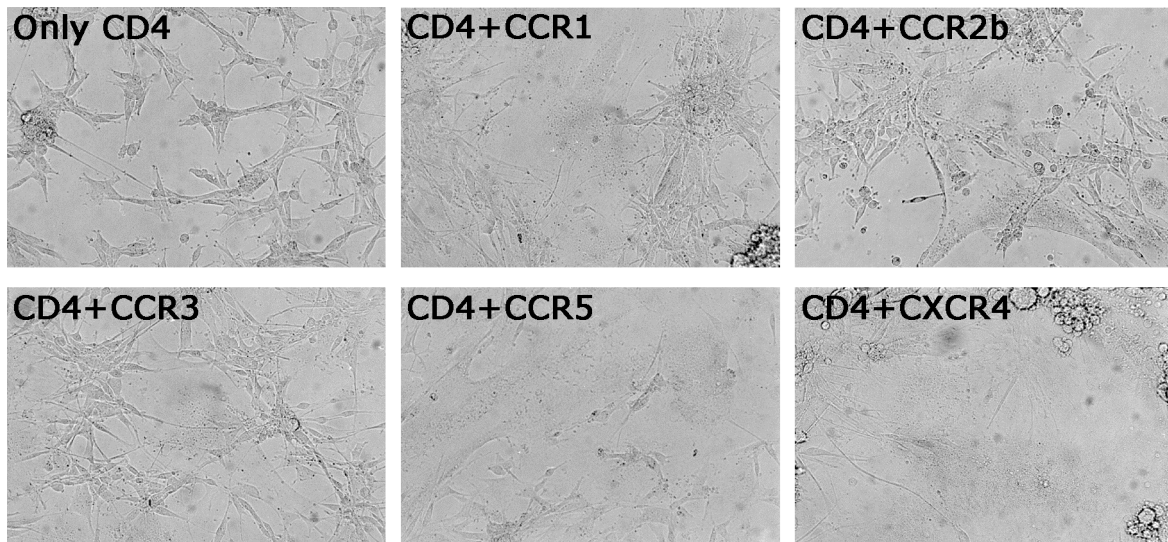

Fig. 8. Infection of U87 cells expressing CD4 and chemokine receptors by the primary HIV-2 isolate 1010. Massive syncytia were formed in cultures with cells expressing CCR1, CCR2b, CCR3, CCR5 or CXCR4. No syncytia were observed in the parental cell line, expressing only CD4.

During progress of the study, two new 7TM receptors, BOB (GPR15) and Bonzo (STRL 33), were discovered and shown to function as coreceptors for selected SIV, HIV-2 and HIV-1 strains (68). Also, since several isolates induced syncytia in the U87.CD4 parental cells, known to endogenously express low levels of Bonzo (68), it was of interest to test the coreceptor usage of these isolates in another indicator cell line system, devoid of endogenous Bonzo expression. Therefore, we tested our isolates for use of these receptors in GHOST(3) osteosarcoma cells expressing CD4 and the either of the CCR5, CXCR4, BOB and Bonzo, and engineered to express GFP upon HIV infection. The pattern of CCR5 and CXCR4 usage confirmed our results in the U87 cells. Five HIV-2 isolates used BOB in addition to CCR5, whereas only one isolate efficiently used Bonzo. However, it can not be excluded that there was some background fluorescence/antigen production for CXCR4 using isolates (1010 and 6669) due to endogenous CXCR4 expression in the GHOST(3) cells.

Since infection and replication in MT-2 cells in our panel of primary HIV-2 isolates was not restricted to CXCR4 usage, we investigated expression of alternative HIV-2 coreceptors in MT-2 cells. The observation that the isolate with the most prominent ability to use BOB, isolate 2297, was also the only non-CXCR4 using isolate able to induce syncytia in MT-2 cells prompted us to investigate BOB mRNA expression in MT-2 cells. Indeed, low levels of BOB mRNA could be detected in MT-2 cells by RT-PCR, but not by Northern blot analysis. In contrast to MT-2 cells, neither CEM nor U937-2 cells expressed BOB mRNA at levels detectable by RT-PCR or Northern blot. These findings, coupled with the relatively frequent ability of our isolates to use BOB, indicate that BOB usage may be one of the pathways for HIV-2 infection of MT-2 cells. If this assumption is correct, it could explain why phenotypically distinct groups of HIV-2 could not be distinguished clearly in MT-2 cells, and why for the HIV-2 isolates tested there was no clear correlation between MT-2 tropism and replicative capacity in CEM and U937-2 cells.

The role of the V3 region as a determinant for coreceptor usage of HIV-1 suggests the possibility for a similar function of the HIV-2 V3 region. Among the HIV-2 isolates tested in this study, mutations to positively charged amino acids at positions 314 and/or 313 were associated with CXCR4 usage, although the number of isolates was too limited to establish a definitive correlation.

The *in vivo* relevance of this promiscuity in coreceptor usage is unclear. The lower pathogenicity of HIV-2, as compared to HIV-1, is difficult to explain by a broader coreceptor

usage. Hence, other viral properties are strongly suggested to be responsible for determining the difference in virulence between these two viruses. Moreover, the observation that coreceptor usage by HIV-1 often broadens with disease progression (55, 204) does not seem to apply to HIV-2, since in our material all isolates, whether from AIDS patients or asymptomatics, were equally promiscuous. However, as for HIV-1, CXCR4 usage appears to be most frequent in late stage infection.

CCR5 or CXCR4 are required for efficient HIV-2 infection of PBMC *in vitro*

In Paper III, we found that most primary HIV-2 isolates used CCR5 and that viruses able to use CXCR4 can be isolated from some patients with late stage disease, but also that the majority of HIV-2 isolates appeared to be promiscuous in coreceptor usage. However, the *in vivo* relevance of this *in vitro* promiscuity in coreceptor usage remains unclear. The importance of CCR5 as the primary coreceptor for HIV-1 host cell entry is supported by clinical data linked to a polymorphism, a 32 bp deletion (Δ32), within the CCR5 gene. Homozygosity for Δ32 (CCR5-/-), which is found in approximately 1% of the Caucasian population, results in lack of CCR5 cell surface expression and is associated with resistance to HIV-1 infection (66, 136, 198). Since this polymorphism is not prevalent in West Africa, where HIV-2 is endemic, the role of CCR5 in HIV-2 transmission and disease progression cannot be determined in a similar fashion.

The experimental systems used for testing coreceptor usage involve cell lines engineered to express high levels of CD4 and the coreceptor, and may not accurately reflect the situation in primary cells. In Paper IV, to address the issue of coreceptor requirements for HIV-2 infection of primary cells, we set out to evaluate the relative importance of CCR5 and CXCR4 for HIV-2 infection of activated PBMC. The requirement for CCR5 in HIV-2 *in vitro* infection of PBMC was determined by comparing $TCID_{50}$ of ten primary HIV-2 isolates previously characterised for coreceptor usage in CCR5 +/+ vs. CCR5 -/- PBMC. These cells were obtained from three blood donors, one with CCR5 wild-type alleles (CCR5 +/+) and two with a homozygous CCR5Δ32 genotype (CCR5 -/- A and CCR5 -/- B). Isolates incapable of using CXCR4 showed a clear preference for infection of CCR5 +/+ as compared to CCR5 -/- PBMC, with a difference in $TCID_{50}$ ranging from about one to three logs. However, donor-dependent variation was observed, since CCR5 -/- PBMC from donor B were consistently more sensitive to infection than CCR5 -/- PBMC from donor A, which were completely resistant to infection with four isolates. In contrast, isolates able to use CXCR4 (1010 and

6669) displayed only minor differences in $TCID_{50}$ between CCR5 +/+ and CCR5 -/- PBMC. Thus, even though CCR5 and CXCR4 independent infection could be observed with high titer HIV-2 isolates, efficient infection of PBMC was highly dependent on the ability to utilise either CCR5 or CXCR4.

Comparison of growth kinetics revealed that the replication of non-CXCR4 using isolates was markedly delayed in CCR5 -/- vs. CCR5 +/+ PBMC. In contrast, the two isolates capable to use CXCR4 exhibited almost identical growth kinetics in cells from both donor genotypes. The growth rate of the CXCR4-using isolates was accelerated and reached higher magnitude during the first week of infection in both cells types as compared with the replication of non-CXCR4 using isolates. Surprisingly, three of four non-CXCR4 using isolates finally replicated to higher levels in CCR5 -/- than in parallel CCR5 +/+ PBMC cultures. These results show that CXCR4 independent replication of primary HIV-2 isolates in activated PBMC is significantly faster in the presence of cell surface CCR5. However, late replication to high levels in CCR5 deficient PBMC occurs with some isolates.

Passage of virus isolates in CCR5 -/- PBMC revealed that the ability to replicate in these cells was not due to the presence of minor previously undetected viral variants able to use CXCR4, since the passaged virus was still unable to replicate in U87.CD4 CXCR4 cells. The passaged virus also showed a similar delay in replication in CCR5 -/- PBMC as unpassaged virus. In addition, all CCR5 using isolates tested retained the ability to use CCR5 after passage in CCR5 -/- PBMC, showing that these isolates included viral variants with truly multitropic envelopes.

Taken together, our results suggest that one or several coreceptors other than CCR5 and CXCR4 can be used by HIV-2 to infect PBMC. However, CCR5 and CXCR4 independent infection of PBMC was inefficient, although late occurring replication to high levels was observed for some isolates. These results emphasise the importance of CCR5 and CXCR4 as the major coreceptors for HIV-2 infection of activated PBMC *in vitro*, despite the promiscuity in coreceptor usage exhibited by HIV-2 in more artificial experimental settings. Initially low cell surface density of the alternative coreceptor followed by a slowly upregulated expression may explain our observation that some non-CXCR4 using HIV-2 isolates after initial slow replication in CCR5 deficient PBMC reach elevated levels of replication.

In line with our observations, emerging data suggests that the role of the alternative coreceptors *in vivo* or in primary cells is limited. Using a coreceptor-specific inhibitor approach, Zhang *et al.* recently found that infection of PBMC *in vitro* by most HIV-2 isolates was mediated via entry through CCR5 and/or CXCR4 (245). The same conclusion was made for a pediatric HIV-1 isolate able to use Bonzo (247). Interestingly, a report on HIV-1 infection of monocyte derived macrophages by isolates with promiscuous coreceptor usage revealed that infection was exclusively mediated by CCR5 and CXCR4 (214). Furthermore, no evidence was found for a contribution of any additional coreceptors to viral pathogenicity or viral replication in lymphoid tissue cultured *ex vivo*, neither in HIV-1 (207) nor in HIV-2 (206) infection. In the SIV_{mac} model, BOB (GPR15) has been shown to have a minor influence on *in vivo* replication and pathogenicity (182).

However, it cannot be excluded that alternative coreceptors may play a role in HIV infection of different tissue compartments, such as the brain and mucosal surfaces. Such coreceptors may be expressed at higher levels in these compartments, potentially enabling them to function as relevant coreceptors for HIV *in vivo*. Also, strong selective pressure caused by the use of effective CCR5 and CXCR4 blocking agents may select for virus variants with preference for alternative coreceptors. Interestingly, SIV from red-capped mangabeys used CCR2b as its major coreceptor (42), most likely as a result of a frequent inactivating CCR5 polymorphism among red-capped mangabeys. Therefore, the use of alternative coreceptors should remain a serious consideration in the development of HIV entry inhibitors.

Productive HIV-2 infection in the CNS is restricted to macrophages/microglia

HIV-2 infection of the brain has so far been poorly studied. For HIV-1 the main target cells are CD4$^+$ macrophages/microglia (122, 229, 234). HIV-2 has the capacity to use a broader range of coreceptors than HIV-1 *in vitro* (34, 100, 103, 149, 156, 217), and is less dependent on CD4 for infection (185). The aim of Paper V was to detect productive HIV-2 infection in encephalitic brain specimens, collected in Côte d'Ivoire (139), from HIV-2 or HIV-1/HIV-2 dually seropositive individuals who died in AIDS, and to investigate whether HIV-2 has an expanded tropism for brain cells *in vivo*, as compared to HIV-1. The only report, until now, describing productive HIV-2 infection in the brain, to our knowledge, was a case report, which did not address the question of infected cell types (72). In the study from which we obtained the brain samples, it was observed that HIVE was more prevalent among individuals infected with HIV-2 than HIV-1 (139). Encephalopathy as the primary AIDS defining illness

has also been reported to be higher in HIV-2 than in HIV-1 infected patients (2.8% vs. 0.8%) in Portugal (153).

Detection of components produced late in the HIV life cycle, such as Gag and Env proteins, is a sign of productive infection of the cell, whereas regulatory or accessory proteins, such as Tat Rev or Nef, can be detected in latently infected cells (33, 120). To facilitate detection of productive HIV-2 infection in the paraffin embedded brain tissue we screened Mabs against Gag proteins and regulatory proteins for HIV-2 reactivity. One anti-HIV-2 and 25 anti-SIV Mabs (due to the general lack of available anti-HIV-2 Mabs) were tested on formalin fixed and paraffin embedded chronically HIV-2 infected H9 cells by an immunohistochemical assay. One Mab, SIV27f, directed to SIV p27, was identified, which showed strong cytoplasmic and membrane staining of HIV-2 infected, but not HIV-1 infected or uninfected cells. No Mab directed against any regulatory or accessory protein tested showed significant reactivity against the HIV-2 infected cells.

CNS specimens from four of six HIV-2 seropositive and one of two HIV-1/HIV-2 dually seropositive individuals with HIVE were positively immunostained for HIV-2 p26. Of the two dually HIV-1/HIV-2 infected patients with HIVE, one, 24D, stained positive for HIV-2 but not for HIV-1, while the other, 240A, showed positive staining for HIV-1 p24 but not for HIV-2. p26 positive cells were found perivascularly, in microglial nodule-like structures and in ramified parenchymal cells. In addition, p26 positive MNGC were frequently seen, typically found around small blood vessels, but also scattered in the neuropil and in microglial nodules. Stainings with SIV27f on brain sections from three HIV-1 seropositive patients, two with and one without HIVE, as well as sections from two HIV seronegative patients were uniformly negative. Staining with an isotype matched control antibody produced consistently negative results on all brain sections.

To determine the phenotype of productively HIV-2 infected brain cells we used antibodies against cell markers for monocytes/macrophages, leukocytes and astrocytes. The anti-CD45/Leukocyte common antigen (LCA) antibody stained infiltrating lymphocytes strongly and, more weakly, macrophages and activated microglia. Anti-CD68 gave both perivascular macrophages, cells in microglial nodules and parenchymal microglia an intense granular cytoplasmic staining. Intensely glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes were abundant in sections from patients with HIVE, reflecting the astrocytosis that is usually seen in the brains of patients dying with AIDS. In double staining experiments,

colocalised immunoreactivity with HIV-2 p26 was seen for both CD45 and CD68. In fact, we found no HIV-2 p26 positive cells that were negative for CD45 and/or CD68. In contrast, a mutually exclusive pattern of immunoreactivity was seen in HIV-2 p26 and GFAP double stainings. Thus, productive infection was in this patient material restricted to CD45/CD68 positive cells, whereas no signs of other productively infected cell types, including astrocytes, were seen.

In conclusion, we report that HIV-2, like HIV-1 and SIV, is confined to productive infection of microglia and macrophages in the brain. Thus, the observation that encephalitis appears to be more common in HIV-2 infection than in HIV-1 infection cannot be explained by the involvement of more infected cell types in the brain. We were unable to exclude latent infection of astrocytes or other cell types since none of the antibodies we tested could detect early HIV-2 proteins. Productive infection by a laboratory adapted CD4 independent HIV-2 isolate (ROD B) of primary adult and fetal astrocytes *in vitro* has been reported (185), yet in this *in vivo* study we saw no evidence of productive infection of astrocytes. Furthermore, CD4 negative neurons and microvascular endothelial cells express chemokine receptors (CXCR4 and CCR5) that can be used by HIV-2 primary isolates to infect cells *in vitro* (104), and thereby represent possible targets for HIV-2 infection. Yet we did not observe productive infection in these cells types in any of the samples tested.

Indirect mechanisms of neuronal damage need to be invoked to explain HAD since there is no evidence for direct, productive infection of neurons. Apoptosis of neurons and astrocytes is induced by HIV-1 infection *in vitro* (210) and has been shown in autopsy brain tissue from infected patients with AIDS (5, 89, 175, 210). The ability of HIV-1 to induce apoptosis *in vitro* maps to the viral envelope (166), and envelope proteins with a broader coreceptor usage, R5X4, tend to induce apoptosis in primary human brain cultures more frequently than R5 envelope proteins (166). This is interesting since many isolates of HIV-2 have envelope proteins capable of a broad coreceptor usage.

HIV-2 can *in vitro* infect CD4 independently with reasonable efficiency, yet appears to be unable to bypass the use of CD4 *in vivo*, at least in the brain compartment. There may be constraints to CD4 independent infection *in vivo*. One inhibiting factor may be that of neutralising antibodies. It has been shown that CD4 independent infection by both TCLA and primary isolates of HIV-2 is more sensitive to neutralising antibodies and that most HIV-2

patient sera contain high titres of neutralising antibodies to CD4 independent infection but not to CD4 dependent infection (Thomas *et al,* unpublished).

GENERAL CONCLUSIONS

In the studies included in this thesis, we have characterised different biological properties of HIV-2. This is important, not only for the understanding of HIV-2 pathogenesis *per se*, but also because identification of the underlying mechanisms for the differences in pathogenic potential between HIV-1 and HIV-2 may aid in the development of preventive and therapeutic strategies against the HIV-1 pandemic.

By peptide immunisation of guinea pigs, the central and C-terminal part of the V3 region of HIV-2 gp125 was confirmed to be an important target for neutralising antibodies. However, subtle changes in the sequence and length of peptides resulted in major differences in the ability to elicit HIV-2 neutralising antibodies. The conserved F-315, H-316, W-329 and C-330 amino acid residues were suggested to participate in a conformational neutralising epitope. The capacity to elicit antibodies directed to the V3 region of HIV-2 might be desirable in a future HIV-2 vaccine, in contrast to HIV-1, where anti-V3 antibodies appear to be of little clinical relevance.

Production of recombinant human antibody Fab fragments by combinatorial library/phage display was shown to be a suitable method to obtain anti-HIV-2 antibodies with neutralising capacity. Six Fabs that neutralised the homologous strain SBL6669 were obtained, of which one also neutralised a heterologous virus isolate. Future studies should include investigation of the neutralising capacity of these Fabs against divergent primary HIV-2 isolates, mapping of their binding regions in gp125 and testing their protective capacity in a suitable animal model, for example hu-PBL-SCID mice.

Primary HIV-2 isolates were shown to frequently use CCR5, but were often promiscuous in coreceptor usage. Broadening of coreceptor usage was not associated with disease progression, as has been suggested for HIV-1. CXCR4 usage was observed for some isolates recovered from patients with advanced disease, and appeared to correlate with positively charged amino acid residues at positions 314 and/or 313 in the V3 loop. Low level BOB expression in MT-2 cells coupled with promiscuous coreceptor usage among HIV-2 isolates was suggested to account for difficulties in clearly distinguishing distinct phenotypic groups in MT-2 cells. The *in vivo* relevance of HIV-2's broad coreceptor usage *in vitro* remains to be clarified.

CCR5 or CXCR4 were shown to be required for efficient infection of PBMC by primary HIV-2 isolates *in vitro*. However, CCR5 and CXCR4 independent infection of PBMC was observed for the majority of isolates tested, showing that the use of alternative coreceptors should remain a serious consideration in the development of HIV entry inhibitors.

Productive HIV-2 infection in the brain was shown to be restricted to macrophages or microglia. Thus, the broad coreceptor usage and relative CD4 independence of HIV-2 *in vitro* appears to have little influence on the *in vivo* tropism, at least in the brain compartment. Other factors are therefore suggested to account for the higher frequency of encephalopaty observed in HIV-2 than in HIV-1 infection.

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