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Recombinant CXCR4/CCR5 hybrid receptors as tools for studies of HIV-1 receptor usage

Antonsson, Liselotte

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Recombinant CXCR4/CCR5 hybrid receptors as tools for studies of HIV-1 receptor usage

Liselotte Antonsson

LUND UNIVERSITY

Department of Experimental Medical Science
Faculty of Medicine
Sweden

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Faculty Opponent:

Professor Birgitta Åsjö
Center for Research in Virology
The Gade Institute, University of Bergen
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Abstract:
The chemokine receptors CCR5 and CXCR4 are required, together with CD4, for the entry of HIV-1 into target cells. CCR5 using HIV-1 dominates during transmission and the asymptomatic phase of infection. During progression, virus phenotypes with the ability to use CXCR4 emerge in about 50% of the infected individuals. Individuals continuously harbouring CCR5-restricted isolates still progress to AIDS. Differences among CCR5 using isolates, has been found and an evolution towards an altered mode of CCR5 coreceptor use and a reduced sensitivity to inhibition by natural CCR5 ligands has also been described.

With the aim to study interactions of natural ligands and HIV-1 isolates with these chemokine receptors, a set of hybrid CXCR4/CCR5 receptors were constructed. Signalling response to their respective natural ligands, SDF-1 and RANTES were studied and prototypic R5 and X4 isolates (HIV-1_{BaL} and HIV-1_{IIIB}) were tested for their ability to use these chimeric receptors. The results showed that ligands and virus isolates use different receptor epitopes which, in turn, vary between the two receptors.

Further, the evolution of primary HIV-1 isolates was studied. A total of 246 sequential primary HIV-1 isolates were studied. Using our chimeric CXCR4/CCR5 receptors, we showed that R5 isolates from immunosuppressed individuals are distinct from those isolated from individuals with higher CD4+ T-cell counts, with regards to coreceptor usage. The analysis also showed that the ability to utilize chimeric receptors correlated inversely with the sensitivity to RANTES inhibition of infection. The R5 isolates used receptor chimeras to various degrees. Based on these results, the R5 viruses could be subdivided into two groups: the $R5^{narrow}$ phenotype and the $R5^{broad}$ phenotype. The $R5^{narrow}$ phenotype is defined as viruses that use wt CCR5 but no chimeric receptors, whereas viruses using at least one chimeric receptor in addition to CCR5, are designated $R5^{broad}$ viruses.

The mode of coreceptor use by paired plasma and CSF isolates from HIV-1 infected individuals with varying degree of immunodeficiency and neuropathology were studied. The R5 viral phenotypes predominated both in plasma and in CSF. We were able to identify discordant plasma/CSF wt coreceptor use but also, varying R5 viral phenotypes in the paired isolates within individual patients. There were no characteristic patterns of receptor use that could distinguish CSF from plasma isolates. R5 virus use of chimera FC-2 correlated highly with immunosuppression. Efficient chimeric receptor use also correlated, with an increased resistance to inhibition by the CCR5 antagonist TAK-779.

In conclusion, our findings propose that alterations in the mode of CCR5 use may be a key event in R5 virus pathogenesis. We believe that R5 virus ability to utilize these CXCR4/CCR5 chimeric receptors reflects a more flexible and more efficient CCR5 usage, which may include a reduced dependency upon interactions with the N-terminal of the receptor for infection. The findings are important, not only with regards to R5-virus pathogenesis and optimization of emerging treatment with CCR5 antagonists, but also for HIV-infection within the CNS.

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To Peter and Simon,

Liselotte Antonsson
Department of Experimental Medical Science
Faculty of Medicine
Lund University
BMC A12
SE-221 84 Lund
Sweden
Liselotte.Antonsson@med.lu.se

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CONTENTS

ABBREVIATIONS

7TM	7-transmembrane
aa	Amino acid
AC	adenylyl cyclase
ADC	AIDS dementia complex
AIDS	Acquired immuno-deficiency syndrome
AZT	3'-Azido-3'-deoxy-thymidine (zidovudine)
BBB	Blood-brain barrier
B-cell	B-lymphocyte
bp	Base pair
CCL	CC chemokine ligand
CCR5	CC chemokine receptor 5
CD	Cluster of differentiation
CHO	Chinese hamster ovary cells
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T-lymphocyte
CXCR4	CXC chemokine receptor 4
DAG	Diacyl glycerole
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EC_{50}	50% Effective concentration
ECL	Extracellular loop
ELISA	Enzyme-linked immunosorbent assay
GALT	Gut associated lymphatic tissue
GDP	Guanosine diphosphate
gp	Glycoprotein
GPCR	G protein-coupled receptor
G-protein	GTP-binding protein
GTP	Guanosine triphosphate
HAART	Highly active anti-retroviral therapy
HIV	Human immuno-deficiency virus
IC_{50}	50% Inhibitory concentration
ICL	Intracellular loop
IL	Interleukin
IP_3	Inositol triphosphate
kb	Kilobases
kD	KiloDalton
mAb	Monoclonal antibody
MIP	Macrophage inflammatory protein
NK-cell	Natural killer cell
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NSI	Non-syncytium inducing
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PI	Protease Inhibitor
PKC	Protein kinase C
PLC	Phospholipase C
R5	CCR5-using virus
RANTES	Regulated on activation normal T-cell expressed and secreted
RNA	Ribonucleic acid
RT	Reverse transcriptase
T-cell	T-lymphocyte
TM	Transmembrane
SDF	Stromal derived factor
SI	Syncytium inducing
SIV	Simian immuno-deficiency virus
U87	Astroglioma cell line
wt	Wild type
X4	CXCR4-using virus

LIST OF PUBLICATIONS

This thesis is based on the following papers.

Paper I

Molecular mapping of epitopes for interaction of HIV-1 as well as natural ligands with the chemokine receptors, CCR5 and CXCR4. Antonsson, L., Boketoft, Å., Garzino-Demo, A., Olde, B., Owman, C. (2003) AIDS 17, 2571-9.

Paper II

Coevolution of RANTES sensitivity and mode of CCR5 receptor use by human immunodeficiency type 1 of the R5 phenotype. Karlsson, I., Antonsson, L., Shi, Y., Öberg, M., Karlsson, A., Albert, J., Olde, B., Owman, C., Jansson, M., Fenyö E. M., (2004) J Virol. 78, 11807-15.

Paper III

Discordant HIV-1 phenotypes in paired plasma and cerebrospinal fluid samples - clinical implications of varying mode of coreceptor use. Antonsson, L., Karlsson, U., Repits, J., Ljungberg, B., Kidd-Ljunggren, K., Hagberg, L., Svennerholm, B., Jansson, M., Gisslen, M., Owman, C. (2007) *Submitted manuscript*

INTRODUCTION

Brief history

In the early 1980's clusters of cases of *Pneumocystis carinii* pneumonia and Kaposis sarcoma, were observed among previously healthy homosexual men in the United States. These severely immunosuppressed patients were identified and reported by the Centers for Disease Control (CDC) and Gottlieb et al (2, 64) and were eventually recognized as the first cases of acquired immunodeficiency syndrome (AIDS). In 1983 the virus causing this disease was identified as a lentivirus, belonging to the *Retroviridae* family (14, 57). The virus was eventually named Human Immunodeficiency Virus (HIV) in 1986 (35).

In 1996 certain cell membrane receptors were shown to be necessary, in addition to the CD4 molecule, for the virus to enter its target cells (6, 30, 41, 43, 44, 52). These virus coreceptors were chemokine receptors, and members of the superfamily of G protein-coupled receptors (GPCRs). This discovery was the beginning of extensive studies, opening a whole new field of research. The understanding of HIV infection and the pathogenesis of AIDS is essential for the development of new antiretroviral therapies.

G protein-coupled receptors

A family of cell surface receptors

Cell surface receptors facilitate cell communication. Cells need to respond rapidly to changes in their environment. In multicellular organisms, cells also have to coordinate their actions.

The superfamily of GPCRs is the largest family of cell surface receptors and also the largest protein superfamily in the mammalian genome (56). About 2% of the human genome encodes GPCRs. There are about 800 identified human GPCR sequences. These receptors are involved in virtually all physiological processes and approximately 40% of the modern drugs act on GPCRs (21). These receptors transduce signals in response to a variety of stimuli such as peptides, ions, neurotransmitters, hormones, lipids, odorants or even photons. Additionally, the fact that these receptors are found in such diverse organisms as *e.g.* zebrafish, fruit fly *(Drosophila melanogaster)*, a

nematode *(Caenorhabditis elegans)* and plants, further illustrates their crucial importance for most complex living organisms and also the evolutionary success of this signalling mechanism (75).

Common features of GPCRs

GPCRs are highly conserved and recognized by their seven hydrophobic transmembrane spanning helices connected by three hydrophilic intracellular loops (ICL1-3) and three hydrophilic extracellular loops (ECL1-3). The receptor proteins, also called 7-transmembrane receptors (7TM) are composed of about 300-1000 amino acid (aa) residues. Bundle of helices are formed in the cell membrane, with an extracellular amino (N-) terminal and an intracellular carboxy (C-) terminal of variable length (Figure 1). Other common characteristics for these receptors, are their highly conserved cysteines in ECL1 and ECL2, allowing disulfide binding in order to stabilize the receptor structure, the DRY motif in the intracellular side of TM3 and the NPXXY motif in TM7. The C-terminal loop can form a fourth ICL with the cell membrane, through palmitoylation at C-terminal cysteine residues (137).

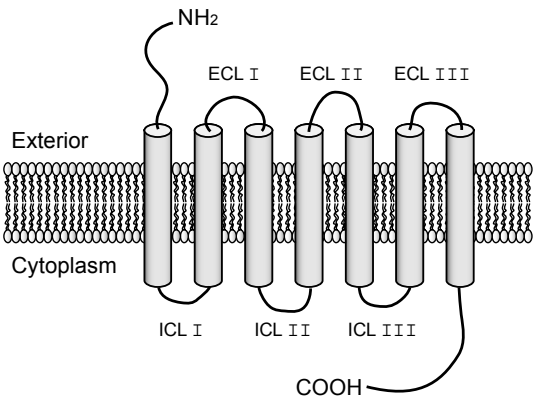

Figure 1. Schematic representation of a GPCR. The seven transmembrane spanning helices are connected by three intracellular loops (ICL1-3) and three extracellular loops (ECL1-3). The bundle of helices is placed in the plasma membrane, with an extracellular N- terminus and an intracellular C- terminus.

Several common characteristics are maintained in the GPCRs, but their ligands differ greatly in size and structure. The size and aa sequence composition in extra-cellular regions also vary between GPCRs.

The human GPCRs were recently classified, based on phylogenetic analysis, into five different groups; glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin, of which the rhodopsin family contains the most members (56). The receptors studied in this research project belong to the rhodopsin family.

Signalling

GPCRs transmit extracellular stimuli into the cell. As their name implies, GPCRs induce their signal through interaction with a G-protein (GTP binding protein). The G-protein is composed of three subunits, α, β and γ. There are four classes of G-protein α-subunit families, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. Each of these G-protein classes, regulate specific classes of effector molecules. $G\alpha_s$ stimulates adenylyl cyclase (AC), $G\alpha_{i/o}$ inhibits AC and activates K+ channels, and $G\alpha_{q/11}$ stimulates phospholipase C (PLC). $G\alpha_{12/13}$ are mainly involved in the small G-protein, Rho mediated responses (153).

The mechanism of signal transduction through a GPCR was originally described as a simple model (Figure 2) where ligand binding causes a conformational change in the receptor, which then recruits intracellular heterotrimeric G-proteins. As a result, the G-protein exchanges GDP for a GTP on the α-subunit. This replacement induces dissociation of the subunits. Both the α subunit and the βγ subunit regulate downstream intracellular effectors like enzymes or ion channels. The GTP is then hydrolized to GDP and the α and βγ subunits re-associate (110).

The picture is now complicated by studies showing that the G-proteins are central but that other associated proteins also are important. The intracellular events following receptor activation, engagement of different pools of G-proteins, non-G-

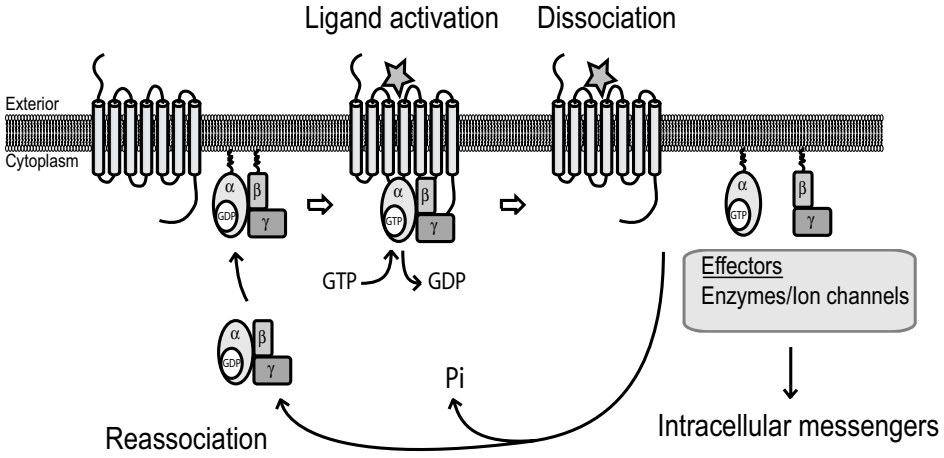

Figure 2. GPCR activation and signalling.

protein effectors and multiple active receptor states gives the impression of a complex signal diversity (101). The existence of receptor dimers and oligomers, influencing receptor maturation, trafficking and ligand binding, further adds complexity to the system (55).

Fine-tuning of the receptor response occurs through different processes. Agonist binding to the GPCR starts a chain of events at both receptor level and further downstream, that regulate the ability of the receptor to response. The receptor can be desensitized, internalized and also recycled again. The regulation of signalling can also occur through modulations of G-proteins or effectors (101).

Chemokines and their receptors

Chemokines are small chemoattractant cytokines found in *e.g.* mammals, birds, amphibians and fish. They comprise a superfamily of structurally similar (20-50% homology), 8-15kD, (70-120 aa) polypeptides involved in immunity and inflammation (91, 115, 116, 135). Most chemokines are secreted and they act on GPCRs, induce chemotaxis and recruit cells into sites of inflammation. They are also involved in a variety of other activities such as maturation, homing, hematopoesis and organogenesis (12, 84, 100, 114).

Chemokines are classified (CXC, CC, CX_3C and XC) based on the number and spacing of the first two of four conserved cysteine residues in the N-terminal of the molecule. X stands for an aa residue other than cysteine. Most chemokines belong to the CC or CXC chemokines. There are now approximately 50 known human chemokines (24).

The chemokine receptors belong to the rhodopsin family of GPCRs. They are usually 340-380 aa in length with 25-80% sequence identity (19, 114). There are approximately 19 known chemokine receptors (24). Chemokine receptors have two conserved cysteine residues, in the N-terminal and in ECL3, which are believed to form a disulfide bridge, stabilizing the structure together with the putative bridge between ECL1 and ECL2, common in all GPCRs (18).

Homeostatic chemokines are constitutively expressed in specific organs, whereas inflammatory chemokines tend to be produced by many cell types in inflamed tissues. Chemokines induce chemotaxis in neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts and keratinocytes. Dysfunction of the chemokine system has been

implicated in various conditions like multiple sclerosis, type-1 diabetes, rheumatoid arthritis, asthma and allergy (10, 19, 61, 122).

There is an overlap and redundancy in the chemokine system, since most chemokine receptors can bind more than one ligand and chemokines can often bind to and activate more than one receptor. Cells can also express multiple chemokine receptors (19).

In addition to their role as mediators of receptor signalling, many chemokines have also demonstrated *in vitro* antimicrobial potency, highlighting another property for the group of chemokines (165).

CCR5

The CC-chemokine receptor CCR5 was cloned in 1996 (133) and is a receptor for the natural ligands, Regulated upon activation normal T-cell expressed and secreted (RANTES, CCL5), Macrophage inflammatory protein-1α (MIP-1α, CCL3) and MIP-1β (CCL4). CCR5 is involved in inflammation and is expressed on monocytes, macrophages, memory/effector T-cells, Natural Killer cells (NK-cells) and immature dendritic cells (DCs). The ligands are predicted to interact with the CCR5 N-terminal, but also with regions in ECL1 and ECL2. Like for most chemokine receptors, CCR5 activation engages $G\alpha_i$ proteins, which activates phospholipase C (PLC) producing the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG), leading to intracellular calcium mobilisation and activation of protein kinase C (PKC). Other G-protein independent pathways may also be activated (94, 117, 133). This receptor is also the main coreceptor for entry of R5 strains of HIV-1 (44).

CXCR4

The CXC-chemokine receptor CXCR4 was originally cloned in 1993 (51, 99). The receptor has only one known ligand, Stromal derived factor (SDF-1), which exists in at least two isoforms, SDF-1α and β. CXCR4 is more of a house-keeping chemokine receptor, expressed on leukocytes, especially on naïve T-cells, B-cells, monocytes and also at lower levels in many other tissues (89, 106). SDF-1 is constitutively expressed in many tissues, which together with results from CXCR4/SDF-1 knockout mice show their essential role for different aspects of embryonic development (107). CXCR4 activation engages $G\alpha_i$ proteins. This receptor is also the main coreceptor for entry of X4 strains of HIV-1 (52).

HIV

History

HIV, a lentivirus belonging to the *Retroviridae* family was isolated in 1983 (14, 57) and found to be the causative agent for AIDS. It was further divided into two types; HIV-1 and HIV-2, when a related virus was found in 1986 (32). HIV-1 is spread worldwide, but HIV-2 which is slightly less virulent, is mainly prevalent in certain regions of West Africa. Molecular phylogenetic studies have shown that HIV-1 originates from a strain of simian immunodeficiency virus (SIV) found in a subspecies of chimpanzees, a result of several occasions of cross-species transmission. HIV-2 originates from a SIV strain from sooty mangabeys. Based on sequence analyses, HIV-1 viruses are divided into three groups, M (most HIV-1 isolates), O (rare outliers) and group N. Group M has diverged to subtypes (clades) A-K. The subtypes have different geographic distribution with subtype C being most prevalent in the world and subtype B being most common in North America and Europe (69) (www.unaids.org).

Virus structure

The mature virus is approximately 120 nm in diameter (Figure 3). The characteristic cone-shaped nucleocapsid composed by the viral capsid protein p24 in the core of the virus, contains the HIV-1 genome consisting of two copies of the plus stranded 10 kb RNA molecule, encoding nine genes. The RNA is closely associated with the stabilizing nucleocapsid protein p7 and the viral enzymes, reverse transcriptase (RT), integrase and cellular tRNA. The virus envelope consists of a lipid bilayer, originating from the host cell when the new virus budds off. The matrix protein, p17 lines the inside of the envelope. The viral glycoproteins, gp120 and gp41 are organized as trimers, forming spikes protruding the bilayer (145). Three of the nine genes, gag, pol and env encode structural proteins. The gag encodes a precursor protein, which among other things give rise to p24, p17 and p7 proteins. The env encodes the precursor gp160 which is cleaved to form gp120 and gp41 proteins. Pol

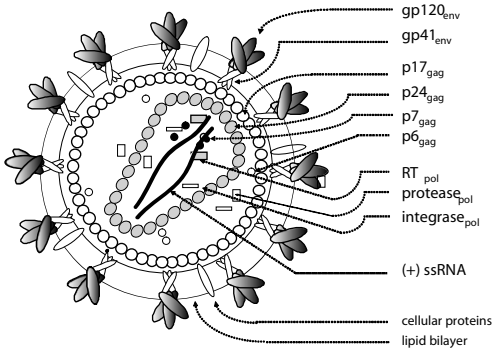

Figure 3. Structure of HIV-1.

encodes three viral enzymes, RT, protease and integrase. The other six genes encode regulatory and accessory proteins involved in the virus replication cycle and infection (145, 156).

G protein-coupled receptors as coreceptors for HIV

In 1995 the natural ligands of the receptor CCR5, were shown to inhibit HIV-1 cell entry (33). It was further shown, in 1996, that in addition to a CD4 molecule, virus interaction with either of the chemokine receptors CCR5 or CXCR4 was required for entry into target cells *in vitro* (6, 30, 41, 43, 44, 52).

The central role of these coreceptors *in vivo* was proven, when a mutant allele for CCR5, with a 32bp deletion within the coding region of CCR5, was identified. Homozygous individuals are strongly protected against infection of viruses using CCR5 for entry. This deletion causes a frame shift resulting in a truncated protein, not expressed on the cell surface. The allele is most common in northern Europe where about 15% of the population is heterozygous and approximately 1% is homozygous, for this mutation (40, 73, 98, 134).

In the years after the discovery of the major corecptors of HIV-1, several other related GPCRs, were found to support fusion of different HIV-1 strains *in vitro*. These receptors are *e.g.* CCR2b (43), CCR3 (43), CCR8 (79), CXCR6 (46, 168), Gpr1 (46), Gpr15 (46), ChemR23 (132), BLT1 (118), RDC-1 (143), CX_3CR1 (168) and APJ (168). The importance of these receptors *in vivo* is debated. CCR5 and CXCR4 seem to be the major coreceptors *in vivo* (166, 167, 169).

Viral entry and the replication cycle

The HIV-1 enters its target cells by fusion with the cell membrane (Figure 4). The entry process begins with the interaction of trimeric viral gp120/gp41 with CD4 on the host cell. A conformational change is triggered in gp120 which allows a hidden region of gp41 to interact with one of the coreceptors, CCR5 or CXCR4. Further, a fusion peptide of gp41 is inserted in the target cell membrane and a six-helix bundle structure is formed, bringing the membranes together, leading to membrane fusion of viral envelope

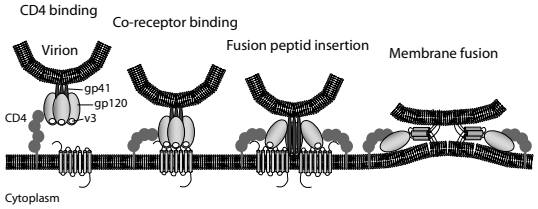

Figure 4. HIV binding and fusion with the target cell membrane.

and target cell membrane. Following fusion, the viral nucleocapsid is relased into the cytoplasm (145). The RNA is uncoated and transcribed to double-stranded DNA by the virus encoded RT. The resulting proviral DNA is transported into the cell nucleus, where it is integrated into the host cell chromosomal DNA by the viral enzyme integrase. The integrated viral genome, the provirus, is transcribed by host cell RNA polymerase into new viral genomes and also mRNA transcripts which are translated into new viral proteins. Host cell transcription factors regulate the transcription. Viral RNA copies and proteins are transported to the cell membrane and assembled to immature virions. The budding triggers the virus encoded protease, to process precursor proteins, generating mature virus particles (145).

The coreceptor tropism is controlled by the env gene (74). The envelope protein gp120 contains five constant regions (C1-C5) and five variable regions (V1-V5). The V3 loop is suggested as the main determinant of biological phenotype (142, 144, 161) and small changes in the aa composition can alter the coreceptor use. However, other regions of gp120 have also been suggested as important for coreceptor usage (22, 77).

Evolution of virus

HIV is one of the fastest evolving organisms. The viral RT has no proofreading and as a consequence generates about 0.2 errors per genome in each replication cycle. There are also further errors done during transcription. The "HIV-1 viral generation time, defined as the time from release of a virion until it infects another cell and causes the release of a new generation of viral particles" (123) is about 2.5 days and approximately 10^{10} virions are produced every day even in asymptomatic HIV-1 infected individuals. These properties together with recombination events and genetic selection create a huge antigenic variation and a challenge for the host immune system and for development of treatment strategies (123, 129).

Biological phenotypes

The biological phenotype of primary HIV isolates was originally described by their cell tropism, *in vitro* syncytium inducing capacity or cell culture replication kinetics. Isolates were defined as macrophage-tropic, non-syncytium inducing (NSI), slow/low virus and T-cell line tropic, syncytium inducing (SI) or rapid/high viruses (11, 53, 150). These classifications were only partly synonymous. When the corecepors CCR5 and CXCR4 were discovered and shown to be in large responsible for different biological phenotypes, a new classification based on coreceptor use was established.

CCR5-using isolates were called R5 viruses, CXCR4-using isolates were described as X4 viruses and viruses able to use both receptors for entry were called R5X4 viruses (15).

R5 viruses are almost invariably isolated from patients with asymptomatic HIV-1 infection (36, 159, 172). During disease progression viruses able to use CXCR4 alone (X4-viruses) or in combination with CCR5 (R5X4) emerge in about 50% of infected individuals (16, 81, 150, 151). CXCR4-using viruses are considered more virulent and their appearance is linked to disease progression (11, 25, 53, 150, 151).

Individuals continously harbouring CCR5-restricted isolates still progress to AIDS (39). Differences among primary R5-isolates have been described, as well as a progress towards a reduced sensitivity to C-C chemokines and especially RANTES inhibition of infection, during disease progression (76, 78). Increased cytopathicity of R5-isolates from individuals with disease progression, has also been observed (90, 138).

Why R5 viruses are transmitted and dominate during the asymptomatic phase, and why there is a subsequent switch from CCR5-using to CXCR4-using viruses is poorly understood. The switch is associated with declining CD4+ T-cell levels, but disease progression still occurs in individuals only harbouring CCR5-using viruses. Different hypothesis have been outlined trying to explain these events (105, 130).

In short, the dominance of R5 viruses early in infection is suggested to be a result of differences in coreceptor expression on target cells during pathogenesis (38) or the expression pattern of chemokines at sites of transmission (3). The switch of coreceptor use may also be explained by chance events of mutations in the predominated R5 viruses, changes of the viral fitness, further influenced by altered selection pressures occurring in immuno-compromized individuals (130).

Transmission and pathogenesis

In January 2006, UNAIDS (Joint United Nations Programme on HIV/AIDS) and WHO estimated that AIDS had killed more than 25 million people, since 1981. The total number of people living with HIV had reached 39.5 million (www.unaids.org).

The major routes of HIV transmission are through hetero and homosexual intercourse, sharing contaminated needles, vertical transmission from mother to child during pregnancy, breast-feeding, or receiving infected blood products (95).

Target cells

The presence of CD4 and the coreceptors CCR5 or CXCR4 are the basis for making cells permissive for viral entry and infection. CD4+ T-cells, macrophages, microglial cells, and DCs are the main targets for HIV-1 infection.

Dendritic cells

Immature DCs in the mucosa are believed to be important for the sexual transmission of HIV-1. These cells express low levels of CD4, CCR5 and CXCR4 and may be infected by HIV-1 but productive infection is restricted. Following virus binding to C-type lectins, like DC-SIGN and the mannose receptor, DCs migrate to lymphoid tissues and transmit HIV-1 to CD4+ T-cells. The DCs also present HIV-antigen and initiate immune responses (59, 163).

T-lymphocytes

CD4+ T-cells can be divided in three subsets; (i) antigen naïve (T_N) which circulate between blood and secondary lymphoid tissues and the memory cells, (ii) the antigen experienced, central memory (T_{CM}) and (iii) effector memory cells (T_{EM}). T_N express CXCR4, but litte or no CCR5 whereas memory cells often express both CCR5 and CXCR4. T_{CM} are circulating in blood and and lymphoid tissues and T_{EM} are found in large numbers in gut, liver and lung. There are numerous of CCR5 expressing T_{EM} cells. Located within and below genital and gastrointestinal tract epithelial layers this population is easily available at sexual transmission. The T_{EM} cells in gut-associated lymphatic tissues (GALT) seem to be responsible for the massive viral replication which can be detected in plasma analysis of HIV-1 RNA levels in acute HIV-1 infection. The loss of memory cells is followed by an increased differentiation of T_N cells and thus a suggested fatal reduction in the T_N pool (66, 67, 97, 102, 125).

Mononuclear phagocytes

Macrophages are among the first cells to be infected by HIV-1. Although the number of infected macrophages are 10-100-fold lower, compared to CD4+ T-cells (47), they are considered as an important viral reservoir throughout infection and may contribute substantially to virus production in individuals with low CD4+ T-cells (AIDS) (149). Infection occurs primarily via the CCR5 receptor but CXCR4 facilitated macrophage infection also occurs (62, 86, 108). Macrophages express low levels of CD4, CXCR4 and CCR5 and virus isolates which replicate efficiently in macrophages may have adapted to the use of low receptor levels (152, 158). Monocytes have a low susceptibility to HIV-1 infection *in vitro*, but infection *in vivo*

is evident from recently presented work (173). Macrophages and microglial cells are the major target cells for HIV-1 in the central nervous system (CNS) (65, 152).

Stages of infection

The pathogenesis of HIV-1 infection is a slow process, with the average time from infection to symptomatic AIDS and death, without therapy, being about 10 years (119). Although considered slow, HIV-1 causes an active progressive disease.

The acute phase of infection in general lasting for a few weeks, is characterized by a massive viral replication reflected by high plasma levels of HIV-RNA, and a substantial loss of CD4+ memory T cells (Figure 5). Some people are asymptomatic, but many develop flue-like symptoms during this phase (31, 80, 125).

After a few weeks a partial control of infection is normally achieved, and the CD4+ T-cell count is partially restored. Simultaneously, the plasma viral load decreases to a steady level, the viral set point, which is a predictor for the future rate of disease progression, where a high set point predicts for a faster progression (103). This chronic, usually asymptomatic phase, where replication is partially controlled, often lasts for several years. The rate of memory T-cell proliferation is elevated, but the average life-span of the cells is shortened. The persistent loss of memory CD4+ T-cells requires a continuous differentiation of naïve CD4+ T-cells into memory CD4+ T-cells. The pool of naïve T-cells are eventually believed to be exhausted (111). After several years, the continuous CD4+ T-cell depletion, most often leads to the collapse of the immune system. At this point CD4+ T-cell counts rapidly decrease and plasma viral load increase again (20, 125, 146). CD4+ T-cell counts <200 cells/µl and/or the occurrence of opportunistic infections, lymphomas and certain cancers are AIDS defining criteria (1).

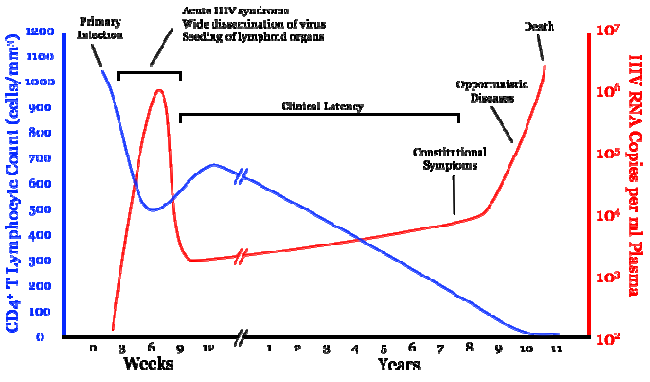

Figure 5. General time-course of HIV-1 infection. The figure shows CD4+ T-cell count (blue) and plasma virus load (red).

There are a few HIV infected individuals, who remain asymptomatic and who have no

signs of immunological deprivation for several years without antiretroviral treatment. These long-term non-progressors, are clinically healthy after having been infected for >8 years, with CD4+ T-cell counts >500 cells/μl. Some of them eventually progress to AIDS but a few have remained healthy for more than 15 years. The explanation for their unique control of infection is incompletely understood, but it probably includes a mix of favourable host and viral factors (37, 120).

Central nervous system infection

HIV-1 enters the CNS early in the course of infection (27, 28, 128). In the absence of anti-retroviral treatment, HIV-1 frequently causes neurological abnormalities such as AIDS dementia complex (ADC) (93, 162).

Symptoms of HIV-1 associated encephalitis are common and observed early in patients, suggesting that the neuropathological changes are a gradual process that may begin early. However severe ADC is more generally observed in later stages of infection (93, 162).

The blood-brain-barrier (BBB) separates the CNS from the circulatory system. A compartmentalization of HIV-1 infection seems to exist, since evidence of a separate viral evolution, differing from that in peripheral immune cells, has been found in phenotypic (26, 29) and genotypic studies (48, 58, 87, 112).

HIV-1 mainly invades the CNS via infected monocytes and/or T-cells. The HIV-1 infection in CNS persists at low levels until the onset of AIDS. Resident macrophages and microglial cells are the main HIV producing cells in the brain (88, 124). A viral adaption to replication in these target cells may include changes in coreceptor use. HIV-1 enter these cells mainly through the coreceptor CCR5 (5, 62, 68, 141, 147). Other cells in the CNS, such as astrocytes and neuronal cells, may also be infected and serve as a reservoirs for the virus even though infection seem to be non-producive (88).

The compartmentalization in CNS may reduce effectiveness and durability of drug treatment, as the BBB hampers the accessibility of antiretroviral agents. CNS may also serve as a sanctuary for drug resistant mutants (88). Compartmentalized differences in HIV-1 coreceptor use may affect the efficiency of emerging treatment with coreceptor antagonists.

Damage to the brain is suggested to be produced by the virus itself in combination with cellular factors released by activated and infected cells (88). Furthermore, viral gp120 cause apoptotic responses and neuronal damage through its interaction with chemokine receptors (23, 70, 170).

Immune responses and escape

The immune response against infections is made up of surface barriers, the innate and the adaptive immune response. The adaptive immune response is composed of the humoral and the cellular response.

The innate defence against HIV-1 mainly is composed of soluble factors *e.g.* cytokines, the complement system, and effector immune cells. TNF-α and IFN-γ are examples of cytokines controlling viral replication. Effector cells of the innate immune system are macrophages, DCs, neutrophils and NK-cells. Chemokines recruit and affect cytotoxic function of NK-cells, T-cells and macrophages and can inhibit viral replication. The innate immune system is rapid and present at the major site of HIV-1 entry, the mucosal surface. It also activates the adaptive immune system against HIV-1 (96).

The humoral immune system is effectuated by HIV-specific antibodies produced by B-cell derived plasma cells. HIV specific antibodies (often non-functional) are detected soon after initial infection, within a few days or weeks. Eventually a small proportion of the antibodies are neutralizing with the ability to control infection of cells. There are epitopes for neutralization in both the variable and conserved regions of gp120 envelope protein. Broadly inhibiting antibodies recognize conserved epitopes and strain specific neutralization antibodies recognize variable loops (148, 164).

Within a few weeks after infection, HIV-specific Cytotoxic T-lymphocytes (CTLs) appear. Studies of SIV infection have shown that these cells partially control the viral load (136). CTLs are found in high numbers during the chronic phase and then decline in late stages of disease. These cells recognize and kill infected cells but also produce cytokines and chemokines, influencing and inhibiting infection (42, 139). Expression levels of CC-chemokines has been shown to affect risk of infection and disease progression (34, 157).

During the chronic phase of the disease, the viral replication continues at a level determined by the balance between immunological control and viral escape. HIV

neutralizing antibodies and CTL responses are important for the initial decrease in replication to the viral set-point. Viral escape occurs through evolution in and by concealing of neutralization epitopes, through heavy glycosylation and steric hindrance of conserved receptor binding sites. The gp120 tolerates a high level of escape mutations, and neutralization antibodies are often formed to an earlier virus strain (13, 42, 139).

Treatment options

In 1987 a nucleoside reverse transcriptase inhibitor (NRTI), zidovudine (AZT), became available for HIV infected individuals (49, 104). The drug appeared to prolong life and reduce the mortality (54), but the promising results were soon hampered by the invariable appearance of virus strains with a reduced sensibility to the drugs (92).

The development of other drugs, such as non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PIs) and the combinatorial use of drugs from different classes (Highly active anti-retroviral therapy, HAART), dramatically improved the prognosis for HIV infected patients with access to the drugs and have heavily reduced the morbidity and mortality (140). Though more successful than monotherapy, drug resistant virus still appear, and potentially serious side effects are a major concern.

Antiretroviral therapy is generally initiated when there is a high risk of opportunistic infections, usually at a CD4+ T-cell count level of about 200-300 cells/µl (U. Karlsson, MD personal communication).

In addition to several new approved NRTIs, NNRTIs, and PIs, new classes of drugs are under development, including integrase inhibitors and entry inhibitors. The only approved entry inhibitor, enfuvirtide (T-20), targets a gp41 region of the envelope and inhibits the fusion process (121, 146).

Also, chemokine receptor antagonists are currently developed for therapy. Several small molecule antagonists for CCR5 and CXCR4 have been tested for their ability to inhibit HIV-1 replication and a few have progressed to clinical trials. Since the drug target is a receptor, it will not undergo mutations in response to drug pressure, but the virus may evolve to change its use of CCR5. Developing drugs targeting CXCR4 has been difficult, since CXCR4 and its natural ligand SDF-1 seem essential for cellular processes (155).

A vaccine is probably the best strategy to prevent disease and the transmission of HIV. Several vaccines have been in clinical trials, but the work is challenging (45).

There are still no drugs available that are able to completely eliminate virus. HIV infection is chronic and life-long treatment is needed. New drugs and treatment strategies are, thus required (146).

AIMS OF THIS THESIS

Paper I To construct CXCR4/CCR5 receptor chimeras (also called hybrid receptors) in order to characterize and compare epitopes used by the natural chemokine ligands, RANTES and SDF-1, and prototypic R5 and X4 HIV-1 isolates.

Paper II To study the evolution of HIV-1 biological R5 phenotypes in switch and non-switch patients, by the use of CXCR4/CCR5 receptor chimeras. Also, to investigate the correlation between mode of CCR5 use and disease progression. RANTES inhibition of infection in relation to chimeric coreceptor use was also included.

Paper III To evaluate wt and chimeric coreceptor use of paired plasma and cerebrospinal fluid HIV-1 isolates and further correlate these results with the degree of immunosuppression and the sensitivity to the CCR5 antagonist, TAK-779.

METHODOLOGY

This section gives a short summary of the main methods used. For detailed information, see respective paper in the Appendix (Paper I-III).

Construction of chimeric receptors (Paper I, II)

The receptor chimeras of CXCR4 and CCR5, were made by stepwise exchanging portions of CCR5 with corresponding regions of CXCR4. They were constructed using a variant of the single overlap and extension PCR method where the final sequence is made up of two pieces, each containing an overlapping sequence of the joint region of each receptor segment. The joint region was placed in conserved parts of TM regions, in order to avoid disturbance of protein conformation.

Cell lines and receptor expression (Paper I, II)

Chinese Hamster Ovary cells (CHO-K1) were stably transfected with pCMV.IRES.AEQ plasmid using calcium phosphate precipitation. The clone with the highest luminescence function was chosen for further experiments. The IRES element of the vector ensured a high frequence of positive clones, since the gene of interest and the gene for selection are transcribed as one mRNA.

For signalling studies, CHO-K1.AEQ cells were transiently transfected using Lipofectamine. A pIRES-Puro vector containing the gene for the wild-type (wt) receptor or receptor chimeras was introduced into the cells. Flow cytometric analyses were used to evaluate cell surface expression of one cell aliquot. The other cell aliquot was used for calcium mobilisation assay. The high level of receptor expression and reproducability allowed for transient transfections, as opposed to the more time-consuming stable transfections used for the infections experiments.

Human astroglioma U87.CD4 cells were stably transfected with receptor constructs and individual clones were tested for cell surface expression. Flow cytometric analyses were used to select a set of clones with similar receptor expression. Monoclonal cell lines were chosen for further experiments.

Generation of CXCR4 monoclonal antibody (Paper I)

The monoclonal antibody (mAb) recognizing CXCR4, was generated by immunizing Balb/c mice with a synthetic peptide consisting of the N-terminal aa 2-16 of CXCR4. Positive clones were evaluated in cell-ELISA and the best clone, 5/5B5, was purified by protA affinity chromatography and tested on cell surface expressed CXCR4 receptors using flow cytometry. This antibody allowed for testing cell surface expression of all chimeric receptors and CXCR4, using one antibody.

Flow cytometry (Paper I, II, III)

Flow cytometric analyses, were performed using the in-house CXCR4 antibody, or commercially available CCR5 and CD4 antibodies. The CD4 antibody was applied to evaluate the CD4 expression of all established U87 cell lines. A CCR5 antibody was used to evaluate the wt CCR5 expressing CHO-K1.AEQ or U87 cells.

Virus isolates (Paper I, II, III)

Prototypic isolates HIV-1_{BaL} and HIV-1_{IIIB} used in Paper I, were a kind gift from professor Eva Maria Fenyö, Lund University, Sweden.

The 34 patients studied in Paper II were selected from a larger cohort of 53 HIV-1 infected individuals (76, 81). They were selected on basis of different rates of CD4+ T-cell decline and biological phenotypes, according to cell line assays. The study began in the mid-1980s and few were on antiretroviral therapy at onset of study.

The 28 HIV-1 infected patients in Paper III were retrospectively selected from a longitudinal study cohort at the Department of Infectious Diseases, Sahlgrenska University Hospital, Göteborg, Sweden (60). Fourteen patients were severely immunodeficient and seven patients were diagnosed with ADC. Twenty-five patients were anti-retroviral treatment naïve, and none had received anti-retroviral medication during at least nine months prior to virus isolation.

Virus was isolated from peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid (CSF), according to standard procedures (4, 7).

The prototypic HIV-1 isolates (Paper I) and the primary patient isolates (Paper II, III) were passaged in phytohemagglutinin-P (PHA-P) and interleukin-2 (IL-2)

stimulated PBMCs. Virus was grown and harvested at days 7 and 10. The virus stocks were stored at -140°C. Virus content was evaluated in terms of p24 assays. Chosen isolates were also evaluated for RT activity.

Cell signalling (Paper I)

Receptor-mediated cell function was analyzed using the aequorin assay. The photoprotein aequorin is stably expressed in CHO-K1 cells. Briefly, CHO-K1.AEQ cells transiently expressing receptor protein were incubated with coelenterazine and portions of the cells were stimulated with different concentrations of ligands. The calcium response was then evaluated in a luminometer, and 50% effective concentration (EC_{50}) was calculated.

Infection assay (Paper I, II, III)

U87.CD4 cell lines stably expressing wt or chimeric receptors were seeded in 48-well plates and after three days they were incubated with HIV-infected PBMC culture supernatants. The cells were washed and fresh medium added. Supernatants from the infected cell cultures were collected at day 0 (after washing) and day 5 or 7 of infection and assayed with p24 antigen ELISA. The cell cultures were inspected regularly and syncytia formation was also recorded.

Inhibition of infection (Paper II, III)

PHA stimlated PBMCs were infected in the presence of RANTES. This was diluted in three-fold steps starting at a final concentration of 600 ng/ml. After three days the medium was changed and RANTES concentrations restored. The 50% inhibitory concentrations (IC_{50}) were calculated seven days after infection, using results of p24 ELISA.

RT-normalized virus was used for the TAK-779 inhibition experiments. PHA-activated PBMCs were infected in triplicates with virus in the presence of TAK-779. TAK-779 was serially diluted in three-fold steps, starting at the concentration of 990 nM, and simultaneously added to the cells and virus. Infected PBMCs were washed on day 1 and fresh inhibitor at concentrations corresponding to the setup were added. Supernatants were harvested on day 7. The sensitivity to TAK-779 was evaluated as IC_{50} and IC_{90}, calculated from p24 antigen release in the control cultures.

SUMMARY OF RESULTS

For details, see respective paper in the Appendix (Paper I-III).

Paper I

We set out to compare the epitopes of CCR5 and CXCR4 used by the natural ligands for signalling, and the epitopes used by prototypic R5 and X4 HIV-1 strains for entry (8). A set of seven hybrid CXCR4/CCR5 receptors were constructed, where increasing portions of CCR5 were replaced with the corresponding parts of CXCR4. The cell surface expression was analyzed by flow cytometry after the constructs were transfected into cell lines. An in-house mAb, recognizing the N-terminal of CXCR4 was generated and could be used to study the cell surface expression of all chimeras. Five of the chimeras, FC-1, FC-2, FC-5, FC-6 and FC-7, were successfully expressed. Two of them, FC-3 and FC-4, did not reach the cell surface. Three closely related variants, FC-3a, FC-3b and FC-4b, were constructed and one of them, chimera FC-4b showed cell surface expression. Thus, a set of six chimeras, FC-1, FC-2, FC-4b, FC-5, FC-6 and FC-7 (Figure 6), were used in the further experiments.

The aequorin-based assay was used for signalling analysis with the natural ligands, SDF-1 and RANTES. CHO-K1.AEQ cells, stably expressing the aequorin protein, were transiently transfected with the receptor constructs. Cell surface expression was verified in each experiment before stimulation with different concentrations of each ligand. The cells expressed similar amounts of receptors, and EC_{50} values of each receptor ligand-stimulation, were calculated.

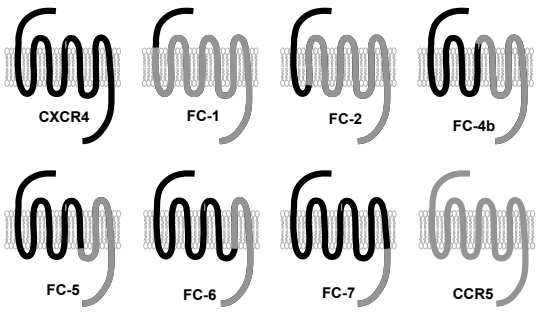

Figure 6. Schematic representation of CXCR4 (black) and CCR5 (grey) and the CXCR4/CCR5 chimeric recptors FC-1, FC-2, FC-4b, FC-5, FC-6 and FC-7.

Both RANTES and SDF-1 stimulated their cognate wt receptor in a concentration-dependent manner, giving EC_{50} values in the normal physiological range. Only two of the chimeras, FC-1 and FC-2, showed signalling in response to RANTES. The EC_{50} values were 100-1000 times higher than for the wt CCR5 receptor. Only the chimera FC-7 signalled in response to SDF-1.

The same set of chimeric receptors, were stably transfected into U87.CD4 cells, and cell lines expressing similar amounts of receptors were selected for subsequent experiments. The set of U87.CD4 cell lines expressing wt and chimeric coreceptors, was tested for HIV-1 entry. Cells were infected with the laboratory strains, HIV-1_{BaL} and HIV-1_{IIIB}. Infection with HIV-1_{BaL}, but not HIV-1_{IIIB}, was strongly supported by wt CCR5. The ability of HIV-1_{BaL} to infect the test cells was lost when the N-terminal of CCR5 had been exchanged with that of CXCR4 (chimera FC-1). However, when larger segments of CXCR4 were introduced (chimera FC-2 and FC-4b), infection was partly resumed. The other chimeras, as well as wt CXCR4, did not show coreceptor activity in the presence of HIV-1_{BaL}.

Insertion of the N-terminal from CXCR4 into CCR5 (chimera FC-1) did not support infection with HIV-1_{IIIB}. In the remaining chimeras, with increasing contribution of CXCR4, a varying degree of HIV-1 infection was resumed. The chimera FC-5 differed somewhat from the others in that the p24 levels resulting from infection were quite variable. The coreceptor function of the chimera containing the CCR5 C-terminal in CXCR4 (chimera FC-7) was as efficient as for wt CXCR4. HIV-1_{IIIB} did not infect cells expressing wt CCR5.

There was a considerable difference in the way the chimeric constructs interacted with the natural ligands RANTES and SDF-1, compared to the interactions with the two HIV-1 strains. The results with RANTES and HIV-1_{BaL} suggested that relatively small parts of the receptor are of critical importance, but that the epitopes involved differ. For RANTES there seemed to be a complementary use of both the N-terminal and the first two ECLs. SDF-1 required an essentially complete CXCR4, whereas HIV-1_{IIIB} was less demanding in its use of the receptor.

The results show that the two prototypic HIV-1 strains tested, interact with different epitopes on their respective coreceptors, and that the interaction of the respective ligands with corresponding receptors diverges from the viral interaction. These findings would provide a basis for tailoring future drugs that block viral entry through the two major coreceptors without interfering with their physiological function.

Paper II

In this paper (83) the evolution of primary HIV-1 isolates was studied. A total of 246 isolates from 31 HIV-1 infected individuals were tested. The patients were selected based on their different rates of CD4+ T-cell decline in the first five years of their

infections. The viral evolution was then further correlated to pathogenesis. Isolates were tested for wt coreceptor use. Virus isolations from 17 patients yielded R5 isolates throughout the study and 14 initially yielded R5 isolates but later switched to X4 isolates. The set of six CXCR4/CCR5 chimeras in U87.CD4 cells were infected with these sequentially isolates.

The R5 isolates used FC-1, FC-2, and FC-4b chimeras to various degrees, but did not use the other chimeric receptors, or CXCR4. Based on these results, the R5 viruses could be subdivided into two groups: those with the $R5^{narrow}$ phenotype and those with the $R5^{broad}$ phenotype. The $R5^{narrow}$ phenotype is defined as viruses that use wt CCR5 but no chimeric receptors, whereas viruses using at least one chimeric receptor in addition to CCR5 are designated $R5^{broad}$ viruses. Depending on the number of chimeric receptors used, the $R5^{broad}$ viruses were further divided into $R5^{broad(1)}$, $R5^{broad(2)}$, and $R5^{broad(3)}$ phenotypes.

The R5 phenotype and its relation to pathogenesis was evaluated. In short, R5 phenotypes from first and last R5 isolates, obtained from 26 patients and isolated at least 18 months apart, were tested. An evolution of the $R5^{narrow}$ phenotype towards a broader use of CCR5 was found. The R5 phenotype was also correlated with the degree of immunosuppression, as measured by the CD4+ T-cell counts. Broader R5 isolates were more often isolated from patients with low CD4+ T-cell counts than from patients with high CD4+ T-cell counts. The evolution of R5 phenotypes occurred in both the switch and non-switch group, but was more pronounced in the switch group.

Infectious titers of R5 isolates with different phenotypes were tested. The $R5^{broad}$ phenotypes showed an increased infectivity, compared to R5 ^{narrow} isolates, in studies on CCR5 expressing cells.

Different R5 phenotypes were also evaluated for their sensitivity to inhibition by the CCR5 ligand RANTES. The analysis showed that the ability to utilize chimeric receptors correlated inversely with the sensitivity to RANTES inhibition.

The work indicates that various R5 phenotypes isolated from different stages of infection differ in their mode of coreceptor use. Phenotypic changes during viral evolution may reflect a more flexible or efficient use of CCR5. While some viruses require the N-terminal of CCR5 ($R5^{narrow}$), others can utilize alternative receptor

epitopes ($R5^{broad}$). Importantly, the mode of coreceptor use correlated with the sensitivity to inhibition by the CCR5 ligand, RANTES.

Paper III

In this paper (9) we set out to study the mode of coreceptor use by paired plasma and CSF isolates from HIV-1 infected individuals with varying degree of immunodeficiency and neuropathology. Twenty-eight HIV-1 infected patients were selected from a longitudinal study cohort at the Department of Infectious Diseases, Sahlgrenska University Hospital, Göteborg, Sweden (60). Fourteen patients were severely immunodeficient and seven patients were diagnosed with ADC. Twenty-five patients were anti-retroviral treatment naïve, and none had received anti-retroviral medication during at least nine months prior to virus isolation.

The paired plasma and CSF isolates were tested for their ability to infect U87.CD4 cells expressing CCR5 or CXCR4. The R5 viral phenotypes predominated both in plasma and in CSF. CXCR4 using viruses were found in plasma samples from seven patients. In three of the corresponding CSF-isolates only R5 phenotypes were detected and one CSF isolate (R5X4) utilized CXCR4 30 times less efficiently as compared to the corresponding (X4) plasma isolate.

The mode of CCR5 use was also studied using the chimeric CXCR4/CCR5 receptors. The chimeras FC-1, FC-2 and FC-4b used by R5-isolates in Paper II were applied in the study. We were able to identify discordant plasma/CSF R5 viral phenotypes in six of 21 patients, but there were no characteristic patterns of chimeric receptor use that could distinguish CSF-isolates from plasma isolates. Further, R5 phenotypes ranging from $R5^{narrow}$ to $R5^{broad(3)}$ were represented in both compartments in a non-specific manner. In seven patients with ADC, no specific patterns of chimeric coreceptor use by CSF-isolates or plasma isolates were found. CSF neopterin levels did not correlate with the mode of coreceptor use.

To evaluate a possible relationship between mode of coreceptor use and susceptibility to inhibition by the CCR5 antagonist, TAK-779, we selected paired R5-virus isolates from seven patients with varying degree of immunodeficiency and chimeric receptor use, including three patients with ADC. Virus isolates with a moderate to high ability to utilize chimeric receptors were all relatively resistant to inhibition by TAK-779.

The mode of CXCR4/CCR5 chimeric receptor use was correlated with CD4+ T cell counts and viral load for each individual. Individuals harbouring plasma $R5^{broad(2-3)}$ phenotypes had significantly lower CD4+ T-cell counts as compared to individuals with $R5^{narrow}$ or $R5^{broad(1)}$ phenotypes. The strongest association with immune suppression was found when comparing individuals with FC-2 using (FC-2+) R5 plasma isolates to those with FC-2 negative phenotypes (FC-2-). The presence of X4 or R5X4 phenotypes was, as expected, linked to immunosuppression. Looking at viral phenotypes within the CSF, we found a significant correlation between the presence of FC-2+ R5 isolates and elevated CSF viral load.

The R5 virus dominance in CSF isolates may be explained by a lower capacity of X4/R5X4 variants to replicate in target cells within CNS. We further believe that the ability to utilize our CXCR4/CCR5 chimeric receptors reflects a different mode of CCR5 use. Efficient chimeric receptor use correlated to increased resistance to TAK-779 inhibition. The chimera FC-2, was shown to be a useful tool for studying the R5 phenotypes during disease progression.

DISCUSSION AND PERSPECTIVES

CCR5-using isolates are transmitted and can be isolated at all stages of disease progression, but in approximately 50% of HIV-1 infected individuals CXCR4 using viruses emerge during progression to AIDS (16, 81, 150, 151). The CXCR4 using isolates are associated with disease progression and increased virulence (11, 25, 53, 150, 151).

With the aim of mapping the epitopes by which HIV-1 interacts with its major target cell receptors, CCR5 and CXCR4, and further to compare them with the epitopes used by the natural ligands SDF-1 and RANTES during receptor activation, hybrid (*i.e.* chimeric) CXCR4/CCR5 receptors were constructed (Paper I). We wanted to study the dynamics of the shift by virus and the natural ligands, respectively from one receptor to the other. Therefore, the two receptors were mixed in various proportions. We successively replaced larger segments of CCR5 with corresponding segments of CXCR4, and the junctional region was hence moved along the receptor structure in an unbiased manner to include an additional TM region in each new segment. A set of six receptor chimeras was used in the experiments. The chimeras were stimulated with the natural chemokine ligands SDF-1 and RANTES and also with prototypic R5 and X4 isolates, respectively. The ligands and virus were found to use different epitopes which, in turn, varied between the receptors. The results open up for a possiblity to develop inhibitiors of HIV-1 entry, without interfering with the physiological function of these receptors.

CCR5 and CXCR4 are differently targeted during disease progression and these receptor chimeras made it in addition possible to detect an unprecedented variation in coreceptor use during HIV-1 pathogenesis (82).

The evolution of HIV-1 coreceptor use during disease progression and the switch from CCR5 to CXCR4 use has been studied extensively. However, the mechanisms behind the disease progression in individuals that develop AIDS due to infection with exclusive R5 phenotypes are less understood. An increased sensitivity to RANTES inhibition, was shown to appear in R5-isolates from patients developing AIDS (76, 78). R5 viruses were also shown to evolve into more virulent phenotypes, in patients with progressive disease (90). In addition, evolution of CCR5 use has also been shown *in vitro*, during selection pressure of a small-molecule CCR5 antagonist (154). Also, Gorry et al have reported on HIV-1 variants with increased affinity for CCR5

and reduced dependence of CCR5 and CD4 (63). Together, these results have suggested evolutionary changes within the group of R5 viruses.

Using our chimeric CXCR4/CCR5 receptors, we could show that R5 isolates from immunosuppressed individuals are distinct from those isolated from individuals with higher CD4+ T-cell counts with regard to coreceptor usage (Paper II). Broad CCR5 usage, as measured by the use of chimeric coreceptors, was also associated with a decreased sensitivity to inhibition by the natural CC-chemokine, RANTES.

The CNS is invaded early in the course of HIV-1 infection, (27, 28, 128) and replication in macrophages and microglial cells eventually induces neuropathologic conditions. Due to the exixtence of a BBB the CNS is considered to be a restricted compartment, where a separate viral evolution has been described (26, 29, 48, 58, 87, 112). We set out to study coreceptor usage of paired plasma and CSF isolates (Paper III). The chimeras FC-1, FC-2 and FC-4b were applied in the study. Discordant CXCR4 and CCR5 use was found in paired isolates. In addition, the chimeras allowed us to detect discordance in chimeric receptor use between the two compartments. Furthermore, the ability of R5 isolates to use chimera FC-2 correlated with immunosuppression. Efficient chimeric receptor use also correlated with an increased resistance to the CCR5 antagonist, TAK-779.

The findings from Paper II and III are also in agreement with more recent studies by others that have demonstrated an increased viral resistance to entry inhibitors, including TAK-779 and natural CCR5 ligands, by R5 isolates from individuals with AIDS (65, 85, 113, 131). We have expanded upon these observations by showing, in more detail, how the mode of coreceptor use is linked to the sensitivity by these inhibitors.

We believe that the ability of R5 viruses to utilize these CXCR4/CCR5 chimeric receptors reflects a more flexible and more efficient CCR5 usage. This may include a reduced dependency upon interactions with the N-terminal residue of the receptor during infection. R5 variants with the ability to use CCR5 lacking the N-terminal has been described (126, 127). Also, the N-terminal of CCR5 has been shown to be important for signalling (Paper I) (17, 71, 109, 171), and this may be one explanation for the correlation between chimeric receptor use and viral sensitivity to inhibition by RANTES.

In our studies of R5 isolates we applied the CXCR4/CCR5 receptor constructs. Since both receptors are HIV-1 coreceptors, parts of CXCR4 may compensate for exchanged parts of CCR5. To address this issue, new chimeras were constructed, and a combination of CXCR2 and CCR5 was chosen. Unfortunately, these CXCR2/CCR5 hybrid receptors could not be expressed on the cell surface.

The evolution of R5 isolates possibly reflects changes in expression patterns of the natural ligands during disease progression. The viral envelope may be selected for changes in coreceptor density, conformation, or post-translational modifications. Naturally occurring R5 env variants, with distinct mode of CCR5 and CD4 interaction and varying sensititity to inhibiton by RANTES, has been reported (72). Moreover, glycosylation and sulfation patterns of receptors have also been shown to affect coreceptor function (50, 160). This viral selection possibly reflects changes in the access to target cells and their particular way of presenting the coreceptors. Late in disease progression the main available target cells, the T-cells, are limited and macrophages may well be the main source of virus production. This R5 virus evolution may be a consequence of a collapsed immune system which allows for the appearance of HIV-1 phenotypes with a specific mode of coreceptor use.

The CXCR4/CCR5 chimeric receptors have proven to be valuable tools in studying coreceptor use and especially R5 viral evolution. They made it possible to explore the existence of R5 phenotypes that were not possible to distinguish in studies using only wt CCR5 or CXCR4 receptors, respectively. The chimeras have thus provided the basis for a new nomenclature of HIV-1, where R5 viruses can be further subdivided, based on chimeric receptor use. According to which R5 isolates can be subdivided into the $R5^{narrow}$ and the $R5^{broad(1-3)}$ isolates.

In Paper III we found that R5 virus use of the chimeric receptor, FC-2, correlated higly with immunosuppression. The chimera FC-2 alone may be a useful tool in future studies on R5 pathogenesis, and also for the optimization of emerging treatment with CCR5 antagonists. The possible correlation between chimeric receptor use and cell tropism is a subject for future studies, as this would help to further clarify the mechanisms behind R5 virus pathogenesis. The correlation between chimeric receptor use and sensitivity to inhibition by CCR5 antagonists needs to be verified in larger studies with clinical isolates. It may well be that individuals harbouring R5 isolates can be tested and further subdivided for different treatment strategies. The possibility that CCR5 antagonists may give rise to viral escape mutants of the $R5^{broad}$ phenotype should also be considered. Our studies have

relevance not only with regards to R5-virus pathogenesis and optimization of treatment strategies, but also for HIV-infection within the CNS.

In conclusion, our findings propose alterations in the mode of CCR5 use that may be a key event in R5 virus pathogenesis. HIV-1 disease progression is paralleled by an increased capacity of R5 viral phenotypes to utilize CXCR4/CCR5 chimeras and a concomitant decreased viral sensitivity to inhibition by RANTES and TAK-779. Further studies of R5 isolates should be carried out to broaden the understanding of HIV-1 pathogenesis and to develop new treatment strategies.

POPULÄRVETENSKAPLIG SVENSK SAMMANFATTNING

Människan är uppbyggd av ett stort antal celler. För att kroppens olika organ ska kunna samverka och för att kroppen ska kunna fungera som en helhet, måste de olika cellerna kommunicera med varandra.

Cellernas kommunikationssystem är uppbyggt av mottagare, s.k. receptorer, på de olika cellernas yta och signaler som skickas runt mellan cellerna. Receptorerna och signalerna fungerar som nyckel och lås, d.v.s. när rätt signal når rätt receptor så får just den cellen information från andra celler och omgivningen. På det sättet når en viss information bara de celler som behöver just den upplysningen. Olika delar av kroppen dirigeras för att fungera på ett visst sätt, vid ett tillfälle. Signalerna kan exempelvis bestå av luktsignaler, hormoner, ljus som lyser in i ögat eller ämnen som påverkar kroppens immunsystem. De flesta receptorer tillhör en familj som heter GPCR.

Man har uppskattat att det finns ungefär 800 olika GPCR-receptorer i vår kropp varav ungefär hälften bearbetar luktsignaler som kommer in via näsan. Ett flertal andra signaler och receptorer gör att vårt immunförsvar samarbetar. Immunförsvaret får signaler om främmande ämnen som kommit in i kroppen, eller från en skada som skett i något av kroppens organ. Med hjälp av olika signaler kan sedan immunsystemet agera för att ta bort ämnet eller för att reparera skadan i kroppen.

År 1981 började man lägga märke till att ett flertal människor i världen drabbades av en ny okänd sjukdom som bröt ner immunförsvaret. Dessa tidigare friska människors immunsystem fungerade inte längre och de blev sjuka och dog av olika infektionssjukdomar och cancersjukdomar. År 1983 fann man att viruset HIV (Human immunodeficiency virus) orsakade denna nya sjukdom, AIDS (Acquired immunodeficiency syndrome). Sedan dess har många människor i världen blivit infekterade och avlidit i AIDS.

Detta arbete handlar om två GPCR-receptorer, CCR5 och CXCR4, som normalt fungerar i kroppens immunförsvar. 1996 visade det sig att dessa två receptorer utnyttjas av HIV när det tar sig in och infekterar olika celler i kroppen. Utan dessa receptorer på cellytan kan cellerna inte infekteras. Denna nya kunskap ledde till att intresset för hur HIV använder sig av och kommer in i cellerna via dessa receptorer, blev mycket stort.

När en person blir infekterad använder HIV nästan alltid den ena receptorn CCR5. Hos personer som är svårt sjuka i AIDS har man kunnat visa att de virus som de har i kroppen då, ofta använder den andra receptorn, CXCR4. Det verkar alltså som att HIV förändras under den tid det tar från att man smittats, tills man är svårt sjuk i AIDS, något som ofta tar många år.

I det här arbetet har vi tagit reda på mer om hur det går till när HIV tar sig in i cellerna genom att använda dessa receptorer. För att kunna göra detta tillverkade jag blandreceptorer (hybrider), som består av delar från både CCR5 och den andra receptorn CXCR4.

I ett första arbete visade vi att HIV använder receptorerna på andra sätt än immunförsvarets naturliga signalämnen. Resultatet visade att man kan utveckla HIV-läkemedel som blockerar användandet av dessa receptorer utan att störa de naturliga signalerna som överförs via receptorerna.

Genom samarbeten med Lunds universitetssjukhus och Sahlgrenska universitets-sjukhuset i Göteborg har vi kunnat ta fram HIV från ett flertal patienter. HIV från smittade, fortfarande relativt friska personer, samt från patienter som är svårt sjuka i AIDS, har isolerats. Med hjälp av de konstgjorda hybridreceptorerna har vi sedan kunnat visa att dessa olika HIV-isolat använder receptorerna på olika sätt för att ta sig in i cellerna. Det sker alltså en utveckling av viruset under sjukdomsprocessen. Viruset förändrar sitt sätt att använda receptorn, CCR5, när personen blir sjukare och det blir också svårare att blockera infektionen med vissa typer av HIV-läkemedel.

Vi har dessutom isolerat HIV från hjärnan för att jämföra med HIV som finns i kroppens blodbanor utanför hjärnan. Även här har vi kunnat se skillnader i hur viruset använder CCR5 och att viruset i hjärnan ibland skiljer sig från viruset i blodbanan, hos en och samma person. Detta ökar förståelsen för den speciella AIDS-demens som patienter kan drabbas av. Även i denna studie hittade vi virus med olika känslighet för blockering med läkemedel.

Hybridreceptorerna som har använts i de tre studier som avhandlingen bygger på, har visat sig vara mycket bra verktyg för att förstå infektionen och sjukdomsprocessen. Genom att lära sig mer om hur HIV fungerar och tar sig in i den infekterade personens olika celler i kroppen, kan man öka kunskapen om sjukdomen, optimera behandlingen, samt lättare utveckla nya läkemedel mot HIV och sjukdomen AIDS.

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