HUMAN IMMUNODEFICIENCY VIRUS TYPE 2: PATHOGENESIS AND ANTIRETROVIRAL THERAPY

Humaan immunodeficientie virus type 2: pathogenese en antiretrovirale therapie

Proefschrift

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## CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>General introduction</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>HIV-2 infection in 12 European residents: virus characteristics and disease progression. <em>AIDS 1996;10:1649-1655</em></td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>Low viral load in plasma and lymphoid organs of HIV-2 infected patients. <em>Submitted</em></td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>CD4⁺ T cells remain the major source of HIV-1 during end stage disease. <em>AIDS 1999;13:1015-1021</em></td>
<td>63</td>
</tr>
<tr>
<td>5</td>
<td>Coreceptor usage of human immunodeficiency virus type 2 primary isolates and biological clones is broad and does not correlate with their syncytium inducing capacities. <em>J Virol 1998; 72:6260-63</em></td>
<td>73</td>
</tr>
<tr>
<td>7</td>
<td>Antiviral resistance of biological HIV-2 clones obtained from individuals on nucleoside reverse transcriptase inhibitor therapy. <em>Submitted</em></td>
<td>99</td>
</tr>
<tr>
<td>8</td>
<td>Development of a real-time quantitative RT-PCR for the detection of HIV-2 RNA in plasma. <em>Submitted</em></td>
<td>113</td>
</tr>
<tr>
<td>10</td>
<td>Summarising discussion</td>
<td>131</td>
</tr>
</tbody>
</table>

Nederlandse samenvatting 143
Dankwoord 151
Curriculum vitae 153
## CHAPTER 1

**General introduction**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Lentiviruses and their hosts</td>
<td>6</td>
</tr>
<tr>
<td>I.1 Non-primate lentiviruses</td>
<td>6</td>
</tr>
<tr>
<td>I.2 Primate lentiviruses</td>
<td>9</td>
</tr>
<tr>
<td>I.2.1 Classification</td>
<td>9</td>
</tr>
<tr>
<td>I.2.2 Virus properties and replication</td>
<td>10</td>
</tr>
<tr>
<td>I.2.3 Cell receptor usage</td>
<td>12</td>
</tr>
<tr>
<td>I.2.4 HIV pathogenicity in small animal models</td>
<td>13</td>
</tr>
<tr>
<td>I.2.5 Pathogenesis of HIV-1 versus HIV-2 infection</td>
<td>15</td>
</tr>
<tr>
<td>I.2.6 Epidemiology and transmission of HIV-2 infection</td>
<td>17</td>
</tr>
<tr>
<td>II. Antiretroviral therapy</td>
<td>19</td>
</tr>
<tr>
<td>III. Aim of this study</td>
<td>21</td>
</tr>
</tbody>
</table>
I Lentiviruses and their hosts

Human immunodeficiency virus type 1 (HIV-1), human immunodeficiency virus type 2 (HIV-2), and simian immunodeficiency virus (SIV) have been identified as hitherto unknown primate members of the Lentivirinae subfamily of the family Retroviridae in 1983, 1986 and 1985 respectively. HIV-1 and HIV-2 were identified as the causative agents of the newly emerging acquired immunodeficiency syndrome (AIDS) of humans (1-3) and SIV was shown to cause AIDS in certain primate species. HIV-1 is clearly an emerging virus, which is expected to have infected between 30 and 40 million people by the year 2000. Although lentiviruses of different animal species share many biological features, the natural course of the disease they cause in their respective host species varies considerably. Table 1 summarises the currently known lentiviruses and their pathogenic characteristics in different host species. Elucidation of the differences in the pathogenesis of infection with different lentiviruses as well as the underlying mechanisms, may be expected to lead to a better understanding of the course of the natural infection with any of these viruses and will provide new tools for the development of intervention strategies. Most notably, the understanding of the marked differences between the natural diseases caused by the respective primate lentiviruses HIV-1, HIV-2 and SIV, may lead to the identification of new therapeutic and preventive measures for AIDS in humans, which in the light of the current pandemic spreading of this disease are more needed than ever.

1.1 Non-primate lentiviruses

Non-primate lentiviruses cause persistent lifelong infections, which usually remain subclinical in their natural host, although some infected animals develop slowly progressive inflammatory lesions within target organs like central nervous system, lymphoid tissues, lungs and joints (4). After a long incubation period, which may range from many months to years, the onset of disease symptoms is usually insidious. All lentiviruses infect monocytes and macrophages, but other cells like lymphocytes may also be infected (5). The majority of infected cells harbour the virus in a latent, often defective form as proviral DNA. Active viral replication is usually restricted to less than one per hundred or thousand cells (6). It has been suggested that latently infected cells, such as the blood monocyte, may transport the virus to the various target organs, concealing the virus from immunologic surveillance (6). Once in the tissue, these cells may transfer the virus to other target cells. Approximately four to eight weeks after infection, neutralising antibodies directed to the envelope glycoproteins as well as antibodies to the major core protein of the virus usually develop (7,8). Despite the in vitro effectiveness of antibodies in neutralising virus infectivity, studies in several primate and non-primate lentivirus systems have shown that their in vivo protective effect is limited (7).
<table>
<thead>
<tr>
<th>Host</th>
<th>Virus</th>
<th>Disease</th>
<th>Onset of chronic disease</th>
<th>Primary target organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>VISNA MAEDI virus</td>
<td>Wasting, paralysis, pneumonitis</td>
<td>insidious</td>
<td>Brain, lung (joints, mammary glands)</td>
</tr>
<tr>
<td>Goats</td>
<td>CAEV</td>
<td>Arthritis, leukoencephalitis</td>
<td>insidious</td>
<td>Brain (lung, joints, mammary glands)</td>
</tr>
<tr>
<td>Horses</td>
<td>EIAV</td>
<td>Anaemia</td>
<td>insidious</td>
<td>?</td>
</tr>
<tr>
<td>Cattle</td>
<td>BIV</td>
<td>None</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>Cats</td>
<td>FIV</td>
<td>AIDS, wasting, leukoencephalitis</td>
<td>insidious</td>
<td>Lymphoid organs, brains</td>
</tr>
<tr>
<td>Various African</td>
<td>SIV</td>
<td>None</td>
<td>Chronic carrier</td>
<td></td>
</tr>
<tr>
<td>monkey species</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macaques (Exp.</td>
<td>SIV HIV-1 HIV-2</td>
<td>AIDS</td>
<td>insidious</td>
<td>Lymphoid organs, brains</td>
</tr>
<tr>
<td>infections)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chimpanzees</td>
<td>SIV&lt;sub&gt;CPZ&lt;/sub&gt; HIV-1&lt;sub&gt;Exp.&lt;/sub&gt;</td>
<td>None</td>
<td>Chronic carrier</td>
<td>Lymphoid organs, brains</td>
</tr>
<tr>
<td>humans</td>
<td>HIV-1 HIV-2</td>
<td>AIDS</td>
<td>insidious</td>
<td>Lymphoid organs, brains</td>
</tr>
</tbody>
</table>

Table 1. Lentivirus-associated disease characteristics and their hosts

The lentiviruses of the ungulate species, Maedi/Visna virus, caprine arthritis encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and bovine immune deficiency virus (BIV), have the macrophage as their primary target cell, and do not cause primarily immunodeficiency. Maedi/Visna and CAEV cause a slowly progressive, usually fatal disease in small ruminants, involving the brain, lungs, joints and mammary glands (4,9). EIAV causes a slow disease in horses, which is characterised by a slowly progressive anaemia (10). BIV has so far not been associated with a disease in cattle (11). In contrast to the ungulate lentiviruses, FIV, which occurs in several feline species as a group of closely related viruses, has the same spectrum of primary target cells as the primate lentiviruses including lymphocytes, monocytes, macrophages and follicular dendritic cells (FDC). Interestingly, FIV also causes an immunodeficiency syndrome.
in cats, which is usually referred to as feline AIDS. This disease has many pathogenic features in common with AIDS in primates (12).

1.2 Primate lentiviruses

1.2.1 Classification

Human lentiviruses are divided into two types, HIV-1 and HIV-2. HIV-1 was first isolated from a patient with generalised lymphadenopathy in 1983 by Luc Montagnier and co-workers at the Pasteur Institute in Paris (1), and his findings were confirmed by Robert Gallo (National Institute of Health, USA) in 1984 (13). HIV-2 was first isolated in 1986, from a mildly immuno-compromised patient in West Africa (2). Primate lentiviruses other than HIV-1 and HIV-2 have been found in a wide range of nonhuman-primates (14), and are known as simian immunodeficiency viruses (SIV) with a subscript to denote their species of origin. Phylogenetic trees have been constructed from the complete lentivirus genome alignment. The tree displayed in Figure 1 was constructed using PHYLIP DNADIST.

* HIV-1/O
* HIV-1/O
SIVcpzGab
* HIV-1/A
* HIV-1/B
SIVMND
* SIVWCM
SIVAGMVer
* SIVBABI
SIVAGMver
SIVAqgri
SIVAGMlan
SIVAGMbab
* SIVPAM
SIVGVK
SIVSM
* SIVMAC
* HIV-2

Figure 1. Phylogenetic relationship among representative primate lentiviruses. For HIV-1, the three groups (M,N, and O) are shown, as well as two of the ten described subtypes of HIV-1 group M. For HIV-2, three of the six known subtypes are represented. The SIV have a subscript denoting the species from which they were isolated: These are chimpanzee (CPZ), mandrill (MND), white-crowned mangabey (WCM), African green monkey (AGM), baboon (BAB), patas monkey (PAM), Sykes' monkey (SYK), sooty mangabey (SM), and macaque (MAC). There are four species of African green monkeys, i.e., vervet, grivet, tantalus, and sabaeus monkeys; viruses from each exhibit similar levels of diversity to that shown for the two examples of SIVAGMver. Probable positions of cross-species transmission are indicated by E. Adapted with permission from P.M Sharp, E. Bailes, F Gao, Hirsch V and B.H. Hahn. Origins and evolutions of AIDS viruses: estimating the time scale. To appear in: Biochem Soc Trans, feb 2000.
Some of the genomes are known to represent recombinants or mosaics between two or more different lineages, so this tree should not necessarily be assumed to reflect the true phylogenetic history of these viruses. There is good genetic, phylogenetic and biological evidence to suggest that the lineage containing HIV-2 was ancestral in sooty mangabeys (Cerocebus torquatus atys) (14-17). Recently, evidence was published that a chimpanzee subspecies (P. t. troglodytes) is the primary reservoir for HIV-1. HIV-1 groups M, N and O are the result of at least three independent introductions of SIVcpz into the human population (18).

Within the major lineages, it is possible to identify further phylogenetic ramifications. The prototypic isolates form a cluster of many subtypes (18). As in HIV-1, the grouping of HIV-2 isolates fall into different subtypes, subtype A-E. There is no obvious correlation of geographical origin within or among each of the various subtypes. The most common HIV-2 strain, subtype A, contains viruses originating from diverse locations across West Africa, including Senegal (ST, MVP), the Cape Verde Islands (ROD), Mali (BEN), Ghana (GH1), Gambia (ISY, D194), and Guinea Bissau (NIH2, CAM2) (19, 20). Subtype B includes viruses from Ghana (D205, GH2) (21) and Cote d'Ivoire (UC1) (22). Representatives of subtype C (2238) and D (FO78) were identified in Liberians (23) and subtype E in Sierra Leone (24). For this group of HIV-2 viruses, SIV-infected sooty mangabeys are believed to be the source of human infection (14,17,22,23). The natural habitat of SIV-infected Sooty mangabeys coincides with the geographical pattern of HIV-2 endemicity in West Africa (17,23). Approximately 30% of sooty mangabeys in the wild are infected with SIVsm. Mangabeys are often hunted for food and kept as pets. Scratches, bites and exposure to monkey blood while preparing food are possible causes of interspecies transmission to humans (17). Furthermore, simian-simian cross-species transmission has occurred between naturally infected sooty mangabeys and various macaque species in captivity (14,15,25,26). SIVsm is not pathogenic in its natural host, but it is pathogenic in certain macaque species (25-27) and in humans. It is of interest that there are presently only single representatives of subtype C-E (FO784, 2238 and PA), and so far all have failed to replicate at demonstrable levels in tissue culture (23,24). The individuals, in whom these three viruses were identified, were all healthy and came from areas where HIV-2 associated AIDS has not been reported. This suggests that subtypes C-E differ from members of subtype A and B in their pathogenic potential. This could be the result of differences in replication characteristics, transmissibility, tissue tropism, quasispecies complexity, proportion of defective viruses, or combinations of these.

1.2.2 Virus properties and replication
Mature virions produced by cells infected with HIV-1, HIV-2 and various strains of SIV are similar in morphology and composition. The virions are approximately 110 nm in diameter. The viral envelope is a lipid bilayer that buds from the cellular plasma membrane and contains the protruding viral Env glycoproteins. The core viral particle is composed of virion core
proteins MA (p17), CA (p24) and NC (p9), and contains the viral RNA and enzymes (Fig 2). Like in all retroviruses, the RNA genome of HIV-1, HIV-2 and SIV contains the three structural protein encoding regions, gag, pol, and env (Fig 2). The core protein capsid (CA), nucleocapsid (NC), and matrix (MA) are encoded by gag, and are generated by proteolytic cleavage of the gag precursor protein. The pol region encodes the viral enzymes necessary for replication, such as reverse transcriptase (RT), integrase (IN) and protease (PR). The env-encoded glycoprotein contains a surface (SU) and a transmembrane (TM) domain.

Figure 2. Virion structure. A schematic representation of an HIV-1 particle. MA = Matrix; CA = Core capsid; NC = Nucleocapsid; Prot = protease; RT = Reverse transcriptase; Int = Integrase; SU = surface subunit; TM = transmembrane subunit.

Beside the structural genes, additional genes encode accessory proteins. The regulatory genes tat and rev encode small, non-virion proteins, which induce transactivation of viral transcription (tat) and regulate nuclear export of unspliced RNA. Vpu and nef are involved in downregulation of the HIV-1 receptor CD4 and HLA class 1 molecules. Vif is involved in virion assembly and vpr facilitates nuclear transport. The accessory genes of the HIV-1 genome are designated nef, vif, vpu and vpx, whereas HIV-2 and SIV encode nef, vif, vpu and/or vpr.

After entering the cell, retroviral RNA is reverse transcribed into proviral DNA by viral reverse transcriptase (RT). After reverse transcription proviral DNA is integrated into the genome of the host cell, joining the host cell
DNA. The proviral DNA now functions as a mammalian gene and can replicate synchronously with host chromosomal genes. Viral precursor proteins are produced in the endoplasmatic reticulum of the host cell. These structural proteins assemble around viral genomic RNA at the plasma membrane. The Env glycoproteins and the structure formed by the precursors, with two copies of viral RNA, bud through the plasma membrane. The final step consists of proteolytic cleavage of Gag precursor molecules, which occurs as the particle buds free from the cell, incorporating the Env protein and a portion of the plasma membrane into its outer envelope.

HIV-1

\[
\begin{align*}
\text{5'LTR} & \quad \text{gag} & \quad \text{pol} & \quad \text{vif} & \quad \text{3'LTR} \\
& \quad \text{......rev......} & \quad \text{......tat......} & \quad \text{env-SU} & \quad \text{TM} \\
& \quad \text{vpr} & \quad \text{vpu} \\
& \quad \text{vx} & \quad \text{vpr}
\end{align*}
\]

HIV-2

\[
\begin{align*}
\text{5'LTR} & \quad \text{gag} & \quad \text{pol} & \quad \text{vif} & \quad \text{3'LTR} \\
& \quad \text{......rev......} & \quad \text{......tat......} & \quad \text{env-SU} & \quad \text{TM} \\
& \quad \text{vpr} & \quad \text{vpu} \\
& \quad \text{vpx} & \quad \text{vpr}
\end{align*}
\]

**Figure 3.** Genomic organisation of the two human lentiviruses HIV-1 and HIV-2

1.2.3 cell receptor usage

It has been known since 1984 that the CD4 molecule is the primary receptor for the primate lentiviruses (HIV-1, HIV-2 and SIV) (28-31). The CD4 receptor, which belongs to the immunoglobulin (Ig) superfamily, is expressed on T-helper lymphocytes and less densely on macrophages, dendritic cells, and microglial cells (32-37). The highly glycosylated outer surface glycoprotein of HIV-1 (gp120), specifically binds to the CD4 molecule. However, the CD4 receptor itself is not sufficient for entry of HIV-1 into cells. When the cloned gene for human CD4 is expressed on the surface of rodent cell lines, or on certain human cell lines (astrocytes), HIV-1 binds to CD4, but fails to undergo the process of fusion and entry. All strains of HIV-1, HIV-2 and SIV bind to CD4, but need for their entrance into human target cells additional cell surface cofactors (38). The identity of some of these factors was discovered late in 1995 and throughout 1996. The CC-chemokines RANTES, MIP-1α and MIP-1β, secreted by CD8+ T cells, inhibited cell fusion or virus entry *in vitro* of macrophage-tropic strains of HIV-1, indicating the involvement of a chemokine co-receptor (39,40).
From studies on the infectious behaviour of HIV-1, markedly distinct cytotropisms in vitro for different CD4+ human cells at different stages in the course of disease were revealed (41-43). Env fusion specificity is a major determinant of HIV-1 cytotropism. HIV-1 isolates have been categorised into two groups, based on their ability to infect distinct target cell populations: macrophage-tropic or T-tropic viruses. A number of chemokine receptors have shown to cooperate with the CD4 receptor to facilitate virus entry through interaction with gp 120 (44-46). It was discovered that a seven-transmembrane protein, 'fusin' or CXCR4, when co-expressed with CD4, allowed infection of non-human cells by T-cell line tropic HIV-1 variants (46). CXCR4 chemokine receptors are expressed in continuous T-cell lines and most human tissues, with high expression levels in the lung and the heart, and moderate levels in the brain. They are also present on resting T cells, B cells, and surprisingly on monocytes/macrophages.

Subsequently a second chemokine receptor, CCR5, was reported (47-48). This receptor was required for entry of macrophage-tropic isolates of HIV-1. The co-receptor CCR5 is expressed on monocytes, macrophages, activated and memory T cell subsets, and microglial cells. Primary T cells express significant levels of both CCR5 and CXCR4, accounting for their susceptibility to both T-cell line and macrophage-tropic viruses.

Investigators then identified additional chemokine receptors, such as CCR-2b, CCR-3 (49,50), Bonzo and BOB (51,52). The clinical relevance of the co-receptors is not yet fully understood. It has been stated that co-receptor usage of HIV-1 clones correlate with the viral phenotype: macrophage-tropic, non-syncytium-inducing (NSI) isolates exhibit CCR-5-restricted entry, whereas T cell line tropic, SI isolates can also use CXCR-4 (53). Longitudinal studies have shown a shift towards usage of CXCR-4 as coreceptor for HIV-1 during disease progression, suggesting that the broadening of coreceptor usage contributes to the cytopathic potential of HIV-1 strains in vivo (43,54,55). However, CXCR-4 is expressed on macrophages, but is not used as a receptor by so-called X-4 tropic viruses in these cells. More importantly, it was demonstrated that a genetic variation in the CCR-5 receptor might offer protection from sexually and parentally transmitted HIV-1. In Caucasians, approximately 1% is homozygous for a defect on the CCR5 gene, and about 15% are believed to be heterozygous (56,57). This homozygous deletion is not associated with any known clinical disease, but it proved to be strongly protective against HIV-1 infection, despite expression of other co-receptors (CCR-2b, 3, BOB, Bonzo). The very closely related HIV-2 appears to be able to use CXCR4 (58,59). Studies comparing the requirements for HIV-1 and HIV-2 entry may contribute to the understanding of the pathogenicity of these human lentiviruses.

1.2.4 HIV pathogenicity in small animal models

Studies on lentivirus infections of animals and humans illustrate that there is a close association between virus characteristics and disease progression (53). The characteristics used to classify HIV strains in vitro include
replication rate, ability to induce the formation of multinucleated cell (syncytium inducing (SI) capacity), coreceptor requirement and the ability to infect different target cells (54,55). SI strains in general have a high replication rate, infect targets via α- and β chemokine receptors, may infect immortalised T cell lines and, albeit inefficiently, macrophages. HIV-1 NSI strains have a low replication rate, require expression of β chemokine receptors, do not infect T cell lines and replicate efficiently in macrophages (53). Animal model systems have proved to be useful to evaluate host-cell interactions that define the ability to infect and cause disease in the host. They might also provide a means of assessing the varying roles of humoral and cellular immunity and the pathogenic course as well as the participation of host or viral genetic factors in disease induction. Furthermore, accelerated animal model systems may provide more rapid disease models in which to evaluate antiviral drugs or vaccines. Studies on the interaction between HIV-1 and the host have been hampered by the lack of a suitable animal in which both vireamia and disease can be studied. Both HIV-1 and HIV-2 have been inoculated into several primate species in attempts to mimic the disease course with these virus infections in humans. Chimpanzees, rhesus monkeys, macaques, pig-tail macaques, mangabeys and baboons have been infected with HIV-1 or HIV-2, and varying results have been noted, and an AIDS-like syndrome has been induced in some of the animals (60-62). These models remain restricted to studies on development of vireamia. This led to the development of a number of experimental mouse systems, using the concept of immunodeficient mice, which permit survival and growth of transplanted human mononuclear cells or hematopoietic tissues (63).

**Chimeric murine HIV models**

HIV-1 does not infect normal mice. CB 17 mice with a genetically defined severe combined immunodeficiency syndrome (SCID) accept human foetal tissue transplants (SCID-hu mice) or peripheral blood mononuclear cells (PBL) from humans (hu-PBL-SCID mice) (63). Human tissues and cells in these chimeric mice create a human type haematological environment to support HIV-1 replication (64). Because HIV-1 has been shown to replicate predominantly in activated macrophages and T lymphocytes in lymphoid organs, an alternative chimeric human-to-mouse model was developed. Human PBMC were grafted intraperitoneally into gamma-irradiated CBA/N mice. This will result in an acute xenogenic graft versus host disease (xeno-GvHD) providing an environment in which human cells of both the monocytic and the lymphocytic cell lineage's become highly activated (65). This allows high levels of HIV-1 replication (66,67). Depletion of human CD4\(^+\) T cells from the graft results in a complete abrogation of the acute xeno-GvHD reaction. Furthermore, the close interaction between lymphoid cells and antigen presenting cells, which is a hallmark of the development of acute xeno-GvHD, also seems to be essential in the pathogenesis of HIV-1 infection (65). The high replication rate in close association with antigen presenting cells exhibits more similarities with HIV-1 infected lymphoid
tissues than in vitro HIV-1 infected PBMC cultures. After intraperitoneal infection with different strains of HIV-1, with NSI or SI phenotypes, in situ hybridisation studies observed the presence of HIV-1 in macrophages and CD4+ T cells in the peritoneal tissue and the lymphoid organs. The number of HIV-1 RNA+ cells and the phenotype of the infected cells depend, at least in part, on the cytotropism of the virus strain. Therefore, these small animal systems are important for the development and testing of antiviral drugs and vaccines (68), as well as studies concerning post-exposure prophylaxis and possibly the prevention of maternal-foetal transmission.

1.2.5 Pathogenesis of HIV-1 versus HIV-2 infection

HIV-1 infection
The immune pathogenesis of HIV infection is complex. Four mechanisms that contribute to the progression of HIV disease have been identified: (1) lack of elimination of HIV after primary infection (69,70); (2) persistent virus replication in lymphoid organs throughout the course of infection (71,72); (3) chronic stimulation of the immune system, which may cause inappropriate immune activation and progressive exhaustion of the immune system (73); (4) destruction of lymphoid tissue (74).

Primary HIV infection. The SIV models of acute infection have been experimental systems to determine the initial localisation and spread of the virus. Sequential lymph node biopsies showed that virus may be detected in lymph nodes as soon as one week after primary infection (75-77). In this stage the virus is exclusively cell-associated. During this early period intense virus spreading occurs, and is disseminated via blood and lymphoid tissues throughout the body. After 2 to 3 weeks the number of infected virus-expressing cells declines rapidly, probably as a result of the emergence of virus-specific immune responses. In addition, virus particles are trapped in the follicular dendritic network of the germinal centres with antibody and complement. These events coincide with a decrease in plasma viraemia (76). Cross sectional studies in humans indicate that the kinetics observed in the SIV model are comparable to those obtained in humans (78,79). Although the immune system is capable of down regulating virus replication dramatically, HIV-1 is probably never completely eliminated, and progression to a chronic phase of infection occurs in most cases. The level of down regulation of viraemia varies, and may depend on viral characteristics and/or quantitative as well as qualitative differences in HIV-1 specific immune responses, and/or host factors like Δ32CCR5.

The incidence of typical symptoms following primary HIV-1 infection or 'HIV-mononucleosis', is not accurately known, and is believed to be 30-60%. Symptoms vary from those of a non-specific viral infection (fever, sweats, myalgia, headaches, sore throat, erythematous rash, lymphadenopathy), to more defined symptoms or syndromes, like Guillain-Barré Syndrome or meningitis (80,81). Usually these manifestations of acute infection symptoms resolve within 2 to 3 weeks.
Figure 4. The level at which plasma HIV RNA stabilises after primary infection predicted subsequent progression to AIDS in a group of men in the multicenter AIDS Cohort study. (modified from Mellors et al. Science 1996, 272: 1167-1170)

Downregulation of viraemia and resolution of symptoms mark transition from the acute to the chronic phase of HIV-1 infection. However, the presence of cells containing HIV-1 DNA and the levels of HIV-1 RNA* mononuclear cells, indicates that virus replication is continuous (82-84). In 1995 it was calculated that daily about $10^{10}$ virions are produced and cleared from circulation each day (66,67). More than 99% of virus is produced by newly infected CD4* T lymphocytes in the lymphoid tissue (71,72). In 1996 it was demonstrated that there is a relation between plasma viral load and clinical outcome (85). The risk of developing AIDS and death was directly correlated to the plasma viral load, which proved to be a better surrogate marker of HIV-1 disease progression than CD4* T cell counts (fig 4). The median time to develop AIDS after HIV-1 infection is estimated to be 10-12 years. When plasma HIV-1 viraemia is not detected (in less than 0.5% of infected individuals), the risk to develop AIDS within 10 years is less than 5% (long-term non-progressors).

**HIV-2 infection**

Once serological testing for HIV-2 became available, anecdotal reports arose of a long clinical latency period for this infection (86-88). The longest, best-studied HIV-2 cohort has been the cohort of commercial sex workers in Senegal (89). In this cohort (n=136), the incidence rate of AIDS was 0.95 per 100 person-years of observation among HIV-2 infected women, and 5.6 in HIV-1 infected women. Kaplan Meyer analysis comparing HIV-2 and HIV-1 seroincident women showed a 60% rate of AIDS-free survival after 5 years of infection with HIV-1. In contrast, none of the HIV-2 seroincident women developed AIDS, and therefore exhibited a 100% probability of AIDS free survival after 5 years. Cases with an incubation period of 14 and even 27 years have been described. (86-88). These observations
corroborate the findings about the relatively slow decline in immunological function observed in HIV-2 infection. CD4⁺ T cell counts and CD4/CD8 ratios decline less dramatically in HIV-2 infected individuals than in HIV-1 infected individuals (89). The rate of developing an abnormal CD4 cell count from the time of infection onward was 1% per year for HIV-2 infected individuals versus 10% per year for the HIV-1 infected women.

The clinical symptoms of HIV-2 induced disease are similar to those of HIV-1 (91). Studies in Gambia and Guinea-Bissau reported a high mortality among symptomatic HIV-2 patients (92-94). However, studies in Gambia (95) and Paris (96) showed a longer survival for HIV-2 patients after AIDS diagnosis. This is also reflected in autopsy results from AIDS cases associated with HIV-2 infection. In an autopsy study of 294 HIV-infected patients in Ivory Coast, tuberculosis, bacteriaemia and cerebral toxoplasmosis were responsible for a significant proportion of all HIV-related deaths (97). In contrast to HIV-1 related deaths, in HIV-2 positive cadavers three conditions associated with extreme immunodeficiency were observed: severe multiorgan cytomegalovirus infection, multinucleated giant cell encephalitis and intra- or extrahepatic cholangitis, conditions suggesting a prolonged survival with HIV-2 in the terminal stage of infection. Evidence for a lower viral load in HIV-2 infected individuals has been reported from virus isolation and proviral PCR studies (98,99). In individuals with the same genetic and social background, this may be due to essentially different characteristics of both viruses, or a different interaction with the immune response of the host.

1.2.5 Epidemiology and transmission of HIV-2 infection

Urban centres in West African countries (fig 5) have the highest HIV-2 antibody seroprevalence (100,101). The highest rate of HIV-2 infection in the general population has been found in Guinea-Bissau, where seroprevalence was 8.9% among urban and 7.7% among rural residents (102). In Guinea Bissau, Senegal, Gambia and the Cape Verdian Islands the incidence of HIV-2 infection exceeds that of HIV-1. In Guinea-Bissau (103), Ivory Coast (104) and Senegal (105) HIV-2 prevalence has been stable or declining, while in the same populations HIV-1 prevalence has increased. The decline of dual sero-reactivity among female commercial sex workers over the years is striking, from 38% in 1992 to 13% in 1997 (data collected by project RETRO-CI, Abidjan, Cote d'Ivoire). Many places outside West Africa with HIV-2 cases have historical links with Portugal (Brazil, Angola, Mozambique, and India). In North America and Europe infection with HIV-2 is sporadic.

Until recently there was little evidence that HIV-2 was becoming established as a locally growing epidemic anywhere beyond West Africa. However, HIV-2 spread has now been documented within India, primarily via heterosexual intercourse, without apparent direct contact with West Africans (106). Although the global spread may not be as fast as that of HIV-1 infection, HIV-2 has spread beyond the boundaries of the original
African source of the epidemic. Transmission of HIV-1, HIV-2 and SIV has been documented to occur by the same modes: sexually, perinatally and by blood or blood-contaminated products or devices (107-110). However, transmission rates of HIV-2 are significantly lower.

Figure 5 Map of West Africa

Sexual transmission of HIV-2 was studied prospectively among 1452 registered female sex workers in Dakar, Senegal, followed from 1985-1993 (105). The annual incidence of HIV-1 increased 12-fold over the 8-year study period. In contrast, the annual incidence of HIV-2 remained stable over these years, as did the seroprevalence. This occurred in the setting of a higher seroprevalence in the population during the entire study period for HIV-2 (11.2%) than for HIV-1 (6.2%). The annual incidence rate for heterosexually acquired HIV-1 infection in this study in Senegal is similar to that seen in cohorts of homosexual men in the United States (111), although much lower than that reported from a study of female sex workers in Nairobi, Kenya (112). Mathematical modelling of seroincidence data suggests a five- to ninefold decreased infectivity of HIV-2 per sexual act, compared with HIV-1.

The rate of vertical transmission of HIV-1 in the industrialised countries has been reported from 13-32%, and from 25 to 48% in developing countries (113). The best predictor for the risk of perinatal transmission of HIV-1 is plasma HIV-1 RNA levels (114). Although investigation of HIV-2 perinatal transmission is limited, studies indicate that transmission may occur, but is
much less frequent than observed in HIV-1 (115-118). Table 2 shows the results of some prospective studies addressing this issue (119-125). The rate of HIV-1 transmission was in most studies 10- to 20-fold higher than that of HIV-2. Cross sectional surveys in different West African countries have shown paediatric HIV-2 infection to be extremely uncommon (126-129). No information is available on breast milk transmission of HIV-2, but epidemiological data obtained from infants and their HIV-2 seropositive mothers suggest it to be rare.

<table>
<thead>
<tr>
<th>Country</th>
<th>N</th>
<th>% transmitted</th>
<th>n</th>
<th>% transmitted</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea Bissau</td>
<td>53</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
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</tr>
<tr>
<td>France</td>
<td>41</td>
<td>0</td>
<td>260</td>
<td>21</td>
<td>121</td>
</tr>
<tr>
<td>Ivory Coast</td>
<td>93</td>
<td>1.2</td>
<td>138</td>
<td>25</td>
<td>120</td>
</tr>
<tr>
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<td>29</td>
<td>3.4</td>
<td>29</td>
<td>34</td>
<td>122</td>
</tr>
<tr>
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<td>170</td>
<td>4.0</td>
<td>64</td>
<td>21</td>
<td>123</td>
</tr>
<tr>
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<td>15</td>
<td>25</td>
<td>91</td>
<td>19</td>
<td>124</td>
</tr>
<tr>
<td>Portugal</td>
<td>37</td>
<td>5.4</td>
<td>NA</td>
<td>NA</td>
<td>125</td>
</tr>
</tbody>
</table>

Table 2 Perinatal transmission rates of HIV-1 and HIV-2. NA = not addressed

II Antiretroviral therapy (ART)

It has become clear that plasma and tissue HIV-1 loads are substantial and that high-level viral replication occurs at all stages of infection (66, 67, 72). Developing effective and sustained antiviral effects, which allow reconstitution of the depleted immune system or prevent depletion, remains the major challenge for HIV therapy.

Within the HIV-1 and HIV-2 replication cycles are several potential targets for antiviral attack. Conversion of single-stranded RNA genome to double stranded DNA involves DNA polymerisation and ribonuclease H (RnaseH) enzymatic activities of HIV-1 RT. During this process once every 10,000 times a spontaneous mutation develops, and consequently many heterogenetic strains evolve. The DNA polymerisation part of this process is the target of nucleoside and non-nucleoside reverse transcriptase inhibitors. The sensitivity of primary isolates of HIV-2 to zidovudine (AZT), didanosine (ddI) and lamivudine (3TC) was similar to, or slightly less, than that of primary HIV-1 isolates (130-132). In contrast, the other group of agents inhibiting reverse transcriptase, the non-nucleosides, such as nevirapine, has repeatedly shown very minimal to no inhibition in vitro of HIV-2 replication (133, 134). Another target for antiviral therapy is the HIV-protease. The HIV-protease is essential for processing the Gag-Pol precursor proteins in an irreversible manner. In the presence of HIV-protease inhibitors immature virus particles are produced, which are non-infectious.
Preliminary data have shown that protease inhibitors do have in vitro activity against HIV-2, though with less potency than for HIV-1 (135).

In 1998, it became accepted that ART, once started, should aim at full suppression of viral replication. Rapid advances in the management of HIV infection has been achieved by ART, which includes a combination of reverse transcriptase inhibitors and a protease inhibitor, the current standard of care (137). Although ART has only been generally used in industrialised countries during the past few years, it has dramatically reduced morbidity and mortality rates (137). Two factors critical for long-term patient outcome - initial viral response and the durability of that response - have been shown to depend on the potency of the regimen and the therapy compliance of the patients. Studies on convenient regimens, focusing on only once or twice daily dosing schedules, without dietary restrictions, and with minimal side effects are essential. Encouragingly, powerful ART reduces the viral load not only in the plasma compartment, but also in the lymphoid tissues (138). Both the number of virus producing cells and the number of viral particles on the surface of the follicular dendritic cells decline proportional to the decrease in plasma HIV-1 RNA. However, total eradication of infectious HIV from a patient has so far not been achieved. Recently it was suggested that a special sanctuary site for HIV-1 persists in the form of latently infected mononuclear cells (139). These cells would contain integrated proviral DNA, which is not expressed. Replication inhibitors are not applicable here. It has been suggested that additional immunomodulatory therapy (e.g. general immune activation by cytokines or vaccination) is required to clear this compartment.

The efficacy of ART is presently being monitored by high sensitive methods, such as the quantitative (ultrasensitive) HIV-1 RT-PCR. Clinically used goals of successful ART are a decline of HIV-1 RNA to less than 500 copies/ml or less than 20 copies/ml using the ultrasensitive assay. Due to difficulties in establishing a valid technique for measuring plasma HIV-2 RNA loads, the only surrogate parameter available to evaluate ART in HIV-2 infection is the peripheral CD4+ T cell count, which unfortunately has proven to be inadequate. Alertness on loss of effectiveness of ART over time is relevant, since it was quickly learned that reduced effectiveness of ART may result from the cumulative acquisition of multiple mutations, leading to viral escape. Multiple mutations are required for high level resistance to zidovudine and protease inhibitors, while a single mutation is sufficient for high-level resistance to lamivudine and non-nucleoside reverse transcriptase inhibitors. Studies have demonstrated that different combinations of mutations have distinct effects on HIV-1 susceptibility to antiviral drugs (Table 3). As the number of mutations in HIV-1 RT increases to three or four, there can be a 100-fold reduction in susceptibility to zidovudine (140).

Data on HIV-2 susceptibility to antiretroviral drugs are limited. HIV-2 RT has about 60% sequence identity with HIV-1 RT, and catalytic properties that are quite similar (141). The observation that the folding of the "fingers and the palm" of the more distant related RT of murine leukaemia virus is
the same as HIV-1 RT supports the idea that the structure of HIV-1 RT and HIV-2 RT are sufficiently similar to assume that amino acids in equivalent positions in the respective sequences will have similar roles in the two enzymes.

<table>
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<tr>
<th>Mutations introduced</th>
<th>ZDV IC50, µM</th>
<th>Fold resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.01*</td>
<td>1x**</td>
</tr>
<tr>
<td>M41L</td>
<td>0.04</td>
<td>4x</td>
</tr>
<tr>
<td>T215Y</td>
<td>0.16</td>
<td>16x</td>
</tr>
<tr>
<td>M41L, T215Y</td>
<td>0.60</td>
<td>60x</td>
</tr>
<tr>
<td>D67N, K70R, T215Y</td>
<td>0.31</td>
<td>31x</td>
</tr>
<tr>
<td>M41L, D67N, K70R, T215Y</td>
<td>1.79</td>
<td>179x</td>
</tr>
<tr>
<td>D67N, K70R, T215Y, K219Q</td>
<td>1.21</td>
<td>121x</td>
</tr>
<tr>
<td>D67N, K70R, T215F, K219Q</td>
<td>11.4</td>
<td>114x</td>
</tr>
<tr>
<td>ddl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K65R</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>L74V</td>
<td></td>
<td>5-10x</td>
</tr>
<tr>
<td>M184V</td>
<td></td>
<td>2-5x</td>
</tr>
<tr>
<td>3TC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M184V/I</td>
<td></td>
<td>100x</td>
</tr>
<tr>
<td>Multidrug resistance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q151M</td>
<td>0.1</td>
<td>10x AZT; 5x ddl/ddC</td>
</tr>
<tr>
<td>A62V,V75I,F77L,F116Y,Q151M</td>
<td>1.9</td>
<td>190x AZT; 50x ddl;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20x ddC; &gt;10x d4T</td>
</tr>
</tbody>
</table>

Table 3. Susceptibility to AZT, ddl, ddC and 3TC of HIV-1 variants with defined mutations in RT. * 50% inhibitory concentration (IC50), ** fold decrease in IC50

III AIM OF THIS STUDY

During the late fifties a population of experienced West African sailors started to settle in Rotterdam. Today approximately 15,000 West Africans and their relatives live in Rotterdam. They are of Afro-Portuguese ethnic origin, with a lively contact with and frequent travel to their relatives and friends in their country of origin. Both HIV-1 and HIV-2 are prevalent in this, mostly heterosexual, population. The studies presented in this thesis were initiated to increase our understanding of the different clinical courses of HIV-1 and HIV-2 induced disease among this population of West African descent.

In chapter 2 disease progression rates among HIV-2 infected individuals in relation to virus characteristics are assessed and compared with the
disease progression rates among HIV-1 infected individuals in the same population. In chapter 3 we study molecular pathological aspects of HIV-infection by assessing the amount and distribution of productively infected cells in lymphoid tissues by HIV-2 RNA in situ hybridisation and immunohistochemistry. In chapter 4 we assess the source of HIV-1 production during end-stage disease. In chapter 5 we evaluate the capacity of a panel of human cells stably transfected with chemokine receptor genes to support infection with HIV-2 primary isolates from patients at different stages of disease, and compare the results with data from studies on HIV-1 coreceptor usage. Chapter 6 describes our efforts to study the in vivo pathogenic potential of HIV-2 isolates and biological clones in a chimeric human to mouse model for in vivo HIV infection. We studied the direct (killing of infected cells) and indirect (inhibition/modulation of antigen presenting cells) cytopathic effect of HIV-2 strains on the development of acute xeno-GvHD symptoms, CD4/CD8 ration and the ability of the graft to repopulate mouse tissues. In chapter 7, 8 and 9 we describe several aspects related to the management of HIV-2 infection. In chapter 7 we studied genotypic and phenotypic patterns of resistance of HIV-2 virus strains to nucleoside reverse transcriptase inhibitors (NRTIs) from patients before and after antiretroviral therapy with NRTIs. In chapter 8 we describe the development of a quantitative assay to measure plasma HIV-2 RNA, and in chapter 9 we show the response to ART in dually HIV-1/HIV-2 infected patients. Finally, a summarising discussion is presented in chapter 10, which also includes the response observed to triple therapy in patients with infection with HIV-2 monoinfection.

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CHAPTER 2

Human immunodeficiency virus type 2 infection in twelve European residents: virus characteristics and disease progression

Human immunodeficiency virus type 2 infection in twelve European residents: virus characteristics and disease progression

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Objective: Assessment of disease progression rate amongst twelve HIV-2 infected West European residents (nine of West African descent), as compared to disease progression rate among HIV-1 infected individuals of the same population, and characteristics of the HIV-2 strains involved.

Methods: HIV-2 infected individuals were identified by commercially available serological assays, their clinical status and CD4+ cell counts were monitored, and HIV-2 was isolated from their peripheral blood mononuclear cells. T cell line tropism and syncytium inducing capacities of the isolated viruses were determined and their phylogenetic relationships were analysed by comparing PCR amplified nucleotide sequences of reverse transcriptase (RT) gene segments.

Results: Eight of the twelve HIV-2 infected individuals presented with progressive disease and one of them progressed from CDC group A1 to A3 within 36 months after seroconversion. The ratio of asymptomatic versus symptomatic individuals, among residents of the Rotterdam region of West African descent, was 2:7 for HIV-2 and 8:9 for HIV-1 infected individuals. From six of the nine individuals with progressive disease HIV-2 was isolated. The time required for virus isolation correlated inversely with the individuals CD4+ cell counts. Five of the HIV-2 isolates replicated in immortalised T cell lines, and two isolates from patients with AIDS induced syncytia. Five HIV-2 isolates from Cape Verdian Isles born patients grouped together within subtype A. The HIV-2 isolate from a patient of Ghanese origin belonged to subtype B. In the RT genes from HIV-2 isolates of two zidovudine treated patients mutations were identified, one of which has also been shown to be involved in zidovudine resistance in HIV-1.

Conclusion: Disease progression in HIV-2 infection may be as rapid as in HIV-1. HIV-2 isolation and viral phenotype were related to disease status, and mutations identical to those observed in HIV-1 zidovudine resistance were observed in patients treated with zidovudine.
INTRODUCTION

Since the discovery of HIV-2 as the second cause of acquired immune deficiency syndrome (AIDS) in West Africa in 1985 [1,2], the virus has been shown to have virtually spread world-wide [3]. However, sexual and perinatal transmission of HIV-2 is much less efficient than that of HIV-1, probably as a result of low viral burdens during the relatively long asymptomatic period [4-10]. This is probably the reason why, despite its wide distribution, the numbers of HIV-2 infected individuals have remained relatively low [3]. During the long asymptomatic period, it is usually difficult to isolate HIV-2, but like in HIV-1 infection, HIV-2 can be isolated from virtually all HIV-2 seropositive patients with AIDS [8-10]. Most of these viruses show early and high reverse transcriptase (RT) activity and exhibit a syncytium inducing (SI) phenotype in peripheral blood mononuclear cell (PBMC) cultures [8]. It has been suggested that cytopathic HIV-2 strains were only found within subtype A and that attempts to isolate viruses belonging to HIV-2 subtypes C, D and E and to a lesser extent subtype B, usually remained unsuccessful [11].

Here we describe data on the pathogenesis and characteristics of viruses isolated from eleven HIV-2 infected individuals living in the Rotterdam area, which all except two belonged to West African immigrant communities. In addition, the same data are presented for one HIV-2 infected individual born and living in France. By comparing the number of seropositive individuals with and without progressive disease amongst HIV-1 and HIV-2 infected individuals of West African origin in the Rotterdam area, it was suggestive of similar pathogenicity of the predominantly involved HIV-2 subtype A and HIV-1 in the same community.

MATERIALS AND METHODS

Study population

From August 1989 onward, clinical and immunological parameters and from August 1994 onward virological parameters were monitored of eleven HIV-2 seropositive individuals and one individual with a dual HIV-1 and HIV-2 infection, who regularly visited clinics in Rotterdam or Paris. All individuals gave informed consent and the study was approved by the medical ethical committee of the Rotterdam hospital.

Age, sex, country of birth and probable mode of transmission in these individuals is shown in Table 1. Nine out of 12 individuals originated from West Africa, individual RH2-4 was a Dutch born male spouse of individual RH2-5, a Cape Verdian female. RH2-11 was a Dutch born female former regular sexual partner of a Cape Verdian male (serostatus unknown). Patient PH2-1 was a French born homosexual man living in Paris, with a documented HIV-2-seroconversion in 1992.

Two patients had been treated with zidovudine for two years (RH2-2) and six months (RH2-5) respectively at the moment of virus isolation.
All patient sera tested negative for HIV-1, HTLV-I, HBsAg, except for individual RH2-8, who was infected with both HIV-1 and HIV-2.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Country of Birth</th>
<th>Probable Mode of Transmission</th>
<th>Probable Disease State</th>
<th>CD4 Count</th>
<th>Serology</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH2-11</td>
<td>54</td>
<td>F</td>
<td>NL</td>
<td>Heterosexual</td>
<td>C3</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>RH2-9</td>
<td>35</td>
<td>F</td>
<td>CVI</td>
<td>Heterosexual</td>
<td>C3</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>RH2-10</td>
<td>35</td>
<td>M</td>
<td>CVI</td>
<td>Heterosexual</td>
<td>C3</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>RH2-2</td>
<td>36</td>
<td>F</td>
<td>CVI</td>
<td>Heterosexual</td>
<td>C3</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>RH2-6</td>
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<td>F</td>
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<td>C3</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
</tr>
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<td>130</td>
<td>-</td>
</tr>
<tr>
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<td>France</td>
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<td>-</td>
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<td>-</td>
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</tr>
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<td>+</td>
</tr>
<tr>
<td>RH1-2</td>
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<td>CVI</td>
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<td>C3</td>
<td>10</td>
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<td>C3</td>
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<td>C3</td>
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<td>+</td>
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<td>Sen</td>
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<td>C3</td>
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<td>C3</td>
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<td>+</td>
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<tr>
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<td>37</td>
<td>F</td>
<td>CVI</td>
<td>Heterosexual</td>
<td>A2</td>
<td>350</td>
<td>+</td>
</tr>
<tr>
<td>RH1-13</td>
<td>29</td>
<td>F</td>
<td>CVI</td>
<td>Heterosexual</td>
<td>A2</td>
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<td>+</td>
</tr>
<tr>
<td>RH1-14</td>
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<td>CVI</td>
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<td>A1</td>
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<td>+</td>
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<td>M</td>
<td>Ghana</td>
<td>Heterosexual</td>
<td>A1</td>
<td>860</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1. Demographic and Clinical Characteristics of the Study Participants at Entry. ahusband of RH2-5. cCVI Cape Verde Islands, NL the Netherlands, IVC, Ivory Coast, Sen Senegal. bM, male; F, female. d10^6 cells/l

Serology and virus isolation

Sera were tested for antibodies to HIV-1 and HIV-2 (ELFA, bioMerieux, Lyon, France) and by type specific Western Blot (WB) (HIV-Blot 2.2, Diagnostic Biotechnology, and New LAV Blot 2, Sanofi Diagnostics Pasteur). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation as described previously [12]. All PBMC
samples were cocultured with three days phytohemagglutinin prestimulated donor seronegative lymphocytes (PHA-PBMC) according to standard protocols [12]. All cultures were maintained for up to 6 weeks and monitored for the presence of HIV-p24 antigen (V5 ELISA, Organon Teknika, Oss, The Netherlands) in the supernatant at weekly intervals. T cell lines (MT2, SupT1, H9) and monocyte derived macrophages were infected with culture supernatants of the HIV-2 positive PBMC cultures which were twice weekly analysed for the presence of p24 antigen in supernatants and for syncytium formation twice weekly [13]. HIV-1 IIIB kindly provided by the MRC AIDS directed programme [14] and HIV-1 strains ACH 172.BA-L (NSI) provided by Dr. H. Schuitemaker from the Central Laboratory for Blood Transfusion Service in Amsterdam [15] were used for reference purposes.

Polymerase chain reaction (PCR) detection of RT sequences and phylogenetic analysis
High molecular-weight DNA was extracted from MT2 cells that had been co-cultured with PBMC from patients RH2-2, RH2-2, RH2-6, and RH2-7 or directly from PBMC of patients RH2-1 and PH2-1, using cell lite beads [16]. Amplification was performed as described previously [11]. Briefly, RT sequences were amplified in a nested PCR with primers RTC/RT2 and RT3/RT4 for 1.5 minutes at 94°C, 1 minute at 40°C, 2 minutes at 74°C for 30 cycles with 10 minutes extension at the end. The amplified fragments were cloned in pTA cloning vector (Invitrogen, San Diego CA) according to the manufacturers protocol. Clones were sequenced with the Taq Dye Deoxy Terminator sequencing kit on the 373A sequencing system of Applied Biosystems (Foster City, CA). All clones were sequenced on both strands using custom made primers, except for a small (200 bp) part of RH2-2, to which none of the reverse primers hybridised.

The phylogenetic relationships of the viruses were determined from their RT nucleotide sequences using the UPGMA method in the software of Geneworks (Intelligenetics, Mountain View, CA). Alignment of the protein sequences was performed with the Geneworks software.

RESULTS

Clinical status and serology
The clinical status of the 12 HIV-2 seropositive individuals at entry in the study is shown in Table 1. According to the revised classification system of the Centres of Disease Control [17] five patients (RH2-2, RH2-6, RH2-9, RH2-10, RH2-11) were classified in category C3, one (RH2-1) in category C1, two (RH2-5 and RH2-7) in category A3 and three (RH2-3, RH2-4, RH2-8) in category A1. RH2-2 had a history of tuberculosis, recurrent oral candidiasis and cytomegalovirus (CMV) induced retinitis. RH2-6 suffered from extreme wasting. RH2-9 had intra cerebral malignant lymphoma; RH2-10 was diagnosed with disseminated Mycobacterium avium-intracellularare, and RH2-11 had a history of Pneumocystis Carinii and CMV-retinitis. All
five of them suffered from end stage AIDS at the time of entry in the study. RH2-1 suffered from Kaposi's sarcoma since 1989, which was localised on the lower extremities. The other six patients were asymptomatic at entry.

For eight of the individuals no information was available about the time of seroconversion. From three patients (RH2-9, RH2-10 and RH2-11) PBMC were not available. One individual, PH2-1, could be studied for 36 months onward from the time of seroconversion, during which period his CD4+ cell counts decreased from about 600 to 160 cells/mm³ (Fig. 1). No overt clinical signs developed in this patient during the observation period. Western blot analysis of his serum samples collected during the first 16 weeks after seroconversion showed that antibodies against all major HIV-2 proteins developed (Fig. 1).

Figure 1.
Sequential Western blot profiles of HIV-2 specific serum antibodies in patient PH2-1 during seroconversion in October 1992, and his CD4+ cell count of during the 36 months period after seroconversion.

Comparison of clinical and immunological status of HIV-1 infected individuals in the same West African origin in the Rotterdam hospital during the same study period showed that nine out of 17 HIV-1 infected individuals suffered from progressed HIV-related disease at the time of first presentation in the clinic (table 1). The mean age of the HIV-1 infected population was 32 years, and the probable mode of transmission was heterosexual contact.

Virus isolation
HIV-2 was isolated in PHA-PBMC from the PBMC of six of the eight individuals with CD4+ cell counts ranging from about 600 to 10 cells/mm³ (Table 2). The time of first HIV-p24 antigen detection in culture supernatants, which ranged from seven to 42 days after inoculation, proved to correlate inversely with the patients CD4+ cell counts at the time of sampling (r = 0.95, ci = 0.99). The rapidly replicating HIV-2 isolates from patients RH2-2 and RH2-6, who suffered from end-stage AIDS, induced syncytia in the PHA-PBMC, whereas the other four HIV-2 isolates failed to do so. The four HIV-2 isolates from the patients with the lowest CD4+ cell
Counts (130-10 cell/mm³) were shown to replicate in MT2 cells. The two isolates from the end-stage AIDS patients RH2-2 and RH2-6 induced syncytia and p24 antigen production after seven days (Table 2). Isolates from RH2-6, RH2-7 and PH2-1, who all had CD4⁺ counts < 200 cells/mm³, replicated in Sup T1 cells as shown by the presence of detectable p24 antigen in the supernatants within 14 days. The HIV-2 isolates from patients RH2-6 and PH2-1 also induced syncytia in these cells (Table 2). No replication could be demonstrated with any of the HIV-2 isolates in H9 cells.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Day of first p24 antigen detection upon infection of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA-PBMC</td>
</tr>
<tr>
<td>RH2-11</td>
<td>NA</td>
</tr>
<tr>
<td>RH2-9</td>
<td>NA</td>
</tr>
<tr>
<td>RH2-10</td>
<td>NA</td>
</tr>
<tr>
<td>RH2-2</td>
<td>7 (SI)</td>
</tr>
<tr>
<td>RH2-6</td>
<td>7 (SI)</td>
</tr>
<tr>
<td>RH2-5</td>
<td>14 (NSI)</td>
</tr>
<tr>
<td>RH2-7</td>
<td>21 (NSI)</td>
</tr>
<tr>
<td>PH2-1/b</td>
<td>21 (NSI)</td>
</tr>
<tr>
<td>RH2-1</td>
<td>42 (NSI)</td>
</tr>
<tr>
<td>PH2-1/a</td>
<td>c</td>
</tr>
<tr>
<td>RH2-3</td>
<td>–</td>
</tr>
<tr>
<td>RH2-8</td>
<td>–</td>
</tr>
<tr>
<td>RH2-4</td>
<td>–</td>
</tr>
<tr>
<td>HIV-1 IIIB</td>
<td>4 (SI)</td>
</tr>
<tr>
<td>HIV-1 ACH 172.BAL</td>
<td>6 (NSI)</td>
</tr>
</tbody>
</table>

Table 2. HIV-2 Isolation and In Vitro Characteristics. PH2-1/a, seven months after seroconversion, CD4⁺ cell count 570/mm³; PH2-1/b, 36 months after seroconversion, CD4⁺ cell count 200/mm³. (SI), syncytium inducing; (NSI), non-syncytium inducing. c, no p24 antigen demonstrated within six weeks of incubation. dNT, not tested. eNA, no materials available.

PCR amplification of RT genes sequences

To further characterise the HIV-2 isolates, RT gene segments were amplified and sequenced. The open reading frames from all the clones that were fully sequenced (n=11) were intact with the active YMDD site present and no stop codons observed. Fig. 2 shows an alignment of the deduced amino acid sequences of one clone for each of the six HIV-2 isolates. Limited amino acid diversity was observed in the individuals originating from the Cape Verdian Isles (91-94% identity). Phylogenetic analysis indicated that all these isolates grouped together (Fig. 3) and comparison...
with published sequences included them into subtype A [11,18]. The French patient, who had probably been infected by an African immigrant in France of unknown origin, also grouped within subtype A (89-92% identity with the subtypes A from the Rotterdam area). The HIV-2 isolate from patient RH2-6, who is of Ghanese origin, proved to be more distantly related (84-86% identity with the subtype A isolates) and grouped within subtype B [11], which was confirmed by Bootstrap analysis.

**Mutations related to zidovudine resistance**

RT fragments corresponding to the region that is implicated in zidovudine resistance in HIV-1 [19] were amplified for the two HIV-2 isolates from patients who had been treated with zidovudine for two years (RH2-2) and six months (RH2-5) respectively. In HIV-2 isolate RH2-2 mutation K28 -> R28 and in isolate RH2-5 K177-->M177 were identified. The former change has also been implicated in HIV-1 zidovudine resistance while the latter may be specific for HIV-2 isolates.

**DISCUSSION**

In this paper we have presented clinical and virological data from twelve HIV-2 infected individuals living in West Europe. Nine of them were of West African origin and three were born in Western Europe. All except one (PH2-1) had probably been infected through heterosexual contacts. Seven of the West African patients presented with an AIDS indicator disease or a CD4-cell count <200/mm³. Therefore, the number of HIV-2 infected patients of West African origin with progressed HIV-related symptoms upon presentation to the Rotterdam hospital was seven out of nine (Table 3). This ratio seems relatively high, when compared to data of HIV-2 infected patients presented by others [4,5]. It may be speculated that the relatively high number of symptomatic individuals in this group was caused by a reluctance to seek medical attention in the absence of clinical symptoms. This would imply that only the tip of an iceberg of HIV-2 infected individuals amongst the West African immigrant population of the Rotterdam area would have been included in this study. However, during the same observation period only nine of the 17 HIV-1 infected individuals from the same immigrant population presented with progressed HIV-related disease (Table 1). The median age of the HIV-2 and HIV-1 infected population was 37 and 32 years respectively.

Although no data were available on place and duration of both HIV-1 and HIV-2 infection, several observations prompted us to speculate that HIV-1 and HIV-2 infections in our population had a similar rate of disease progression. First the ratios of asymptomatic versus symptomatic individuals were not in favour of a more rapid disease progression in HIV-1 infected individuals within this group with the same social background, the same median time of residence in Europe and the same mean time of follow-up (32 months). Second, the rapid decline in CD4⁺ cell counts
observed in the French individual (PH2-1) over a 36 months observation period which clearly showed that HIV-2 infection may run a rapidly progressive course. This is not in agreement with the relatively slow disease progression reported by others for HIV-2 infected individuals [4,5]. So far we could not identify any predisposing factors, like co-infection with other blood borne viruses or a selective immigration pattern amongst these individuals (data not shown).

Figure 2. Alignment of RT protein fragment sequences from six HIV-2 viral isolates. The nucleotide sequences were determined from PCR amplification products, translated and aligned; ROD (HIV-2 subtype A) and 7312A (HIV-2 subtype B) were used for reference purposes. The arrows (•) indicate mutations corresponding with zidovudine resistance in HIV-1.

The results of virus isolation studies in PHA-PBMC and T cell lines, indicating more successful virus isolation from individuals with progressed disease and low CD4+ cell counts, are in agreement with the data presented by others [8,20-22]. The correlation between a SI virus phenotype in PHA-PBMC and in MT2 cells, with more progressed disease and/or lower CD4+ cell counts, is similar to observations in HIV-1 infected individuals [20-22]. It indicates that like in HIV-1 infection this marker may be of prognostic value. No exact information about geographical origin of the HIV-2 infection of the individuals in our study could be obtained. Nevertheless, in the phylogenetic analysis of the RT gene segments all the viruses from individuals of Cape Verdian origin clustered within subtype A. HIV-2 ROD, which also originated from an individual of Cape Verdian origin
[23], also clusters within this subtype. This phylogenetic analysis also indicated that the HIV-2 isolate from the individual born in Ghana (RH2-6), clustered within subtype B. This is the first subtype B strain reported to have a S1 phenotype. Preliminary sequencing data of the envelope genes of RH2-4, RH2-5 and PH2-1 indicate that RH2-4, who was born in The Netherlands, was infected by his wife RH2-5 of Cape Verdian origin. Furthermore, phylogenetic analysis of the envelope genes confirmed that RH2-4 and PH2-1 clustered within subgroup A, as was also demonstrated by RT gene analysis (R. Gruters, not published). Whether the RT mutations observed in the HIV-2 genes that are involved in HIV-1 zidovudine resistance result in increased resistance to zidovudine remains to be elucidated. To our knowledge this is the first description of mutations in HIV-2 possibly related to zidovudine treatment.

Figure 3. Phylogenetic tree of HIV-2 isolates estimated from their nucleotide sequences of RT fragments (UPGMA); ROD (HIV-2 subtype A), 7312A (HIV-2 subtype B) and HXB (HIV-1) were used for reference purposes.

Further evaluation of the development of clinical signs in the HIV-2 infected individuals of this study population, together with changes in genotypic and phenotypic characteristics of their viruses, may show to what extent more or less variable viral characteristics contribute to, or correlate with, the pathogenesis of HIV-2 induced disease.

Acknowledgements
The authors wish to acknowledge Ms C. Kruyssen for secretarial assistance and Ms N. Langebeek for assistance in patient administration and sampling.
References


CHAPTER 3

LOW VIRAL LOAD IN PLASMA AND LYMPHOID ORGANS OF HIV-2 INFECTED INDIVIDUALS
LOW VIRAL LOAD IN PLASMA AND LYMPHOID ORGANS OF HIV-2 INFECTED INDIVIDUALS

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3Bernhard Nocht Institut für Tropen Medizin, Körber Labor für AIDS Forsschung, Bernhard Nocht Straße 74, Hamburg, Germany
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§These authors contributed equally to the work presented

Summary
Lymphoid tissue specimens collected from 10 HIV-2 infected individuals at different stages of the infection, were analysed by immunohistochemistry and in situ HIV-2 RNA hybridisation. Like in asymptomatic HIV-1 infected individuals, productively infected cells in the lymphoid tissue of asymptomatic HIV-2 infected individuals, were predominantly found in areas with low numbers of CD8+ cells. In lymphoid tissue from asymptomatic HIV-2 infected individuals, we found higher numbers of CD8+ cells in the follicles and five fold lower numbers of productively HIV infected cells, as compared to asymptomatic HIV-1 infection. Furthermore, plasma levels of viral RNA proved to be at least hundred times lower at this stage. In contrast, in HIV-2 infected patients who had developed AIDS, plasma virus levels were only five times lower than in HIV-1 infected patients with AIDS. Productively infected CD4+ T cells were predominantly found around foci of inflammation. Collectively, the data presented indicate that a low level of virus production in lymphoid organs may explain the relatively long asymptomatic stage in HIV-2 infection.
Introduction

The lentiviruses human immunodeficiency virus type 1 and type 2 (HIV-1 and HIV-2), are the etiological agents of acquired immunodeficiency syndrome (AIDS) (1,2). HIV-1 is pandemic and although the number of reported HIV-2 related AIDS cases increases worldwide, high HIV-2 sero-prevalence is still largely confined to West Africa (7-11). The \textit{in vitro} characteristics, like viral tropism for \(\alpha\)- or \(\beta\)-chemokine receptor positive CD4\(^+\) cells, induction of cytopathic changes (3-5), as well as the \textit{in vivo} pathogenesis they induce are similar. The most poignant differences between HIV-1 and HIV-2 infections are a generally slower disease progression (6) and significantly lower transmission rates observed in the latter (8-10). These observations parallel immunological changes observed in the respective infections both infections: CD4\(^+\) T cell counts and CD4/CD8 ratios generally decline less dramatically in HIV-2 infected individuals (6).

The mechanism underlying these differences between HIV-1 and HIV-2 are not known. Virus isolation and proviral PCR studies have indicated that the overall viral burden in HIV-2 infected individuals is lower than that in HIV-1 infected individuals (14,15). Furthermore a recent study showed that plasma HIV-2 viral RNA levels in HIV-2 infected individuals with more than 14% CD4 cells are significantly lower than in HIV-1 infected individuals at the same stage of the disease. In AIDS patients with less than 14% CD4 cells, the plasma levels of HIV-1 RNA and HIV-2 RNA were comparable.

The limited information presently available on the pathogenesis of HIV-2 as compared to HIV-1 infection, prompted us to study the tissue architecture as well as the nature and localisation of HIV-2 infected cells in lymphoid tissues collected from HIV-2 seropositive individuals at different stages of the infection. Similar studies have in the past significantly improved the knowledge on the pathogenesis of HIV-1 infection. It is well established that the lymphoid organs represent a major reservoir for HIV-1 (24,25). The viral burden in the lymph nodes proved to be magnitudes higher than in the peripheral blood (19). As a consequence of permanent infection, lymphoid tissue shows several architectural changes ranging from follicular hyperplasia to lymphocyte depleted nodes.

No published data are available on changes of lymphoid tissue in HIV-2 infection. Our study was done to assess HIV-2 replication in lymphoid tissues obtained at different stages of the infection. The data were compared with plasma HIV-2 RNA levels determined with a recently developed assay, and with lymphoid tissue and plasma samples obtained from both HIV seronegative and HIV-1 seropositive individuals using the same techniques.

Materials and methods

\textit{Patients and materials}

Surgical lymphoid tissue biopsy specimens were collected with informed consent from 10 HIV-2 infected West African individuals (nine from the
Cape Verde Islands, one from Guinea Bissau) residing in Rotterdam, for
diagnostic purposes. From seven patients who exhibited some lymph node
enlargement, a cervical lymph node biopsy was taken and from three
patients with tonsil hypertrophy a tonsil biopsy was taken. Two patients on
anti-retroviral therapy (RH2-17 and RH2-5) failed to show clinical improve­
ment upon therapy as judged from a continuing decline in CD4⁺ T cell
counts (RH2-17) or a new AIDS defining event (RH2-5): a disseminated
*Mycobacterium tuberculosis* (MTB) was diagnosed using lymph node
biopsy material. Clinical data of these 10 HIV-2 infected individuals are
presented in table 1. HIV-2 infected individuals were divided into two
groups on basis of a CD4 count either below (group 1) or above (group 2)
200 cells/µl.

<table>
<thead>
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<th>Tonsil/lymph node</th>
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<td>RH2-12</td>
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<td>990</td>
<td>Tonsil</td>
<td>No</td>
</tr>
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<td>230</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>M</td>
<td>35</td>
<td>A3</td>
<td>110</td>
<td>Lymph node</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 1. Clinical Characteristics HIV-2 Seropositive Patients. F is female and M is male

For comparison lymph node specimens obtained previously from HIV-1
infected patients were selected. Six lymphoid tissue specimens had been
collected from HIV-1 infected patients with little or no lymph node
enlargement and CD4⁺ cell counts >500/µl (16). Four HIV-1 infected lymph­
oid tissue biopsies had been obtained from HIV-1 infected patients with
AIDS (17). Biopsy specimens from 10 HIV-negative individuals were
selected from our previously published studies (16). All HIV-2 seropositive
individuals, regularly attending the clinic in Rotterdam, were selected for
quantitative HIV-2 RNA determinations. Forty-eight HIV-1 and 17 HIV-2
infected individuals, not on highly active antiretroviral treatment (HAART), were sampled and divided in two groups based on CD4 cell counts below or above 200/μl, as was done for the molecular pathological examinations.

**Light microscopy**

Histological procedures were followed as previously described (16). In short, part of the tissue was fixed overnight with 4% neutral buffered formalin and subsequently embedded in paraffin or directly embedded in Tissue Freezing Medium (Leica Instruments, Nussloch, Germany), snap frozen in liquid nitrogen and stored at -70°C. Dewaxed 5 μm paraffin sections were cut and stained with hematoxylin and eosin, Giemsa, Gomori silver impregnation, Ziehl-Neelsen and Grocott's staining.

**Immunohistochemistry**

Dewaxed paraffin sections were boiled in a domestic pressure cooker for 3 min. and chilled down to room temperature. Cryostat 5 μm sections were fixed with 2% paraformaldehyde for 10 min. Paraffin or frozen sections were incubated with the primary antibodies (CD4, Novocastra, Newcastle upon Tyne, UK, 1:40; CD8, Dako 1:10; CD23, Dako 1:100; CD45 RO, Dako 1:100; CD68, Dako 1:10, Km4p, generously gifted by M. Parwaresch, University of Kiel, Germany). Binding of antibodies was visualised by the alkaline phosphatase anti-alkaline phosphatase technique using New Fuchsin as chromogen. The sections were then either counter-stained with hematoxylin and mounted, or dehydrated and subjected to in situ hybridisation to detect HIV RNA.

**In situ hybridisation**

35S-labeled, single stranded, anti-sense HIV-1 and HIV-2 RNA probes (Lofstrand Labs., Gaithersburg, MD, USA) were used. The HIV-2 probe was constructed and kindly provided by C.H. Fox (Molecular Histology Lab, Gaithersburg, MD). The in situ hybridisation was done as previously described (16,18). In short, frozen sections were fixed in 4% paraformaldehyde for 20 min. Paraffin sections were either treated with Proteinase K (0.01 mg/ml) for 8 min. at room temperature or boiled in a pressure cooker in citrate buffer (pH 6) for five minutes and run in duplicate. The sections were incubated with prehybridisation mixture (50% formaldehyde, 0.5 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.02% Ficoll-polyvinylpyrrolidone-BSA and 7 mg of tRNA/ml) for 2 hours at 37°C and then covered with hybridisation mixture (prehybridisation mixture supplemented with 10% dextran sulfate and 2x10^6 dpm of probe/ml) overnight at 45°C. The sections were washed with three changes, each in 50% formamide: 50% 2 x standard saline citrate (SSC), 2 x SSC-0.1% Triton, and finally 0.1x SSC. Sections were digested with RNase (Boehringer Mannheim, GmbH, Mannheim, Germany) at 37°C for 40 minutes. After an additional washing step the slides were dipped into photo emulsion (NTB2; Kodak, Rochester, NY) exposed for 3-7 days and developed, counter-stained with hemalaun and mounted. As a negative
control, sections were hybridised with a $^{35}$S labelled sense probe. The sections were examined with a microscope equipped with epiluminescent illumination (Axiophot; Carl Zeiss Inc., Jena, Germany). Cells were considered positive for viral gene expression if the grain count was more than six times the background.

Figure 1. HIV-2 In Situ Hybridisation and Immunohistochemistry on Lymphoid Tissue. A lymph node biopsy of patient RH2-13 showing irregular follicular hyperplasia with irregular shaped follicles (A). The structure of FDC (arrows), visualised with red staining (CD23), is reasonably intact and HIV-2 ISH overlay is visualised with green epiluminescence (B). A tonsil biopsy of RH2-5 showing HIV-2 producing
cells, visualised by silver grains, in the lymphoid epithelium CD8⁺ cells are stained red (C).

**Semiquantitative Image Analysis of CD4⁺, CD8⁺ or HIV-RNA⁺ cells**
Using a 20x objective, regions of germinal centres (GC) and T cell dependent areas (T zones) were marked on the monitor. Total CD4⁺ and CD8⁺ cells were counted in these areas. Ten fields of GC and T zones were counted on each slide. The percentages of cell subsets were calculated in the designated areas. For the quantification of the number of HIV-RNA⁺ cells per mm², the microscope was equipped with an image analyser (Carl Zeiss Inc., Jena, Germany). The numbers of HIV-RNA⁺ cells located in the GC and extra follicular tissue were counted in the whole cutting level of the tissue specimen. The surface areas occupied by the GC and extra follicular tissue was determined.

**Quantitative viral RNA assays**
For the quantification of HIV-1 viral RNA levels in plasma, the Cobas Amplicor assay (Roche Diagnostics, Almere, The Netherlands) was used. A recently described in-house developed assay was used to quantify the plasma HIV-2 RNA levels (Schutten et al submitted). In short, viral RNA was isolated from EDTA plasma using a slightly modified High Pure Viral RNA Isolation kit (Roche Molecular Diagnostics, Mannheim, Germany). cDNA was made with AMV-RT using the 3′primers HIV-2TMrpr1 5'-CCACACGCTGCCTTTGGTA-3' and HIV-2TMrpr2 5'-TCTGCATGGCTGTTGGATG-3'. The cDNA was detected with the Taqman real time detection PCR method using two different primer/probe sets. Set one encompassed a region between the 5' LTR and the Gag/Pol open-reading frame (nucleotides 380 to 441 of the HIV-2 NIHZ isolate). Set 2 encompassed a region in the gag gene (nucleotides 1078 to 1144 of the HIV-2 NIHZ isolate). As standard for the quantification, an electron microscopy counted virus stock from the HIV-2 NIHZ isolate was used (ABI, catalogue number 10-128-000, Maryland, USA).

**Results**

**Tissue architecture**
Follicular hyperplasia with irregularly shaped follicles (Fig. 1A) were observed in 4 lymphoid tissue samples obtained from HIV-2 infected individuals with peripheral CD4⁺ T cell counts >200/µl. The GC showed a starry sky pattern. The mantle zone was also attenuated in these specimens. In all nodes proliferation of high endothelial venules was observed. The T dependent zone contained elevated numbers of activated lymphocytes. The biopsy taken from patient RH2-5, HIV-2 positive, CD4⁺ T cell count <200/µl, showed multiple granulomas with or without caseation and epitheloid cell layers (Fig.2). Multinucleated cells of Langhans type were observed. The tonsil (right) biopsy taken from this patient after six months of specific MTB treatment showed an increased number of
granulocytes in a part of the squamous epithelium (not shown). This increase was not observed in the left tonsil. On basis of the histological examination, we were unable to identify the causative agent of this focal inflammation. The lymphoid tissue obtained from a cervical lymph node from patient RH2-18 showed evidence of a monocytic B cell reaction in the subcapsular and intermediate sinuses. Multiple giant cells were present in these areas (Fig. 3A). In sections taken at other levels of the same lymph node no aberrant histological observations were made (Fig. 3B).

<table>
<thead>
<tr>
<th>Peripheral blood</th>
<th>Lymphoid tissue</th>
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<tbody>
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<td>CD4 count</td>
<td>CD8 count</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>RH2-3</td>
<td>660*</td>
</tr>
<tr>
<td>RH2-12</td>
<td>990</td>
</tr>
<tr>
<td>RH2-13</td>
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<tr>
<td>HIV*</td>
<td>ND</td>
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</table>

**Table 2.** Immuno-Histopathological Characteristics. * CD4 and CD8 counts are given in cells per μl and mm² in peripheral blood and lymphoid tissue respectively. Mean numbers per group are given in bold face.

**Immunohistopathology and distribution of HIV-2 RNA positive cells in lymphoid tissue biopsies from individuals with peripheral CD4 cell counts >200/μl (group 1)**

In several individuals with low peripheral blood CD4⁺ T cell counts considerable numbers of tissue CD4⁺ T cells were observed (table 2). To further address this issue, we used a previously described semi-quantitative method to count the numbers of CD4⁺ and CD8⁺ T cells in GC and in T
zones (17) (Table 2). For comparison previously obtained data from sero-negative and HIV-1 infected subjects, generated with the same method (16), were used. It could be calculated that the number of CD4⁺ T cells in peripheral blood of HIV-2 seropositive individuals as compared to HIV-negative individuals was 46% lower whereas the CD4⁺ T cell counts in the GC and T cell zone of lymphoid tissues from HIV-2 infected individuals were about 13% and 20% lower than in HIV negative individuals. The mean tissue CD4/CD8 ratios in the GC of tissue biopsies obtained from HIV-1 and HIV-2 infected individuals were 1.7 and 1.0 respectively. The lower CD4/CD8 ratio in the GC area of the HIV-2 seropositive individuals was largely due to higher numbers of CD8⁺ cells (data not shown). The mean CD4/CD8 ratio in the peripheral blood and lymphoid tissue T zones did not differ substantially between the two groups (0.6 both and 1.2 versus 1.4 respectively).

Figure 2. HIV-2 in situ hybridisation and immunohistochemistry of a lymph node of patient RH2-5, coinfected with Mycobacterium tuberculosis. Within the lymph node granulomas are observed with large caseous necrotic centres (CNC) surrounded by an epitheloid cell layer and many macrophages (A). In the perinecrotic zone (PNZ) a large number of HIV-2 producing cells were observed (silver grains, indicated by arrows). Immunolabeling showed an abundance of CD68⁺ macrophages (red staining, A and inset II), but virus producing cells proved to be CD4⁺CD45RO⁺ T cells (red staining, inset I).
Both in the HIV-1 and the HIV-2 infected individuals, HIV-RNA+ cells were mainly confined to GC in lymph nodes and tonsils and to the lymph epithelium of the tonsils (Fig.1B, table 3). In both areas low numbers of CD8+ T cells were found (Table 2). We subsequently determined the phenotypes of the virus producing cells by double labelling for HIV-2 RNA with in situ hybridisation and immunohistochemical staining for CD4, CD45RO, CD68 or lysozyme. All productively HIV-2 infected cells proved to be CD45RO+CD68+lysozyme-, identifying them as CD4+ T cells. When compared with lymphoid tissues from HIV-1 infected individuals, there seemed to be a relatively low number of trapped HIV-2 RNA overlying the FDC network (Fig 1C). For HIV-1, it has been shown that the number of productively infected cells is directly related to both the amount of trapped virus onto the FDC processes and to plasma viral load (20). We therefore counted numbers of virus producing cells in GC and the T zones (table 2). The mean number of HIV RNA+ cells per mm² in the total surface area is approximately five times lower for the tissues obtained from asymptomatic HIV-2 infected patients (0.29) than the number found in tissues from asymptomatic HIV-1 infected patients (1.34) (table 3).

Immuno-histopathology and distribution of HIV-2 RNA positive cells in lymphoid tissue biopsies from individuals with peripheral CD4 cell counts <200/μl (group 2)

In the biopsies from individual RH2-5, high numbers of HIV-2 RNA+ cells were observed around the granulomas in the lymph node (Fig 2) and in the area in the right tonsil where the increase of granulocytes had been observed (data not shown). The number of HIV-2 RNA+ cells was markedly higher than in areas without aberrant histological signs (Table 3). The lymph node biopsy of individual RH2-18 showed many HIV-2 RNA+ cells in sections 2 and 3, where also the monocytic B cell reaction and Langhans type giant cells were observed (table 3, Fig 3A). Section 1 showed fewer HIV-2 RNA+ cells (table 3, Figure 3B). Most HIV-2 RNA+ cells, in both tonsil and lymph node biopsies from individual RH2-5, were CD45RO+CD68-lysozyme- cells, identifying them as CD4+ T cells (Figure 2 inset). In sections 2 and 3 from the lymph node specimen of individual RH2-18 multiple HIV-2 RNA+CD68+ multinucleated cells were observed at the sites of the monocytic B cell reaction (data not shown). In the other parts of the lymph node all productively HIV-2 infected cells were CD45RO+CD68-lysozyme-.

Quantitative HIV-1 and HIV-2 plasma viral RNA loads

Thirty-three HIV-1 and 9 HIV-2 infected individuals with a CD4 cell count below 200/μl and 15 HIV-1 and 8 HIV-2 infected individuals with a CD4 cell count above 200/μl were selected. The results of quantitative HIV-1 and HIV-2 viral load determinations are shown in figure 4. The difference between the mean quantitative plasma viral loads of HIV-1 and HIV-2 infected patients with CD4 count less than 200/μl was significant (Student T test, p=0.014). In six out of eight samples from HIV-2 seropositive indivi-
duals with more than 200 CD4 cells per µl plasma viral RNA levels proved to be below the detection limit (< 500 copies/ml), whereas only one of the HIV-1 samples was below the detection limit of 500 copies/ml. Mean HIV-2 RNA plasma viral loads were at least $2 \log_{10}$ lower than in HIV-1 infected individuals in this stage of the infection (Figure 4).

<table>
<thead>
<tr>
<th></th>
<th>CD4 count</th>
<th>HIV RNA$^+$ cells/mm$^2$</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>GC</td>
</tr>
<tr>
<td>RH2-12</td>
<td>990*</td>
<td>0.28</td>
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<tr>
<td>199/87</td>
<td>914</td>
<td>0.04</td>
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<td>RH2-3</td>
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<td>RH2-15</td>
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<tr>
<td>Mean</td>
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</table>

|            |           |            |              |
| RH2-5      | 150       | 5*         | 7*           | 38**        |
| RH2-5l     | 120       | 1.8        | Ind          | 1.14        |
| RH2-5r     |           | 1.77       | Ind          | 2.4         |
| RH2-18#1   | 110       | 0.36       | 0.62         | 0.55        |
| RH2-18#2   |           | 6.7        | 7.61         | 7.28        |
| RH2-18#3   |           | 7.52       | 2.31         | 2.93        |
| mean       |           | 127        | 3.63         | 2.83        |
|            |            | 2,86       |              |
| HIV-1      | 209/96    | 580        | 1.62         | 0.7         | 1.12        |
|            | 149/96    | 570        | 1.66         | 0.97        | 1.28        |
|            | 1008/88   | 500        | 1.06         | 0.32        | 0.53        |
|            | 126/96    | 500        | 1.93         | 1.27        | 1.62        |
|            | 799/95    | 400        | 0.73         | 0.32        | 0.49        |
|            | 629/97    | 380        | 5.18         | 1.68        | 3.01        |
| mean       |           | 488        | 2.03         | 0.88        | 1.34        |

Table 3. * Peripheral blood CD4$^+$ T cell per µl. Ind = indeterminate, $^+$ = All HIV-RNA$^+$ cells/total surface area, not processed in the mean ** = the mean total number of HIV-RNA$^+$ cells in three granulomas.

**Discussion**

In the present study we have shown that the plasma and tissue viral loads in HIV-2 infected individuals is significantly lower than that in HIV-1 infected individuals at the same stage of infection. Productively infected cells in both
HIV-1 and HIV-2 infected individuals were mainly located in areas with low numbers of CD8\(^+\) T cells. Higher numbers of CD8\(^+\) T cells were found in the GC of the HIV-2 infected individuals than in the GC of HIV-1 infected individuals. Relative to the healthy uninfected control individuals studied, both HIV-1 and HIV-2 infected individuals had higher numbers of CD4\(^+\) cells in their lymphoid tissue as compared to the peripheral blood.

Figure 3. HIV-2 in situ hybridisation of a lymph node of patient RH2-18. HIV-2 particles are visualised with green epiluminescent light. Arrows indicate infected cells.

In HIV-1 infection the number of productively infected cells in lymphoid tissues, as determined by HIV-1 RNA in situ hybridisation, directly correlates with plasma viral load (19). In the HIV-2 infected individuals with a CD4 count above 200/\(\mu\)l, the numbers of productively infected cells proved to be approximately five times lower than in HIV-1 infected individuals at the same stage of the infection. From these data it may be concluded that the mean plasma viral load in the HIV-2 positive individuals also should be about five times lower than in HIV-1 seropositive individuals. However, the mean plasma HIV-2 RNA load, obtained with the recently developed quantitative HIV-2 RT-PCR, proved to be at least 100 times lower. Two observations suggest that a slower replication rate is responsible for the lower plasma viral load in the HIV-2 infected individuals. The in situ hybridisation staining, which is directly correlated with the number of virus particles, in general proved to be less intensive for HIV-2 infected cells than for HIV-1 infected cells (ref.17 and fig 1C, 2, 3B,). We furthermore recently obtained a pre-seroconversion sample from an HIV-2 infected individual which proved to contain approximately \(10^4\) HIV-2 RNA copies per ml. This is significantly lower as compared to pre-seroconversion samples from HIV-1.
infected individuals suggesting a lower production rate of HIV-2 than of HIV-1. This may subsequently result in a low post-seroconversion-nadir and a relatively low stable set point of plasma HIV-2 RNA. In HIV-1 infection the level of plasma HIV-1 RNA is correlated with survival (21). Analogous to HIV-1 infection, the low levels of plasma HIV-2 RNA during and after primary infection may explain the prolonged asymptomatic phase of HIV-2 infection as compared to HIV-1. Both the number of productively infected cells and the plasma HIV RNA levels of the HIV-2 infected patients with CD4^+ cells <200/μl proved to be approximately five times lower than those found in HIV-1 infected individuals with CD4 counts below 200/μl and equal to those found in HIV-1 infected individuals with CD4 counts >200/μl. These data implicate that in contrast to what was generally assumed disease progression in the end stage of the HIV-2 induced pathogenesis is slower as compared to that of HIV-1. These data therefore corroborate findings of Lucas et al (28), who showed that opportunistic infections that are in general only found in HIV-1 infected individuals who are immunosuppressed for a long period of time, are found more often in HIV-2 infection than in HIV-1 infection. This already suggested a prolonged survival of HIV-2 infected individuals in the end stage of the disease.

Figure 4. Plasma Viral RNA Load in HIV-1 and HIV-2 Seropositive Individuals. Plasma HIV RNA levels in individuals with CD4 counts below (left panel) and above (right panel) 200/μl. The mean CD4 cell counts are given. Mean plasma viral
loads ( ) are given and the Student T test P value for the comparison of the plasma HIV-1 and HIV-2 RNA values for the patients <200 CD4/μl. * Calculation of mean plasma viral load is not possible due to non accurate values <500 copies/ml.

It has been shown that during the acute phase of HIV-1 infection, the appearance of HIV-1 specific CD8⁺ T lymphocytes coincides with a drop in plasma HIV-1 RNA (22). Furthermore, antiviral pressure exerted by HIV-1 specific T lymphocytes during this stage, may lead to rapid selection of escape variants (23). It is therefore generally accepted that HIV-1 specific CD8⁺ T cells are a major antiviral component of the HIV specific immune response. This is supported by our observation that the numbers of productively infected cells are significantly lower in areas where relatively high numbers of CD8⁺ cells are present. In HIV-1 infected individuals CD8⁺ T cells are rarely found in the GC, but they can be found in the GC of lymphoid tissues from HIV-infected individuals. It has therefore been suggested that HIV-1 specific CD8⁺ T cells migrate into the GC since high amounts of virus can be found in these areas (24). Here we have shown that the CD4/CD8 ratio in the GC of HIV-2 infected individuals is markedly lower than in HIV-1 infected tissue, due to a higher number of CD8⁺ T cells in the GC of HIV-2 infected individuals. In the T zones and the peripheral blood no significant differences between the two groups were found. It may therefore be speculated that CD8⁺ T cells in HIV-2 infected individuals are more efficient in controlling virus replication than in HIV-1 infected individuals.

In HIV-1 infection it has been shown that viral loads increase during opportunistic infections at end stage disease (25,26). Successful specific treatment for the infectious agent usually results in a return of plasma viral loads to baseline levels. Also in the HIV-2 infected biopsies, one of them co-infected with MTB, increased numbers of productively infected cells were found at the sites of focal inflammation. In HIV-1 infection it has recently been postulated that there is a switch towards macrophages as the major source of virus production during such inflammatory reactions involving high numbers of activated macrophages (27). In the MTB infected lymph node of patient RH2-5, in which high numbers of activated macrophages were found, and in the tonsil of the same patient, which showed increased numbers of granulocytes, productively HIV-2 infected cells all proved to be CD4⁺ T cells. These data are in agreement with our recent findings in HIV-1 infected individuals, co-infected with MTB or Mycobacterium avium complex, in which CD4⁺ T cells remained the major source of HIV-1 production (17). Studies on the lymph node of patient RH2-18, obtained at the time when the histology showed a monocytic B cell reaction, did reveal production of HIV-2 by CD68⁺ multinucleated giant cells of monocytic origin in some areas, whereas in other areas CD4⁺ T cells proved to be the main source.

Collectively, the data indicate that the slower disease progression and the lower transmission rate in HIV-2 infection can be explained by a slower replication rate of HIV-2 as compared to HIV-1.
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CHAPTER 4

CD4 T CELLS REMAIN THE MAJOR SOURCE OF HIV-1 DURING END STAGE DISEASE

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CD4 T CELLS REMAIN THE MAJOR SOURCE OF HIV-1 DURING END STAGE DISEASE

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SUMMARY
Objective: To assess the source of HIV-1 production in lymphoid tissue biopsies from HIV-infected patients, with no prior anti-retroviral protease inhibitor treatment, with a CD4⁺ cell count >150/µl (group I) or <50/µl (group II), co-infected with Mycobacterium tuberculosis or Mycobacterium avium complex.

Design and methods: Lymphoid tissue biopsies from 11 HIV-1 infected patients, taken for diagnostic purposes, were studied by HIV-1 RNA in situ hybridisation and immunohistochemistry.

Results: Patients of group I showed well organised granulomas, in contrast to patients of group II, in which granuloma formation was absent. HIV-1 RNA⁺ cells in group I patients were mainly found around the granulomas, whereas in group II HIV-1-producing cells were confined to areas with remaining intact lymphoid tissue. Despite the abundant presence of macrophages, the productively infected HIV-1-positive cells in both groups were almost exclusively CD4⁺ T cells.

Conclusion: In contrast to previously published data, CD4 T cells appear to remain the major source of HIV-1 production in end-stage disease.
Introduction
Molecular histopathological studies of lymphoid and non-lymphoid tissues of HIV-1 infected individuals have contributed considerably to our knowledge of the mechanisms involved in HIV-1-induced disease. Soon after infection, when HIV-1-specific humoral and cellular immune responses can be demonstrated, virus production is confined largely to CD4 T cells located within lymphoid tissues such as lymph nodes, mucosa associated lymphoid tissue (MALT) and spleen (1-4). During the entire asymptomatic stage of the disease, CD4 T cells are the major source of HIV-1, whereas productively infected cells expressing macrophage or dendritic cell markers are observed rarely in lymphoid tissues. Productively infected macrophages and dendritic cells have been observed in nasopharyngeal lymphoid tissues, cervix and brain (5-10); however, these cells are generally believed to contribute minimally to the high levels of virus produced throughout the course of the infection. During the asymptomatic and early symptomatic stages (CD4>200/µl), follicular hyperplasia with irregularly shaped follicles is often observed (11,12). Large numbers of virus particles within immune complexes are then captured onto the processes of follicular dendritic cells (1,2).

In the intermediate stages of the infection, the numbers of productively infected CD4⁺ T cells within lymphoid tissue increase (1,2). Ultimately however CD4⁺ T cells almost disappear and the architecture of lymphoid tissue, especially with regard to the germinal centres and the follicular dendritic cells network, is disrupted and the ability to trap virus is lost. Also at this stage of the disease, virus production is relatively high (13). The increase of cell-free plasma HIV-RNA and the virtual absence of CD4⁺ T cells, which are thought to be the major source of virus production, is up till now an unresolved paradox. Recently it has been suggested by Orenstein et al, that a possible explanation may be the switch from CD4⁺ T cells to another cell type as the major source of virus during end-stage disease (14). An alternative explanation could be that due to the increased replication rate of HIV-1 at end-stage disease, as well as a higher percentage of infected cells, a small reservoir of CD4⁺ T lymphocytes is sufficient to produce the observed increased levels of cell-free virus in plasma (15).

To address this issue we studied lymphoid tissue biopsies from 11 HIV-1 infected patients co-infected with Mycobacterium tuberculosis (MTB) or Mycobacterium avium-complex (MAC), by HIV-1 RNA in situ hybridisation and immunohistochemistry. Patients co-infected with MTB or MAC were selected because of their lymphoid tissue generally contains large numbers of macrophages. The hypothesis of Orenstein et al, that at end-stage disease the bulk of HIV-1 production shifts from CD4⁺ T cells to macrophages, may be tested by studying these patients.

Material and methods
Patient materials.

Lymph node specimens taken from eleven HIV-1-seropositive patients for diagnostic purposes, were selected for this study. The selection criteria
were: HIV-1 laboratory diagnosis; MTB or MAC laboratory diagnosis; peripheral blood CD4+ T cells <500/μl or AIDS-defining disease; no prior antiretroviral therapy with protease inhibitors. The peripheral blood CD4 cell counts and co-infections are listed in Table 1.

**Immunohistochemistry**

Tissues were fixed overnight with 4% buffered formalin (pH 7) and subsequently embedded in paraffin. The tissues were cut for routine histology (Giemsa, Ziehl-Neelsen, haematoxylin & eosin and Gomori’s silver impregnation). For immunohistochemistry, dewaxed 5μm paraffin sections were placed in a domestic pressure cooker containing 0.01 M sodium citrate solution (pH 6), boiled for 2 min, and chilled to room temperature (16). Primary antibodies towards CD4 (Novocastra, Newcastle upon Tyne, UK), CD68 (KP1), lysozyme and CD45RO (UCHL-1) (Dakopatts, Copenhagen, Denmark) were used as described previously (17). The sections were subsequently either counter-stained with haematoxylin & eosin and mounted, or they were dehydrated and subjected to HIV RNA in situ hybridisation.

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<th>Patient</th>
<th>CD4+ T cells/μl</th>
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<tr>
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**Table 1. Clinical characteristics of 11 HIV-1 seropositive patients.** Group I, patients with CD4 cell count 50-500/μl; Group II, patient with CD4 cell count <50 /μl. ND, not done

**In situ hybridisation**

A [35S]-labelled, single stranded, anti-sense HIV-1 RNA probe (Lofstrand Labs., Gaithersburg, Maryland, USA), which contains 1.4-2.7 kb fragments collectively representing approximately 90% of the HIV-1 genome, was used as previously described (3). In short, paraffin sections were either treated with proteinase K (0.01 mg/ml) for 8 min. at room temperature or heat denatured in citrate buffer (pH 6). The sections were incubated with
prehybridisation mixture (50% formamide, 0.5 M NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5% Ficoll-polyvinylpyrrolidone-BSA and 7 mg of tRNA/ml) for 2 hours at 37°C and then covered with hybridisation mixture (prehybridisation mixture supplemented with 10% dextran sulfate and 2x10^6 d.p.m. of probe/ml) overnight at 45°C. The sections were washed, RNase treated (Boehringer Mannheim GmbH, Mannheim, Germany) for 40 min. at 37°C and rewashed and then dipped into emulsion (NTB2; Kodak, Rochester, New York, USA). After exposure for 3-7 days the slides were developed, counter-stained with haemalaun and mounted as previously described. As negative controls, sections were hybridised with a radio-labelled sense probe. The sections were examined with a microscope equipped with epiluminescent illumination (Axiophot, Carl Zeiss Inc., Jena, Germany). Cells were considered positive for viral gene expression if the amount of grains counted was more than six times the number in the background.

Results
Lymph nodes from patients with 150-500/µl peripheral CD4 T cells, hereafter referred to as group I patients, contained multiple well-organised granulomas occupying 50-90% of the cutting surface. In parts of the tissue that were not involved in the granuloma formation, the nodal architecture with T cell dependent zone and several small inactive germinal centres was preserved. The granulomas were composed of epitheloid cells, multinucleated cells of the Langhans'-type and CD4+ as well as CD8+ T lymphocytes. The central part of the granulomas often showed caseation (data not shown). In contrast, in lymph nodes from patients with peripheral CD4+ T cell counts <50/µl, hereafter referred to as group II patients, well-organised granulomas were absent. The nodal architecture was obscured by heavy confluent infiltrate consisting of macrophages, intermingled with reactive plasma cells. The macrophages contained many acid-fast bacilli. The number of CD4+ T lymphocytes was decreased; they were present only in the remnants of lymphoid tissue seen as small islets at the periphery of the lymph nodes or between the bands of macrophages (Fig. 1a).

In the lymphoid tissues from the group I patients, the number of HIV-1 RNA-positive cells was unevenly distributed. Significant numbers of cells with a heavy in situ hybridisation overlay were observed mainly around the granulomas and occasionally within the granulomas (Fig. 1b). The numbers of HIV RNA-positive cells within the parts of the nodes where granulomas were observed, were approximately 10 fold higher than those in lymphoid tissue areas that did not contain granulomas (data not shown). In lymphoid tissues from end stage disease patients (group II), the numbers of HIV-1 RNA-positive cells were not significantly increased as compared to lymphoid tissue areas where no infiltrate of macrophages was observed. HIV-1 producing cells were confined to areas with remaining lymphoid tissue (Fig. 1a).
Fig. 1 HIV-1 *in situ* hybridisation on lymph nodes co-infected with Mycobacteria. (a) One MAC-infected lymph node from an HIV-1 infected patient No.8. The HIV-1 RNA signal (arrow) is present only in areas with some intact lymphoid architecture (original magnification 40x). (b) A *M. Tuberculosis* infected lymph node (patient No.1) with considerable numbers of CD4*⁺* T cells (red), and a significantly higher number of HIV-1 RNA-positive cells (arrows). The insert (I) shows double staining of HIV-1 RNA and CD45RO positive cells (original magnification 400x). (c) Abundant numbers of macrophages (red) did not correspond with HIV-1 RNA*⁺* cells (arrows). (d) Occasionally an HIV-RNA*⁺*CD68⁺lysozym*⁺* cell was observed (arrow).

The phenotype of the productively HIV-1-infected cells was subsequently determined by double labelling of HIV RNA by *in situ* hybridisation and immunohistochemical staining for CD4⁺, CD45RO⁺, CD68 or lysozyme. Despite the abundant presence of macrophages and Langhans'-type giant cells, the HIV RNA*⁺* cells in the lymphoid tissues from patients of group I
proved to be almost exclusively CD45RO⁺CD68⁻lysozyme⁻ (Fig. 1b, insert I, d), identifying them as CD4 T cells. The HIV-1 RNA-positive cells in lymphoid tissues from end stage disease patients (group II) also proved to be CD4⁺ T cells. Occasionally an HIV-1 RNA⁺CD45⁺CD68⁺lyzosym⁺ cell was observed (Fig 1d), excluding the possibility that productively HIV-1-infected macrophages were not identified due to down-regulation of CD68 and lysozyme expression by HIV-1 infection. Furthermore, the in situ hybridisation grain counts of HIV-1 RNA-positive CD4 T cells were generally much heavier than those on HIV-1 RNA-positive macrophages, suggesting a lower level of virus production in the latter cells (Fig. 1c,d).

Discussion
We have shown that in contrast to previously published data, CD4 T cells remained the major source of HIV-1 production at end stage infection. The histological appearance and cellular composition of granulomas in lymphoid tissue from group I patients did not differ from those seen in immunocompetent patients (18). Granuloma formation is characteristic in the cell mediated immune response to MTB, and it is well established that CD4⁺ T cells play a pivotal role in host defence against intracellular microorganisms (18). Around the granulomas massive HIV-1 production was mainly confined to CD4⁺ T cells. This finding is in agreement with the observed increase of the non-cell associated plasma viral load during opportunistic infections (19,20) and in studies on lymphoid tissue from HIV-1 infected patients co-infected with opportunistic organisms(14). Treatment of opportunistic or bacterial infections in HIV-1-infected patients resulted again in a decrease of plasma viral load levels to the levels observed before the onset of the opportunistic infection (19-21). The abundant presence of macrophages in lymphoid tissue from both groups did not apparently contribute to the viral load. Not only was the number of HIV-1 RNA⁺ infected macrophages low, but also the level of in vivo virus production by macrophages, assessed by in situ hybridisation grain counting, was lower than in CD4⁺ T cells. In addition, in in situ double labelling analyses of biopsies collected from 50 lymphatic tissues of more than 50 individuals at different stages of HIV-1 infection, productively infected macrophages have been observed only scarcely (unpublished observations). Considering the observation that at end-stage disease, in approximately 50% of patients so-called X4/syncytium inducing/rapid-high HIV-1 strains arise, which have shown to be less macrophage-tropic as compared to HIV-1 strains isolated from asymptomatic individuals (22,23), we postulate that at end stage disease CD4 T cells, rather than macrophages, remain the major source of HIV-1 production. Whether these clearly conflicting observations with previously published data (14) may attribute to differences in patient selection or laboratory techniques remains to be determined. The paradox of increasing viral loads with progressively declining circulating CD4⁺ T cells therefore remains. We and others (24-26) have, however, recently shown that in patients with low peripheral CD4⁺ T cells the decrease of tissue CD4⁺ T cell count is slower. It may be assumed that patients with CD4⁺ T cell counts <
50/μl, still have sufficient numbers of CD4+ T cells to allow the production of the high viral loads generally observed in such patients. Recent studies in AIDS patients have shown a sharp increase of the peripheral blood CD4+ T cell count after starting highly active antiretroviral therapy (26,27), probably reflecting redistribution of CD4+ T cells from epitheloid organs such as the lung or the gut. Taking into account the increased percentage of productively infected cells and the increased virus replication rate of HIV-1 strains isolated, it seems likely that also at the end stage of the disease sufficient CD4+ T cells are present in the individual to explain the high viral load observed.

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Coreceptor usage of human immunodeficiency virus type 2 primary isolates and biological clones is broad and does not correlate with their syncytium inducing capacities.

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Coreceptor usage of human immunodeficiency virus type 2 primary isolates and biological clones is broad and does not correlate with their syncytium inducing capacities.

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Summary

Entry of human immunodeficiency virus type-1 (HIV-1) into target cells is mediated by binding of the surface envelope glycoprotein to the CD4 molecule. Interaction of the resulting CD4/glycoprotein complex with \(\alpha\)- or \(\beta\)-chemokine receptors, depending on the biological phenotype of the virus, will then initiate the fusion process. Here, we show that primary HIV-2 isolates and biological clones, in contrast to those of HIV-1, may use a broad range of coreceptors, including CCR-1, CCR-3, CCR-5 and CXCR-4. The syncytium-inducing capacity of these viruses did not correlate with their ability to infect via CXCR-4 or any other coreceptor. One cell-free passage of the intermediate isolates in mitogen-stimulated, CD8\textsuperscript{+} cell-depleted, peripheral blood mononuclear cells (PBMC) resulted in the outgrowth of variants using CCR-5 only, whereas the coreceptor usage of late and early isolates did not change. Since HIV-2 is less pathogenic \textit{in vivo} than HIV-1, these data suggested that HIV pathogenicity \textit{in vivo} is not directly related to the spectrum of coreceptors used in \textit{in vitro} systems.
HIV entry is mediated by the binding of its surface glycoprotein to the cellular CD4 molecule, which acts as the primary receptor (8,18), and the subsequent interaction of the resulting CD4/envelope glycoprotein complex with another specific cellular co-factor (26,28). Several members of the seven transmembrane G-protein-coupled receptor family have recently been shown to be possible coreceptors for HIV-1 or HIV-2 (10,13,15, 22,25). Two members of this family, the chemokine receptors CCR-5 and CXCR-4 (also termed LESTR or Fusin), are the main coreceptors for macrophage and T cell-line tropic HIV-1 variants, respectively (1,2,4,7,9, 12,24). A small subset of dual-tropic HIV-1 strains is also able to use other members of this family as coreceptors, such as chemokine receptors CCR-2b or CCR-3 (4,11,14) or the orphan receptors Bonzo (also termed STRL33) and BOB (10,17). Moreover, longitudinal studies have shown a shift towards the usage of CXCR-4 as coreceptor for HIV-1 during disease progression, suggesting that broadening of coreceptor usage contributes to the cytopathic potential of HIV-1 strains in vivo (6). Compared to HIV-1, HIV-2 is less transmissible, less cytopathic in vivo, and induces generally a slower progression towards AIDS (19). However, we recently showed that rapid progression can be observed in some HIV-2 infected individuals (27). Similar to HIV-1, an inverse correlation was observed between the replication rate of viruses in vitro and the CD4⁺ T cell count of the patient from which they originated (27). Depending on the time required to detect virus after cocultivation of patient PBMC with phytohemagglutinin (PHA)-stimulated PBMC and the CD4 count of the patient, we could distinguish between early (>500 CD4/μl), intermediate (100<CD4/μl<500) and late (<100 CD4/μl) isolates. Studies comparing the requirements for HIV-1 versus HIV-2 entry may contribute to our understanding of the pathogenesis caused by these human lentiviruses.

Here, we have evaluated the capacity of a panel of human cells stably transfected with chemokine receptor genes to support infection with HIV-2 primary isolates from patients at different stages of the disease (27) (table 1). Five of the isolates studied (PH2-1, RH2-1, RH2-2, RH2-5 and RH2-7) belong to the HIV-2 subtype A, whereas one isolate, RH2-6, belongs to the subtype B (27). The mega-glioblastoma astrocytic cell line U87MG, stably transfected with the human CD4 gene and expression plasmids encoding various coreceptors (15), was infected with each of these isolates. Cells expressing both CD4 and chemokine receptors were selected regularly with 250μg/ml Geneticin (Gibco-BRL) and 1μg/ml puromycin (Calbiochem), respectively, and CD4 expression of all these cell lines was confirmed by FACS analysis (data not shown).

Two to 4.10⁴ adherent cells were incubated for 7 to 15 hours at 37°C in 24-wells plates with a viral inoculum of 250μl containing 2000 cpm of RT activity. Cells were then washed once with DMEM 10%FCS and cultured in 1.5ml DMEM/10%FCS. Virus replication was monitored at days 10 or 11 post-infection by quantification of RT activity in 150μl of culture super-
The abilities of HIV-2 primary isolates to infect U87MG/CD4 cells expressing different coreceptors are shown in figure 1.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical status (CDC criteria)</th>
<th>CD4 count/μl</th>
<th>Biological clones</th>
<th>Phenotype</th>
<th>Subtype$^d$</th>
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<td>A</td>
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<td>NSI</td>
<td>A</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>C1</td>
<td>NSI</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C12</td>
<td>NSI</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E6</td>
<td>NSI</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>H8</td>
<td>NSI</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td>D5</td>
<td>SІ$^b$</td>
<td></td>
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<td>H12</td>
<td>SІ$^c$</td>
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<td>NSI</td>
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<td>NSI</td>
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<tr>
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<td>10</td>
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<td>SІ$^c$</td>
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</table>

Table 1: Description of HIV-2 primary isolates or biological clones and patients clinical status. $^a$N.A. Not available, $^b$SI phenotype on PBMC, $^c$SI phenotype on PBMC and MT-2 cells, $^d$on basis of nucleotide sequences of reverse-transcriptase fragments (27)

The early isolate (RH2-1) only used CCR-5 as its coreceptor (figure 1A). Intermediate, NSI isolates (table 1) PH2-1, RH2-5 and RH2-7, and late, SI isolate RH2-2, all belonging to HIV-2 subtype A, appeared to use a broad range of coreceptors: in addition to CCR-5, CCR-1 and CCR-3 were efficiently used (Figure 1A). These isolates were also all able to use...
CXCR-4, and some entered U87MG/CD4 cells via CCR-2b at different levels of efficiency. This proved not to be dependent on their SI/NSI phenotype (figure 1A). No significant differences in coreceptor usage were observed between intermediate and late subtype A isolates (figure 1A and table 1). The late, subtype B, SI isolate RH2-6 (table 1), however only infected U87MG/CD4 cells expressing the CXCR-4 receptor. In contrast with a previous study (25), these isolates were unable to infect U87 MG/CD4 cells not transfected with any coreceptor. Therefore, the broad range of coreceptor usage of HIV-2 primary isolates could not be attributed to their ability to infect U87MG/CD4 cells via an unidentified coreceptor constitutively expressed by these cells.

Virus isolates were passaged with cell-free infection for one week in CD8-depleted, PHA-stimulated PBMC. Progeny viruses were then assayed for their coreceptor usage. Replication of thus passaged NSI isolates was only detectable in CCR-5 expressing cells, whereas the range of coreceptors used by SI isolates did not seem to be influenced by this passage (figure 1B). However, four to seven days prolonged cultivation after the standard eleven days of NSI isolates in U87MG/CD4 cells showed also a low level of virus production in CCR-1 or CCR-3 expressing cells (data not shown). This indicated that CCR-1 and CCR-3 using variants were still present in the isolates after a one week passage in CD8-depleted PHA-PBMC. Thus, a short-term passage in CD8-depleted, PHA-stimulated PBMC increased preferentially the frequency of CCR-5-restricted variants present in the NSI isolates.

The selective expansion of a viral subpopulation in CD8-depleted PHA-PBMC suggested that the HIV-2 isolates tested consisted of a pool of variants with different coreceptor requirements. In order to determine the actual coreceptor usage of such HIV-2 variants, we isolated 16 biological clones from patients PH2-1, RH2-5 and RH2-7 (table 1). In short, PBMC from these donors were plated in 96-well round bottom plates (Coulter) at 2x10^5, 2x10^4 and 2x10^3 per plate with 4x10^6 PHA-stimulated PBMC from seronegative individuals per plate. Each well was further treated individually and new medium and cells were added once a week, according to standard protocols (27). All wells were monitored after two to three weeks for the presence of p24 antigen (V5 ELISA, Organon Technika). P24 positive wells of 96-wells plates in which less than five positive wells were detected, were considered as clonal. These were further co-cultivated with PHA-stimulated PBMC. Before the assessment of their coreceptor usage, these clones were passaged for one week on CD8-depleted, PHA-stimulated PBMC. Of the 16 biological clones isolated, two (PH2-1 #D5 and PH2-1 #H12) exhibited a SI phenotype on MT-2 cells and/or PHA-PBMC, whereas the other 14 were NSI variants (table 1). The two SI biological clones were obtained from the same individual who progressed rapidly towards AIDS (within 3 years), suggesting that the fusogenic potential of the envelope glycoprotein complex contributed to disease progression in this patient.
Figure 1: Coreceptor Usage of HIV-2 Primary Isolates. Infections were performed with primary isolates cultured for two weeks in PHA-PBMC (A) and then passaged for one week in CD8-depleted, PHA-PBMC (B). Each value represents the mean and standard deviation of 3 independent experiments and is expressed in cpm/150μl supernatant. The cut-off value has been set at 2 times the mean of all the background values. N.T.: Not tested.

All the clones were tested for their coreceptor usage by infection of U87MG/CD4 cells and appeared to be able to use CCR-5 at very high efficiency (figure 2). All but one (PH2-1 #C1) were also able to use at least one other chemokine receptor as a coreceptor. CCR-1, CCR-3 and CXCR-4 were used by 13, 15 or 13 clones of the 16 clones, respectively. In contrast, CCR-2b was only used by 3 clones isolated from the same
patient: RH2-7 #C9, #D7 and #G12 (figure 2B). However, the HIV-2 biological clones exhibited a higher efficiency of infection via CCR-5 than via the other coreceptors. This was shown by consistent higher RT values in CCR-5 expressing cells, and detection of virus production two to four days earlier in CCR-5 expressing cells (data not shown). Finally, no consistent differences in coreceptor usage were observed between the SI clones PH2-1 #D5 and #H12 and the 14 NSI clones.

It has been stated previously that coreceptor usage of HIV-1 clones was correlated with the viral phenotype: macrophage-tropic, NSI HIV-1 variants exhibit a CCR-5-restricted entry, whereas SI, T-cell line-tropic isolates can also use CXCR-4 (3,16,24). The importance of CCR-5-mediated entry has moreover been clearly demonstrated in vivo by the resistance to HIV-1 infection of individuals who have a homozygous deletion in CCR-5 (20,21,23). Our experiments showed that all but one (RH2-6) HIV-2 biological clones and primary isolates, of SI or NSI phenotype, can use CCR-5. Therefore, as described for HIV-1, CCR-5 seems the main coreceptor for primary HIV-2 strains. However, our results do not show a difference between HIV-2 SI and NSI clones with respect to their usage of either CXCR-4, or of any other coreceptor. Thus, in contrast to what has been shown for HIV-1 (3,7,16), there seems to be no correlation between any specific coreceptor usage and HIV-2 syncytium-inducing capacity.

A limited set of HIV-1 strains can use other chemokine receptors than CCR-5, such as CCR-2b and CCR-3, as their coreceptor (4,11,14). In contrast to HIV-1, almost all HIV-2 biological clones and primary isolates used in this study exhibited the usage of a broad range of coreceptors, including CCR-1, CCR-3 and/or CXCR-4 in addition to CCR-5. CCR-1 usage by HIV-1 isolates in vitro has never been documented, and is also consistently absent in our system [C.Guillon, manuscript in preparation]. Moreover, 14 out of the 16 clones tested, including the two SI clones, were able to use at least three of these chemokine receptors for entry into U87MG/CD4 cells. Therefore, and in contrast with previous reports (25), broad coreceptor usage seems characteristic of intermediate and late primary HIV-2 variants.

Mitogen-stimulated CD8+ T cells have been shown to produce the natural ligands of CCR-5, RANTES, MIP-1α and MIP-1β, which may therefore compete with the HIV envelope glycoprotein for binding to CCR-5 (5,12). The broad coreceptor usage of the unpassaged HIV-2 isolates may therefore have been the result of a negative pressure exerted by CD8+ T cells on CCR-5 restricted variants. The preferential selection of CCR-5 restricted viruses within one week of cultivation in CD8-depleted PHA-stimulated PBMC implies however that CCR-5-mediated entry is the main mechanism of infection of CD4+ cells in vitro by HIV-2 NSI isolates, and that other coreceptor usage may occur when efficiency of CCR-5 mediated entry has decreased.
Figure 2: Coreceptor Usage of HIV-2 Biological Clones. Infections were performed with biological clones from A) patient RH2-5, B) patient RH2-7, and C) patient PH2-1. Each value represents the mean and standard deviation of at least 3 independent experiments and is expressed in cpm/150μl supernatant.

It has been hypothesised that broadening of HIV-1 coreceptor usage contributes to the in vivo cytopathogenicity of HIV by increasing the amount of potential target cells (6). Despite the broad coreceptor usage of HIV-2 isolates in vitro, disease progression in HIV-2 infected individuals is significantly slower than for HIV-1 patient's (19). Moreover, using HIV in situ hybridisation and in situ histochemistry of lymphoid tissues from early and
intermediate stage disease patients, we have recently obtained evidence that the number of productively infected cells is significantly lower in HIV-2 infection than in HIV-1 infected individuals [M.E. van der Ende, submitted]. This suggests that the broad coreceptor usage observed in vitro does not add to the in vivo cytopathogenicity of HIV-2, in contrast to syncytium inducing capacity or viral load. Therefore, additional longitudinal studies are required to further elucidate the relevance of coreceptor usage in disease progression after HIV-2 infection.

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References


CHAPTER 6

Broadening of coreceptor usage by HIV-2 does not correlate with increased pathogenicity in an in vivo model

Broadening of coreceptor usage by HIV-2 does not correlate with increased pathogenicity in an *in vivo* model

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**SUMMARY**

We have studied the pathogenic properties of four primary HIV-2 isolates and two primary HIV-2 biological clones in an *in vivo* human to mouse chimeric model. The cell associated viral load and the ability to reduce the severity of the induced graft versus host disease symptoms, the CD4/CD8 ratio and the level of repopulation of the mouse tissues by the graft, were determined. All HIV-2 strains irrespective of their *in vitro* biological phenotype replicated to high titres and significantly reduced the graft versus host disease symptoms and the CD4/CD8 ratios. Reduction of graft repopulation induced by the respective HIV-2 strains showed that the *in vitro* replication rate, syncytium inducing capacity and ability to infect human macrophages did influence the *in vivo* pathogenic potential whereas broadening of coreceptor usage did not.
INTRODUCTION
HIV-1 and HIV-2 are the causative agents of the acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Clavel et al., 1986; Levy et al., 1986). The most prominent feature of the pathogenesis of AIDS is the quantitative and qualitative deterioration of the CD4+ T cell subset. Although CD4+ T cells are already affected soon after infection, it may take one to more than fifteen years before immune failure leads to overt clinical symptoms (Gruters et al., 1990; Gruters et al., 1991). Both virological and host-delimited factors have been shown to determine the length of the asymptomatic period (Cheng-Mayer et al., 1988; Dean et al., 1996; Fenyo et al., 1988; van Baalen et al., 1997). Epidemiological studies have shown that, although progression to AIDS may in specific cases be relatively fast, disease progression in HIV-2 infected individuals is in general much slower than disease progression in HIV-1 infected individuals (Marlink et al., 1994; van der Ende et al., 1996; Whittle et al., 1994). Virological factors that underly this difference between HIV-1 and HIV-2 induced disease progression, are presently not known. Studies in in vitro and in vivo systems, specifying viral characteristics which determine the pathogenicity of HIV strains, may aid in the understanding of HIV induced pathogenesis.

The characteristics used to classify HIV strains in vitro include replication rate, ability to induce the formation of multinucleated cells (syncytium inducing (SI) capacity), coreceptor requirement and ability to infect different target cells (T cell lines versus macrophages) (Asjö et al., 1986; Berger et al., 1998; Cheng-Mayer et al., 1988; Schuitemaker et al., 1992b; Tersmette et al., 1989). For HIV-1 it has been shown that these in vitro characteristics exhibit a high degree of correlation. E.g. SI strains in general have a high replication rate, infect target cells via α- and β-chemokine receptors, may infect immortalised T cell lines and only inefficiently infect macrophages. NSI strains on the other hand, have a low replication rate, require expression of β-chemokine receptors, do not infect T cell lines and replicate efficiently in macrophages (Berger et al., 1998; Schuitemaker et al., 1991). It should however be noted that individual virus strains may also display intermediate biological phenotypes (Groenink et al., 1991; Sabri et al., 1998; Schuitemaker et al., 1992a). Several differences in in vitro characteristics of HIV-1 and HIV-2 have become apparent. The replication rate as defined by the time required to measure virus after initiation of standard virus culture from donor peripheral blood mononuclear cells, is generally lower for HIV-2 than for HIV-1 (Albert et al., 1990; van der Ende et al., 1996). Furthermore, we and others have recently shown that clear differences exist in coreceptor requirements (Guillon et al., 1998; McKnight et al., 1998). The linkage between HIV-1 SI phenotype and CXCR-4 coreceptor usage, was not observed for HIV-2. Furthermore, HIV-2 strains in general proved to have a broader coreceptor usage than HIV-1. Taken together, these observations suggest that in vitro usage of the CXCR-4 coreceptor
and broadening of the coreceptor usage by HIV do not add to enhanced in vivo pathogenicity.

To further address this issue we studied the in vivo pathogenic potential of HIV-2 isolates and biological clones in a chimeric human to mouse model for in vivo HIV infection (the xeno-GvHD mouse model) (Huppes et al., 1992; Huppes et al., 1993; Schutten et al., 1996). In this model high numbers of human peripheral blood mononuclear cells (PBMC) are grafted into the peritoneal cavity of immune deficient mice. In these mice an acute graft versus host reaction develops within seven to 14 days. The human lymphocyte population that repopulates the mouse tissues is characterised by high CD4/CD8 ratios (Schutten et al., 1996). Depletion of human CD4+ T cells from the graft results in a complete abrogation of the acute xeno-GvHD reaction. Depletion of antigen presenting cells (APC, macrophages) from the human graft results in lower CD4/CD8 ratios and a concomitant delay of the xeno-GvHD symptoms (Huppes et al., 1993). We therefore set out to study in this model the direct (killing of infected CD4+ T cells) and indirect (inhibition/modulation of antigen presentation) pathogenic effect of different HIV-2 strains and isolates on the development of acute xeno-GvHD symptoms, CD4/CD8 ratio and the ability of the graft to repopulate mouse tissues.

<table>
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<tr>
<th>HIV-2 Strain</th>
<th>replication ratea</th>
<th>Biological phenotypeb</th>
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<td>X4</td>
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<td>14</td>
<td>Intermediate NSI</td>
<td>R1,R3,R5,X4</td>
<td>+</td>
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<tr>
<td>PH2-1 D5</td>
<td>14</td>
<td>Intermediate SI</td>
<td>R5</td>
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</table>

Table 1. Characteristics of the HIV-2 Strains a first day of a positive signal in p24 antigen ELISA after start of virus isolation (van der Ende et al. 1996). b Biological phenotype according to M.E. van der Ende et al. (van der Ende et al. 1996). c coreceptor usage according to Guillon et al. (Guillon et al. 1998, Berger et al. 1998)

MATERIALS AND METHODS

Virus strains and xeno-GvHD mice

Xeno-GvHD mice were prepared as previously described (Huppes et al., 1992; Huppes et al., 1993; Schutten et al., 1996). In short, 3-4 weeks old XID mice (CBA/HNOaHsd, Harlan Netherlands BV. Zeist) were give total body irradiation (9 gray) with syngeneic bone marrow support (5x10⁶ cells/mouse intra veneously.). Subsequently, ficoll gradient isolated human
PBMC from HIV seronegative individuals (blood type A, rhesus +) were given at 2x10^7 per gram mouse bodyweight intra peritoneally (i.p.). These xeno-GvHD mice were challenged i.p. with HIV-2 within one hour after they had been grafted with human PBMC. Mice grafted with human PBMC from one and the same HIV seronegative blood donor were equally distributed over the respective groups of mice receiving different HIV-2 strains. Each group consisted of five mice. The primary HIV-2 strains used in this study had been isolated in mitogen stimulated PBMC only and the in vitro characteristics have previously been described (Guillon et al., 1998; van der Ende et al., 1996). Relevant virus characteristics are given in Table 1. The doses of challenge virus were equilibrated on basis of the counts per minute measured in a RT assay (van Baalen et al., 1998) and equalled approximately 30 mouse infectious doses 50% per mouse.

Parameters of acute xeno-GvHD reaction and HIV-2 infection

Directly after the onset of acute xeno-GvHD symptoms (furry coat, breathing problems and wasting), mice were sacrificed and lymphocytes were isolated from the peritoneal cavity. Human to mouse chimerism was confirmed and the CD4/CD8 ratio of the human graft was calculated, using FACSscan analysis with human CD3 and CD8 conjugates (Becton Dickinson, Leiden, The Netherlands) according to the instructions of the manufacturer. The viral loads of the lymphocytes isolated from the peritoneal cavities were determined in an infectious centre assay (ICT)(Schutten et al., 1996). In short, human lymphocytes isolated from the peritoneal cavity were counted and titrated in duplo from 2x10^5 to 2 cells per well onward using five fold dilution steps in 96 round bottomed plates. PBMC that were pre-stimulated with mitogen for three days, were added as feeder cells (5x10^4 per well). The cells were cultured for seven days in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% foetal calf serum (BioWhittaker), penicillin (100U/ml), streptomycin (10μg/ml) and 50 IU recombinant human IL-2/ml (Eurocetus, Amsterdam, The Netherlands), after which virus was detected by RT assay. The minimal number of cells isolated from the peritoneal cavity required to detect virus was taken as a measure for the cell associated viral load.

HIV-2 in situ hybridisation and immunohistochemistry

Xeno-GvHD mouse tissues including spleen, lung, bowel, hart, liver and bone were prepared from mice, which had been grafted with PBMC isolated from the same individual. Tissues from two mice infected with the same HIV-2 isolate/strain were analysed. Tissues were fixed overnight with 4% formalin and subsequently embedded in paraffin. For routine immunohistochemistry, dewaxed 5 μm paraffin sections were heat denatured with 0.01 M buffered sodium citrate (Norton et al., 1994). The sections were incubated with CD45 (LCA)(Dakopatts, Copenhagen, Denmark) according to the manufacturers' instructions (Tenner-Racz et al., 1998). Binding of antibodies was visualised by the alkaline phosphatase...
anti alkaline phosphatase technique using New Fuchsin as red chromogen. We have previously shown that this conjugate does not stain mouse cells (Schutten et al., 1996) After immuno-staining the sections were either counter-stained with hematoxylin and mounted or dehydrated and subjected to HIV RNA in situ hybridisation. ³⁵S-labeled, single stranded, anti-sense HIV-2 RNA probes (Lofstrand Labs., Gaithersburg, MD, USA), which contained 1.4-2.7 kb fragments, collectively representing approximately 90% of the HIV-2 genome, were used as previously described (Embretson et al., 1993; Fox et al., 1991). In short, paraffin sections were either treated with Proteinase K (0.01 mg/ml) for 8 min. at room temperature or heat denatured. The sections were incubated with prehybridisation mixture (50% Formamide, 0.5 M NaCl, 10 mM Tris HCl, pH 7.4, 1 mM EDTA, 0.02% Ficoll-polyvinylpyrrolidone-BSA and 7 mg of tRNA/ml) for 2 hours at 37°C and then covered with hybridisation mixture (prehybridisation mixture + 10% dextran sulfate and 2x10⁶ dpm of probe/ml) overnight at 45°C. The sections were washed, RNase treated (Boehringer Mannheim GmbH, Mannheim, Germany) for 40 min. at 37°C and further developed, counter-stained with hemalaun and mounted as previously described. As a negative control, sections were hybridised with a radiolabeled sense probe. The sections were examined with a microscope equipped with epiluminescent elumination (Axiophot; Carl Zeiss Inc., Jena, Germany). Cells were considered positive for viral gene expression if the number of grains overlying a cell was more than six times the background.

<table>
<thead>
<tr>
<th>HIV-2 strain</th>
<th>Xeno-GvHD</th>
<th>ICT</th>
<th>CD4/CD8 ratio</th>
<th>CD45⁺ cells</th>
<th>HIV-2⁺ cells</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>160</td>
<td>0.6-0.8</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

Table 2  a Severity of the xeno-GvHD symptoms ++ = severe, + = minor, +/- minor in some of the animals, - = no symptoms observed within the observation period (11 days). b Number of infectious centres per 4x10⁵ cells isolated from the peritoneal cavity. c The range of the CD4/CD8 ratio within each group of mice. d Amount of CD45⁺ cells in the organ +++ = very high, ++ = high, + = low, - = occasionally a positive cell, N.A. = not applicable due to xeno-GvHD induced necrosis of the organ. e Amount of HIV-2 RNA⁺ cells +++ = very high, ++ = high, + = low, - = occasionally a positive cell.
RESULTS

Acute xeno-GvHD symptoms and CD4/CD8 ratio
Grafting human PBMC from HIV seronegative individuals at 2x10^7/gram mouse bodyweight i.p. has been shown to induce an acute xeno-GvHD reaction within 14 days in approximately 100% of the animals (Huppes et al., 1992;Schutten et al., 1996)(Table 2). In general the development of acute xeno-GvHD symptoms is fast and little variation is observed between animals grafted with PBMC from donors with the same ABO/rhesus factor. The acute xeno-GvHD symptoms that were observed in the groups of HIV-2 infected xeno-GvHD mice were in general less severe as compared to the control mice (Table 2). No significant differences were observed between the different groups of HIV-2 infected mice, either with respect to the absolute numbers of human lymphocytes isolated from the peritoneal cavity (data not shown), or with respect to the number of infected centres in the ICT (Table 2). Quite unexpectedly no significant differences in symptoms or CD4/CD8 ratio were observed between xeno-GvHD mice infected with the early HIV-2 RH2-1 (R5) isolate or late isolate HIV-2 RH2-2 (R1,R3,R5,X4). Acute xeno-GvHD symptoms were not observed in the mice infected with the late subtype B isolate HIV-2 RH2-6 (X4) and CD4/CD8 ratios from these mice were lower as compared to mice in the other groups (Table 2). Although the differences between mice infected with the SI and NSI biological clone from PH2-1 were minor, the xeno-GvHD mice infected with the SI biological clone PH2-1 D5 (R5) did show less severe xeno-GvHD symptoms as compared to the mice infected with the NSI biological clone PH2-1 E6 (R1,R3,R5,X4) (Table 2).

Immunohistochemistry of tissues from xeno-GvHD mice
In order to study the ability of the human PBMC graft to repopulate the mouse tissues after infection with HIV-2, tissues from the xeno-GvHD mice (spleen, lung, heart, bone, bowel) were prepared and analysed for the amount of human cells present by immunohistochemical staining for human CD45. Variation in the distribution of the human lymphocytes was low between mice grafted with the same donor PBMC (data not shown). High numbers of human cells, especially in highly vascularised tissues like the lung, were observed in xeno-GvHD mice which were uninfected (Table 2). Only small remnants of necrotised spleen could be obtained from these mice, in which the mouse tissue was almost completely replaced by human cells (Figure 1A). Within the tissues only CD45^+CD68^- cells (T cells) were found and CD45^-CD68^+ cells (macrophages) were exclusively found adhered to and migrating into tissues isolated from the peritoneal cavity (Schutten et al., 1996). In all HIV-2 infected xeno-GvHD mice relatively high numbers of human T cells and macrophages could be observed in the tissues isolated from the peritoneal cavity. Similar to the uninfected mice, macrophages were exclusively found migrating into and adhered to the tissues isolated from the peritoneal cavity (data not shown). The number of
human cells in the lungs of the xeno-GvHD mice infected with HIV-2 RH2-1 (R5), RH2-2 (R1,R3,R5,X4) and PH2-1 E6 (R1,R3,R5,X4), was significantly lower as compared to the number of human cells in the lung of the control mice (Table 2). The structure of the spleen obtained from these mice was relatively intact and contained many CD45+ cells, mainly within the peri-arteriolar lymphocyte sheets (Figure 1B,C and E, Table 2). Hardly any CD45+ cells were found in the lung and spleen of mice infected with HIV-2 RH2-6 (X4) (Figure 1D, Table 2). Similarly the lung of HIV-2 PH2-1 D5 (R5) infected mice was almost completely devoid of human cells and low amounts of human cells were observed in the spleen (Figure 1F, Table 2).

Figure 1.Comparison of the amount of human lymphoid cells and productively HIV-2 infected cells in xeno-GvHD mice as determined by CD45 staining (red alkaline phosphatase staining) and HIV-2 RNA in situ hybridisation (black grains).
A. uninfected control, B. HIV-2 RH2-1 (R5), C. HIV-2 RH2-2 (R1,R3,R5,X4), D. HIV-2 RH2-6 (X4), E. HIV-2 PH2-1 E6 (R1,R3,R5,X4), F. HIV-2 PH2-1 D5 (R5)

HIV-2 infection of xeno-GvHD mice

The ability of the HIV-2 isolates and biological clones to productively infect cells expressing the macrophage marker CD68 was determined by double
labelling (HIV-2 RNA in situ hybridisation with CD68 immunohistochemistry) (Table 1). Productively infected macrophages were observed in all HIV-2 infected xeno-GvHD mice except for mice infected with the late isolate RH2-2 (R1,R3,R5,X4) (Table 1). Also the relative number of productively infected cells was determined in the tissues with high numbers of human lymphocytes (spleen and lung) (Table 2). HIV-2 RNA in situ hybridisation signal above background was absent in the negative control mice. High amounts of HIV-2 RNA+ cells were observed in the peritoneal cavity of all mice infected with the different HIV-2 isolates and biological clones (data not shown). High numbers of productively infected cells could be observed in lung and spleen of xeno-GvHD mice infected with HIV-2 RH2-1 (R5) (Figure 1 C). Despite the fact that no significant differences in the amounts of CD45+ cells were observed between xeno-GvHD mice infected with HIV-2 RH2-1 (R5) (Figure 1 C) or HIV-2 RH2-2 (R1,R3,R5,X4) (Figure 1 D), a significant lower number of productively infected cells was found in the tissues of xeno-GvHD mice infected with HIV-2 RH2-2 (R1,R3,R5,X4) (Table 2). Similar to HIV-2 RH2-1 (R5) infected xeno-GvHD mice, many productively infected cells were found in the tissues of xeno-GvHD mice infected with HIV-2 PH2-1 E6 (R1,R3,R5,X4) (Figure 1E, Table 2). Although the number of cells and therefore also the number of productively infected cells in the peripheral tissues of xeno-GvHD mice infected with HIV-2 PH2-1 D5 (R5) (Figure 1F) were significantly lower as compared to HIV-2 RH2-1 (R5), RH2-2 (R1,R3,R5,4X4) and PH2-1 E6 (R1,R3,R5,4X4), still a relatively high percentage of the cells observed in the peripheral tissues of these mice were productively infected (Table 2).

DISCUSSION

In the present paper we have shown that the SI capacity, replication rate and the ability to infect macrophages influenced the pathogenic properties of HIV-2 strains in the xeno-GvHD mouse model for HIV infection. Moreover a SI macrophage-tropic HIV-2 biological clone that only uses CCR-5 as coreceptor proved to be more pathogenic than a highly homologues NSI macrophage-tropic biological clone with a potentially broader coreceptor usage (CCR-1, CCR-3, CCR-5 and CXCR-4). These data show that broadening of the potential cellular host range does not increase the pathogenic potential of HIV.

Both direct and indirect mechanisms have been suggested to contribute to the pronounced CD4+ T cell depletion observed in HIV infected individuals (for review see Zinkernagel & Hengartner (Zinkernagel et al., 1994). The direct mechanisms include acute single cell lysis of infected cells (Samson et al., 1996) and depletion of uninfected CD4+ cells by fusion with infected cells (syncytium formation) (Tersmette et al., 1993). It has also been shown that cells of the monocyte/macrophage lineage do not function properly, which may result in dis regulation of activation and/or maturation of CD4+ T cells due to functionally disturbed antigen presentation (Meyaard et
al., 1993). This may also indirectly contribute to the decline of CD4+ T cells. Two observations suggest that this last phenomenon contributed to the HIV-2 induced CD4+ T cell depletion in our xeno-GvHD mouse model. First, no significant differences were observed in the CD4/CD8 ratio and the number of CD45+ cells in spleen and lung of mice infected with the early, macrophage-tropic NSI isolate RH2-1 (R5) and the late non macrophage-tropic SI isolate RH2-2 (R1,R3,R5,X4). This despite the fact that the late SI isolate is highly cytopathic for CD4+ T cells in vitro and the early NSI isolate does not induce single cell lysis to a significant extent (personal observations). The fact that the percentage of infected cells was high in the RH2-1 (R5) infected mice and low in the RH2-2 (R1,R3,R5,X4) infected mice also suggests that these virus strains cause CD4+ T cell depletion by two different mechanisms. E.g. it seems likely to assume that HIV-2 RH2-2 (R1,R3,R5,X4) infected cells are directly killed and therefore do not reach the peripheral tissues studied, whereas RH2-1 (R5) infected cells are obviously not killed directly, but rather seem to be functionally affected. Second, RH2-2 (R1,R3,R5,X4) and RH2-6 (X4) are both highly cytopathic for CD4+ T cells in vitro. The macrophage-tropic isolate RH2-6 (X4) proved to be far more pathogenic in vivo with respect to abrogating GvHD symptoms and inhibiting migration of T cells to peripheral tissues, than the non macrophage-tropic isolate RH2-2 (R1,R3,R5,X4).

SI capacity, replication rate and coreceptor requirements have all been shown to positively correlate with rapid disease progression in HIV-1 infected individuals. Because for HIV-1 these factors are generally linked, it has not been possible to distinguish which factors have a causal relation with disease progression. Since SI capacity and coreceptor usage are not linked for HIV-2, we could study these factors independently. The observation that in vitro coreceptor usage of HIV-2 strains is broader as compared to HIV-1 already suggested that broadening of coreceptor usage does not necessarily lead to increased in vivo pathogenicity. Indeed in the xeno-GvHD model RH2-6 (X4) proved to be far more pathogenic with regard to all aspects studied as compared to RH2-2 (R1, R3, R5, X4), suggesting that broadening of the coreceptor usage does not significantly add to the in vivo pathogenicity. This is further supported by the observation that the NSI biological clone PH2-1 E6 (R1, 3, 5, X4) was less well able to inhibit migration of T cells and may therefore be regarded more pathogenic as compared to it's SI counterpart PH2-1 D5 (R5) (R-5).

We have recently shown that the number of productively infected cells in lymphoid tissue from HIV-2 infected individuals is significantly lower than in HIV-1 infected individuals. It has furthermore been shown that the number of productively infected cells is positively correlated with the plasma viral load, which is in turn correlated with disease progression. Interestingly, we did not observe major differences in cell associated viral loads nor in the pathogenicity of the HIV-2 strains as compared to previously studied HIV-1 strains (Schutten et al., 1996). It therefore seems that in the absence of a substantial humoral and cellular antiviral immune response both in vitro and
in our human to mouse chimeric model, HIV-1 and HIV-2 may have comparable replicative and pathogenic potential. We therefore hypothesise that the lower viral loads and slower disease progression in HIV-2 infected humans as compared to HIV-1 infected individuals is related to a difference in the interaction of the virus with the HIV specific immune response.

References


progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus populations *Journal of Virology* 66, 1354-1360.


CHAPTER 7

Antiviral resistance of biological HIV-2 clones obtained from individuals on nucleoside reverse transcriptase inhibitor therapy.

Submitted
ANTIVIRAL RESISTANCE OF BIOLOGICAL HIV-2 CLONES OBTAINED FROM INDIVIDUALS ON NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR THERAPY

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SUMMARY

Objective: To study the development of phenotypic and genotypic resistance of HIV-2 during nucleoside reverse transcriptase inhibitors (NRTI) treatment.

Methods: Biological HIV-2 clones were generated from three patients before and after initiation of antiretroviral therapy with zidovudine (AZT) in patient RH2-7, AZT and didanosine (ddl) in patient PH2-1, and after addition of lamivudine (3TC) to AZT-monotherapy in patient RH2-5. The sensitivity to NRTI of the virus clones, as defined by the 50% inhibitory concentration (IC50), was determined in vitro. The predicted amino acid sequences of the reverse transcriptase proteins from these clones were determined.

Results: Comparing the sensitivity of the biological HIV-2 clones obtained after start of therapy to those from antiviral naïve patients, resistance had developed to AZT (patients RH2-7 and RH2-5) and 3TC (patient PH2-1 and RH2-5). No resistance to AZT was observed in the biological clone from PH2-1 obtained after start of therapy. The resistant clones from RH2-5 and PH2-1 but not RH2-7, contained amino acid mutations at positions where HIV-1 has been shown to mutate after AZT and 3TC treatment.

Conclusions: Phenotypic resistance of HIV-2 to nucleoside analogues, which developed in HIV-2 infected patients treated with NRTI, was associated with genotypic changes. Some of the mutations at amino acid positions in the HIV-2 reverse transcriptase gene corresponded with those involved in HIV-1 resistance, although no conventional mutations associated with resistance to AZT were observed.
Introduction
Human immunodeficiency virus type 2 (HIV-2), the second causative agent of the acquired immunodeficiency syndrome (AIDS), belongs like HIV-1 to the subfamily of lentivirinae of the Retroviridae family (1). In vitro characteristics of both HIV-1 and HIV-2, such as tropism for CD4+ α- or β-chemokine receptor positive cells and capacity to induce cytopathic changes as well as in vivo induced pathogenesis are quite similar (2-6). Despite the similarities, both perinatal and sexual transmission rates of HIV-2 are significantly lower (7-9), which has so far resulted in a more restricted spread of HIV-2 than HIV-1. Although the virus has spread globally, it is only more prevalent than HIV-1 in certain West African countries, such as Guinea-Bissau, the Gambia, Senegal, and the Cape Verde Islands. Via trade routes HIV-2 had limited spread to other parts of the world such as Angola, Mozambique, Gao and Western Europe (10). The natural history of disease development in HIV-2 infection is protracted. Epidemiological studies among cohorts of asymptomatic HIV-1 and HIV-2 infected individuals have demonstrated an AIDS incidence of 0.94 and 0.23 per 100 person years of observation in HIV-1 and HIV-2 infected individuals respectively (11). These observations are in line with the immunological changes observed in HIV-2 infected individuals. CD4+ T cell counts and CD4/CD8 ratios decline slower than in HIV-1 infected individuals (11). Furthermore we have recently showed significantly lower tissue and plasma viral loads in HIV-2 infected individuals than in HIV-1 infected individuals at comparable stages of disease development (submitted for publication).

In HIV-1 infection it has been shown that both the risk of developing disease and the lack of efficacy of antiviral therapy are strongly associated with HIV-RNA plasma levels (12,13). NRTI treatment strategy from 1987 to 1994 was initially limited to AZT monotherapy, which delays disease progression in asymptomatic and advanced HIV-1 disease and decreases plasma HIV-1 RNA by approximately 0.3 log_{10} copies/ml (14,15). Subsequent results from studies on combination NRTI therapy showed slower disease progression and an improved survival for antiviral naive HIV-1 infected patients on combination therapy versus monotherapy (16-18). A more sustained increase in CD4+ cell counts and decrease in plasma HIV-1 RNA of approximately 1 log_{10} copies/ml were observed in individuals receiving duo-therapy with two NRTI. In HIV-1 infected patients it has been shown that the virostatic properties of AZT, as well as the clinical and immunological benefits, decrease during the course of monotherapy (19). Increasing evidence indicates that treatment failure of NRTI therapy is closely linked to levels of phenotypic resistance and an associated genotypic mutation pattern (20). Viral genotyping may therefore be an important tool in choosing anti-HIV therapy regimens (21-23).

Limited data on antiretroviral treatment of HIV-2 infected individuals are available. Only small differences have been observed between the in vitro sensitivity of primary HIV-1 and HIV-2 isolates to AZT, ddI and 3TC (24). No data are yet available on the development of genotypic or phenotypic
resistance of HIV-2 strains from patients on NRTI therapy. We therefore studied biological clones from three HIV-2 infected patients treated with NRTI and determined their phenotypic and genotypic resistance patterns.

**Figure 1.** CD4⁺ cell counts in three HIV-2 seropositive patients treated with NRTIs.

**Material and methods**

**Patients**

Patients in this study regularly visited our outpatient clinics in Rotterdam (n=2) and Paris (n=1). Clinical, immunological and virological parameters were monitored from 1994 onwards. Patient RH2-7 (39-year-old heterosexual male) and patient RH2-5 (a 40-year-old heterosexual female) were both living in Rotterdam and originated from the Cape Verdian Isles. Patient PH2-1 was a French born homosexual man living in Paris. All three patients were asymptomatic at the time of starting therapy. The relevant clinical data on antiretroviral treatment and CD4⁺ cell counts after start of therapy are given in Fig 1.
Isolation of biological virus clones

The isolation of biological HIV clones have been described previously (6). Briefly, participant peripheral blood mononuclear cells were cocultivated at three different dilutions, 2x10^2, 2x10^3, and 2x10^4 cells per well, with three days mitogen-prestimulated (PHA-L, Boehringer Mannheim, Germany), HIV-negative donor PBMC (4 x 10^4 per well) in 96-well microtiter plates (Costar). Every week 50μl of culture supernatant was collected from each well for detection of p24 antigen (V5 ELISA, Organon, Boxtel, the Netherlands). At the same time, 2x10^2 cells from each well were transferred to a new well, and 4 x 10^4 fresh mitogen-prestimulated healthy donor PBMC were added to propagate the culture. Productively infected wells were considered to contain a clonal virus strain, when less then 5 out of 96 wells were tested positive. The biological clones were further cultured using mitogen stimulated PBMC and expanded to high titered stocks by a one-week passage on U87/CCR-5 cells (6). For comparison a previously described HIV-1 molecular clone, obtained from an antiviral-naive individual was used (25,26).

Antiviral drug susceptibility testing

The sensitivity of the biological clones to the NRTI was determined in vitro, using a similar method previously described for HIV-1 drug testing (27). Virus stocks were thawed and titered by RT activity. Mitogen-stimulated CD8-depleted PBMC from HIV seronegative donors were inoculated in triplicate with 50TCID₅₀ in the presence of 10 fold dilutions of AZT, 3TC, and ddi, starting at 10μM. After one week, supernatants were tested for RT activity (28). The concentration of the drug that resulted in a 50% reduction in RT activity, the 50% inhibitory concentration (IC₅₀) was calculated by the median effect equation (27). Virus clones were considered resistant, when the IC₅₀ was >5 times higher than the IC₅₀ of the naive clones (in patients RH2-7 and PH2-1) or the clone before therapy adjustment (in patient RH2-5).

Polymerase chain reaction detection of RT sequences

High molecular weight DNA was extracted with Celite beads (Janssen Chimica, Beersen, Belgium) from PBMC that were infected with the biological virus clones from the patients (29). Amplification was performed as previously described (30). Briefly, RT sequences were amplified in a polymerase chain reaction with primers 5'RD1 (5'CCCCAATCAACATT­TTGGCA G3') and 3'RD3 (3'CCTAGTTCTGCATT-TTTGCCC3') (kindly provided by Dr. C Boucher) for 2 min at 92°C, 1 min at 50°C, 2 min at 72°C for 35 cycles with a final extension of 10 min. at 72°C. The amplified fragments were cloned into the PCR2.1 cloning vector (Invitrogen, San Diego, California, USA) according to the manufacturer's protocol. Clones were sequenced with the Taq Dye Deoxy Terminator sequencing kit on the 373A sequencing system of Applied Biosystems (Foster City, California, USA). All clones were sequenced on both strands using custom-made
primers. Alignment of the protein sequences was performed with the Lasergene software (Dnastar, Madison, USA). A 3-D model structure of the HIV-2 ROD RT protein was generated on basis of the HIV-1 RT protein crystal structure 1RT2 (31) using Swiss-Model 3.0 and PdB Viewer both kindly provided by Drs. M.C. Peitch and N. Guex (GlaxoWellcome Experimental Research, Geneva, Switzerland)(32,33)

Results

Patients
The indication for initiation or adjustment of antiretroviral therapy in all three patients was a low CD4+ cell count. After 20 months of treatment with AZT, patient RH2-7 developed renal failure caused by HIV-nephropathy, a few months later he was diagnosed with disseminated Mycobacterium avium complex. Patient PH2-1 remained asymptomatic during treatment with AZT/ddI, and was switched to a protease inhibitor containing regimen because of a declining CD4 cell count. Patient RH2-5 was treated with AZT monotherapy and in addition AZT/3TC combination therapy for 14 and 18 months respectively until she progressed to AIDS as she developed Mycobacterium Tuberculosis.

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Table 1. Susceptibility of HIV-2 clones to NRTI. For the HIV-2 biological clones the IC50 of the NRTI used in this study are given in micro molar. * N.T. = Not tested

Virus phenotypic sensitivity to NRTI
Biological HIV-2 clones were obtained from patients RH2-7 and PH2-1 before treatment (RH2-7 G12 and PH2-1 E6) and after 20 and 6 months after initiation of antiretroviral therapy respectively (RH2-7 A5 and PH2-1 C12). The first virus clone from patient RH2-5 (RH2-5 G7) was obtained after 6 months of AZT monotherapy and the second clone 12 months after adding 3TC to the regimen (RH2-5 A10)(Figure 1). The sensitivity to AZT of RH2-7 G12 and PH2-1 E6 obtained from antiviral naive patients proved to be comparable to the HIV-1 molecular clone obtained from an antiviral
replication of all HIV-2 clones and the HIV-1 clone was high and therefore did not allow us to determine development of resistance to ddl. This has also been observed by others and may be caused by the inefficient conversion of ddl to the biologically active form ddA-triphosphate when activated peripheral blood mononuclear cells are used for phenotype resistance (34,35).

Figure 2. Alignment of the predicted amino acid sequence from HIV-2 reverse transcriptase (RT) protein fragments. The arrows (▼) indicate mutations corresponding with NRTI resistance in HIV-1.

Sensitivity of the biological HIV-2 clones obtained after start of therapy were compared to those obtained before therapy or, for the clones obtained from patient RH2-5, to clones from antiviral naive individuals. The biological clones from patients on AZT monotherapy (patient RH2-5 and RH2-7) were 10 to 20 times less sensitive to inhibition by AZT. Sensitivity to AZT RH2-5 A10 relative to RH2-5 G7 was unchanged. No resistance to AZT was observed for PH2-1 C12. Both clones from patients on duo NRTI therapy were less sensitive to inhibition with 3TC, clone PH2-1 C12 10x less sensitive and clone RH2-5 A10 45x less sensitive.
Genotypic analysis of biological clones
The RT genes from the biological HIV-2 clones were sequenced (Fig. 2). From several biological clones the sequence was determined directly on the PCR product. In none of these, multiple polymorphism's were observed, supporting the clonality of these biological clones. Multiple differences in amino acid sequences were observed between the clones obtained before and after therapy. For convenience the predicted amino acid differences between the clones before and after therapy are given in table 2.

HIV-1 RT and HIV-2 RT have similar catalytic properties and a 60% sequence identity (36). The observation that the folding of the palm of the more distant related RT of murine leukaemia virus is the same as HIV-1 RT, supports the idea that HIV-1 RT and HIV-2 RT are sufficiently similar that amino acids in equivalent positions in the sequence will have similar roles in the two enzymes (36). Therefore we set out to study positions within the RT gene where HIV-1 mutates in case of resistance to NRTI.

<table>
<thead>
<tr>
<th>RH2-7 A5</th>
<th>RH2-5 A10</th>
<th>PH2-1 C12</th>
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<tbody>
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<td>T14P (ACA→CCA)</td>
<td>K22R (AAA→AGA)</td>
<td>E122K (GAA→AAA)</td>
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<td>K35S (AAA→AGT)</td>
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<td>M184V (ATG→GTG)</td>
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<td>E49R (GAA→AGA)</td>
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<td>V111I (GTA→ATA)</td>
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<td>Q151I (CAG→ATA)</td>
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<td>M184V (ATG→GTG)</td>
<td>E292G (GAA→GGA)</td>
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<td></td>
<td>G213R (GGA→AGA)</td>
<td>Y317C (TAC→TGC)</td>
</tr>
<tr>
<td></td>
<td>T237N (ACC→AAC)</td>
<td></td>
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<tr>
<td></td>
<td>V350L (GTA→TTA)</td>
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</table>

Table 2. Changes of amino acids on the RT gene obtained from three HIV-2 clones from patients after therapy with NRTI. Mutations associated with HIV-1 resistance to NRTI are printed in bold face

The predicted amino acid sequence of RH2-7 A5 showed no mutations relative to RH2-5 G12 at positions analogous to those associated with resistance to AZT in HIV-1 infection. According to the predicted protein structure one mutation, K223R, was found near the active site of RT (Figure 3). Mutations of RH2-7 A5 relative to RH2-7 G12 at positions 14, 51, 84 and at positions 249, 286, 287 were at more distant sites respectively in the fingers and thumb of the HIV-2 RT protein.

Genotypic analysis of the first biological clone from patient RH2-5, 6 months on AZT, revealed a methionine at position 151, which may explain the low susceptibility to AZT. As we do not have virus strains from patient RH2-5 before initiation of treatment, we cannot be sure whether this mutation was indeed induced by AZT selection pressure. However, in none of the hitherto described HIV-2 virus isolates of naive patients mutations at this position have been observed (37).
Genotypic analysis after duo therapy with NRTI was done on the clones obtained from PH2-1 (AZT/ddI) and RH2-5 (AZT/3TC). Both virus clones showed a M184V mutation. For HIV-1 this mutation is associated with a strong reduced sensitivity to 3TC in phenotypic assays (23). The second clone obtained from patient RH2-5 showed isoleucine instead of methionine at position 151. Furthermore, a M41L mutation appeared, which is for HIV-1 associated with low-level resistance to AZT (20).

Discussion
In the present paper we have described the development of phenotypic and genotypic resistance to NRTI during treatment of HIV-2 patients. Mutations associated with resistance, although in some cases occurring at similar positions as in HIV-1, proved to be different with respect to topography and time of appearance when compared to mutations generally observed in HIV-1.

In HIV-1 infection the genotypic resistance pattern for NRTI correlates with the level of phenotypic resistance (20). Loss of antiviral efficacy acquired under selection pressure of AZT mono-therapy involves a gradual and stepwise accrual of mutations in functional regions of the RT gene at positions K70R, T215Y and M41L respectively. Despite the fact that the second virus clone from patient RH2-7, obtained after 20 months of AZT monotherapy, was 20 fold less sensitive to AZT in the phenotypic assay, genotypic analysis did not show any mutations associated with resistance to AZT. To further elucidate this discrepancy an additional biological clone, from the same patient and the same time point, was sequenced. Also in this biological clone no mutations were found at positions associated with HIV-1 AZT resistance (data not shown). According to the predicted HIV-2 RT protein structure, the R223K mutation was positioned near to the active site, and therefore seems a likely candidate to confer AZT resistance. It can however not be excluded that the other mutations of RH2-7 G12 indirectly conferred AZT resistance. Site-directed mutagenesis should be done to be able to define regions and positions that are involved in resistance to zidovudine HIV-2 RT. In patient RH2-5 the virus clone obtained after 6 months of AZT monotherapy was phenotypically resistant to AZT. Genotypic analysis showed the appearance of methionine at position 151. The Q151M mutation requires a change of two nucleotides within one codon. In HIV-1 infections this mutation develops in asymptomatic individuals after more than one year of combination therapy with AZT and zalcitabine (ddC) or ddI (38,39). Recently this mutation has been shown to induce resistance to multiple NRTI in HIV-1 infection, and is associated with a set of other mutations, including A62V, V75I, F77L, F116Y (38,39). Among these five mutations, Q151M is thought to be the first mutation to develop. Up till now the factors which predispose virus strains to develop the set of multidrug resistance mutations in favour of AZT resistance mutations are not known. It has been suggested that certain nucleotide or amino acid sequences predispose these viruses not to acquire any AZT-related mutations (39).
the virus clone RH2-5 A10 no new mutations associated with multidrug NRTI resistance in HIV-1 infection were observed. According to the predicted protein structure, several mutations are positioned in the fingers of the RT protein. It is not excluded that they also contribute to resistance.

Figure 3. The predicted protein structure of reverse transcriptase of HIV-2, according to the Swiss-Prot database. Amino acid residues in red mark the active site; yellow amino acids residues are mutations of RH2-7 A5 relative to RH2-7 G7.

In HIV-1 infection the benefit of combination therapy with two NRTI compared to monotherapy may be explained by constraints on coincidental developing resistance mutations for two NRTI (40,41). Furthermore it has been shown that mutations that confer resistance to one NRTI may prevent mutations associated with resistance to other NRTI in the regimen (42,43). Susceptibility of the virus PH2-1 C12 to 3TC, obtained after 6 months of AZT/ddI combination therapy, was significantly lower as compared to PH2-1 E6. This decrease most probably resulted from the change of methionine to valine at position 184. This mutation was unexpected and early, since in HIV-1 infection the M184V mutation is in general observed after 3TC or AZT/3TC therapy and rarely after AZT/ddI combination therapy. In HIV-1 infection, the M184V mutation may develop within 4-12 weeks after initiation of AZT/3TC treatment, whereas genotypic resistance to AZT is in general rare or absent after 24 weeks (42,43). Monotherapy with either AZT or ddl induces mutations associated with resistance to AZT or ddl within 48 weeks in the majority of the patients (40). During duo therapy with AZT and ddl mutations associated with resistance to ddl (codon 69, 74 or 184) were rarely observed. The number of mutations associated with AZT resistance was comparable to the number found in the AZT monotherapy group. It has therefore been suggested that selection for AZT resistance may impose a
background that restricts evolution of ddl resistance. The genotypic resistance pattern in patient PH2-1, the M184V mutation and the absence of AZT resistance, seems to be more in agreement with AZT/3TC therapy in HIV-1 infected individuals than to AZT/ddl therapy. It may be hypothesised that for optimal enzymatic activity of HIV-2 RT the M184V mutation is preferable.

In conclusion, genotypic mutations in the HIV-1 RT gene are predictive for phenotypic resistance to NRTI and clinical failure, and are relevant for making rational effective drug choices. In our study we have shown that this correlation of phenotypic and genotypic resistance is not so clear in HIV-2 infection. Switching NRTI therapy in HIV-2 infected individuals cannot be decided only on genotypic analysis results. As the number of HIV-2 infected patients on antiretroviral treatment is increasing, further studies are needed.

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CHAPTER 8

Development of a Real-Time Quantitative RT-PCR for the Detection of HIV-2 RNA in Plasma
Development of a Real-Time Quantitative RT-PCR for the Detection of HIV-2 RNA in Plasma


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SUMMARY

We describe an assay for the quantification of HIV-2 RNA in EDTA plasma based on RT-PCR using the Taqman real-time PCR detection method. As standard an electron microscopically counted virus stock of HIV-2 strain NIH2 was used. The lower detection limit is 5x10^2 HIV-2 RNA copies per ml of EDTA plasma. The assay is linear within the range required (5x10^2-10^6 HIV-2 RNA copies/ml of EDTA plasma) with an intra assay variability of 2.5% and an inter-assay variability ranging from 2% at 10^6 copies to 7.5% at the lower detection limit. Three primer/probe combinations were developed to circumvent false negative samples due to nucleotide variation in the target sequence. Using these primer/probe sets we were able to detect HIV-2 DNA sequences from all HIV-2 seropositive individuals and two out of five dual HIV-1 and HIV-2 seropositive individuals visiting our clinic.
Introduction

The human immunodeficiency virus type 1 and 2 (HIV-1 and HIV-2) are the causative agents of the acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al. 1983; Clavel et al. 1986). They both belong to the genus of the lentiviruses. In contrast to the worldwide spread of HIV-1, HIV-2 infections are more confined to West-Africa (Marlink, 1996). A significant percentage of the AIDS cases in Senegal, the Gambia, and Ivory Coast are caused by HIV-2 infection (Ariyoshi et al. 1996; Marlink, 1996; Norrgren et al. 1997; Norrgren et al. 1999; Pieniazek et al. 1999; Poulsen et al. 1997). HIV-2 infected individuals are also found in West European countries with former colonial and/or trade links with West-African countries such as the Netherlands, France, Portugal, and Spain (Machuca et al. 1999; Marlink, 1996; van der Ende et al. 1996).

Molecular diagnostic assays currently used to quantify the number of HIV-1 RNA molecules in plasma and serum are highly type specific (Nkengasong et al. 1998; Nkengasong et al. 1999). They have proven to be of significant clinical relevance since it was shown that the rate of disease development is directly related to the plasma viral RNA level (Mellors et al. 1996). The current guidelines as set out by the Panel on Clinical Practices for Treatment of HIV Infection for starting anti-retroviral therapy (available at http://hivatis.org/) are therefore based on both clinical parameters and on a cell-free plasma or serum viral load above $2 \times 10^4$ HIV-1 RNA copies per ml. HIV-1 infected individuals are believed to fail on anti-retroviral therapy regimens if their cell-free viral load after 12-16 weeks of treatment is above the detection limit of the standard quantitative HIV-1 plasma viral load assays.

Of the 650 HIV-infected patients attending our hospital, 20 are HIV-2 seropositive (van der Ende et al. 1996), data not shown. They all belong to, or have direct links with a population of West African immigrants residing in the Rotterdam area. Six of them proved to be dually HIV-1/HIV-2 seropositive by Western blot analysis according to WHO guidelines (Genelabs Diagnostics Ltd, Singapore). Since it has been shown that the antiviral activity and drug-resistance patterns of drugs against HIV-1 not always parallel those against HIV-2, more research in this area is required (Kempf et al. 1995; van der Ende et al. 2000). We therefore developed a quantitative molecular assay for the measurement of HIV-2 viral RNA in plasma, based on real-time detection of HIV-2 RNA enabling us to detect HIV-2 RNA over a 4 log range in clinical samples.

MATERIALS AND METHODS

Patients and viruses.

Peripheral blood mononuclear cells (PBMC) and EDTA plasma were collected from HIV seropositive individuals visiting our out-patient clinic, using vacutainer CPT and hemogard K3 EDTA tubes respectively (Beckton Dickinson, Franklin Lakes, USA). HIV-1 and HIV-2 seropositivity was
determined with the HIV BLOT 2.2 and the HIV-2 western blot version 1.2 from Genelabs Diagnostics (Genelabs Diagnostics Ltd., Singapore) using WHO criteria. Patients PBMC were aliquoted and stored at -135°C until use. As a "gold standard" an electron microscopically counted virus stock from the HIV-2 NIHZ strain was used (Advanced Biotechnologies Incorporated, Maryland, USA). Prior to lysis with 10mM Tris, 150mM NaCl, 1mM EDTA and 1% Triton X-100 pH7.5 the stock contained 1.84x10^{10} virus particles per ml.

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<th>Composition</th>
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<tr>
<td>HIV-2TMrpr1</td>
<td>422-440</td>
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</table>

**Table 1.** Primers and Probes. ^1 The nucleotide position of the oligonucleotide within the HIV-2 NIHZ strain is given.

**Primers and probes.**

The primers and probes were designed on basis of the HIV-2 subtype A consensus sequence using the Primer Express software (PE Biosystems,
Nieuwerkerk aan de IJssel, The Netherlands). Due to the relatively high variability of HIV-2, we chose to develop two primer/probe sets for subtype A (set 1 and set 2) and one primer/probe set for subtype B (set B) (Table 1). Set one and set B encompass a region between the 5' LTR and the Gag/Pol open-reading frame (nucleotides 380 to 440 of the HIV-2 NIHZ isolate). For set B the composition of the primers and probe were slightly modified to fit the HIV-2 subtype B consensus sequence. Set 2 encompassed a region in the gag gene (nucleotides 1083 to 1149 of the HIV-2 NIHZ isolate). Primers were synthesised by Isogen Bioscience BV (Isogen Bioscience BV, Maarssen, The Netherlands) and the probes were labelled at the 5' end with the fluorochrome FAM and at the 3' end with the quencher TAMRA and synthesised by PE Biosystem (PE Biosystem, Nieuwerkerk aan de IJssel, The Netherlands). Primers just outside the Taqman primer/probe sets were designed for the evaluation of the variability within the Taqman primer/probe sets in the Rotterdam cohort of HIV-2 seropositive individuals. For set 1 and set B the primers HIV-2TM1sfpr and HIV-2TM1srpr were used and for set 2 HIV-2TM2sfpr and HIV-2TM2srpr were chosen (Table 1).

**Nucleic acid isolation.**

For the isolation of HIV-2 RNA, a modification of the High pure viral RNA isolation kit (Roche Diagnostics, Almere, The Netherlands) was used. Briefly, 800 μl of binding buffer (6M guanidine hydrochloride, 50 mM Tris-HCl, 30% Triton X-100 (w/v) and 10μg/ml poly (A) carrier RNA, pH 6.6) was added to 400 μl sample. The mixture was incubated for 10 minutes at room temperature and subsequently loaded onto the filter columns. The columns were centrifuged at 8000x g, the flow-through was discarded and the column was washed once with an inhibition removal buffer (5 M guanidine hydrochloride, 20 mM Tris-HCl and 36% EtOH, pH 6.6) and twice with a wash buffer (20mM NaCl, 2mM Tris-HCl and 75% EtOH, pH7.5). The viral RNA was eluted from the columns with 50μl redistilled H₂O and stored at -80°C until further use. DNA from patient PBMC's was isolated using the High pure viral nucleic acid kit according to the manufacturers instructions (Roche Diagnostics, Almere, The Netherlands).

**Taqman HIV-2 RNA quantification.**

The number of HIV-2 RNA copies was determined using the Taqman real-time PCR measurement system(Kimura et al. 1999; Martell et al. 1999; Niesters et al. 2000). For reverse transcription, 15 μl viral RNA was incubated with 2 μl 35pmol/μl of either HIV-2TMpr1 or HIV-2TMpr2 for two minutes at 80°C. The complementary DNA (cDNA) reaction (final concentration 2 units AMV-RT (Promega, Leiden, the Netherlands), 50 mM Tris-HCl, 50 mM KCl, 3mM MgCl₂, 4 mM DTT and 10mM of each dNTP) was performed in a final volume of 25μl for 30 minutes at 55°C. For the set 1 Taqman reaction 1μl 35pmol/μl HIV-2TMfpr1, 1μl 35pmol/μl HIV-2TMpr1, 1 μl 5pmol/μl TMprobe1, 2μl H₂O and 30 μl 2x Taqman Universal PCR mastermix (PE Biosystems, Nieuwerkerk aan de IJssel, The Netherlands)
was added to the HIV-2TMrpr1 initiated cDNA. For the set 2 Taqman reac-
tion 1 µl 30 pmol/µl HIV-2TMfpr2, 1 µl 20 pmol/µl HIV-2TMpr2, 2 µl H2O, 30 µl
2x Taqman Universal PCR mastermix and either 1 µl 5 pmol Tmprobe2.1 or
1 µl 10 pmol/µl Tmprobe2.2 was added to the HIV-2TMrpr2 initiated cDNA.
For the set B Taqman reaction 1 µl 25 pmol/µl HIV-2TMfprB, 1 µl 25 pmol/µl
HIV-2TMprB, 1 µl 5 pmol/µl TmprobeB, 2 µl H2O and 30 µl 2x Taqman
Universal PCR mastermix was added to the HIV-2TMprB initiated cDNA.
The real-time PCR amplification and detection were performed on an ABI
Prism 7700 Sequence Detection System (PE Biosystem, Nieuwerkerk aan
de IJssel, The Netherlands) using standard cycling conditions (2 min. 50
°C, 10 min. 95°C and 50 two step cycles of 15 seconds at 95°C and 60
seconds at 60°C).

RESULTS AND DISCUSSION

Detection limit, linearity and intra-/interassay variability.
The efficacy of the assay was initially determined using serial dilutions of
the HIV-2 NIHZ standard and Taqman set1. The PCR cycle at which the
FAM fluorescent signal exceeded a limit of 0.04 was taken as threshold
cycle (Ct) value. The Ct value was determined in six independent assays of
serial dilutions of the HIV-2 NIHZ standard made in diethyl pyrocarbonate
(DEPC) treated water. The sensitivity of the assay was 50% at 50 virus
particles/ml input and 100% at all higher input samples (10^2-10^6 virus
particles/ml, Figure 1A). The detection limit of the assay was therefore set
at 10^2 virus particles/ml, although samples with 50 virus particles/ ml are
regularly detected at Ct values above 40. At these Ct values however,
the chance of false positive samples increases to approx. 5% (data not shown).
The Ct values of virtually all samples above this detection limit fell within
the 10% confidence interval (Figure 1A). The linear range of the assay was
at least 4 logs with a Spearman regression coefficient of 0.99 and the inter-
assay variability based on the dilution series Ct values ranged from 2% at
10^6 virus particles/ml to 7.5% at the detection limit (10^2 virus particles/ml).
The intra-assay variability was determined by processing 32 samples of 10^4
virus particles/ml and proved to be 2.5%.
Viral RNA detection in EDTA plasma.
Since the assay should predict the number of virus particles in plasma of
individuals, we determined the efficiency of the assay to detect virus RNA
isolated from plasma relative to virus RNA isolated from DEPC treated
water. The HIV-2 NIHZ standard was diluted in EDTA plasma pooled from
six HIV seronegative volunteers and in DEPC treated water and
subsequently processed according to the standard procedure. Figure 1B
represents the average from three independent assays. The HIV-2 NIHZ
standard dilution series Ct values were taken to generate a standard curve
and the Ct values obtained with the plasma dilutions of the HIV-2 NIHZ
particles, were used to calculate the output number of virus particles. The
regression curve of the plasma dilution series proved to be almost parallel
to but 2.7 times lower (y=0.36x, Spearman regression coefficient of 0.97) than the water dilution series (by definition y=x). Since each HIV-2 particle contains two RNA copies, it can be calculated that the cut-off of the assay for the detection of HIV-2 RNA copies per ml of EDTA plasma is \((2) \times (2.7) \times (10^2)= 5 \times 10^2\).

**Figure 1** A) Regression curve based on the mean of six independently processed serial dilutions of the HIV-2 NIHZ EM counted standard. The error bars indicate the 90% confidence interval of the mean. The regression curve is given for the samples above the detection limit of the assay (100 virus particles/ml). —: individual data points, : mean of six independent samples. B) Regression curve of three indepen-
dent processed serial dilutions of the HIV-2 NIHZ EM counted standard diluted in DEPC treated water and EDTA plasma Δ: HIV-2 NIHZ particles diluted in water. ■: HIV-2 NIHZ particles diluted in EDTA plasma.

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Table 2. Efficacy of HIV-2 detection with the Taqman primer/probe sets. $^1$ Proviral DNA from 16 patients was tested for it's fit with the Taqman primers/probe sets. $^2$ Serological reactivity as determined by Western blot. $^3$ Ct value measured with a standard amount of target input. Ct values more than three Ct values higher than the lowest Ct value obtained with a set are shown in grey. $^4$ Ct values shown in grey are considered a misfit.

**Nucleotide variation within HIV-2 from Rotterdam patients**

To overcome possible variation within the target HIV-2 RNA sequence of the Taqman set 1 primers and probe, two additional sets were developed. The second set was located within a relatively conserved region of the Gag p24 gene. Furthermore, for the detection of subtype B HIV-2 strains, the primers and probe from set 1 were slightly modified to fit subtype B sequences. In order to determine which Taqman set had the best fit with HIV-2 sequences from HIV-2 seropositive individuals visiting our outpatient clinic, a PCR was carried out on proviral DNA in PBMC's from these individuals with primers covering approximately 400 base pairs including the Taqman set 1 and set B target sequences (forward primer HIV-2TM1seqfpr and reverse primer HIV-2TM1seqrpr) and the Taqman set 2
target sequence (forward primer HIV-2TM2seqfpr and reverse primer HIV-2TM2seqrpr). The amount of the amplicon generated for each patient was estimated by agarose gel separation using ethidium bromide staining. For all patients an approximately similar amount of the amplicon covering the Taqman set 1 region was subsequently analysed in the Taqman procedure with Taqman set 1. In this way we ensured that from each patient approximately the same amount of target was present in the Taqman assay. Differences in Ct output are therefore directly related to nucleotide sequence differences between the target sequence and the primers or probe used in the Taqman assay. For Taqman set 2 and set B the same procedure was followed. Ct values obtained in this way are shown in table 1. A Ct value that differed more than 3.0 from the lowest Ct value obtained with that same set was considered a misfit. Set 1, B and 2 proved to detect respectively seven, one and twelve proviral DNA sequences out of the 16 patients tested (Table 2). The proviral DNA detected with the Taqman primer/probe set B, which was designed to fit with subtype B HIV-2 strains, was isolated from a patient originating from Ivory-Coast, where subtype B HIV-2 strains is most prevalent (Pieniazek et al. 1999). Three out of the 16 HIV-2 seropositive individuals were not covered with the three Taqman sets used. Interestingly all three patients were both HIV-1 and HIV-2 seropositive with a high CD4 count (>600/μl). Whether these patients are indeed HIV-2 infected or HIV-1 infected with serological HIV-2 cross-reactivity therefore remains to be elucidated.

In summary, our data show that the quantitative molecular assay may be used to quantify the plasma HIV-2 RNA load in the vast majority of the HIV-2 seropositive patients living in the Rotterdam area visiting our outpatient clinic.

References


CHAPTER 9

Antiretroviral Therapy in Patients with Dual HIV-1 and HIV-2 Infection

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Antiretroviral Therapy in Patients with Dual HIV-1 and HIV-2 Infection

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Increasing numbers of patients with HIV-2 infection are being seen in Western Europe. 630 HIV-1, 14 HIV-2 and 6 dual HIV-1 and HIV-2 seropositive patients attend our hospital. The HIV-2 seropositive patients all belong to, or have direct links with a group of West African immigrants residing in the Rotterdam area. Six of the previously untreated HIV-2 seropositive patients who all started on zidovudine 300 mg twice daily, lamivudine 150 mg twice daily and indinavir 800 mg thrice daily, have so far shown clinical improvement with plasma HIV-2 RNA levels <5x10^2 copies/ml (data not shown). Here we describe the response to antiretroviral therapy of two previously untreated dually HIV-1 and HIV-2 infected patients whom both unexpectedly failed on antiretroviral therapy.

One was a 55-year-old, asymptomatic Cape Verdian man. At baseline his CD4+ cell count was 50/µl and his plasma HIV-1 RNA level was 7,5x10^3 copies/ml (Cobas Amplicor 1.5, Roche Diagnostics, Capelle a/d IJssel). He was treated with ritonavir 400 mg twice daily and saquinavir 400 mg twice daily. His plasma HIV-1 RNA was undetectable (<5,0x10^2 copies/ml) within 4 weeks and has remained undetectable for 18 months. The second patient was a 28-year-old, asymptomatic man from the Ivory Coast. At baseline his CD4+ cell count was 40/µl and his plasma HIV-1 RNA level was 5,4x10^3 copies/ml. He was treated with zidovudine 300 mg twice daily, lamivudine 150 mg twice daily and nelfinavir 1250 mg twice daily. His plasma HIV-1 RNA level decreased to <5,0x10^2 copies/ml within 4 weeks and has remained undetectable for 9 months. The reduction in plasma HIV-1 RNA levels suggested adequate viral suppression in both patients. However, retrospective analysis of HIV-2 RNA levels revealed no decrease of HIV-2 RNA in patient 1 (>2,5x10^4 copies/ml during follow-up) and in patient 2 an initial decrease in HIV-2 RNA levels from 5,0x10^3 too undetectable (<5,0x10^2 copies/ml) followed by a rebound above baseline (2,1x10^4 copies/ml)(figure).

HIV-2 is thought to have a protective effect on HIV-1 super infection, so that either HIV-1 or HIV-2 but not both are thought to actively replicate in dually infected patients. In these two patients however both HIV-1 and HIV-2 actively replicated at the same time. Given the different susceptibilities of HIV-2 strains to protease inhibitors and the more limited options for treatment of HIV-2 infection (3,4), we recommend caution before the
initiation of antiretroviral therapy in dual HIV-1 and HIV-2 seropositive individuals. Especially since plasma HIV-2 RNA levels >1,0x10^4 copies/ml during antiretroviral therapy as measured in the two patients described above are expected to result in progression to end stage disease. Serological testing for HIV-2 infection and monitoring HIV-1 and HIV-2 plasma viral loads in dually HIV-1/HIV-2 seropositive individuals are therefore essential for adequate management of disease in HIV seropositive individuals.

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Plasma HIV-1 and HIV-2 RNA Levels and CD4⁺ Cell Counts before and during Antiretroviral Therapy in Two Patients with HIV-1 and HIV-2 Infection. Solid line indicates detection limit of the HIV-1 and the HIV-2 plasma viral RNA tests (5,0x10^2 copies per ml for both assays)

References

SUMMARISING DISCUSSION
General discussion and summary

Infection with human immunodeficiency virus type 2 (HIV-2) predominantly occurs in West African countries around the Gulf of Guinée, but has spread to many countries outside Africa through immigrants and sexual partners of West Africans (1). Although the structure and the replication strategy of HIV-2 are similar to those of HIV-1, the pathogenesis (2-5) and transmission rate - both sexual (6) and perinatal (7-12) - differ from those of HIV-1 infection. The mechanism involved in the apparently lower pathogenicity of HIV-2 infection is currently unresolved. During this study, which lasted from 1995 to 1999, the population visiting the HIV-clinic in Rotterdam (n=766) was multinational and consisted of patients from 60 different nationalities. Among these, 67 individuals from seven nationalities originated from West Africa. Forty percent (n = 23) of this patient population proved to be HIV-2 seropositive and 60 percent (n = 42) HIV-1 seropositive. From the HIV-2 seropositive individuals, about one fifth (n=5) proved to be dually HIV-1/HIV-2 seropositive by Western blot analysis, according to WHO guidelines. This study was started to increase our understanding of the different clinical courses observed for HIV-1 and HIV-2 induced disease. This understanding may lead to a better disease management and choice of antiretroviral treatment of individuals infected with HIV-2.

First we were interested in the rates of disease progression amongst the HIV-2 and HIV-1 infected individuals from West African origin, and in the characteristics of the HIV-2 strains involved. The ratios of asymptomatic versus symptomatic individuals were 2:7 for HIV-2 and 8:9 for HIV-1 infected individuals in our cohort. Like in HIV-1 infection, the success of HIV-2 isolation from PHA-stimulated PBMC correlated with disease progression, low CD4+ T cell counts, and the ability of the virus to induce syncytia in T cell lines (13, chapter 2). We also observed that HIV-2 infection may have a rapid progressive course, judged by a CD4+ T cell decline from 600/µl to less than 200/µl within 36 months after acute HIV-2 infection in a French homosexual individual, who did not belong to the Rotterdam cohort. The more favourable asymptomatic /symptomatic ratio for HIV-1 infected patients may have been influenced by our patient selection. However, the observed rapid progressive course of HIV-2 infection in a patient with a known seroconversion date, led us to believe that the course of HIV-2 infection may be as rapid as in HIV-1 infection.

In HIV-1 infection plasma HIV-1 RNA is an important prognostic parameter for disease progression (14). Several studies have reported on tissue HIV-1 RNA load before and after initiation of antiretroviral treatment, the tissue HIV-1 RNA load proved to correlate with plasma HIV-1 RNA load (15,16). It is generally accepted that plasma HIV-1 RNA load is an important tool in the decision-making on starting antiretroviral therapy. For HIV-2 infection a reliable quantitative assay to measure plasma HIV-2 RNA
load was missing, so we set out to study lymphoid tissue biopsies from HIV-2 infected individuals by in situ hybridisation and immunohistochemistry in order to determine tissue HIV-RNA load (chapter 3). The histological characteristics, like irregular follicular hyperplasia upon disease progression, were in general similar to the observations made in HIV-1 infection (for review see reference 17). The number of productively infected cells per mm$^2$ of tissue was found to be five times lower as compared to quantitative data obtained from HIV-1 infected individuals during comparable stages of disease. Immunohistochemistry studies furthermore revealed a significantly higher percentage of CD8$^+$ T cells in the germinal centres (GC) of the lymphoid tissues (LT) taken from HIV-2 infected individuals as compared to those of HIV-1 infected individuals. In HIV-1 infection, CD8$^+$ T cells are considered a major antiviral component of the HIV-1 specific immune response (18,19). Therefore, we conclude that the relatively low numbers of productively HIV-2 infected cells may be related to the presence of the relative high numbers of CD8$^+$ T cells. From the tissue viral load data it was estimated that the mean plasma viral load in asymptomatic HIV-2 infected individuals is five-fold lower than in HIV-1 infected individuals at the same stage of the disease. This would result in a slower disease course in HIV-2 infected individuals. Recently we developed an assay to assess quantitative plasma HIV-2 RNA loads (chapter 7), and found the plasma HIV-2 RNA levels to be even 100 times lower at this stage. This discrepancy between tissue and plasma viral load differences in HIV-1 and HIV-2 infected individuals could either be due to a slower replication of HIV-2 or to a higher clearance of HIV-2 virions (20). There are several arguments in favour of a more efficient control of virus replication in HIV-2 infected individuals. The observed amount of produced virions per infected cell by in situ hybridisation analysis proved to be considerably higher in HIV-1 infection than in HIV-2 infection (P. Racz personal observation). Moreover, we recently observed in an individual during acute HIV-2 seroconversion, HIV-2 RNA levels before seroconversion as low as 10,000 copies/ml, arguing against a more efficient clearance.

Production of HIV-1 in macrophages is lower than in CD4$^+$ T cells, as judged by a lower overlay of virion particles (Racz, not published). As CD4$^+$ T cells were predominantly the major source of HIV-2 production in the study described in chapter 3, the low plasma HIV-2 RNA load could not be attributed to a switch from CD4$^+$ T cells to macrophages as the major source of virus production. For HIV-1 infection this switch has been reported during opportunistic infections (21). In order to confirm this difference in the nature of virus producing cells, we looked into lymphoid tissue samples from HIV-1 infected patients during opportunistic infections and end-stage disease (chapter 4). We could not confirm this finding in our lymphoid tissue samples. Therefore we concluded that CD4$^+$ T cells remain the major source of virus production during late stage disease in both HIV-1 and HIV-2 infection.

Chapters 2, 3 and 4 were based upon ex vivo and in vivo studies on
HIV-2 infection. Virus characteristics of HIV-1 and HIV-2 were comparable, however, tissue and plasma HIV-2 RNA were five and at least 100 times lower respectively than tissue and plasma viral load in HIV-1 infection. This may be due to the high numbers of CD8+ T cells - possibly controlling virus replication - found in the GC of LT from HIV-2 infected individuals. We were interested to determine whether specific virus properties of HIV-2 contributed to the observed differences found in chapters 3 and 4.

Entry of HIV-1 into target cells is mediated by binding of the surface envelope glycoprotein to the CD4 molecule (22). This binding triggers conformational changes in gp120 allowing the subsequent interaction of gp120 with an α- or β-chemokine receptor. (23) The latter depends on the biological phenotype of the virus. Subsequently the fusion process is initiated. CCR-5 and CXCR-4 are the main coreceptors for macrophage and T-cell-line tropic variants of HIV-1 respectively (24,25). In HIV-1 infection longitudinal studies have shown a shift towards the usage of CXCR-4 as co-receptor for HIV-1 during disease progression (26,27). This suggested that broadening of coreceptor usage contributes to the cytopathic potential of HIV-1 strains in vivo. In chapter 5 we have evaluated the capacity of primary isolates and biological clones of HIV-2 infected individuals at different stages of the infection to use coreceptors. To this end we used a panel of human cells stable transfected with chemokine receptor genes. Like primary HIV-1 strains, all biological clones and primary HIV-2 isolates, of SI or NSI phenotype, were able to use CCR-5. Thus we concluded that CCR-5 is the main coreceptor for HIV-2. There was no difference between HIV-2 SI and NSI clones with respect to their usage of CXCR-4, or any other coreceptor. So, in contrast to HIV-1, there seems to be no correlation between specific coreceptor usage and HIV-2 syncytium-inducing capacity. Furthermore, almost all primary isolates and biological clones used in this study exhibited the usage of a broad range of coreceptors, including CCR-1, CCR-3 and/or CXCR-4 in addition to CCR-5. This suggested that the broad coreceptor usage in vitro does not add to the in vivo cytopathogenicity of HIV-2.

To further address this issue we studied the in vivo pathogenic potential of HIV-2 isolates and biological clones in a chimeric human to mouse model for in vivo HIV infection (the xeno-GvHD mouse model) (chapter 6). Although it may be argued that this model functions as a refined test-tube, a clear advantage of the model is that large numbers of antigen-presenting macrophages and activated T-cells are present (28). In this model high numbers of numbers of human peripheral blood mononuclear cells (PBMC) are grafted in the peritoneal cavity of immune deficient mice. Within seven to 14 days an acute graft versus host reaction develops. The human PBMC repopulate mouse tissue, eventually resulting in human cell populations with a high CD4/CD8 ratio (29). Depletion of human CD4+ T cells from the graft results in a complete abrogation of the acute xeno-GvHD reaction. Depletion of antigen presenting cells results in lower CD4/CD8 ratios and a concomitant delay of the xeno-GvHD symptoms. In this model direct killing
of CD4+ T cells as well as indirect pathogenic effects of different HIV-2 strains and primary isolates could be studied. It was found that a SI macrophage-tropic clone, using only CCR-5 as its coreceptor, proved to be more pathogenic than a NSI macrophage-tropic biological clone with a broad coreceptor usage (CCR-1, CCR-3, CCR-5 and CXCR-4). This suggested a broadening of cellular host-range does not increase the pathogenic potential of HIV-2. From two HIV-2 strains using CXCR-4, both cytopathic for CD4+ T cells in vitro, the macrophage-tropic isolate proved to be far more pathogenic than the non-macrophage tropic one, indicating that the ability to infect human macrophages influences in vivo pathogenicity. Also, the replication rate and the relatively low CD4/CD8 ratio of an early HIV-2 NSI in the xeno-GvHD mouse model did not differ significantly from several other HIV-1 and HIV-2 isolates from end-stage AIDS patients. It therefore seems that in both in vitro and in our chimeric animal model, HIV-1 and HIV-2 may have comparable replicative and pathogenic potential.

From the data presented in chapters 2 to 6 we conclude that the lower viral load and pathogenicity observed in HIV-2 infected individuals as compared to HIV-1 infected individuals, are rather related to a different interaction of the virus with the specific immune response, than solely to virus characteristics.

In chapter 7, 8 and 9 we studied issues related to antiretroviral therapy (ART) in HIV-2 infected patients. First, we examined the development of phenotypic and genotypic resistance of HIV-2 during nucleoside reverse transcriptase inhibitors (NRTI) treatment. NRTI treatment strategy from 1987 to 1994 was initially limited to AZT monotherapy, which temporarily delays disease progression in asymptomatic and advanced HIV-1 disease and decreases plasma HIV-1 RNA loads by approximately 0.3 log10 copies/ml (30,31). Subsequent results from studies on combination NRTI therapy showed slower disease progression and an improved survival for antiviral naive HIV-1 infected patients on combination therapy versus monotherapy (32-34). In HIV-1 infection this degree of virus suppression is usually insufficient, resulting in the rapid selection of NRTI-resistant virus mutants, with specific genotypic mutations on the RT gene, which correlates with diminished phenotypic sensitivity to NRTI (35). We found that phenotypic resistance of HIV-2 to nucleoside analogues, which developed in HIV-2 infected patients treated with NRTI, was also associated with genotypic changes. Although some of the mutations at amino acid positions in the HIV-2 reverse transcriptase gene corresponded with those involved in HIV-1 resistance, no conventional mutations associated with resistance to AZT were observed. A noteworthy observation was the appearance of a Q151M mutation after 6 months of monotherapy with AZT. In HIV-1 infection this mutation emerges only in 2% of patients treated with multiple NRTI (36,37).
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Table 1. Response to antiretroviral therapy. ND = not done.

*HIV-2 RNA could not be quantified. ** Moment of last follow-up in months. Rtv is ritonavir, sqv is saquinavir, nfv is nelfinavir, ind is indinavir.
In vivo monitoring of the effectiveness of ART in HIV-2 infected patients has been hampered by the absence of validated assays to measure plasma HIV-2 RNA. We have recently developed and validated an assay which allows us to quantify plasma HIV-2 RNA levels with a detection level of 500 copies/ml (chapter 8). From 1994 on 14 HIV-2 infected individuals received ART. The response to therapy is shown in table 1. Monotherapy or dual NRTI therapy resulted in failure in all three patients, as judged from the kinetics of their plasma HIV-2 RNA loads. Two out of two patients failed double protease-inhibitor (PI) therapy: one of them (RH2-19) was treatment naive and had an HIV-1/HIV-2 dual infection (chapter 9) and the other patient (RH2-5) was AZT/3TC experienced. A combination of AZT/3TC and nelfinavir failed in a naive HIV-1/HIV-2 infected patient (RH2-20). AZT/3TC and indinavir induced sustained plasma HIV-2 RNA suppression in 6 out of 9 patients. Two of the three failing patients (AH2-1, RH2-25) did so after stopping the indinavir because of side effects. One failing patient (AH2-2) proved to have low indinavir plasma levels, probably because of insufficient compliance.

After failing double PI therapy (saquinavir/ritonavir) and triple therapy consisting of two NRTI and one PI, we did a sequence analysis of the RT and protease gene in some of the patients. The wild type HIV-2 protease gene and the reverse transcriptase gene differed from HIV-1 on some positions in amino acids. For the RT gene it concerns position 101, 106, 181, 188, 190, 210, 215 and 219. On the positions 181, 188 and 190 the amino acids of HIV-2 RT are the same as those found in HIV-1 strains that are resistant to non-NRTI (NNRTI). The most important difference of wild type HIV-1 and HIV-2 in the protease protein is the amino acid valine on position 71. In HIV-1 a mutation at position 71 (A→V) is associated with resistance to nelfinavir. In addition, there are several different amino acids on positions that are considered secondary mutations for HIV-1 PI resistance, associated with some resistance to various PI's. It concerns the positions 10, 20, 32, 36, 46, 47, 77 and 82. The relevance of several of the mutations at positions 210, 215 and 219 in the RT protein and positions 20, 63, 77, 82 in protease remains unclear, since mutations in HIV-1 associated with resistance involve mutations to other amino acids than those observed in the wild type HIV-2 protease. No resistance associated mutations were found in the protease proteins of patients RH2-20 and AH2-1 failing on AZT, 3TC and nelfinavir and AZT, 3TC and indinavir respectively. For patient AH2-2 a mutation was found at position 90 (L→M) and for patient RH2-5 the I84V and L90M mutations were observed. These data show that only few mutations relative to HIV-1 suffice for resistance to PI's.

We furthermore described a different response to ART of two ART naive patients, who were dually HIV-1/HIV-2 infected (chapter 9). One patient received double PI combination therapy, ritonavir and saquinavir 400 mg bid, the second patient received AZT 300mg bid, 3TC-150 mg bid and nelfinavir 1250 mg bid. The observed reduction of HIV-1 RNA loads below the detection limit of 500 copies/ml suggested adequate sustained viral
suppression in both patients. However, retrospective analysis of HIV-2 RNA loads showed inadequate viral suppression in both patients.

Both observations, the rapid unusual development of a Q151M mutation and the failure on double PI therapy (ritonavir/saquinavir) and triple therapy (two NRTI plus nelfinavir) suggests that ART in HIV-2 seropositive individuals should be carried out with great caution. Non-nucleoside reverse transcriptase inhibitors, such as nevirapine, have shown minimal in vitro inhibitory effect on HIV-2 replication (38,39). Obviously these findings seriously limit the choice of ART combinations in HIV-2 infected individuals. Probably, in contrast to the situation for HIV-1 infected individuals, for HIV-2 infected individuals only one possible ART combination is available. Given the reluctance of many individuals to adhere for many years faithfully to an ART regimen, and knowing the setpoint of plasma HIV-2 RNA after acute infection in general is comparable to that of non-progressors in HIV-1 infection (14), ART in HIV-2 infected patients should not be started too early. Most of our HIV-2 infected patients with CD4+ T cells >200/μl, did not have detectable plasma HIV-2 RNA levels. This is comparable to HIV-1 infected individuals with a long-term non-progressive infection. Recently an HIV-1 infected individual has been described, infected with only nef-deleted forms of HIV-1, which proved to be associated with a long-term non-progressive infection (40,41). He had been asymptomatic for 15 years. However, his CD4+ T cells were observed to decline, with non-detectable plasma HIV-1 RNA levels. It may be speculated that viral replication at levels that cannot be detected by current plasma HIV-RNA assays, is sufficient to cause the loss of CD4+ T cells. It therefore seems wise to start ART in HIV-2 infected individuals around 300 CD4 cells per μl, before the rise in plasma HIV-2 RNA level and before the occurrence of AIDS defining opportunistic infections. Furthermore, in contrast to what is generally believed, our data show that both HIV-1 and HIV-2 may actively replicate at the same time in dually infected patients. Therefore we are convinced that both HIV-1 RNA and HIV-2 RNA should be monitored before and during ART in dually infected individuals.

Taken together, we conclude that the relatively low in vivo cytopathogenicity of HIV-2 as compared to that of HIV-1, is not primarily dependent on currently determined differences in virus characteristics between HIV-1 and HIV-2, but is rather the result of a difference in the mutual interaction between the virus and the host immune system. This apparently results in a long, non-progressuve course of the infection, with prolonged periods of low viral loads and consequently low sexual and low perinatal transmission rates and different epidemiological characteristics of HIV-2 infection compared to HIV-1 infection. Undoubtedly, the relatively low in vivo replication rate of HIV-2 and the difference in amino acid composition of the RT and protease genes, contribute to the observed differences in susceptibility of HIV-2 strains to ART, and in the development of resistance towards antiretroviral compounds.
References

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Nederlandse samenvatting
Samenvatting

Besmetting met het humaan immunodeficiëntie virus type 2 (HIV-2) treft men vooral aan in Westafrikaanse landen rond de Golf van Guinee. Het heeft zich echter ook verspreid naar vele landen buiten Afrika via immigranten en seksuele partners van Westafrikanen (1). Hoewel de structuur en de wijze van vermenigvuldigen van HIV-1 en HIV-2 overeenkomen, is het verloop van de ziekte (2-5) en de kans op overdracht - zowel seksueel (6) als verticaal van moeder op kind (7-12) - verschillend. Het mechanisme dat verantwoordelijk is voor het duidelijk tragere ziektebeloop van HIV-2 is onopgehelderd. Gedurende de periode van deze studie, 1995-1999, was de samenstelling van de HIV-geïnfecteerde patiëntenpopulatie (n=766) zeer divers, en bestond uit 60 verschillende nationaliteiten. Zeventien procent personen waren afkomstig van 7 verschillende Westafrikaanse landen. Zestig procent hiervan (n=42) was HIV-1 seropositief, terwijl veertig procent (n=23) HIV-2 seropositief bleek. Van de 23 HIV-2 seropositieve individuen waren er in de Western Blot 5 seroreactief tegen zowel HIV-1 als HIV-2.

Deze studie had als doel inzicht te verkrijgen in factoren die samenhangen met het verschil in ziektebeloop van HIV-1 en HIV-2 geïnfecteerde individuen. Een grotere kennis hiervan biedt hulp bij het maken van keuzes met betrekking tot antiretrovirale therapie en draagt bij aan een betere behandeling van HIV-2 geïnfecteerde patiënten.


In HIV-1 infectie is plasma HIV-1 RNA een belangrijke prognostische parameter voor ziekteprogressie (14). Meerdere studies hebben daarbij ook laten zien dat de hoeveelheid HIV-1 RNA in het weefsel correleert met het plasma HIV-1 RNA (15,16). Voor de beslissing om antiretrovirale therapie te starten is de hoogte van het plasma HIV-1 RNA belangrijk. In het geval van HIV-2 infectie was zo'n kwantitatieve test niet beschikbaar, zodat we besloten biop ten van lymfoid weefsel, verkregen van HIV-2 geïnfecteerde
patiënten, te bestuderen met behulp van in situ hybridisatie en immuno­histochemie, om zodoende de hoeveelheid virus van een individu te kunnen inschatten, en daarmee de indicatie voor wel of geen therapie te kunnen vaststellen. Histologische karakteristieken, zoals onregelmatige folliculaire hyperplasie tijdens ziekteprogressie, bleken in het algemeen overeen te komen met observaties die gedaan zijn bij HIV-1 infectie (voor een review zie ref.17). Het aantal productief geïnfecteerde cellen per mm^2 bleek in HIV-2 geïnfecteerd lymfoïd weefsel van asymptomatische individuen 5 keer lager te zijn dan in HIV-1 geïnfecteerd lymfoïd weefsel verkregen van individuen in hetzelfde stadium van de infectie. Bovendien bleek het percentage CD8^+ cellen in de kiemcentra van het lymfoïd weefsel van HIV-2 geïnfecteerde individuen significant hoger ten opzichte van HIV-1 geïnfecteerde individuen. In HIV-1 infectie worden CD8^+ cellen als een belangrijk onderdeel gezien van de HIV-1 specifieke afweer reactie. Mogelijk dat er een relatie is tussen het hoge percentage CD8^+ cellen en het lage aantal productief geïnfecteerde cellen in HIV-2 infectie. Vanuit de aanwezige hoeveelheid HIV-2 RNA in het weefsel schatten we dat het plasma HIV-2 RNA vrijvoudig verlaagd zou zijn (20). Dit zou passen bij een trager ziektebeloop. Recent is door ons een kwantitatieve test ontwikkeld om het plasma HIV-2 RNA te meten (hoofdstuk 7), en het bleek dat het plasma HIV-2 RNA zelfs 100 keer lager was dan het plasma HIV-1 RNA van patiënten in dezelfde fase van de infectie. Dit verschil in geschatte plasma HIV-2 RNA en gemeten plasma HIV-2 RNA zou veroorzaakt kunnen worden door ofwel een tragere replicatie of een verhoogde klarings van HIV-2 virionen (20). Er is een aantal argumenten die pleiten voor een tragere replicatie. Ten eerste is het aantal virionen wat per HIV-2 geïnfecteerde cel gezien wordt m.b.v. in situ hybridisatie, kleiner dan dat bij een productief geïnfecteerde HIV-1 cel (Racz, niet gepubliceerd). Bovendien bleek tijdens een geobserveerde acute HIV-2 infectie het plasma HIV-2 RNA, nog voor seroconversie, slechts 10.000 copies/ml te zijn, hetgeen tegen een effectievere klarings pleit. Vervolgens bleek de viral load in HIV-2 geïnfecteerde patiënten met AIDS ook significant lager te zijn dan in patiënten met AIDS en een HIV-1 infectie. Dit ondersteunt eerdere berichten, dat de overleving met HIV-2 en AIDS langer is dan die van HIV-1 en AIDS.

De productie van HIV-1 virionen is hoger in CD4^+ T cellen dan in macrofagen (Racz, niet gepubliceerd). In onze studie waren CD4^+ T cellen de voornaamste bron van HIV-2 virionen, zodat een trager productie niet geweten kan worden aan een productie switch van CD4^+ T cellen naar macrofagen. Voor HIV-1 is deze switch beschreven tijdens het optreden van opportunistische infecties (21). Wij hebben geprobeerd dit te bevestigen, maar in tot onze beschikking bestaand HIV-1 geïnfecteerd lymfoïd weefsel bleven CD4^+ T cellen, ook tijdens opportunistische infecties, de belangrijkste bron van virusproductie (hoofdstuk 4).

Hoofdstuk 2, 3 en 4 zijn gebaseerd op in vivo studies van HIV-2 infectie. Viruskarakteristieken van HIV-1 en HIV-2 waren gelijk, echter weefsel en plasma viral load waren vijf en respectievelijk 100 keer lager tijdens HIV-2
mogelijk is er een verband met de hoge aantallen CD8⁺ T cellen - deze kunnen virusreplicatie beperken, die in de kiemcentra van de lymfekliieren worden aangetroffen. We waren vervolgens geïnteresseerd in virusspecifieke eigenschappen van HIV-2, die konden bijdragen aan de verschillen die we gevonden hadden in hoofdstuk 3 en 4.

Het binnenkomen van HIV-1 in target cellen wordt mogelijk gemaakt door het binden van het glycoprotein (gp120) dat aanwezig is op het oppervlak van het virus en het CD4 molecuul van de target cel (22). Deze binding zet een reeks veranderingen op gang, waardoor het gp120 een interactie aangaat met een α of een β chemokine receptor (23). Welke gekozen wordt hangt af van het biologische fenotype van het virus. Vervolgens kan het fusieproces beginnen. CCR-5 en CXCR-4 zijn de voornaamste coreceptoren voor respectievelijk macrofagen en T-cellijn­trope virussen (24, 25). In HIV-1 infectie hebben studies laten zien dat er tijdens ziekteprogressie in toenemende mate gebruik wordt gemaakt van CXCR-4 als coreceptor (26, 27). Dit ‘bredere’ gebruik van coreceptoren suggereert daarmee bij te dragen aan het cytopathische vermogen van HIV-1 in vivo. In hoofdstuk 5 hebben we primaire isolaten en biologische kloons van HIV-2 geïnfecteerde individuen in verschillende stadia van de infectie getest op hun vermogen om gebruik te maken van coreceptoren. Hiertoe kozen we voor een aantal menselijke cellijnen die de verschillende receptor genen bezaten. In tegenstelling tot HIV-1 waren alle primaire HIV-2 isolaten en biologische kloons, zowel van het SI als het non-SI fenotype, in staat CCR-5 als coreceptor te gebruiken. Wij concludeerden hieruit dat CCR-5 de belangrijkste coreceptor is voor HIV-2. Er was geen verschil tussen HIV-2 SI en HIV-2 non-SI in het gebruik van CXCR-4 (of welke andere receptor dan ook) als coreceptor. Er bleek dus geen correlatie tussen een specifiek coreceptor gebruik en syncytium-inducerend vermogen, zoals dat bij HIV-1 wel is gevonden. Voorts lieten bijna alle primaire isolaten en biologische kloons in deze studie een breed gebruik van coreceptoren zien, inclusief CCR-1, CCR-3 en/of CXCR-4, in aanvulling op CCR-5. Dit suggereert dat een breed gebruik in vitro niet bepalend is voor het cytopathisch vermogen van HIV-2 in vivo.


In hoofdstuk 7, 8 en 9 hebben we een aantal therapie-gerelateerde onderwerpen bestudeerd. Ten eerste hebben we de ontwikkeling van fenotypische en genotypische resistentie voor en na het gebruik van nucleoside reverse transcriptase remmers (NRTI) onderzocht. De reguliere behandeling van HIV-1 geïnfecteerde patiënten in de periode van 1987 tot 1994 bestond uit NRTI's, en was aanvankelijk beperkt tot zidovudine (AZT) monotherapie, wat tijdelijk de ziekteprogressie remt in asymptomatische en gevorderde stadia van HIV-1 infectie. Het verlaagt de plasma HIV-1 RNA met circa 0.3 log_{10} copies/ml (30,31). Resultaten van daaropvolgende studies in therapie-naieve HIV-1 geïnfecteerde patiënten lieten zien dat combinaties van 2 NRTI's tot betere resultaten leidden dan monotherapie (32-34). Echter suppressie van de virusreplicatie van HIV-1 is meestal nog onvoldoende met 2 NRTI's, zodat er een selectie optreedt van NRTI-resistente mutanten, met specifieke mutaties op het RT gen, wat correleert met verminderde fenotypische gevoeligheid voor NRTI's (35). Wij vonden dat fenotypische resistentie van HIV-2 voor nucleoside-analogen zich ook in HIV-2 geïnfecteerde patiënten ontwikkelde die met NRTI's behandeld werden. Er werden genotypische veranderingen op aminozuur posities in het HIV-2 RT gezien die in HIV-1 infectie geassocieerd zijn met resistentie voor NRTI's. Er werden echter geen conventionele mutaties gevonden passend bij resistentie voor AZT. In één patiënt werd na slechts 6 maanden monotherapie AZT een Q151M mutatie gevonden. In HIV-1 infectie verschijnt die mutatie slechts in 2% van patiënten die gedurende langere tijd met meerdere NRTI's behandeld werden (36,37).

Het in vivo monitoren van HIV-2 infecties beperkte zich tot controles van CD4 aantallen, wegens het ontbreken van een test om plasma HIV-2 RNA te meten. Recent werd deze in ons laboratorium ontwikkeld. De detectiegrens van de test ligt bij 500 copies/ml (hoofdstuk 7). Vanaf 1994 kregen 14 HIV-2 geïnfecteerde patiënten antiretrovirale therapie voor- geschreven. Zie voor de resultaten tabel 1 pagina 137. Mono- en duo therapie met NRTI's faalde (3/3); therapie met twee protease remmers (PI) faalde (2/2); één patiënt (RH2-5) was tevoren met AZT/lamivudine (3TC) behandeld, en één patiënt (RH2-19) was met HIV-1 en HIV-2 tegelijk
geïnfecteerd. AZT/3TC/nelfinavir faalde (1/1) in een andere patiënt (RH2-20) met een HIV-1/HIV-2 dubbel infectie, en AZT/3TC/indinavir faalde in 3 van 9 patiënten met een HIV-2 mono-infectie. Bij de twee patiënten met een dubbel HIV-1/HIV-2 infectie, liet het plasma HIV-1 RNA wel een goede respons zien (Hoofdstuk 9). Twee van de 3 patiënten die op het indinavir bevattende regiemen faalden (AH2-1, RH2-25), waren gestopt met de medicatie i.v.m. intolerantie. Eén patiënt (AH2-2) had een slechte compliance met lage indinavir spiegels, De andere 6 patiënten behielden ook bij verdere follow-up (tot 30 maanden) ondetecteerbare plasma HIV-2 RNA spiegels.

Na het falen op dubbel PI therapie (ritonavir/saquinavir) en triple therapie bestaande uit 2 NRTI's en één PI hebben we bij een aantal patiënten het protease gen gesequenced. Hierbij bleek dat in wild type HIV-2 een aantal aminozuren van het RT gen en het protease gen verschillend zijn tot het HIV-1 protease gen, waaronder ook op plaatsen die bij HIV-1 geassocieerd zijn met verminderde gevoeligheid voor non-nucleoside analoog RT remmers en proteaseremmers. Het belangrijkste verschil is de Valine groep op positie 71. In HIV-1 betekent een mutatie van 71A naar 71V een intermediate resistentie voor nelfinavir. Verder is er een aantal verschillen op posities die bij HIV-1 als secundaire mutaties geduikt worden en geassocieerd zijn met licht verminderde gevoeligheid voor meerdere proteaseremmers. Deze laatste twee observaties zijn mogelijk de reden voor het falen van RH2-5, RH2-19 en RH2-20 op therapie. Verder werden er geen resistentie geassocieerde mutaties gevonden in het protease eiwit van RH2-20 en AH2-1 die respectievelijk faalden op AZT, 3TC en Nelfinavir en AZT, 3TC en Indinavir. Bij patiënt AH2-2 werd een mutatie op positie 90 (L90M) en bij patiënt RH2-5 werden mutaties op posities 84 (184V) en 90 (L90M) gevonden. Deze observaties tonen aan dat relatief weinig mutaties in HIV-2 protease ten opzichte van HIV-1, resistentie kunnen geven tegen protease inhibitors.

Non-NRTI's, zoals nevirapine, hebben in vitro geen remmend effect op HIV-2 replicatie (38,39). Het kleinere aantal middelen dat beschikbaar is en de snellere ontwikkeling van resistentie, beperken het aantal mogelijkheden van antiretrovirale therapie voor HIV-2 geïnfecteerde patiënten. Op dit moment is er waarschijnlijk slechts één kans op een werkzame combinatie beschikbaar. Er van uitgaand dat bij vele patiënten in de loop der tijd de therapie trouw afneemt, door ingewikkelde regiems, diëttaire beperkingen en/of bijwerkingen, en wetende dat bij de meeste HIV-2 geïnfecteerde patiënten de plasma viral load pas gaat stijgen wanneer het CD4 aantal beneden de 200/µl daalt, is het aan te bevelen behandeling niet te vroeg te starten. Het ziektebeloop van HIV-2 geïnfecteerde patiënten is vergelijkbaar met dat van long term non-progressors onder de HIV-1 geïnfecteerde populatie (14). Recent is een dergelijke patiënt, geïnfecteerd met HIV-1 waarbij het nef ontbreekt, beschreven. HIV-1 zonder nef gen wordt geassocieerd met een non-progressief ziektebeloop (40,41). De betreffende patiënt had een asymptomatisch ziektebeloop gedurende 15 jaar. Al die tijd was er geen detecteerbaar plasma HIV-1 RNA, terwijl het
CD4 aantal toch bleek te dalen. Hieruit zou geconcludeerd kunnen worden dat replicatie van HIV-1 op een niet detecteerbaar plasma-niveau uiteindelijk ook voldoende is om het aantal CD4 cellen te doen verminderen. Het starten van antiretrovirale therapie lijkt gerechtvaardigd bij 300 CD4⁺ T cellen, voordat er een forse stijging van het plasma HIV-2 RNA volgt, en voordat er kans is op opportunistische infecties.

Tenslotte hebben we, in tegenstelling tot wat algemeen wordt aangenomen, aangetoond dat HIV-1 en HIV-2 naast elkaar actief kunnen repliceren in patiënten die met beide virussen besmet zijn. In patiënten, die seroreactief zijn voor HIV-1 en HIV-2, dient voor en tijdens therapie zowel het HIV-1 RNA en het HIV-2 RNA gemeten worden.

Samenvattend kunnen we stellen dat het relatief lage in vivo pathogeen vermogen van HIV-2 in vergelijking met dat van HIV-1, niet primair afhankelijk is van verschillen in eigenschappen van de twee virussen. Het is meer een gevolg van verschillende interacties tussen de virussen en het immuunsysteem van de gastheer. Dit resulteert voor HIV-2 in een lang ziektevrij beloop van de infectie met daarbij behorende langdurige periodes met lage plasma HIV-2 RNA’s. De relatief lage snelheid van vermenigvuldigen van het virus in vivo, de verschillen van een aantal aminozuren op het RT gen en het protease gen, dragen mogelijk bij aan de waargenomen verschillen in gevoeligheid voor antivirale middelen en het tempo waarin resistent mutanten geselecteerd worden.
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Curriculum Vitae
