THE PATHOGENESIS OF AIDS: LESSONS FROM THE SIV-MACAQUE MODEL

(DE PATHOGENESE VAN AIDS:
LESSEN VAN HET SIV MAKAAK MODEL)

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ABBREVIATIONS

AIDS	:	Acquired Immuno Deficiency Syndrome
bp	:	Basepair
CTE	:	Cis-acting transport element
EIAV	:	Equine Infectious Anaemia Virus
FACS	:	Fluorescence Activated Cell Sorter
FIV	:	Feline Immunodeficiency Virus
huCBL	:	Human Cord Blood Lymphocytes
HIV-1	:	Human Immunodeficiency Virus Type-1
IN	:	Integrase
kDa	:	Kilodalton
lacZ	:	ß-galactosidase
LTR	:	Long Terminal Repeats
MID_{50}	:	Monkey Infectious Dose 50
MDM	:	Monocyte Derived Macrophage
mø	:	Macrophage-tropic
NF-κß	:	Nuclear Factor-Kappa B
NSI	:	Non-syncytium inducing
PCR	:	Polymerase Chain Reaction
p.i.	:	post infection
PBMC	:	Peripheral Blood Mononuclear Cells
PR	:	Protease
RT	:	Reverse Transcriptase
RT-PCR	:	Reverse Transcription Polymerase Chain Reaction
Rev	:	Regulator of viral expression
Rh	:	Rhesus
RNP	:	Ribonucleoprotein
RRE	:	Rev-responsive element
(S)AIDS	:	(Simian) Acquired Immuno Deficiency Syndrome
SU	:	Subunit
SI	:	Syncytium inducing
SIV	:	Simian Immunodeficiency Virus Type
SRV-1	:	Simian Retrovirus Type-1
SHIV	:	Simian Human Immunodeficiency Virus
TAR	:	*Tat* activation response element
TID_{50}	:	Tissue Infectious Dose 50
TM	:	Transmembrane
X-Gal	:	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

In the mid-eighties it became apparent that a human retrovirus of the lentivirus subfamily (4), later designated human immunodeficiency virus type 1 (HIV-1), was the etiological agent of the acquired immune deficiency syndrome (AIDS) (124). This disease was characterised by unusual opportunistic infections, neurologic abnormalities, gastrointestinal disorders and malignancies due to an insidious decay of the immune system (79). The urgent need to gain insight in certain aspects of the pathobiology of this infection demanded relevant animal models. As a consequence of this need the search for similar lentiviruses present in other animal species intensified. Several lentiviruses have been identified to induce AIDS-like disease in a variety of animals (75), however, only simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV), which cause AIDS-like symptoms in macaques and cats, respectively, have been commonly used as animal models (76).

In this chapter an overview of the molecular biology of SIV and the utilisation of the SIV-macaque model for AIDS research is given. In particular, the use of molecular clones of SIV to elucidate the pathogenesis of AIDS is described.

1.2 ORIGINS OF SIMIAN AND HUMAN IMMUNODEFICIENCY VIRUSES

In 1969, at the California Regional Primate Research Centre a large number of rhesus monkeys were diagnosed with a high incidence of lymphoma. Animals having this type of lymphoma had unusual opportunistic infections such as *Mycobacterium avium*. Studies suggested a viral etiology of this disease, especially when lymphocyte-associated herpesviruses, adenoviruses and retroviruses could be detected in cultured tumour tissue (42). In the early 1980's comparable cases began occurring at the New England and Tulane regional primate centres as well (78). However, it was not until 1985 that lymphoma transmission studies, morphological comparisons and growth characteristics suggested that the observed disease was associated with the presence of a lentivirus: STLV-III, later designated SIV, the simian counterpart of HTLV-III (HIV-1) (6). Although type D retroviruses (known as simian retroviruses, SRV) were also found to be associated with simian AIDS (SAIDS) in several species of macaques (*Macaca mulatta, M. cyclopis, M. nemestrina, M. fascicularis, M. fuscata* and *M. nigra*), it was excluded as the etiologic agent for the observed disease in these rhesus macaques, since 1) experimental infection with a cell-free type D retrovirus stock failed to induce disease in healthy animals, 2) it was impossible to isolate type D retroviruses from the majority of these monkeys upon cocultivation and, 3) T-cell lines producing SIV did not yield a type D retrovirus when cocultured on cell lines (42, 45, 77). Studies revealed that SIV_{mac} (22), and closely related isolates from various other Asian macaque species, were all derived from SIV_{sm}, a naturally occurring infection of sooty mangabeys (42, 93) (Figure 1).

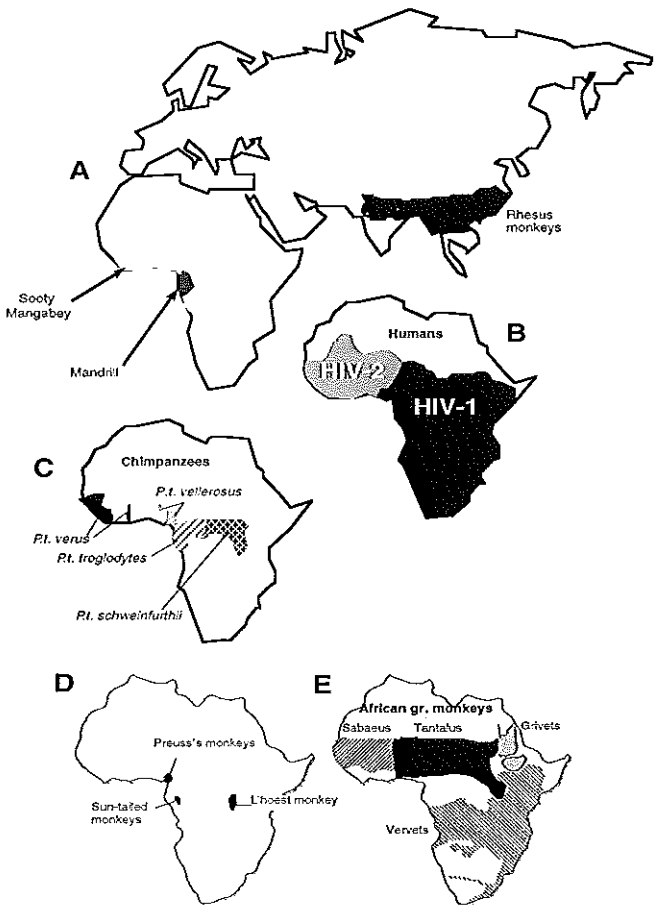

Figure 1. Distribution of African primate species naturally infected with lentiviruses that are resistant to disease (with the exception of humans (B, C, D, and E)). The geographic separation of Asian rhesus macaques which are susceptible to AIDS is shown in A. The approximate distribution of humans infected with HIV-1 versus HIV-2 early in the epidemic is depicted in B.

SIV_{sm}, like lentiviruses isolated from other naturally infected African nonhuman primates, does not cause AIDS in its natural host (50, 99). Lentivirus infections have not only been found in sooty mangabeys (*Cercocebus torquatus atys)* (56), but also occur in African green monkeys (*Cercopithecus aethiops*) (2, 99), mandrills (*Mandrillus sphinx*) (133), L'Hoest's monkeys (*Cercopithecus lhoesti*) (54), sun-tailed monkeys (*Cercopithecus solatus*) (8), Sykes' monkeys (*Cercopithecus mitis albogularis*) (55), and chimpanzees (*Pan troglodytes).* (58, 106). Based on sequence analysis these African nonhuman primate lentiviruses can be classified into 5 main lineages represented by; 1) SIV_{agm} from different subspecies of African green monkeys, 2) SIV_{mnd} from Mandrills, 3) SIV_{syk} from Sykes' monkeys, 4) SIV_{sm} from sooty mangabeys, SIV_{mac}, and HIV-2 of humans, and 5), SIV_{cpz} from the chimpanzees and HIV-1 from humans (Figure 2). African nonhuman primates

were found to harbour SIV asymptomatically while inoculation of SIV_{sm} into rhesus macaques (Asian in origin) induced a disease remarkably similar to AIDS in humans (77). Retrospective evidence suggested that SIV_{mac} was introduced to the New England rhesus colony through a cohort of ten female rhesus macaques obtained from the California colony in 1970 (42). The high degree of genetic homology between SIV isolated from captive rhesus macaques (SIV_{mac}) and the virus found in naturally infected sooty mangabeys (SIV_{sm}) suggested that SIV_{mac} originated from SIV_{sm} by cross-species transmission (40, 42, 56, 93). Similar evidence suggests that HIV-1 may have been introduced into the human population from a specific subspecies of chimpanzees (*Pan troglodytes troglodytes*) (39) (Figure 1).

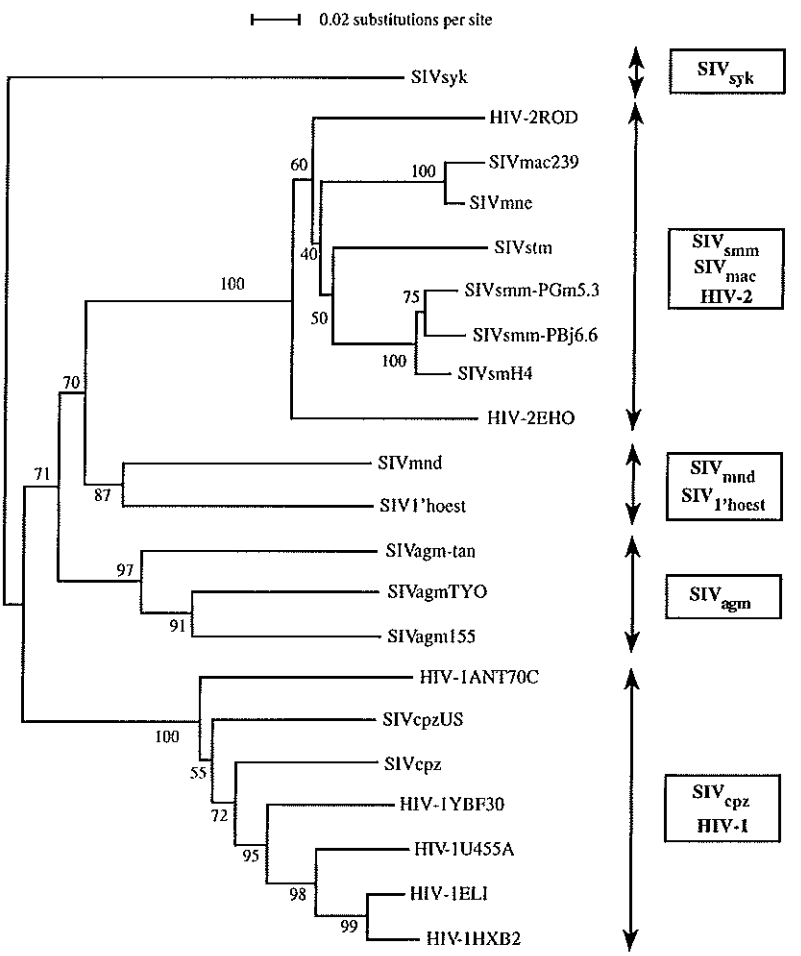

Figure 2. The genetic relatedness of the five currently identified lineages of primate lentiviruses (141).

1.3 GENETIC ORGANISATION

The linear SIV provirus in host cells is 9.2-kb in length and contains structural genes (*gag* and *env*), non-structural genes (*pol*, *tat*, *rev*) and accessory genes (*vif*, *vpx* and/or *vpr* and *nef*), and is flanked by two long terminal repeats (LTR) (Figure 3). The *gag* open reading frame encodes the core proteins p7, p9 (NC), p17 (MA) and p24 (CA), whereas *pol* codes for the viral enzymes reverse transcriptase (RT), RNAse H, protease (PR) and integrase (IN). The envelope gene encodes the envelope glycoprotein (precursor) that is proteolytically cleaved into gp130 (SU) and gp41 (TM). The viral precursor RNA code for Gag, Pol and Env proteins as well as for the accessory Vif, Vpr, Vpx and Nef proteins which are not required for viral growth *in vitro* but are essential for viral replication and pathogenesis *in vivo* (132).

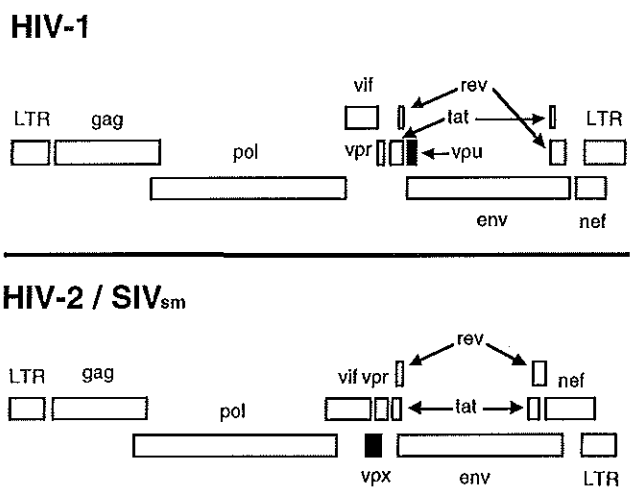

Figure 3. Comparison of the genetic organisation of proviruses of the HIV-1/SIV_{cpz} lineage versus the $SIV_{mac/sm}$/HIV-2 lineage. The organisation of the structural and regulatory genes is highly similar with the exception of *vpu* and *vpx*, respectively.

The LTR flank either side of the SIV proviral genome and contain a TATA box for initiation of viral transcription, which is carried out by the cellular RNA polymerase II. Other regulatory elements include high affinity binding motifs for the nuclear factor-kappa B (NF-κβ) and the cellular transcription factor SP1 (64). Molecular clones of SIV mutated in these particular regions still showed efficient viral replication in primary lymphoid cell cultures and in rhesus macaques (60, 145). This is in contrast with the hypothesis that NF-κβ and SP1 are required for persistent infection and for the induction of AIDS in rhesus macaques. It also suggests that the SIV LTR promoter contains multiple functionally redundant elements capable of supporting sufficient transcription to allow productive viral

replication (109). The LTR also contains signals for integration, transcription-regulation and poly-adenylation of mRNA. Transcriptional activation of LTR-driven gene expression as well as elongation of newly initiated transcripts requires the transactivator protein (Tat), encoded by a highly conserved regulatory gene. Tat interacts both with cellular factors (46) and binds to the Tat activation response element (TAR) sequence that is located in the U5 region of the LTR. The regulator of viral expression (Rev) is encoded by the regulatory gene *rev*, consisting of two exons. The 19 kDa Rev protein exerts its function by interacting with the Rev-responsive element (RRE), located within the *env* mRNA. This element is about 200 base pairs in size and forms a secondary structure necessary for its recognition by Rev (11). It has been shown by mutational analysis that a putative activation domain could be assigned to the carboxy-terminus (second exon) of the Rev protein (103). The function of Rev has been associated with the switch from the synthesis of (multiple) spliced messengers coding for the regulatory proteins to the unspliced and single-spliced messengers, (coding for the Gag, Pol and Env proteins), taking place in the early and late phase in viral replication, respectively. As a consequence Rev down-regulates its own expression and that of Tat and Nef as well (34). Studies in which Rev regulation could be replaced by the introduction of the cis-acting transport element (CTE) of the type D simian retroviruses 1 (SRV-1) suggested that Rev functions by promoting transport of single spliced and unspliced viral messengers out of the nucleus.

Vpr and Vpx are two small auxiliary proteins of about 15 kDa that are incorporated into virus particles (126). Vpr together with the MA protein facilitate transport of the viral pre-integration complexes from the cytoplasm into the nucleus of infected cells (21, 65, 82, 144). The *vpx* gene also encodes a small protein, which is hypothesised to act in nuclear import of viral DNA. Vpr has also been associated with cell cycle arrest and is dispensable for the infection of macrophages, while Vpx has no effect on the cell cycle but is required for virus replication in macrophages (35, 108). Vpx and Vpr are highly related and able to substitute for each other, as was shown by studies in which SIV clones mutated in either *vpr* or *vpx* genes were able to induce disease in rhesus macaques while the same clone mutated in both genes did not. Both *vpr* and *vpx* genes are only present in members of the HIV-2 and the SIV_{sm} family while the HIV-1, SIV_{cpz}, SIV_{agm}, SIV_{mnd} and SIV_{syk} primate lentivirus lineages have only the *vpr* gene as depicted in figure 3.

The *nef* open reading frame extends from the 3'end of *env* into the 3' LTR. Structural studies of the *nef* gene product showed that it is a 280 residue multifunctional regulatory protein with a molecular mass of 34 kDa. Research on the function of Nef showed its ability to down-regulate the expression of cell surface CD4 receptor molecules (10, 41) and MHC class I surface antigens (125). Studies in SIV infected macaques also showed that SIV Nef is essential for maintaining high virus loads and is required for viral pathogenesis (67). Furthermore, the Nef proteins of HIV-1 and SIV have been suggested to play a fundamental role in altering T-cell signalling pathways (1, 7, 128), to enhance viral infectivity, and to increase the state of viral replication in lymphocytes (20, 74, 88, 130). These functions of Nef may be attributed to the interaction of a SH2 binding domain (YxxL/YxxR) present in Nef with Src tyrosine cellular kinases (104, 120). It was demonstrated that by generating an

SH2 motif in the Nef amino terminus of the wild type SIV_{mac239} molecular clone that this new mutant was able to replicate in resting rhesus peripheral blood mononuclear cell (PBMC) cultures, due to an altered state of lymphocyte activation (28).

1.4 THE VIRUS LIFE CYCLE

After entry of the cytoplasm of the infected cell the viral capsid is disrupted and a ribonucleoprotein (RNP) complex is released consisting of viral RNA, NC, MA, RT and IN (14). During reversed transcription the two RNA molecules in the virion are converted to a linear double-stranded DNA (92, 136, 137). This complicated process requires priming by binding of tRNA to the primer binding site (PBS) of the viral RNA (13, 30), RNA-dependent DNA synthesis, degradation of the RNA in the DNA-RNA hybrid intermediate by viral Rnase H (89, 106), two template transfers and elongation of plus strand DNA (9). It is mediated by reverse transcriptase and results in the formation of a linear double-stranded DNA molecule with one complete LTR at each end. Since the reverse transcriptase enzymes of retroviruses lack proofreading activity, the mutation rate is extremely high. This results in the rapid accumulation of vast numbers of highly-related but different virus genotypes, called a quasispecies (57, 110, 115, 143). The formation of a pre-integration complex (double stranded DNA complexed with MA and Vpr) promotes the transport of viral DNA to the nucleus were it randomly integrates in the host genome. Proper integration requires particular sequences at the end of both LTR and is catalysed by viral integrase (IN) (15, 32, 112, 122, 140).

1.5 GENE EXPRESSION

The 5' LTR promoter, in combination with cellular factors, present in the integrated double-stranded DNA (provirus) generates the first viral transcript. This transcript serves both as the genomic RNA that is encapsidated in the virion, and as mRNA encoding viral proteins. Early after infection multiple-spliced messengers are synthesised coding for viral regulatory proteins (Tat, Rev and Nef). Later in infection synthesis of unspliced and single spliced messengers predominate encoding the Gag-Pol proteins and the Env, Vif, Vpu, Vpr and Vpx proteins, respectively. This shift which occurs rapidly after infection (within hours) is mediated by the Rev / RRE interaction which specifically changes the transport of the different transcripts to the cytoplasm, away from the host cell splicing machinery in the nucleus.

1.6 VIRUS ASSEMBLY AND RELEASE

During transport to the cell membrane the envelope polyprotein becomes glycosylated and is cleaved into separate TM and SU proteins (31). Upon budding of the virus particles SU proteins are exposed on the surface of the virions. These glycoproteins react with the Gag and Gag-Pol precursor proteins. The internal proteins (MA, CA, NC, and p6) and the

enzymatic proteins (PR, RT, and IN) are derived from Gag protein and the Gag-Pol fusion protein, respectively. The nature of their transport to the cytoplasmatic face of the membrane is not known. However, balanced ratios of Gag and Gag-Pol proteins are incorporated and drive the formation and release of virions from the infected host cell. Two copies of the viral RNA genome are encapsidated, presumably through specific interactions with the Gag protein, together with tRNA and Vif, Vpr and Nef. The final process, the proteolytic cleavage of Gag and Gag-Pol proteins, is essential for producing infectious virions and occurs just before or immediately after virus release (140).

1.7 NON-HUMAN PRIMATE LENTIVIRUSES

Studies conducted on biological material from HIV-1 infected humans and AIDS patients have contributed enormously to the current knowledge of the biochemistry and molecular biology of the virus and about the pathology of the disease it induces. However, since it is difficult to identify humans early, at the time of primary HIV infection, animal models have been invaluable in providing insights into these early events (16, 96). Primate models have enabled the detailed studies of the target cells for viral infection within the first days of infection and have assisted in correlating this information with the biological phenotype of the infecting virus (131). Animal models have also been invaluable for elucidating the role of particular viral variants in pathogenesis (69), and have proved essential in experimental vaccine development and evaluation (97, 127, 142). As a member of the retrovirus subfamily Lentiviridae, HIV-1 is morphologically and genetically related to other, non-human primate lentiviruses. Indeed, the morphological similarity of HIV-1 to Equine Infectious Anaemia Virus (EIAV) led to its identification as a lentivirus. Since then lentiviruses have been discovered in cattle (47, 95, 119), cats (105) and several African primate species (Figure 1). Lentiviruses naturally infect a number of African non-human primates such as chimpanzees (SIV_{cpz}) (106), African green monkeys (SIV_{agm}) (23), sooty mangabeys (SIV_{sm}) (56), Mandrills (SIV_{mnd}) (133), L'Hoest's monkeys (SIV_{rst}) (54), and Sykes monkeys (SIV_{syk}) (33). These viruses cause a persistent but asymptomatic infection when transmitted naturally amongst their natural hosts (50, 98, 138, 139). The pathogenic potential of these infections was not realised until molecular evidence revealed that Asian rhesus monkeys (Figure 1A), which had developed and died from an AIDS-like disease had been accidentally infected with material derived from a sooty mangabey (59, 78). Thus SIV_{mac}, the virus isolated from rhesus macaques was really SIV_{sm} which had undergone cross-species transmission to a species previously naïve to lentiviruses. A number of years later several lines of research based on compelling molecular similarities came to the same conclusion regarding HIV-2 infection in man (18, 40). The remarkable molecular similarity between the sequences of HIV-2 in humans and SIV_{sm} of sooty mangabeys makes them almost indistinguishable (56). Although direct proof is still lacking, the circumstantial evidence is compelling. More recently, similar sequence evidence has strongly suggested that SIV_{cpz} infection from a subspecies of chimpanzees (*Pan troglodytes troglodytes*) is the source of HIV-1 infection in the human population (Figure 2) (39).

1.8 EXPERIMENTAL NON-HUMAN PRIMATE MODELS FOR AIDS RESEARCH

When the magnitude of the evolving HIV-1 epidemic in the human population was realised, it became clear that animal models would be needed to develop vaccines, therapies and to better understand the pathogenesis of AIDS. HIV-1 proved to be very species specific and only great apes (chimpanzees and gibbon apes) could be successfully infected. Chimpanzees were, and still are, the model of choice for HIV-1 infection, vaccination and immune prophylactic studies (52). This is largely because they are biologically and, most importantly, genetically more similar to humans than any other primate species. The majority of chimpanzees are, however, resistant to AIDS (50, 51) making them of interest for comparative pathobiology and immunisation strategies (94), but limiting their utility when the development of AIDS or its treatment requires an animal model.

For the study of AIDS pathogenesis, therapy and the study of vaccines to prevent disease development the rhesus macaque model has been most widely used. In addition to rhesus macaques, cynomolgus and pig-tailed macaques are also susceptible to AIDS when given SIV_{sm} and are also used, but to a lesser extent. This is largely due to the fact that for rhesus monkeys the immunological reagents are best developed, and the immunogenetics are more extensively studied. The models using SIV_{sm}/SIV_{mac} or related strains are, however, only surrogate models for HIV-1 induced AIDS.

1.9 THE CHIMERIC SIMIAN-HUMAN IMMUNODEFICIENCY VIRUS (SHIV) MODEL

To develop an alternative model for the use of chimpanzees, chimeric viruses based on molecular clones of $SIV_{sm/mac}$ were constructed. Either HIV-1 RT-encoding sequences (for antiviral drug studies) (134) or the HIV-1 *env* gene (for HIV-1 based envelope vaccine studies) (49, 80, 81, 113, 114) were cloned into the SIV_{mac239} infectious molecular clone. The first generation of *env*-SHIV chimeras was not pathogenic (i.e. could infect but did not cause AIDS in rhesus monkeys), whereas the RT-SHIV proved to be moderately pathogenic (134). *In vivo* passage of SHIV chimeras has resulted in certain mutations occurring in the SHIV genome which are assumed to have contributed to their ability to cause an AIDS-like disease in rhesus macaques (17, 61, 62, 113). The patterns of CD4 loss with these *in vivo* passaged SHIV are, however, atypical. For example, infection with the env-$SHIV_{89.6p}$ variant results in an acute and profound loss of $CD4^+$ T-cells. In contrast, the chimeric RT-SHIV causes disease only after a long period of infection and with only slight decline in $CD4^+$ T-cells.

The SHIV model is very useful for addressing specific questions of HIV-1 pathogenesis. For example, HIV-1 utilises two major co-receptors, CXCR4 and CCR5 in addition to a large number of potential minor co-receptors (12, 27, 116). To address the significance of HIV-1 co-receptor use in AIDS pathogenesis, SHIV chimeras were constructed from HIV-1 molecular clones with distinct CCR5 or CXCR4 usage, designated R5 and X4 viruses,

respectively. These specific SHIV chimeras were used to infect rhesus macaques, which were then observed to develop quite distinct pathological lesions (48).

1.10 THE USE OF MOLECULAR CLONES TO STUDY GENETIC DIVERSITY WITHIN SIV ISOLATES

The early cases of reported disease in Asian macaques were caused by cross-species transmission from sooty mangabeys. The resulting viral isolates were designated based on the species they were isolated from, for instance from rhesus macaques (SIV_{mac}), stump tailed macaques (SIV_{stm}) and pigtailed macaques (SIV_{mne}). Several SIV isolates, varying in pathogenic potential, have been characterised over the years (26, 42, 44, 73, 83, 111) and have been used in preclinical evaluation of antiviral agents, vaccines and in immunotherapeutic studies (43, 44, 142). In addition, they were useful in mother-to-infant viral transmission studies (87, 118, 129, 135), in defining viral and host determinants involved in various stages of disease, and in correlating certain emerging virus variants with disease progression (68, 69).

Lentiviruses are highly variable RNA viruses. The high error rate of the viral reverse transcriptase results in the rapid evolution of genetic variants following initial infection of the host. These variants, called a quasispecies, continue to accumulate mutations *in vivo* resulting in the maintenance of a genetically diverse population throughout the infection. In addition, template switching during reverse transcription can result in recombination contributing to the high levels of HIV/SIV genetic variability. The heterogeneity in SIV/HIV inocula represents a drawback in studies that require a well-defined virus genotype. The use of molecular clones of SIV or HIV has been important as starting material in such studies because of their high genetic uniformity. HIV develops the highest heterogeneity in the early asymptomatic stages of disease and the lowest levels at later stages (ARC/AIDS) of disease (24). Virus variants, which emerge late in infection, display characteristics being significantly different from variants evolving shortly after infection. The early variants are in general macrophage-tropic, the later variants are T-cell-tropic and induce formation of syncytia. These late variants are commonly believed to contribute significantly to the clinical outcome of infection (69). As a consequence, the characteristics of a molecular clone derived from an infected animal greatly depends on the particular variant that was selected for the cloning procedure and this may be influenced by both the stage of disease and the cell type from which the virus was isolated. Over the years several molecular clones for SIV have been generated (26, 36, 53, 66, 70, 100, 107). The virulence of those molecular clones in macaques ranges from highly pathogenic (26) to non-pathogenic (107). Thus, the clinical disease they cause following infection is highly variable which makes some clones more suitable for certain experiments than others.

Molecular clones have been used to determine genetic determinants responsible for certain pathogenic properties of the virus. Studies have revealed that the determinants for viral virulence (i.e. macrophage tropism) are multiple and are dispersed throughout the genome (91, 101). Other studies have identified the *env* gene as the major determinant

responsible for differences in biological properties such as cellular tropism and cytopathogenicity (3, 63, 90). More recently, the importance of the *nef* gene on cellular activation *in vitro* and disease progression *in vivo* was confirmed using molecular clones (28, 71, 72, 121).

1.11 OVERVIEW OF $SIV_{SM/MAC}$-STRAINS AND RELATED MOLECULAR CLONES

Several molecular clones of $SIV_{sm/mac}$ have been generated over the years. The first virus isolates and molecular clones were generated at the New England Regional Primate Research Centre with the injection of rhesus monkey 251-79 with tissue of animal 78-72 (Figure 4). The SIV isolate from this particular rhesus monkey (251) served as the source of several molecular clones. Since then the original virus isolates and clones have been unwittingly designated SIV_{mac} and repeatedly passaged *in vitro* resulting in altered properties. For that reason comparisons between viral isolates or molecular clones can only be evaluated in their historical context. The origins and relationships of commonly used isolates and their derivatives are depicted in figure 4 and described below.

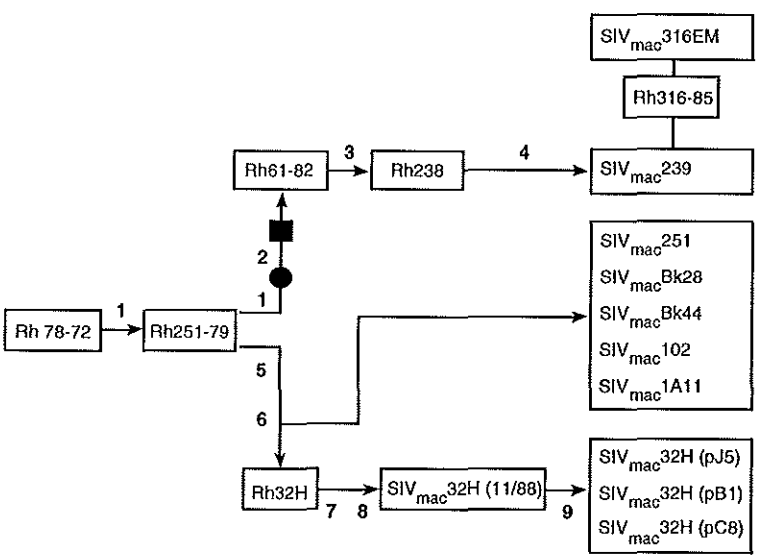

Figure 4. Overview of current SIV_{mac} molecular clones. Rh indicates animals infected with the SIV_{mac} strain. The molecular clones are all listed in the boxes on the right hand side of the chart. **1** = filtered lymphoma homogenate, **2** = PBMC pool from three different macaques, **3** = plasma, **4** = Hut78 cell culture supernatant, **5** = low passage SIVmac251 inoculum prepared on C8166 cells, **6** = H9 cell culture supernatant, **7** =Rh PBMC/huCBL coculture, **8** = C8166 coculture, **9** = JM cell culture. ● = inoculation of seven rhesus macaques. ■ = inoculation of six rhesus macaques (including Rh 61-82).

SIV_{mac251} molecular clones: The virus isolate derived from the rhesus monkey 251 was designated SIV_{mac251}. This was the source for several different molecular clones of SIV_{mac}. A first group of clones (SIV_{mac251}, $SIV_{macBK28}$, SIV_{mac102} and $SIV_{mac1A11}$) was derived after SIV_{mac251} was propagated on H9-cells, a human T-cell line. The second group of clones (SIV_{mac239} and $SIV_{mac316EM}$) was derived after SIV_{mac251} had been passaged in animals 239 and 316-85, respectively. A third group of clones (SIV_{mac32H} (pJ5), SIV_{mac32H} (pB1) and SIV_{32H} (pC8)) was generated from the 251 isolate after inoculations into rhesus monkey 32H following *in vitro* culture (117).

$SIV_{smPBj14}$ molecular clones: The virus isolate SIV_{sm-9} which had been used to inoculate the pigtailed macaque (PBJ), had been passaged once on human PBMC following isolation from a seropositive sooty mangabey. After infection of animal PBJ with this isolate, virus was isolated by coculture with human PBMC. Similar virus preparations were made of spleen and lymph node tissues obtained after monkey PBJ died 8 days after infection. The biological clone $SIV_{smPBj14}$ had acquired increased virulence, which approached 100% mortality in *M. nemestrina* following intravenous inoculation (38). Mortality rates of 33%, 75% and 33% were observed for *M. mulatta, C. atys* and *M.nemestrina*, respectively (38). Animals inoculated with $SIV_{smPBj14}$ developed profuse hemorrhagic diarrhea within five days and died within two weeks post-infection (37). Three molecular clones ($SIV_{smPBj-1.9}$, $SIV_{smPBj-4.9}$ and $SIV_{smPBj4.14}$) were derived from $SIV_{smPBj4.14}$ and induce similar disease symptoms (102). In rhesus macaques, however, these clones do not cause an acute, but rather a more chronic disease progression (37).

$SIV_{mac1A11}$: Virus derived from cells transfected with this clone are cytopathic for rhesus PBMC, replicates in cultures of rhesus macrophages, and infects rhesus macaques when inoculated intravenously. Six macaques inoculated with $SIV_{mac1A11}$ all became infected and produced antibodies to viral envelop glycoproteins that neutralise virus. This molecular clone is markedly attenuated (84, 85). No clinical evidence of disease was observed throughout a 7 months experimental period. Other reports described even longer disease-free periods (86).

$SIV_{smPGm5.3}$: Blood transfusion from a SIV/STLV positive sooty mangabey to rhesus and pigtailed macaques resulted in induction of a neurologic disease and AIDS. Lesions in infected animals include extensive SIV-RNA positive giant cells in brain parenchyma and meninges. Based on morphology infected cells proved to be from the macrophage lineage. $SIV_{smPGm5.3}$ shows high levels of replication in pigtailed and rhesus macaque PBMC and macrophage cultures (100).

$SIV_{smE593.3}$: The biological isolate SIV_{smE543} was obtained late in disease from an immuno-deficient rhesus macaque which suffered from SIV-induced encephalitis. Then, molecular clone $SIV_{smE543-3}$ replicates well in macaque PBMC and monocyte-derived macrophages and resists neutralisation by heterologous sera which broadly neutralise genetically diverse SIV variants *in vitro* (53).

SIV_{mne027}: Uncloned virus isolate SIV_{mne} was used to infect pigtailed macaques and the molecular clone SIV_{mne027} was derived from mesenteric lymph node tissue. This molecular clone is minimally cytopathic and not syncytium-inducing, replicates well in non-stimulated PBMC and is highly cytopathic for $CD4^+$ T-cell subpopulations in stimulated PBMC (70).

1.12 PATTERNS OF PROGRESSION TO AIDS IN SUSCEPTIBLE OUTBRED POPULATIONS

If HIV-1 infection is left untreated the mean survival time is approximately 10 to 12 years post-infection. Because of host differences and genetic polymorphisms of key regulatory genes such as MHC, some individuals will progress slower or faster than the majority of the population (Figure 5). Similarly, in younger individuals such as juveniles or neonates or very old individuals such as geriatrics, the disease can progress more rapidly, in part due to an immature or weakened immune system. While the mean time to death due to AIDS was approximately 10 to 12 years in humans. In Indian rhesus macaques the mean survival time of an animal infected with a strain of medium virulence (SIV_{mac251}) is approximately 1.5 to 2 years (Figure 5).

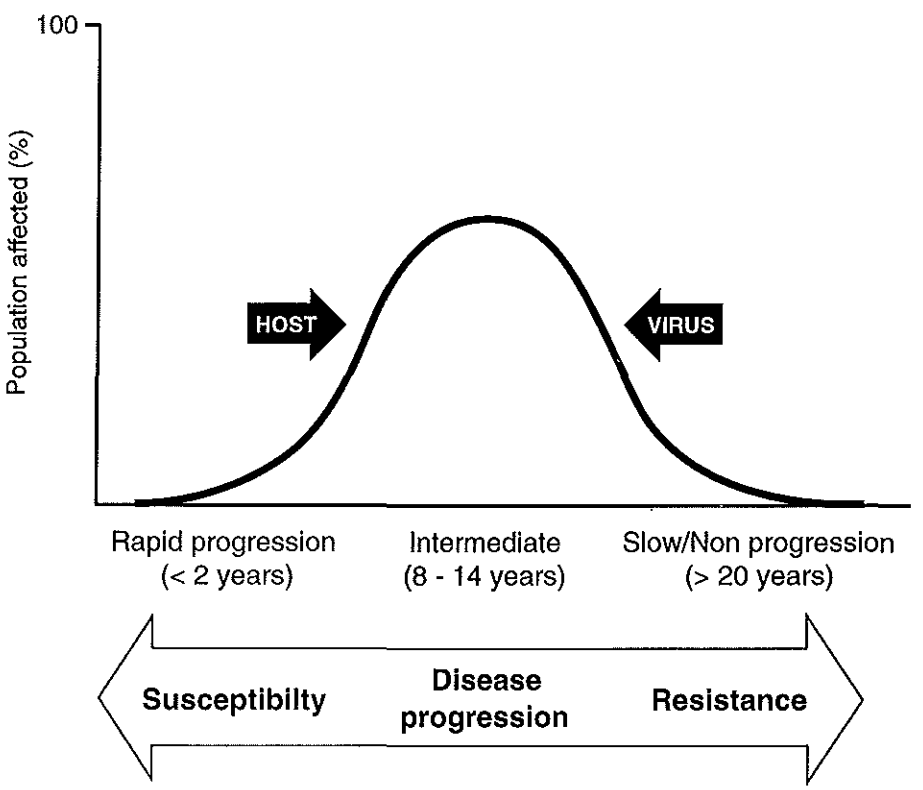

Figure 5. A scheme depicting the different rates of progression to disease in an outbred population with survival being dependent on the interaction of both viral and host factors.

Similar to humans, in an outbred population of macaques there is a host dependent variation in the time to development to AIDS which, together with the virulence of the inoculum virus, determines if an animal will be a rapid, intermediate or slow progressor (Figure 5). In rhesus macaques, a highly virulent strain of $SIV_{sm/mac}$ will likely cause a high and sustained viral load (Figure 6A). This in general is accompanied by a more rapid decline in $CD4^+$ T cell levels than in animals infected with a less virulent strain of SIV (Figure 6B). Viral strains which have an intermediate virulence potential have a lower steady state level of plasma viral RNA than those of higher virulence (Figure 6A).

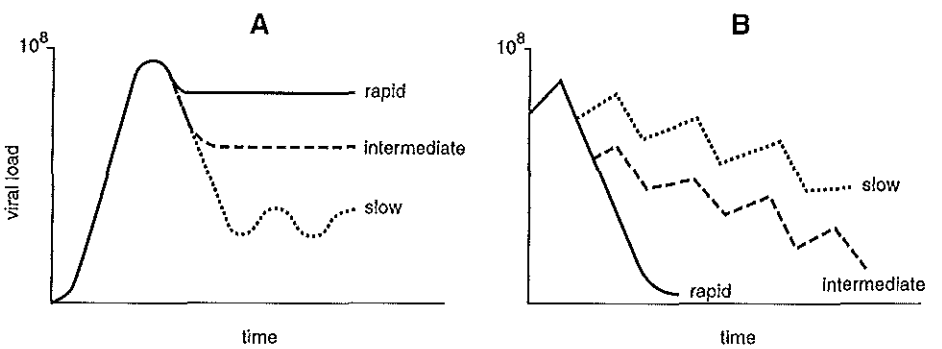

Figure 6. Two schemes depicting A, the virus loads in plasma (concentration of viral RNA per ml plasma) and the influence of virus load on the rate of disease progression, which is inversely correlated with "B", the rate of $CD4^+$ T-cell decline.

Similar to HIV-1, in rhesus monkeys the principal cell targets for $SIV_{sm/mac}$ are the $CD4^+$ T cells and cells of the monocyte/macrophage lineage. Infection of both cell types by HIV-1 is thought to be important for the pathogenesis of AIDS. Cell tropism of HIV-1 has been correlated with the type of biological variants of HIV-1 and the co-receptors these viruses use to enter cells. HIV-1 utilises two major co-receptors and this co-receptor use also correlates with the two different biological variants of HIV-1 observed in patients (12). In the early stages of infection of man viral variants tend to be non-syncytium inducing (NSI). These viruses are macrophage-tropic (mø) and use the CCR5 co-receptor (12). In later stages towards the development of AIDS, syncytium inducing (SI) variants of HIV-1 emerge and become dominant (123). SI variants are primarily T-cell-tropic and use the CXCR4 (3) co-receptor, or they may be dual-tropic for both macrophages and T-cells (R5X4). The conversion from NSI to SI variants in infected patients indicates development of AIDS and thus a poor prognosis (123).

$SIV_{sm/mac}$ also infects both T-cells and macrophages (25). Indeed multinucleated giant cells are a frequent observation in SIV infected monkeys which develop AIDS (5). $SIV_{sm/mac}$ isolates are also capable of infecting $CD4^+$ T cells, even resting T-cells in some cases (28). This seems to be caused by utilising a co-receptor, other than CXCR4. In fact to date no $SIV_{sm/mac}$ group viruses have been shown to be CXCR4 utilising, but all strains are using CCR5 co-receptor (19, 29).

In conclusion, there are a vast number of important similarities between the SIV-macaque model of AIDS and HIV-1 infection of humans, though in some instances, interesting differences exist. The similarities are, however, overwhelming, especially with regard to the correlation of virus load patterns (Figure 6A) with disease progression, and to the individual variation in susceptibility for the development of AIDS in outbred monkey populations (Figures 5 and 6).

1.13 SCOPE OF THIS THESIS

This thesis addresses the issue of increased viral virulence as a possible cause of rapid progression to AIDS. The working hypothesis was that additional virulence factors could be acquired by viral variants which would accelerate the progression to AIDS faster than occurs in the majority of individuals infected with HIV-1.

A study was performed in the rhesus model of AIDS which supported this hypothesis. Efforts were then directed at acquiring proof by developing a new strategy to derive truly representative molecular clones of SIV from an animal which had very rapidly progressed to AIDS (less than one month). One molecular clone was then studied at the molecular level, as well as *in vitro* and *in vivo* to determine its unique biological characteristics.

Studies were initiated to examine possible virulence factors encoded by the *nef* gene by utilising a unique *nef* mutant identified during the molecular cloning of this virus. Finally, a strategy was developed to deliver this molecular clone as DNA directly to cells *in vivo* in lymph nodes.

By using this technique in the accumulation of underived mutations, due to in vitro propagation of the virus, can be circumvented. This will enable us to better correlate genotypic changes within altered phenotype, and may lead to the identification and characterisation of molecular determinants responsible for the accelerated progression to AIDS.

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CHAPTER 2

SPECIFIC PASSAGE OF SIMIAN IMMUNODEFICIENCY VIRUS FROM END-STAGE DISEASE RESULTS IN ACCELERATED PROGRESSION TO AIDS IN RHESUS MACAQUES

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2.1 ABSTRACT

To determine if passage of late stage variants of SIV would lead to a more virulent infection and rapid progression to AIDS, we designed a study in *Macaca mulatta*. In a uniform group of ten age-matched animals from the same genetic breeding stock infected with SIV_{B670}, it took seven months before one of the ten animals developed AIDS. Passage of virus taken immediately prior to death from this animal resulted in death due to AIDS developing within four months in the recipient. Again subsequent passage of virus taken late in disease resulted in an accelerated disease course with AIDS developing in 2.5 and 1.8 months, respectively. The fourth passage of virus taken late in disease from the most rapid progressor (1.8 months) resulted in AIDS developing in this recipient within 1 month of infection. During each consecutive passage *in vivo*, the loss of memory T-cells became more acute. Evidence that the virus became more virulent with selective passage of late stage variants was the markedly increased level of both plasma antigen and viral RNA. The pathology of acute AIDS in these cases closely resembled that seen after a chronic disease course.

2.2 INTRODUCTION

The rate of progression to AIDS is highly variable, influenced by both viral and host factors. The median time to AIDS if left untreated has been estimated to be between 11 and 14 years dependent on the cohort studied (41). At each end of the survival spectrum in a population infected with HIV-1, individuals can be identified which either develop the disease more rapidly (rapid progressors, disease within three years of infection) or become long-term survivors or long-term non-progressors (40). In addition to host factors (i.e. co-receptor mutations) (2, 7, 22, 30, 48, 57), viral factors have been identified (i.e. deletions in *nef*) which contribute to a less pathogenic disease course (25, 45). However, despite the well documented host factors which may contribute to a more rapid disease course, the viral virulence factors responsible for rapid progression to AIDS have not been identified. In contrast to viral virulence factors associated with rapid progression, several viral virulence factors associated with chronic progression to AIDS in the SIV macaque model are being localised to particular regions of the genome (11, 23, 34). To develop a model to evaluate the possible role of viral virulence on rapid progression to AIDS, we turned to the use of non-human primates. Rhesus monkeys (*Macaca mulatta*) when infected with certain strains of SIV, develop a disease which in almost all respects mimics AIDS in humans (17).

The simian immunodeficiency viruses (SIV) are a group of human immunodeficiency virus (HIV) related, but distinct lentiviruses (33) isolated from several different African primates including Chimpanzees (SIV_{cpz}) (43), sooty mangabeys (SIV_{sm}) (12, 42), Mandrills (SIV_{mnd}) (54) and African Green monkeys (SIV_{agm}) (1, 21). The infection of Asian macaques with different SIV strains derived from sooty mangabeys results in the development of an AIDS-like disease remarkably similar to AIDS in humans infected with HIV-1 (47). The SIV macaque model has proven important for investigating the

pathogenesis of lentivirus induced immunodeficiency (4, 8, 20), transmission (37, 53), viral-host interactions (31, 46) as well as the evaluation of antiviral therapy (10, 56) and candidate vaccines (17).

A number of different SIV strains which cause AIDS in Asian macaques have been described (47), which have different disease causing potential (39). Certain commonly studied SIV strains such as SIV_{mac251} cause AIDS over a variable period of time in outbred macaques with 50% mortality ranging from 1-2 years (5, 6, 32, 39). Yet other isolates which have been passaged more extensively in human T-cell lines become highly attenuated (5, 29) and some molecular clones, for instance, are non-pathogenic (38). This attenuation is frequently attributed to passage of SIV on human T-cell lines which may result in truncations in the SIV transmembrane open reading frame (19) and in a growth advantage of certain *in vitro* adapted variants over others (15, 26, 36).

In contrast to the more chronic AIDS causing SIV isolates, a particular isolate designated SIV_{smmPBJ} has been described which causes rapid death of pigtail macaques. $SIV_{smmPBJ14}$ was derived from SIV_{sm} (13). The histopathology of the acute disease syndrome, however, does not resemble AIDS. SIV_{smmPBJ} infection results in an acute hemorrhagic diarrhea culminating in metabolic acidosis and death in experimentally infected pigtailed macaques within 10 to 14 days (9, 14, 58). Since this syndrome is distinct from the AIDS-like illness observed in rhesus macaques and since infection of pigtailed macaques does not result in long-term progressive disease, this $SIV_{smmPBJ14}$ isolate is considered an atypical lentivirus (14). Interestingly, if the acute hemorrhagic diarrhea is treated successfully, animals may recover and then only after a protracted period develop a chronic disease course resembling a more classic AIDS-like disease only after more than a year following infection (44).

The aim of this study was to determine if primate lentiviruses become more virulent with *in vivo* evolution, as was supported by the recent observations of Kimata et al. (18, 24). We proposed that by selective end-stage disease passage of SIV a true AIDS-like disease may develop acutely in rhesus macaques. Using the strategy of serial *in vivo* passage we attempted to increase the virulence of the SIV strain used and to determine how rapidly AIDS could develop in a uniform group of juvenile macaques. The result was a SIV strain designated SIV_{8980} which was capable of inducing a highly accelerated AIDS-like syndrome with extremely high viral loads and rapid loss of CD4 T-cells within weeks of infection. The histopathological lesions observed in all acute cases were indistinguishable from those found following a chronic disease course resulting in AIDS.

2.3 MATERIALS AND METHODS

2.3.1 Animals and viruses

The study population consisted of a uniform group of sixteen captive bred, retrovirus free and male juvenile macaques (*Macaca mulatta*) which were approximately 18 months of age at time of infection. The animals were from a common outbred stock of Indian origin and bred in captivity at the Biomedical Primate Research Centre (BPRC). All were raised maternally until six months of age, then weaned and group housed in preparation for the study. In the first *in vivo* passage (P1) ten age-matched male juvenile *Macaca mulatta* were infected with 5 x 10^2 monkey infectious doses (MID_{50}) of the SIVsm derived strain, SIV_{B670} (3, 42) From the first animal which developed AIDS (seven months post-infection) 2 x 10^6 peripheral blood mononuclear cells (PBMC) were used to passage the infection consecutively to the following group of animals in each subsequent passage. Animals were monitored hematologically to measure T helper/memory cell loss, as well as to determine levels of plasma viraemia for evidence of development of AIDS. Based on clinical and hematological evidence of AIDS animals were euthanised followed by comprehensive autopsy with complete histopathological and bacteriological assessment.

2.3.2 Biochemical- and hematological analysis

Routine clinical biochemistry and hematological analysis were performed at routine intervals to support the clinical diagnosis of AIDS. FACs analysis was performed using Leu 3a (anti-CD4), Leu 2a (anti-CD8) and 4B4 (anti-CD29) to monitor changes in T-helper/memory subsets. Staining of mononuclear cells for lymphocyte subsets was performed by double labelling using FACs analysis Leu 3a and 4B4 as previously described. Plasma antigen levels were determined by SIV p27 antigen capture assay (Coulter Corp. Hialeah, FI.) according to manufacturers recommendations.

2.3.3 Viral RNA levels in plasma

In order to determine SIV RNA levels in plasma of infected macaques a highly sensitive and reproducible quantitative competitive (QC) RT-PCR assay has been developed (49). Briefly, 200 µl of plasma was added to 600 µl of guanidine-isothiocyanate-based lysis solution containing 300 copies of internal standard RNA. The RNA was precipitated by propanol-2 and was reversed transcribed and amplified with rTth DNA polymerase. The amplification products were hybridised in six five-fold dilutions to a capture probe that was streptavidin-horseradish-peroxidase-mediated colorimetric reaction. The amplified internal standard was hybridised to a rearranged 26-bp capture probe in separate microwells. The number of RNA copies in the plasma sample was calculated from the optical density of the sample wells compared to that of the corresponding internal standard. Data was plotted for each individual animal involved in the sequential *in vivo* passage in RNA eq/ml (Figure 3). Furthermore, to compare survival with virus load, the concentration of plasma viral RNA at the threshold achieved at the set point (defined for this model by ten Haaft et al, 1998)) (49), was plotted against the number of weeks each animal survived post-infection (Figure 4).

2.3.4 Preparation of SIV_{8980} virus stocks

PBMC from rhesus monkey 8980 (P4) were isolated from EDTA-treated blood by banding over Lymphocyte Separation Medium and washed twice with RPMI supplemented with 10% FCS. Cells were cultured in an autologous system using only the animal's own PBMC (16) to avoid culture bias or attenuation as has been reported when SIV is propagated on human T-cell lines. After 14 days of culture, cells were transferred to fresh feeder plates and grown for another 14 days. The supernatant from each well was collected and measured for the presence of SIV Gag antigen, using a p27 antigen capture assay (Coulter). Supernatants from wells containing the highest amounts of p27 were pooled, clarified to remove cellular debris, filtrated over a 0.22 um sterile filter (Millipore) and aliquoted. Virus was administered intravenously to 8 rhesus macaques as performed previously. Animals were followed for disease progression for comparison of survival with animals (P1) infected with the pre-passage isolate, SIV_{B670}.

2.3.5 Statistical analysis

The survival of animals infected with the two viral strains (SIV_{B670} and SIV_{8980}) were compared by plotting Kaplan-Meier curves. The difference in the survival times between these two viral isolates in separate groups of monkeys was compared to demonstrate the increase in virulence following serial *in vivo* passage. The significance of this observation was tested using logrank analysis.

2.4 RESULTS

Each consecutive passage in age matched juvenile rhesus monkeys resulted in a reduction of the asymptomatic period and a dramatic acceleration in the progression to AIDS until the most rapid progression time to AIDS of 1 month was reached by the fourth passage. In the first passage (P1) in ten monkeys, the time to death due to AIDS ranged from seven months (P1a and P1b) to more than three years (P1j) (Table 1). The first animals to develop AIDS in P1 were asymptomatic for approximately five and a half months. Disease development in the first animals to succumb to AIDS was characterised by loss of T-helper/memory (CD4/CD29) cells and persistent plasma antigenemia. Table 1 lists the AIDS defining pathological diagnosis of all animals included in the study based on complete histopathological and microbiological workup. Although the length of the asymptomatic period decreased, the clinical and pathological manifestations of the disease did not change. Surprisingly, the same spectrum of pathological lesions that developed after chronic SIV infection was also found after more rapid disease course (Table 1). By the third and fourth passages (P2 and P3 respectively), only a brief asymptomatic period could only be observed for a period of days. In the ten monkeys infected in passage 1, plasma antigen levels were predominantly below the detection level of the antigen capture assay (< 10 ng) (Table 1). T-helper cell numbers, and most prominently T-helper/memory cells declined slowly but persistently after four to five months of infection in animal P1a, the first animal in passage 1 to develop AIDS (not shown).

Table 1. Progression time to death due to AIDS, plasma antigenemia and principal findings at autopsy in sequential passage of SIV in macaques

Monkey	Time to death (months)	Plasma antigenemia SIVp27 (ng/ml)*	Duration	Principle histopathological findings
Passage 1. Infected with SIV_{B670} stock				
P1a	7	<10	Persistent	pneumocystis, candidiasis, CMV, lymphoid atrophy
P1b	7	<10	NP	interstitial pneumonia, CMV, cryptosporidium, candidiasis
P1c	9.5	<10	NP	meningitis, candidiasis, pulmonary abscesses
P1d	13.5	<10	NP	interstitial pneumonia, cryptosporidium, candidiasis
P1e	16	<10	NP	interstitial pneumonia, meningitis, glomerul osclerosis
P1f	20.8	<10	NP	persistent encephalitis, interstitial pneunomia, CMV, candidiasis
P1g	23	<10	NP	peritonitis, cholangitis, interstitial pneumonia
P1h	32.7	<10	NP	disseminated CMV, interstitial pneumonia, gl. sclerosis
P1i	32.8	<10	NP	interstitial pneumonia, candidiasis, giardiasis
P1j	alive	<10	NP	
Passage 2. Infected from P1a				
P2	4.1	15.5	NP	persistent interstitial pneumonia, CMV, glomerulonephritis, cryptosporidium
Passage 3. Infected from P2				
P3a	1.8	35.5	Persistent	encephalitis/meningitis, cryptosporidium, interstitial pneumonia
P3b	2.5	39.9	Persistent	enteritis/wasting, encephalitis/meningitis
Passage 4. Infected from P3a				
P4	1.0	104.8	Persistent	interstitial pneumonia, CMV, diarrhea / wasting, encephalitis / meningitis
Passage 5. Infected from P4				
P5a	2.0	45.3	Persistent	interstitial Pneumonia, CMV, encephalitis / meningitis
P5b	2.0	12.7	Persistent	interstitial Pneumonia, CMV / generalised lymphoid atrophy

*Maximum concentration of plasma antigen measured during the course of the disease.

NP, Not persistent; CMV, cytomegalovirus.

The second passage (P2), consisted of a single animal inoculated with cryopreserved PBMC taken from the first animal to develop AIDS in P1 at six and a half months post infection. This animal, P2, was only asymptomatic for three months during which $CD4^+/CD29^+$ T-cells declined (Figure 1) and plasma antigen levels slowly increased and persisted until AIDS developed at 17 weeks (4.1 months) (Figure 2). PBMC taken from P2 from the last time point before death were administered to two animals, P3a and P3b. These animals developed levels of plasma antigen which were persistent and rose to higher concentrations (Figure 2) than the previous animals P1 or P2 from which they had acquired the virus (35.5 and 39.9 ng p27 for P3a and P3b) respectively (Table 1). During the

asymptomatic period in these animals, which ranged from four to six weeks, T-helper/memory cell numbers declined rapidly. AIDS developed in animals P3a and P3b, by 1.8 and 2.5 months respectively.

In the fourth passage PBMC were administered to animal P4 from animal P3a which had developed AIDS the fastest in passage 3. Within two weeks plasma antigen levels rose to levels greater than 100 ng/ml (Figure 2) with an abrupt decline of $CD4^+/CD29^+$ T-cells (Figure 1). This animal P4 died with AIDS within one month of infection with histopathological lesions characteristic of AIDS (Table 1). PBMC from P4 taken from the last time point before death were administered to animals P5a and P5b. Progression to AIDS more rapid than 1 month post-infection was not observed. It appeared that a maximum virulence had been achieved by the fourth passage since the approximate time to AIDS in the fifth passage was similar to that observed in the third passage. Both animals P5a and P5b progressed to rather uniformly to AIDS within two months with a characteristic rapid loss of T-helper/memory cells (Figure 1) as well as a very high and persistent plasma antigenaemia (Figure 2). To rule out that other pathogens, opportunistic infections or cofactors were not transmitted during the *in vivo* passage all animals were carefully studied for seroconversion of herpesviruses post SIV infection. There was no evidence of transmission of herpesviruses with the SIV inoculum. Furthermore, all animals remained free of STLV and type D retroviral infections during the course of these studies. By all criteria the accelerated disease course was due solely to an increase in virulence of SIV following *in vivo* passage.

One of the most important predictors of the rate of progression to AIDS in humans is level of viral RNA in plasma (35). In the rhesus SIV and SHIV models we have also demonstrated that there is a critical threshold of viral RNA in plasma which is required for a pathogenic disease course. Furthermore a correlation with the magnitude of plasma viral RNA maintained and viral virulence was observed (49). Retrospectively we examined all available plasma samples from this passage study and compared them with the rate of disease progression and the level of passage (Figure 3). A relationship was observed between the maximum or sustainable level of plasma viral RNA and the rate of progression to AIDS (Figure 4) although statistically this would require more animals to strengthen this analysis. The animal which developed AIDS most rapidly had the highest overall level of plasma viral RNA (Figure 3) whereas those which survived longer had lower RNA loads (Figure 4). Not unexpectedly there was a relationship between plasma RNA loads and plasma Ag levels (Table 1, Figure 2 and Figure 3). An inverse relationship was found with the final level of plasma RNA and the $CD4^+CD29^+$ T-cell levels per animal. In general, the higher the sustainable plasma RNA loads obtained, the more rapid T-helper/memory T-cells declined (Figures 1 and 3). Taken together these data suggest that the virulence of the SIV strain increased substantially with each passage. Further proof of the increased virulence acquired by sequential end-stage disease passage was obtained by infecting eight more animals with SIV from the fourth passage animal (P4). Comparison of the survival of macaques infected with the original pre-passage SIV_{B670} strain with animals infected with the post-passage virus strain (SIV_{8980}) isolated from the P4 acute AIDS case (animal 8980) (Figure 5), revealed distinctly different Kaplan-Meier curves with a significantly ($p=0.0036$)

poorer survival in the group of animals infected with the passaged SIV_{8980} strain. Remarkably, the virulence of the passage SIV_{8980} strain was even more pathogenic in mature rhesus monkeys than in the juvenile animals infected with the pre-passage SIV_{B670} stock (Figure 5). If it had been possible to infect more juvenile macaques with the passaged SIV_{8980} stock derived form the P4 animal, then a more acute disease course similar to the P5 animals would have been expected.

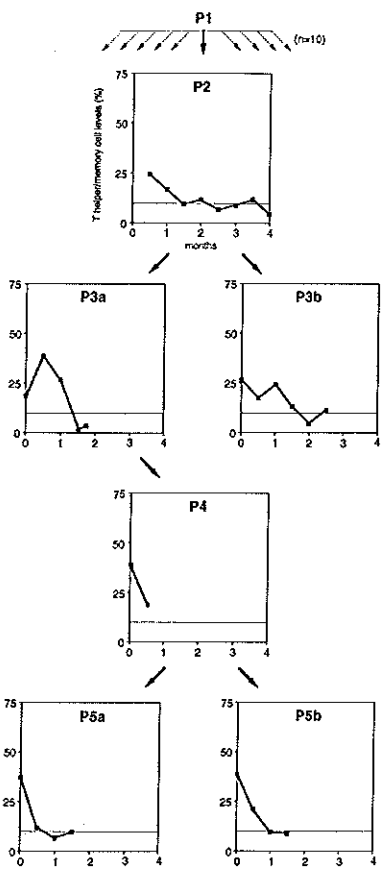

Figure 1
Changes in the CD4+/CD29+ T-cell population during progression to AIDS in animals infected with each subsequent passage of virus.

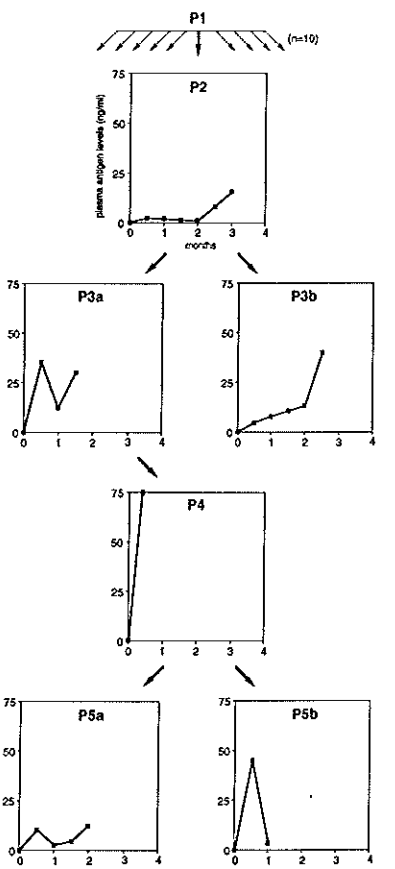

Figure 2
Plasma antigen concentrations (ng/ml) during progression to AIDS in animals infected following subsequent in vivo passage of SIV.

2.5 DISCUSSION

This study demonstrated that serial *in vivo* passage of SIV from end stage disease resulted in an accelerated disease course of AIDS in rhesus monkeys. The ten animals infected with SIV_{B670} in the first passage developed terminal AIDS in a period of time ranging from seven months to more than three years. In contrast, during the passage of this virus a marked reduction in the time to develop AIDS was observed which ranged from 4.1 to 1 month post infection. Our experiments also demonstrated that the accelerated development of AIDS following SIVsm passage in Asian monkeys was associated with the development of very high concentrations of plasma antigen and RNA in the host. These data strongly support the hypothesis that AIDS causing lentiviruses become more virulent upon *in vivo* evolution and acquire the capacity to accelerate the progression to AIDS (18, 24).
SIV infection is a naturally occurring asymptomatic lentivirus infection restricted to several African primate species including sooty mangabeys (SIVsm) (12, 42), African green monkeys (SIVagm) (1, 21) and Chimpanzees (SIVcpz) (43). SIV infection does not occur naturally in Asian primate species such as macaques, yet when experimentally infected with SIV strains derived from sooty mangabeys they develop AIDS with remarkable similarity to the disease in man (19, 47). This model has enabled the study of possible causes of rapid progression to AIDS in a uniformly susceptible host population. Certain host factors such as infection in the neonatal or geriatric period, immunosuppressive treatment for autoimmune disease or transplantation or patients with other concurrent chronic diseases are potential risk factors for rapid progression to AIDS. In an absence of host risk factors some infected individuals still become rapid progressors to AIDS. We propose that such individuals who do not have such host risk factors but become rapid progressors are infected with particularly virulent strains. Several reports suggest that more virulent HIV-1 variants emerge late in the course of the disease as AIDS develops (27, 28). The vast majority of HIV-1 transmissions occur unknowingly from apparently healthy individuals relatively early in infection before symptoms of AIDS develop. Occasionally however, transmission of more virulent variants may occur from individuals with more advanced HIV infection and infection with such variants may give rise to a rapid progression in the absence of host susceptibility factors (50-52). Although it can be expected that transmission from advanced AIDS patients is currently unlikely now that the disease is adequately diagnosed and that treatment and counselling take place in developed countries, it may still occur in some instances. Indeed, certain individuals such as IV drug users and sexworkers may continue to share needles or sell unprotected sex despite having developed AIDS. Infections acquired from individuals with advanced disease may result in a more virulent infection and rapid disease course. To model this scenario we selectively transferred blood animals with a from late stage infection to healthy naive animals of the same age, sex, and breeding stock. Although not inbred they were from the same origin and bred in the same facility under the same conditions. By 1) selecting the first animal in a group to develop AIDS and, 2) selecting late stage samples (2 weeks prior to AIDS) for passage we eventually acquired a virus (SIV_{8980}) which caused a very rapid progression to AIDS.

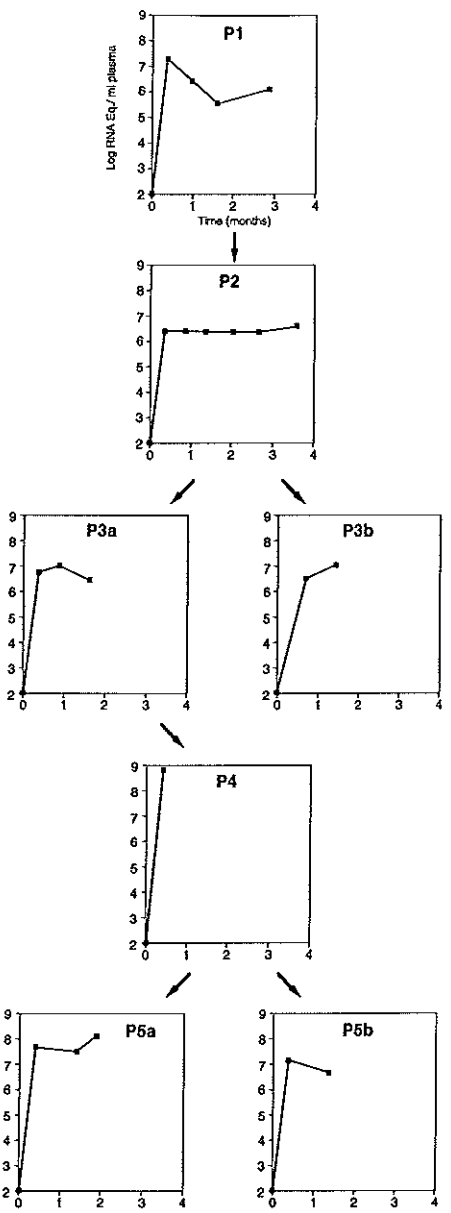

Figure 3.
Plasma viral RNA concentrations (RNA Eq/ml) during progression to AIDS in animals infected following subsequent in vivo passage of with SIV.

Although the number of rhesus monkeys used were relatively limited, after several *in vivo* passages of SIV a remarkably accelerated progression time to AIDS was noted which was not observed in the initial group of ten animals. These results suggested that our experimental late stage transmission from the most rapid progressors selected for particularly virulent viral variants. Finally, the importance of controlling for host variables became apparent when we examined the virulence of the derived SIV strain in older animals (data not shown). When the same virus inoculum was administered to animals of a different age group or geographic origin, the pattern of disease observed was somewhat different from this group, emphasising the importance of controlling for host variables in studies on disease progression. With passage there was a marked increase in maximum plasma antigen levels observed (from <10 ng/ml p24 to >100 ng/ml). These results were similarly reflected when plasma RNA levels were examined. In passage 3 virus loads rapidly reached levels of $1x10^7$ RNA Eq/ml and tended to persist at these relatively high levels until death ensued shortly after. In passage 4 a markedly high peak reaching almost $1x10^9$ RNA Eq/ml was accompanied by death of the animal (Figure 3). A plot of the time to AIDS against the last plasma virus load measured before death was also indicative of the relationship between a very rapid disease course and high virus loads (Figure 4). Further evidence of selection of more virulent variants was obtained at the molecular level. On studying the variation of envelope during each successive passage there was a narrowing of the quasispecies over time with the emergence of a dominant genotype.

In the first two passages there was a decrease in the ratio of synonymous to non-synonymous mutations suggestive that immune responses played a role in selective pressure (55). However, as the virulence of the inoculum increased and the progression time to AIDS decreased to less than two months, then a dominant variant emerged in which only synonymous mutations were observed (55). This is likely due to the marked degree of viral replication as evidenced by the very high levels of viral RNA reached in the most rapid progressors (Figure 4). These animals progressed to AIDS before an effective immune response could be mounted (Table 1).

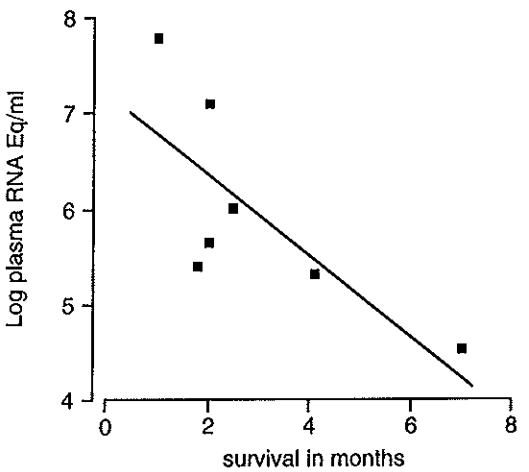

Figure 4. Correlation of survival in months with virus load (RNA Eq/ml) in animals. Virus load was based on the value observed at the "set point" as previously documented in this model as being between 6 and 8 weeks. If animals died before 2 months, then the last plasma load value before death was used.

The pathology observed in these cases was relatively indistinguishable from AIDS observed after a chronic disease course. Importantly, the pathology observed in these cases was distinctly different from the rapid disease syndrome observed with SIV_{pbj} (9) in pigtail macaques (14, 58). The acute disease syndrome observed in SIV_{pbj} infected animals are characterised by hemorrhagic enteritis with death in approximately 10 to 14 days post-infection. If these animals are treated symptomatically they can survive the acute enteritis, to later develop a chronic disease course resulting in AIDS one to two years later (44). That end-stage AIDS can develop as rapidly as one month post-infection after infection with this post-passage strain of SIV, which distinguishes it from SIV_{PBJ}, is in itself of pathologic importance. The study of the pathogenic events that precipitate such an acute disease course may provide more fundamental understanding into the mechanisms of the disease itself. To further understand the pathogenesis of the acute AIDS disease course, a molecular clone has been derived from the most rapid progressing animal. Further studies are in progress with this molecular clone to define the viral virulence factors associated with rapid progression to AIDS.

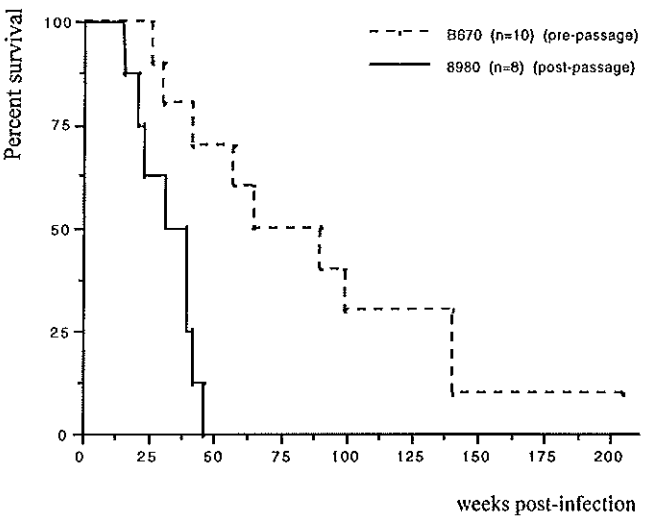

Figure 5. Kaplan-Meyer plot comparing the percent survival of animals infected with the pre-passage isolate (SIV_{B670}) versus the post-passage isolate (SIV_{8950}) derived from the animal P4 over time.

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CHAPTER 3

THE VIRULENCE OF THE PRIMARY INOCULUM DICTATES THE RATE OF PROGRESSION TO AIDS, INDEPENDENT OF DOSE

CHAPTER 3.1

THE RATE OF PROGRESSION TO AIDS IS INDEPENDENT OF VIRAL DOSE IN SIV INFECTED MACAQUES

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3.1.1 ABSTRACT

Of the viral factors that are proposed to influence the rate of progression to AIDS, the role of infectious dose remains unresolved. Intraveneous infection of outbred *Macaca mulatta* with varying doses of SIV_{8980} revealed an endpoint from which an Infectious Dose 50 (ID_{50}) of $5x10^{-6}$ was defined. In the six infected animals, the time to develop AIDS was variable with a spectrum of rapid, intermediate and slow progressors. High and sustained plasma viremia with marked loss of $CD4^+$ T-cells was a distinguishing feature between rapid versus intermediate and slow progressors. Animals that received the highest doses did not develop the highest sustained viral loads, nor did they progress more rapidly to disease. Similarly animals infected with lower doses did not uniformly develop lower viral loads or progress more slowly to AIDS. Furthermore, compiled data from more than 21 animals infected with different doses of the same virus administered by the same route failed to reveal any correlation of infectious dose with survival. Indeed, host factors of these outbred animals, rather than dose of the initial inoculum were likely an important factor influencing on the rate of disease progression in each individual animal. Comparison of animals infected with SIV_{B670}, from which SIV_{8980} was derived, revealed marked differences in disease progression. Clearly, although dose did not influence viral loads nor disease progression, the virulence of the initial inoculum was a major determinant of the rate of progression to AIDS.

3.1.2 INTRODUCTION

The observation that SIV_{sm} infection of Asian macaques resulted in the development of AIDS similar to that seen in HIV-1 infected humans has resulted in the development of an important primate model for the understanding of AIDS pathogenesis (4, 20, 31, 48). The evolution of HIV-1 infection and the development of AIDS varies considerably, with some individuals progressing to AIDS within three years of infection while others remain clinically asymptomatic without evidence of $CD4^+$ T-cell decline for more than 18 years (7, 26, 32, 39, 43). Studies of rapid progressors and long-term non-progressors have revealed that host immunogenetic (8, 24) and immunologic responses (7, 9, 10, 30, 33, 38), as well as certain virological factors (13, 15, 25, 36) (12, 35, 42) may delay the onset of SIV or HIV-induced disease (17, 18, 44, 49).

Viral factors such as infectious dose, route of infection, repeated exposure and viral virulence have been proposed to influence the rate of progression to AIDS (1, 11, 27, 34, 35, 40, 43, 47). To date only the issue of viral virulence has been addressed in depth, but only with regard to attenuation of viral virulence. The Nef protein of SIV and HIV is one of the best defined viral factors and is widely considered to be a critical factor for the pathogenesis of AIDS in both humans (HIV-1/HIV-2) and rhesus macaques ($SIV_{sm/mac}$) (27, 29, 48). The molecular determinants of virulence appear to be encoded by several viral genes in different regions of the genome and in general appear to evolve in an interrelated fashion (14, 34). In a series of SIV studies using single Nef and multiple deletion mutants,

Baba *et al.* showed that AIDS could develop in neonatal animals when given high doses of the virus (2). Subsequently, the viral threshold hypothesis was proposed to explain in part the pathogenic potential of attenuated viruses if high enough levels of viremia were achieved (45). More recently, a pathogenic threshold of plasma viral load has been defined which not only distinguishes between pathogenic and non-pathogenic infections, but also predicts the rate of disease progression (46). Based on these as well as observations from other infectious disease models, we set out to determine the influence of the dose of the inoculum on the initial viral load, the threshold achieved, and thus the influence on disease progression. To address this question, different dilutions of the SIV_{8980} isolate were administered intravenously to ten mature rhesus monkeys. Animals were followed for evidence of infection, plasma viral load, $CD4^+$ T-cell decline and the rate of progression to AIDS. These results were compared to data compiled from other animals infected previously with different doses of the same virus stock.

3.1.3 MATERIALS AND METHODS

3.1.3.1 Animals, clinical and pathological observations

Two separate studies were performed with mature, outbred rhesus monkeys (*Macaca mulatta*) free of SIV, STLV, and type D retroviruses. In the first study 10 animals, two animals per group, received an intravenous administration of serial ten-fold dilutions of 2 ml of SIV_{8980} ranging from $1x10^{-7}$ to $1x10^{-3}$ of the original virus stock (21). Following inoculation, animals were monitored daily for clinical evidence of disease. At two-week intervals for the first two months and at monthly intervals thereafter, animals were lightly anaesthetised for physical examination and blood sampling. Haematological analysis included measurements of $CD4^+$ and $CD4^+/CD29^+$ T-cell subsets, in addition to complete blood cell counts including differential leukocyte analysis. Serum and plasma samples as well as peripheral blood mononuclear cells (PBMC) were stored for retrospective virological analysis. Upon clinical and haematological evidence of AIDS animals were euthanised to avoid unnecessary suffering. A full autopsy was performed at the time of euthanasia. Necropsy and histopathological findings were compiled to confirm the diagnosis of AIDS in all cases.

The second study consisted of retrospective data of rhesus monkeys which had been infected with the same stock virus by the same route of administration but by using different doses. The clinical and pathological follow-up of each animal had been performed using the same standardised criteria.

3.1.3.2 Virus stock and virological analysis

All animals were infected with the same SIV_{8980} stock ($TCID_{50}$ $1x10^{-4.8}$) for comparative purposes. The stock was derived from animal 8980 (P4) following four in vivo passages of SIV_{B670} in juvenile rhesus monkeys born and purpose bred from the BPRC's breeding colony of Indian rhesus monkeys (21). The SIV_{8980} stock was propagated on autologous PBMC. Within 10 days of autologous 8980 PBMC cultivation the supernatant was

harvested, clarified to remove cellular debris, aliquoted and preserved at –135°C.
Virological analysis of inoculated animals included plasma antigen (p27 pg/ml), ELISA for anti-SIV antibodies, virus isolation, DNA-PCR on PBMC and quantitative RT-PCR on plasma. Plasma antigen and anti-SIV ELISA (SIV antigen kindly provided by MRC / programme EVA reagent repository, Potters Bar, UK) were measured as described previously (5). The DNA-PCR assay was performed on genomic DNA isolated from PBMC from inoculated animals. Genomic DNA was isolated from separated and washed PBMC by protein K / Triton X-100-based lysis followed by ethanol precipitation. Nested PCR was performed on each sample using SIV-gag primers as follows: SIV-gag 5' (outer) TTGAAGCATGTAGTATGGGCAGC (1139-1161 nt), SIV-gag 3' (outer) TGCCACCTA-CTTGCTGCACTGGG (1453-1475 nt), SIV-gag 5' (inner) TGGATTAGCAGAAAGCCT-GTTGG (1180-1202 nt) and SIV-gag 3' (inner) CCTCCTCTCGACACTAGGTGGTGC (1424-1446 nt). Outer reaction PCR mixes contained 1µg genomic DNA, 20 mM Tris-HCl (pH 8.3), 50 mM KCl. 0,01 % gelatine, 2.5 mM $MgCl_2$, 200 uM each dNTP, 250 nM each primer and 2U of *Taq* DNA polymerase (AmpliTaq, PE Biosystems, The Netherlands) in a total volume of 50 µl. Cycling conditions for outer primers consisted of an initial denaturation step (95°C, 3 min), followed by five cycles (95°C, 30 sec; 50°C, 30 sec; 70°C, 30 sec), thirty cycles (95°C, 30 sec; 55°C, 30 sec, 72°C, 30 sec), and finally by one cycle (72°C, 7 min.; 4°C, 7 min.; 20°C, 1 sec). From the outer reaction mix, 5µl of product was transferred to an inner reaction mix containing 20 mM Tris-HCl (pH 8.3), 50 mM KCl. 0,01 % gelatine, 2.5 mM $MgCl_2$, 200 µM each dNTP, 250 nM each primer and 2U of *Taq* DNA polymerase in a total volume of 50 µl. Cycling conditions for inner primers were identical to the first PCR except that 20 cycles (95°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec) were carried out. PCR products were analysed by agarose gel electrophoresis.

3.1.3.3 Quantitation of plasma viral load

A quantitative competitive RNA-PCR was used to estimate the virus load in plasma (46). In brief, RNA was extracted from 200 µl of serum or EDTA plasma using guanidine isothiocyanate mediated lysis, followed by propanol-2 precipitation of the RNA. A known amount of synthetic internal standard RNA was added prior to the RNA purification and was co-purified to monitor the efficiency of the purification. The RNA was reverse transcribed and amplified in a single reaction protocol using rTth DNA polymerase (PE Biosystems, the Netherlands) and biotinylated primers. The internal standard RNA was co-amplified to monitor the amplification efficiency. The amplified fragments were detected by a capture probe that was covalently bound to Nucleolink microwells (NUNC A/S, Denmark). The amplification products were detected by a streptavidin-horderadish peroxidase-mediated colorimetric reaction. The amplified internal standard was hybridized to a different capture probe in separate microwells. The number of RNA copies in the sample was calculated from the optical density of the sample wells compared to that of the corresponding internal standard well.

3.1.4 RESULTS

Each pair of animals receiving each virus dilution became infected (table 1) until the doses of $1x10^{-5}$ and $1x10^{-6}$, where only one of each animal became infected. This indicated that the infectious dose 50 (ID_{50}) was between these two dilutions at approximately $3.2x10^{-6}$. Thus, a clear dose-effect and infection was achieved. To determine if animals which received higher doses also developed higher virus loads in plasma, either at the peak or set point of infection, we quantified the plasma virus RNA concentration on sequential samples from each of these infected monkeys. The kinetics of the plasma virus RNA load of each of the infected animals is plotted in figure 1.

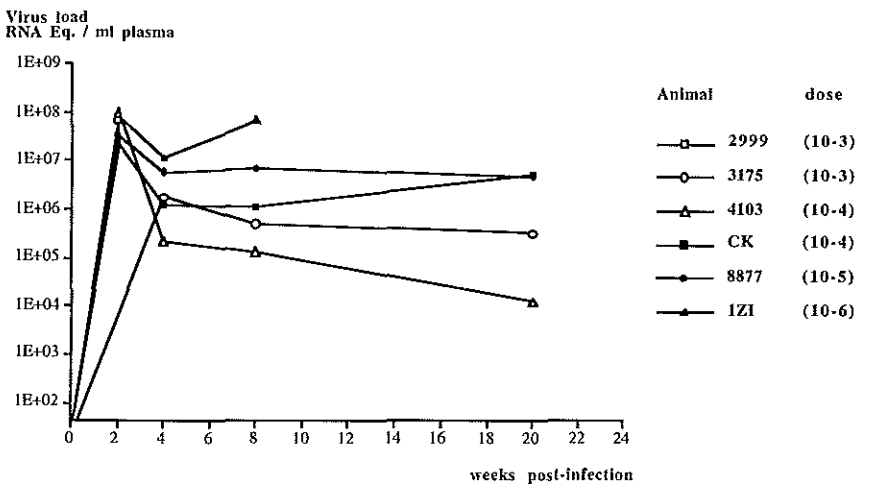

Figure 1. Plasma viral RNA levels in rhesus monkey after intravenous inoculation with different doses of SIV_{8980} (table 1). Plasma virus RNA concentrations are presented as RNA equivalents per ml of plasma (RNA Eq/ml of plasma).

At two weeks post-infection there was no difference in the pre-seroconversion peak of plasma viral load between animals receiving different doses of virus (figure 1). The so-called steady state or set point has been correlated with the rate of progression to disease in HIV-1 infected patients (37) and SIV or SHIV infected rhesus monkeys (46). Comparison of the viral load levels established following the initial peak, when the apparent set points of plasma viral loads were reached also did not reveal any correlation with dose. For instance, animal 1ZI which received the lowest dose of virus had the highest viral load far above the pathogenic threshold as defined by ten Haaft et al. (46). Similarly animal 3175 which received the highest dose of virus had one of the lowest set point values (figure 1). There was also no correlation between dose, the viral peak, or the set point of the plasma viral load. Furthermore, the $CD4^+$ T-cell subsets in the infected animals did not decline inversely with the dose of virus (figure 2).

To ensure that a possible correlation was not overlooked because of the relatively small number of animals studied in this titration study, we retrospectively compiled data from 39 mature rhesus macaques exposed to intravenous doses of SIV_{8980} ranging from $5x10^{-3}$ to 100 ID_{50}. Of these 39 animals 21 became infected and the survival time until onset of AIDS, as was confirmed by necropsy was documented (table 2). The dose and the survival period post-infection clearly did not correlate in rhesus monkeys which became infected with this pathogenic strain of SIV.

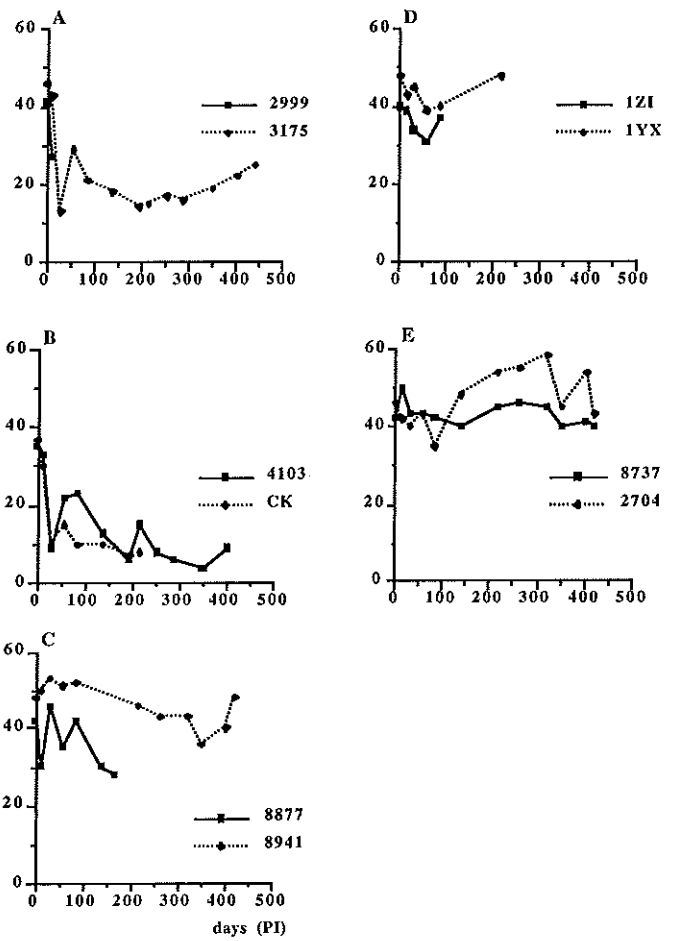

Figure 2. Percentage of $CD4^+$ T-cells in circulation in mature rhesus macaques following intravenous inoculation of SIV_{8980} at different dilutions; A) $1x10^{-3}$, B) $1x10^{-4}$, C) $1x10^{-5}$, D) $1x10^{-6}$ and E) $1x10^{-7}$. Animals 8941 (C), 1YX (D) and 8737 and 2704 (E) remained free of infection and maintained $CD4^+$ T-cell numbers within the normal range.

Table 1 Virological readouts from *Macaca mulatta* inoculated with ten-fold dilutions of SIV_{8980}

Animal	Dilution of	Plasma Ag (pg/ml)#			Virus isolation¶		Anti-SIV Ab§		Proviral‡
		0.0	0.5	1.0	0.5	1.0	0.5	1.0	PCR
2999	10^{-3}	-	+	n.d.	+	n.d.	n.d.	n.d.	+
3175	10^{-3}	-	+	-	+	n.d.	+	+	+
4103	10^{-4}	-	+	-	+	+	+	+	+
CK	10^{-4}	-	+	-	+	+	+	+	+
8877	10^{-5}	-	+	+	+	+	+	+	+
8941	10^{-5}	-	-	-	-	-	-	-	-
IZI	10^{-6}	-	+	+	+	+	-	-	+
IYX	10^{-6}	-	-	-	-	-	-	-	-
8737	10^{-7}	-	-	-	-	-	-	-	-
2704	10^{-7}	-	-	-	-	-	-	-	-

*: diluted virus stock, #: plasma antigen at 0.0/0.5/1.0 months, ¶: virus isolation from PBMC at 0.5/1.0 months, §: anti-SIV env at 0.5/1.0 months, ‡: *pol* PCR from PBMC

3.1.5 DISCUSSION

A number of host, as well as virus factors, interact to influence the rate of progression to AIDS. The effect of the virus dose on the rate of progression to AIDS has been suggested but not proven. The effect of viral dose on disease development in animal models has been reported for cytomegalovirus (23), adenovirus (19) rabies virus (41), hepatitis B virus (22) and herpes simplex virus type 2 (16). To study the effect of HIV-1 dose on disease in humans has proven difficult, even in documented cases of contaminated blood transfusions in cohorts of haemophiliacs. To address the hypothesis that higher doses of HIV-1 may accelerate progression to AIDS we turned to the SIV_{sm} model of AIDS in rhesus macaques. This model mimics the pathogenesis of HIV-1-induced AIDS in almost all respects (48).

Two studies were undertaken using this model. Firstly in a prospective study ten mature rhesus monkeys were inoculated with serial ten-fold dilutions of the pathogenic SIV_{8980} isolate. There was a clear correlation with the dose that the animal received and infection (table 1). However, there was no correlation between the infectious dose and peak virus loads or the set point (steady state) virus loads in plasma (figure 1). Furthermore, there was no inverse correlation between the decline of $CD4^+$ T-cells (figure 2) and the SIV dose the animal received. Lastly, a retrospective analysis of survival data (table 2) of 39 animals exposed to different doses of SIV_{8980} administered intravenously, failed to reveal any correlation between the infectious dose administered and the time to development of AIDS.
A number of host factors which may influence the rate of progression to AIDS have been documented, including age, concurrent disease, and host immunogenetics. We have previously found a correlation between survival and the MHC type of the SIV-infected animals (3, 6). Similarly, several viral virulence factors have been reported to influence the rate of disease progression in man as well as in monkeys.

Table 2 Compiled results of survival of rhesus macaques inoculated with different doses of SIV_{8980}

SIV DOSE MID_{50}	Survival Months	DNA PCR	Results Ab	Ag	VI	Clinical outcome	Macaque
100	18.10	+	-	+	+	AIDS	9001b
100	16.30	+	-	+	+	AIDS	8984b
100	15.00	+	-	+	+	AIDS	8928b
100	10.50	+	+	+	+	AIDS	8771
100	7.00	+	+	+	+	AIDS	8606
100	5.30	+	+	+	+	AIDS	1XI
100	9.00	+	+	+	+	AIDS	1VS
50	0.50	+	-	+	-	AIDS	2999
50		+	+	+	+	Asymptomatic	3175
5	14.50	+	+	+	+	AIDS	4103
5		-	-	-	-	not infected	HQ
5	7.20	+	+	+	+	AIDS	CK
5	4.80	+	+	+	+	AIDS	3991
1.6		-	-	-	-	not infected	4091
1.6		-	-	-	-	not infected	9001a
1.6		-	-	-	-	not infected	8986a
1.6		-	-	-	-	not infected	8984a
1.6		-	-	-	-	not infected	8977a
1.6	7.40	+	+	+	+	AIDS	8967
1.6		-	-	-	-	not infected	8928a
1.6		+	+	+	+	not infected	8754
1.6	30.75	+	+	+	+	AIDS	8604
1.6	3.50	+	-	+	+	AIDS	1RO
1.6	9.00	+	+	+	+	AIDS	L65
1.6	6.90	+	-	+	+	AIDS	CS
0.5		-	-	-	-	not infected	8941
0.5	5.50	+	+	+	+	AIDS	8877
0.5		-	-	-	-	not infected	3215
0.5	9.60	+	+	+	+	AIDS	3049
0.5		-	-	-	-	not infected	CY
0.5		-	-	-	-	not infected	CF
0.16		-	-	-	-	not infected	3532
0.16		-	-	-	-	not infected	3598
0.05	3.50	+	-	+	+	AIDS	1ZI
0.05		-	-	-	-	not infected	1YX
0.05		-	-	-	-	not infected	HT18
0.05		-	-	-	-	not infected	3974
0.005		-	-	-	-	not infected	8737
0.005		-	-	-	-	not infected	2704

Certain SIV_{mac} *nef* mutations may either cause an attenuated disease course and prolonged survival or cause acute haemorrhagic enteritis. But a viral virulence factor that is capable of actually accelerating the disease progression to AIDS has not yet been identified. In a previous study (21) the *in vivo* passage of SIV_{B670} led to an acute progression to AIDS following four *in vivo* passages in monkeys. Comparison of the Kaplan-Meier plots of the survival of animals infected with pre-passage stock versus the post-passage stock clearly revealed that the virus had acquired a significant increase in virulence capable of causing an accelerated disease course (21). Our data indicate that infectious dose is important for establishing SIV infection but does not influence the rate of progression to disease. Once infection has occurred, it is most likely that the replication rate and the virulence properties of the dominant viral variant in the viral inoculum determinant the post-seroconversion level of viral load established (28, 46). Together, viral virulence factors and host responses appear to dictate the rate of progression to AIDS once infection has been established.

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CHAPTER 3

THE VIRULENCE OF THE PRIMARY INOCULUM DICTATES THE RATE OF PROGRESSION TO AIDS, INDEPENDENT OF DOSE

CHAPTER 3.2

VACCINE PROTECTION AND REDUCED VIRUS LOAD FROM HETEROLOGOUS MACAQUE-PROPAGATED SIV CHALLENGE

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3.2.1 ABSTRACT

The efficacy of vaccine protection afforded by live attenuated vaccines was tested by heterologous SIV_{8980} challenge following successful protection against homologous SIV_{mac32H} challenge. Animals immunized with the attenuated $SIV_{macBK28}$ molecular clone were asymptomatic and virus isolation negative by quantitative virus isolation prior to challenge. Two groups of four animals previously immunised five years and four months (respectively) were challenged with 100 MID_{50} of SIV_{8980} as was a third group of four naive controls. All control animals that were challenged developed high levels of plasma antigenemia within two weeks of challenge and developed rapid $T_{h/m}$ cell loss whereas vaccinated animals did not. Quantitative virus isolation from peripheral blood mononuclear cells revealed that one out of four animals in each group became virus isolation positive but that the virus load in the two vaccinated animals was markedly lower than in non-vaccinated controls. Studies are underway to determine the duration and immunological correlates of protection from AIDS.

3.2.2 INTRODUCTION

The SIV-macaque model is currently the only suitable primate lentivirus model in which vaccine protection from infection as well as disease (AIDS) can be studied. The significance of the early successes achieved using whole inactivated virus vaccines was diminished by the demonstration that human cell components incorporated into both the immunogen and challenge virus preparations (1, 5, 8, 9) appeared to be responsible for the protective immunity (2, 11). In fact, no animals have been protected from infection with cell-free, rhesus-grown challenge virus by immunization with whole inactivated virus. Protection against challenge with infected monkey peripheral blood mononuclear cells (PBMC) has been achieved using such immunogens (7), whereas atttenuated live virus vaccines appear to protect against cell-free virus grown in monkey cells (4). In both instances vaccine protection was achieved against an intravenous challenge with virus derived from homologous SIV_{mac251} strains.

This study was performed to determine whether vaccine protection from heterologous rhesus macaque-propagated SIV challenge could be achieved with an attenuated live vaccine, and if so, to determine if the duration of the immunization period was a factor in establishing broader protection against heterologous challenge.

3.2.3 MATERIALS AND METHODS

3.2.3.1 Immunisation

Mature outbred rhesus monkeys bred in captivity at the TNO Primate Center (Rijswijk, The Netherlands) were infected with 2.5 ml of tissue culture supernatant of Hut-78 cells transfected with the BK28 molecular clone of SIV_{mac251} (10). The $TCID_{50}$ (50% tissue culture infectious dose) of the attenuated BK28 stock was approximately 6.3×10^3 per ml

when titrated on C8166 cells. Seven animals that had remained asymptomatic since May 1988 were divided into two groups for the first (n = 3) homologous and second (n = 4) heterologous challenge experiments, respectively. Animals were monitored routinely for T-cell subset analysis, Western blot, and plasma antigen levels as previously described (7). In March 1993 a second group of four animals was infected with the same dose of the attenuated BK28 stock to provide additional immunised animals for the second experiment.

Almost two years post-immunization (February 1990) a homologous SIV_{mac251} challenge was performed on three of the 1988 immunized animals and two naive controls, hereafter designated as experiment 1. The challenge was performed with 50 MID_{50} (50% monkey infectious dose) of the November 1988 pool of the 32H reisolate of SIV_{mac251} grown on C8166 cells as previously described (3). Post-challenge follow-up included bleeding every two weeks for the first two months, with bleeding at two to four month intervals thereafter.

3.2.3.2 Heterologous challenge

Four animals immunized in 1988 and four animals immunized in March 1993 were challenged along with four naive controls with a highly virulent serial *in vivo*-passaged SIV_{8980} strain derived in our laboratory and preserved as a virus stock. An additional four naive unchallenged control animals were added to complete this group of 16 animals, hereafter known as experiment 2. The SIV_{8980} stock was prepared from autologous PBMC from macaque 8980, which died due to AIDS 28 days after infection with SIV_{smB670} passaged four times *in vivo* in juvenile macaques. This stock chacateristically causes a high level plasma viremia and rapid loss of T helper/memory ($T_{h/m}$) cells in juvenile rhesus monkeys. The 8980 stock was prepared using PBMC taken from macaque 8980 two weeks prior to death and cultured without cocultivation in a T-cell culture system (see Quantitative Virus Isolation, below). Within ten days culture supernatant was harvested, clarified to remove cellular debris, aloquoted and preserved at –135°C. *In vivo* titration was performed to establish the MID_{50}. The three groups of animals in experiment 2 were challenged with 100 MID_{50} of this stock by intravenous administration of 2 ml of 8980. Post-challenge follow-up was performed once every two weeks for the first two months, and once every two to four months thereafter. At each time point differential blood cell counts and subset analysis was performed for absolute CD8, CD4 T-cells and $T_{h/m}$ subset analysis.

3.2.3.3 Serological assays

Virus neutralization (VN) titers were determined in U-shaped microtiter plates by incubating in quadruplicate 10 µl of heat-inactivated plasma at different dilutions with 20 infectious particles of $SIV_{mac251\text{-}32H}$ in 10 µl. After shaking and incubating at 37°C 2,000 C8166 cells in 200 µl were added to each well and the plates incubated for 7 days at 37°C, 4% CO_2, 95% humidity. Subsequently, cells from each well were transferred to poly-L-lysine-coated flat-bottomed microtiter plates, fixed in methanol, and immunohistochemically stained with SIV_{mac} specific antiserum and goat anti-human IgG-peroxidase conjugate. Wells containing no stained cells were scored as negative and the number of negative wells used to calculate the neutralisation dose 50% end point (ND_{50}) by the method of Spearmann-Karber.

Antigen capture assay for SIV p27 was performed on serum, and/or plasma using a commercial antigen capture kit (Coulter, Hialeah, FL). Samples were serially diluted to determine levels (pg/ml) of SIVp27 in plasma as previously described (7).

3.2.3.4 Quantitative virus isolation

Prior to heterologous challenge the two groups of animals vaccinated with attenuated BK28 in 1988 and 1993 were virus isolation negative, using a novel autologous PBMC virus isolation assay. This assay is performed using a T-cell culture system in which 1.0 x 10^4 autologous rhesus PBMC are plated per well in sterile 96-well U-shaped tissue culture plates over irradiated feeder cells. This number of PBmCs per well was previously shown to provide suitable sensitivity, as well as a good working range for quantitation. Cells were cultured in an autologous system using only the animal's own PBMC to avoid culture bias resulting from the fact hat many human cell lines are not permissive for all SIV isolates. A feeder system consisting of irradiated human PBMC, two human B cell lines, leukoagglutinin, and indomethacin was used to optimize the growth of a mature T-cell population for virus propagation. After 15 days of culture cells were transferred to fresh feeder plates and grown for another 15 days. The supernatant from each individual well was collected and measured for the presence of SIV gag antigen, using a p27 antigen capture assay (Coulter). The number of infected cells per 106 PBMC was measured by enumerating the number of positive wells per plate after a minimum of 15 days of culture and calculated based on Poisson distribution. We have evaluated and validated this quantitative virus isolation method in the chimpanzee model with quantitative virus RNA and DNA PCR techniques (6).

3.2.4 RESULTS

3.2.4.1 Homologous challenge

Three immunized and two naive animals challenged with 50 MID_{50} of the 32H reisolate of SIV_{mac251} were followed post-challenge for evidence of infection and disease progression. Both naive controls became infected, as evidenced by plasma antigen levels, which peaked at weeks two and three in each of the controls, respectively. Neutralization titers in naive animals were below background on the day of challenge and rose in one of the animals (1XC), which survived the longest after challenge. In both naive controls circulating T_h cell levels droped sharply within the first ten weeks after challenge. The animal (8683) that developed the highest concentration of SIV_{p27} in plasma post-challenge and that failed to mount a neutralizing antibody response within three months after challenge (Table 1, experiment 1) died within five months due to lymphoma attributable to SIV infection. The other control animal developed a slow progressive decline of T_h cell numbers and was humanely euthanised at 11 months post-challenge, showing low-level plasma antigenemia and wasting at this time. Histopathology revealed multiple opportunistic infections consistent with AIDS.

In contrast immunized animals in experiment 1 (see Table 1) had neutralizing titers between 900 to greater than 3,000 on the day of challenge that persisted at the same levels until three months post-challenge. They failed to develop plasma antigenemia, maintained T_h cell numbers greater than 500 x 10^3/ml and have remained assymptomatic for more than three years post-challenge.

Table 1. Neutralisation titers of challenged rhesus monkeys

Immunized 1988			Naïve challenged		
Experiment 1: Challenged with homologous SIV_{mac32H}					
Animal	DC	PC	Animal	DC	PC
K98	905	4,305	1XC	20	2,560
A30	1,290	4,305	8683	20	14
IVM	3,620	6,089			
Experiment 2: Challenged with heterologous SIV_{T8980}					
1YO	6,084	14,482	1VS	14	34
1YR	2,153	4,305	1XI	14	17
2BR	1,522	1,076	8606	14	17
1VY	1,810	453	8771	14	95
Immunized 1993					
1RZ	1,280	2,153	8637	14	17
1SV	3,044	6,089	8680	14	14
1XY	80	3,044	8710	20	14
1YC	640	269	8711	17	17

a Virus neutralising titers measured on the day of challenge
b Virus neutralising titers measured 12 weeks postchallenge

3.2.4.2 Heterologous challenge

The second experimental group, which received the heterologous SIV_{smB676} macaque derived, PBMC grown, cell free challenge, consisted of four macaques immunized with BK28 in May 1988 and four immunized only four months previously. In addition, four animals served as naive controls for challenge and an additional four naive animals were unchallenged controls. On the day of challenge all immunized had relatively high neutralizing antibody titers as shown in Table 1 (experiment 2), ranging from 1522 to 6084 in the 1988 immunized animals, and from as low as 80 to 3044 in the animals immunized four months prior to challenge. Naive animals had background levels on the day of challenge, but these increased to 34 and 95 in two of the four animals. In the animals immunized 5 years earlier neutralizing titers doubled in two of four animals, stayed

approximately the same in one, and dropped the fourth animal (Table 1, experiment 2). In contrast, the neutralizing titers in animals immunized four months earlier increased in three of four animals and persisted at the same high levels as at the day of challenge.

Differences were also seen in the SIV envelope antibody titers between the two immunized groups prior to challenge. The animals immunized five years earlier had the highest SIV envelope antibody titers prechallenge, whereas the animals immunized four months earlier still had rising titers and had not reached maximum levels. Twelve weeks postchallenge three of four animals in each immunised group developed an increase in VN titer. All naïve controls challenged rapidly developed anti-SIV envelope titers post-challenge.

As in the earlier homologous challenge experiment, immunized animals failed to develop evidence of plasma antigenemia, in contrast to naive, challenged controls. Similarly, challenges controls developed rapid $T_{h/m}$ cell losses immediately post-challenge, whereas immunized animals retained relatively normal levels of $T_{h/m}$ cells post-challenge, suggesting that these immunized animals may be protected from disease progression even if sterilizing immunity was not achieved.

3.2.4.3 Quantitative virus isolation

The results of the PBMC virus isolation assay revealed significant differences in virus load in those animals receiving the heterologous SIV challenge. Naive control animals (SIV negative by all assays prior to challenge) developed high numbers of SIV-producing PBMC at levels greater than 80 x 10^6/ml measured at one and three months post-challenge. Virus-infected PBMC were found in only one of four animals in each of the two vaccine groups (Figure 1). At four weeks post-challenge none of the animals immunized in 1988 were virus isolation positive, wheras at twelve weeks post-challenge one animal became virus isolation positive, but was estimated to have only one infected cell in 10^6 PBMC at this time point. Similarly, only one of the animals immunized four months prior to challenge became infected. This animal was first found to be virus isolation positive with 1 infected cell per 10^6 PBMC at one month post-challenge; this increased to 22 infected cells per 10^6 PBMC by three months post-challenge. These results suggest a reduced virus load in cases where sterilizing immunity was not achieved.

3.2.5 DISCUSSION

Previous studies have reported protection from homologous cell-free and cell-associated macaque cell-derived SIV challenge (4, 7). Heterologous protection from rhesus macaque-derived challenge has not been previously reported until this study. Because data from an earlier study (4) using attenuated live virus vaccines suggested that the duration of the immunization in the period prior to challenge was important for protection, we designed an experiment to determine if a longer immunization period (five years) would provide broader protection against a heterologous macaque-propagated cell-free challenge than a shorter immunization period (four months).

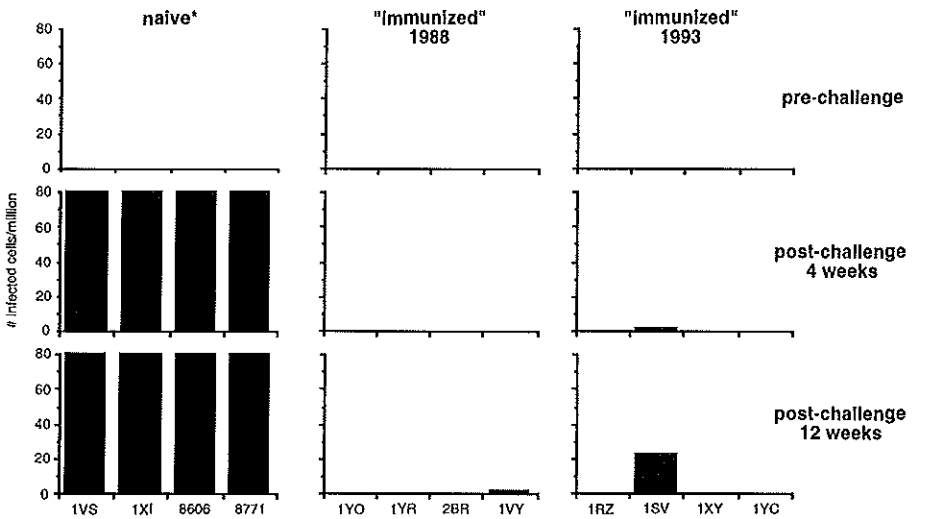

Figure 1. Quantitative virus isolation of animals challenged with SIV8980. Data are shown as the number of infected cells/106 PBMC. *, Naïve controls were negative by all criteria prechallenge.

Our results suggest that four months of immunization was as effective as a 5-year immunization period in which three of four animals from both groups were virus isolation negative. Animals immunized for only 4 months had, despite lower anti-SIV envelope antibody titers in general, homologous neutralizing titers similar to those of animals immunized 5 years prior to challenge. Importantly, the virus load in both groups of immunized animals challenged with heterologous virus was dramatically lower than in naive controls. Furthermore, the virus load in the infected animal immunized for five years was delayed and lower than in the infected animal immunized for 4 months prior to heterologous challenge. Hence, these results would suggest that with this system the duration of the immunization period did not appear to broaden immunity, or to increase the number of animals that were protected from infection with this heterologous challenge. However, a slight benefit in reduced virus load was observed.

We have previously reported that vaccination reduced virus load in homologous cell-free challenged chimpanzees that became infected, and that animals that had higher neutralization titers at the time of challenge had lower virus loads (6). Interestingly, in this SIV study there was no apparent correlation between neutralizing antibody titers and virus load, suggesting that other mechanisms may be mediating protection in animals receiving attenuated vaccines.

The mechanisms involved in this type of vaccine protection are not yet clear and require further study to determine if they are clearly immunological or related to the phenomenon of virus interference. They must also be considered in the light of other so-called "super-infection" experiments. Vaccinated animals that became infected had much lower virus loads than naïve controls, suggesting clearance of virus infected cells postinfection. Clearly, the attenuated live vaccines appear to be the most efficacious, giving the most consistent and

broadest protection based on results from the SIV model. However, important safety issues such as recombination of the challenge strain with the attenuated vaccine strain *in vivo* remain to be addressed.

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CHAPTER 4

DEVELOPMENT OF A MOLECULAR CLONE OF SIV DIRECTLY FROM SERUM

CHAPTER 4.1

DIRECT AMPLIFICATION AND CLONING OF UP TO 5-KB LENTIVIRUS GENOMES FROM SERUM

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4.1.1 ABSTRACT

To produce large cDNA strands from biological samples containing limited numbers of template molecules, it may be essential to minimise both non-specific primer attachments in first strand synthesis and secondary structure in RNA molecules. Failure to do so could result in the accumulation of shortened cDNA strands and therefore may reduce the yield of large cDNA molecules, sometimes below detection level. We show that 5.0-kb cDNA fragments can be generated from simian immunodeficiency virus RNA in a specific reverse transcription (RT)-PCR reaction by increasing the stringency of the primer-annealing conditions followed by the elimination of excess free primer. Since this method utilises a relatively long primer in the first strand cDNA synthesis, it is possible to heat-denature the non-specific RNA/primer complexes and RNA secondary structure without dissociating the primer from the specific template. In contrast to classic RT assays, in which an excess of primer is annealed to denatured RNA just prior to and during reverse transcription at relative low temperatures (37-42°C), this method eliminates false priming. To optimise the yield and fidelity of full-length cDNA molecules, two PCR amplifications are first performed using both *Taq* and *Pfu* polymerase, followed by *Pfu* alone in the second amplification.

4.1.2 INTRODUCTION

Biologically relevant molecular clones of primate lentiviruses are important for the study, development and evaluation of therapeutic and vaccine strategies for the prevention of AIDS. Experimental infection of various species of macaques with Simian immunodeficiency virus (SIV) induces AIDS closely resembling human immunodeficiency virus (HIV) infection in man (13, 21, 28) and is used extensively to evaluate vaccine strategies for HIV (5, 8, 14, 19, 33). To date, only molecular clones derived from cultured virus have been available. In an effort to fully utilise this animal model, it is important to have a technique for acquiring relevant, representative molecularly cloned viruses directly from biological materials.

The use of conventional strategies, such as cloning from lambda phage libraries, has proven to be very difficult for the molecular cloning of SIV and other lentiviruses directly from in vivo samples, because of relatively low frequencies of infected cells (10, 22). Attempts to increase the number of infected cells by in vitro propagation caused selection for a limited subset of viral variants during growth in culture (18, 40). More recently, polymerase chain reaction (PCR) amplification has been used to generate molecular clones of SIV (14) and HIV (27).

The reasons for deriving molecular clones directly from virus present in biological fluids such as serum are twofold. First, the predominant virus population in serum or plasma at any one point in time is most likely to represent an abundantly replicating viral population. Since their predominance is likely the result of escape from immune surveillance, they are of particular biological importance. Second, proviral DNA, is expected to be enriched in defective proviruses (13, 25, 41).

Although reverse transcription (RT)-PCR allows us, in principle, to clone RNA viruses directly from biological fluids, it is difficult to produce large (first and second strand) cDNA molecules by reverse transcription and by PCR, respectively. RT-PCR cloning can be optimised by the isolation of high-quality (polyadenylated) RNA, efficient reverse transcription to generate a cDNA template for the PCR step and an efficient method to clone the PCR product into a suitable vector. Various methods have been described for the isolation of high-quality RNA for use as PCR template (4, 6, 32), as well as for the improvement in the synthesis of first- and second-strand cDNA. Ligation of blunt-ended PCR products in pre-digested plasmids in the presence of a suitable endonuclease can increase the cloning efficiency considerably. This cloning procedure seems preferable to the ones that use restriction recognition sites in the primers, since the latter techniques have proven to be inefficient (16) and/or to generate complicated cloning artefacts (20). Unfortunately, short products tend to accumulate during the RT reaction as a result of non-specific priming. Here we report on a method to minimise these undesired products by using a modified primer extension and a long RT primer in combination with stringent annealing conditions. Moreover, a supplement of spermidine to the first-strand reaction (26) and the use of a *Taq* and *Pfu* polymerase mixture in the second-strand synthesis assist in elongation and efficiency of PCR (1, 7).

By applying this modified assay to monkey serum, we were able to amplify and clone the 5-kb 3' and 5' halves of the SIV_{8980} genome. We anticipate that this method will be of interest for the cloning of large cDNAs from low-abundance RNA molecules and particularly for the generation of molecular clones of RNA viruses directly from clinical samples.

4.1.3 MATERIALS AND METHODS

4.1.3.1 RNA isolation and first-strand cDNA synthesis

The SIV_{8980} isolate was derived from $SIV_{deltaB670}$ (2) after a series of subsequent in vivo passages in rhesus macaques. Serum was taken from an SIV_{8980} infected monkey, that had P27 (nucleocapsid protein) antigenemia (24.5 ng/mg), as measured by a commercially available SIV antigen capture assay (Coulter, Hialeah, FL, USA). For a variety of diverse biological assays, viral RNA from serum is frequently more suitable than that obtained from plasma, because the anticoagulant disturbs the particular assay or because after thawing a fibrin-clot is formed. Hence, for retrospective analysis from frozen samples, serum may be more readily available.

RNA from 100 µl serum was isolated as previously described (4). RNA pellets were dissolved in 20 µl of 40mM piperazine-N-N'-bis (2-ethane-sulfonic acid) (PIPES) (pH6.5), 1mM EDTA, 0.4 M NaCl and 2.5pM (synthesis) primer No. 10120: GAGTGGCTCCACGCTTGCTTGCTTAAAGACCTCTTCAATAAAGCTGCCAATTAGA AGTAAGC (nucleotide [nt] 10058-10120 of the SIV_{smmh4} genome) (17) and incubated for 10 min at 85°C in the presence of 60% (wt/vol) formamide. Specific annealing of the primer to the template was carried out by slowly cooling the incubation mixture to 42°C and

keeping it at this temperature for 16 h. To eliminate the formamide and to change from annealing buffer to synthesis buffer, the RNA/primer complexes were precipitated with 0.1 volume (3M) of sodium acetate and 2.5 volumes of ethanol, followed by a single 70% ethanol wash.

The complexes were dissolved in 13 µl of KCl (90mM) and subsequently heated to 70°C for 10 min and snap-cooled on ice, immediately followed by the addition of 7µl of the synthesis mixture: 10mM $MgCl_2$, 1mM dNTP (each), 1mM dithiothreitol (DTT), 10 U Rnasin (Promega, Madison, WI, USA), 0.5mM spermidine (Sigma, Chemical, St. Louis, MO, USA) and 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (SuperScript II RT; Life Technologies, Breda, The Netherlands). First-strand synthesis was performed during a 60-min incubation at 45°C. Finally, the enzyme was heat-inactivated at 90°C for 10 minutes. Control experiments were performed without reverse transcriptase.

4.1.3.2 PCR amplification of cDNA templates

To evaluate the effect of modified reaction conditions on the length of the synthesised cDNA, test RT-PCR amplifications were performed on RNA aliquots obtained from infected monkey serum. In all RT-PCRs, the cDNA synthesis started at the 3' end of primer 10120 (located in the R region of the 3' long terminal repeat [LTR]). The length of the cDNA was determined by a subsequent PCR amplification. PCR primer pairs consisted of the same 3' primer (No. 10077) combined with different 5' primers (9026; 8023; 7254; 6551; 6059; or 5081) that were located more upstream each time than the previous one, producing fragments of increasing lengths with increased cDNA synthesis. Primers were designed from conserved regions between different SIV strains (Los Alamos database). The names of the primers correspond with the nucleotide positions of the SIV_{smmh4} genome (17) (Figure 1). All primers were obtained from Pharmacia Biotech, Roosendaal, The Netherlands, and all chemicals were obtained from Sigma Chemical. PCR fragments were generated by using a *Taq/Pfu* DNA polymerase mixture on cDNA templates, followed by a second PCR with *Pfu* polymerase (Stratagene, La Jolla, USA) on a small fraction (5-10 µl) of the first amplification. All PCR amplification reactions (100 µl) were performed in a Perkin-Elmer DNA Thermal Cycler 480 (Norwalk, CT, USA). A mixture of AmpliTaq (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and *Pfu* DNA polymerase was used as recommended by Perkin-Elmer with two exceptions. The primer and $MgCl_2$ concentrations were adjusted to 15 pM and 2 mM, respectively, and one unit of both polymerases was used. The program used in these amplifications consisted of 10 cycles of denaturation at 94°C for 1 min; annealing at 59°C for 50 s; extension at 72°C for various times depending on the desired fragment length (usually 1-kb/minute elongation).

Final extension at the end of the program for 10 min. *Pfu* DNA polymerase amplification was as recommended by Stratagene Europe, The Netherlands, except that 15 pM of phosphorothioated (11) primers and 3 units of *Pfu* DNA polymerase were used. In this case, the primers were extended with the following program: 5 min. at 94°C, then 35 cycles of denaturation at 94°C for 1 min; annealing at 58°C for 1 min, followed by extensions as described earlier. A hot start protocol (17) using Ampli-Wax PCR Gems (PE Biosystems,

Nieuwerkerk a/d IJssel, The Netherlands) was used in all PCRs as described by Perkin-Elmer (3). The PCR products produced in the second reaction were analysed by electrophoresis on 1% agarose gels (30).

4.1.3.3 Cloning and analysis of 5.0-kb PCR fragments

PCR fragments were recovered from agarose gels by a freeze-squeeze method (38, 39) and cloned into a (Srf) (35) pre-digested bluescript vector using the PCR-Script SK(+) cloning kit (Stratagene Europe, The Netherlands). Cloning conditions were based on the recommendations of the supplier, except that the vector was gel-purified and co-precipitated with the fragment, facilitated by tRNA. Ligation was carried out in the presence of Srf endonuclease (Stratagene Europe, The Netherlands) (4 U) at 20°C for 14 h in a total volume of 10 µl. MAX. Efficiency STBL2 competent cells (Life Technology) were transformed according to the instruction manual supplied with the bacteria. Recombinant plasmids were isolated with a standard minipreparation method (12). Internal PCR fragments (Figure 1C) were derived from recombinant plasmids and subjected to endonuclease restriction analysis using: *Bcl*I, *Pst*, *Bam* HI, *Hind*III, *Eco*RV, *Sst*-I and *Eco*R1 (Boehringer Mannheim, Almere, The Netherlands). The same fragments were used as oligolabelled probes (10^6 cpm/µg) in dot blot hybridisations with membrane-bound SIV_{8980} RNA (Figure 1D). Nylon filters (Zeta-Probe; Bio-Rad, Veenendaal, The Netherlands) were hybridised in 6x standard saline citrate (SSC)/ 5x Denhardt's at 65°C and washed in 0.1x SSC/1% sodium dodecyl sulphate (SDS) at 45°C (30).

4.1.4 RESULTS AND DISCUSSION

To clone long SIV RNA sequences directly from serum, a primer extension method was optimised to generate cDNA molecules under various reaction conditions. To determine the progression in first-strand cDNA synthesis, we designed PCR primers for the 3' half (5-kb) of the SIV genome. The sequences of the primers along with their positions are shown in figure 1A. The products amplified with these primers are indicated in figure 1B. For the present study, we used purified SIV RNA from serum of an experimentally infected rhesus monkey. RNA was reversed-transcribed into cDNA using both the optimised protocol and the standard one provided by the supplier. Briefly, RNA together with the synthesis primer (No.10120), was heat-denatured at 80°C and snap-cooled on ice. Reverse transcriptase was added and cDNA was synthesised at 42°C for 60 min. The enzyme was inactivated at 90°C. The reaction was then subjected to PCR. The RT-PCR results obtained with this standard protocol are presented in figure 2A. The standard protocol was performed four times and enabled us to efficiently generate double stranded cDNA fragments up to 3-kb. However 4-kb and 3.5-kb products could be detected only faintly (one and two times, respectively). In all cases these products were present in lower amounts than the smaller non-specific products and tended to accumulate, likely at the expense of the larger amplimers. Cloning of 3.5- and 4-kb fragments failed in all cases using the standard technique.

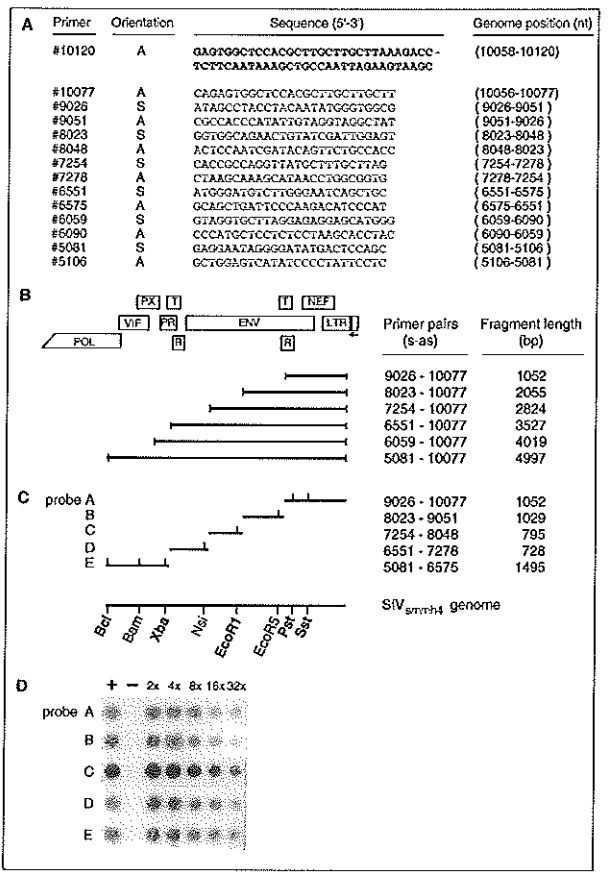

Figure 1. Panel A: Names and sequences of the PCR primers used for 3' half genome amplifications. The cDNA synthesis primer (No. 10120) is printed in bold. Sense (s) and antisense (a) orientations of the primers are indicated in the table. The nucleotide positions (nt) positions correspond with the SIV_{smmh4} genome (14). Panel B: PCR amplification of increasing larger fragment. PCR amplification of increasing larger fragment was used to determine the specificity and length of c-DNA strand synthesis. The horizontal lines represent PCR products generated from (single strand) c-DNA templates of different size. The black bar in the viral Long Terminal Repeat (LTR) (R region) represents the position of the cDNA synthesis primer (No. 10120). The horizontal arrow indicates the direction of the cDNA synthesis. Panel C: Cloned (5-kb) fragments were subjected to restriction site analysis. Internal fragments, represented by the horizontal bars, were generated by PCR amplification from recombinant plasmid DNA containing the full-length amplimer and examined for the presence of specific endonuclease restriction sites (vertical lines). The position of restriction sites is positioned schematically in relation to the SIV_{smmh4} genome. Conserved sites between SIV_{smmh4} and SIV_{PBJ14} are shown in bold. Sites unique to SIV_{smmh4} are in normal print. Panel D: Dot blot. Dilutions of viral RNA, isolated from (50µl) Rhesus serum were bound to nitro-cellulose filters and filter strips separately hybridised with probe A-E. Probes A-E were PCR amplimers from a recombinant plasmid containing the full-length PCR product. Uninfected serum probed with probes A-E was negative (data not shown). Positive controls (+): Recombinant Bluescript SK(+) containing probes A-E (10 ng). Negative controls (-): Bluescript SK(+) (40ng).

Apparently, under these conditions, when primer annealing takes place at temperatures well below the melting temperature (T_m) of the primer, short(er) cDNA molecules are generated because of mispriming, resulting in short products after RT-PCR with primer No. 5106. In our attempt to define optimised conditions for production of full-length cDNA, we modified the annealing conditions for both the RT synthesis of first-strand cDNA and the subsequent PCR conditions.

By using annealing temperatures as high as, or close as possible to, the T_m of the cDNA synthesis primer, we favoured specific primer hybridisation and reduced mispriming (15). Since formamide reduces the melting temperature of DNA/DNA and DNA/RNA duplexes in a linear fashion by 0.72°C for each percent formamide, it became possible to stringently anneal long primers (T_m> 42°C) at a decreased temperature of 42°C. The formamide concentration of 60% gave the highest yield of full-length product during RT-PCR when using synthesis primer No. 10120. In principle, cDNA synthesis should thus start from only one hybridised primer per template and proceed to the 5' terminus of the RNA template. To remove the formamide from the annealing buffer, RNA/primer complexes were precipitated and washed. After cDNA synthesis and PCR, only the DNA product of the expected molecular weight was generated. Syntheses of smaller amplimers due to non-specific primer hybridisation were not observed. However, cDNAs less than full-length may still be generated because of secondary structure in the RNA that tends to cause the reverse transcriptase to pause or stop (23). Since we used long primers (62-mer; 45% CG) with high T_m in the first-strand cDNA, it permitted heat-denaturation of some secondary structures to 70°C before synthesis, without dissociating RNA/primer complexes. In our experiments, we tested avian myeloblastosis virus (AMV) and M-MLV (SuperScript II RT) reverse transcriptases. The latter enzyme yielded the largest products in the best clonable amounts in the modified RT-PCR. Secondary structures may also cause RNAse H to cleave the RNA near the 3'-OH end of the growing cDNA chain. This results in premature termination of cDNA synthesis. Since SuperScript II RT, lacking RNAse H activity, has a higher progression rate than the wild type enzyme (AMV), we assume that this accounts for the better results of this enzyme in our test. Alternatively *Thermus thermophilus* (*Tth*) DNA polymerase, in the presence of Mn^{2+}, possesses an intrinsic reverse transcriptase activity. Because of its high temperature optimum, this enzyme should perform well in regions containing secondary structures. However, since *Tth* polymerase has been shown to be less sensitive relative to M-MLV reverse transcriptase (9), we did not consider this enzyme useful for our purpose. The addition of spermidine in the reverse transcriptase buffer showed a slight increase in the amount of 5-kb product and a decrease in background products. The concentration used here (0.5mM) was based on earlier observations (26) and experience from other groups (J. Gietema, Invitrogen, personal communication), and so we did not further test other concentrations. To minimise the error rate during PCR amplification of the synthesised cDNA the heat stable *Pfu* polymerase containing proofreading activity (29) was used because of its (16x) higher fidelity (31). Unfortunately, we were not able to successfully perform PCR directly on cDNA using only *Pfu*.

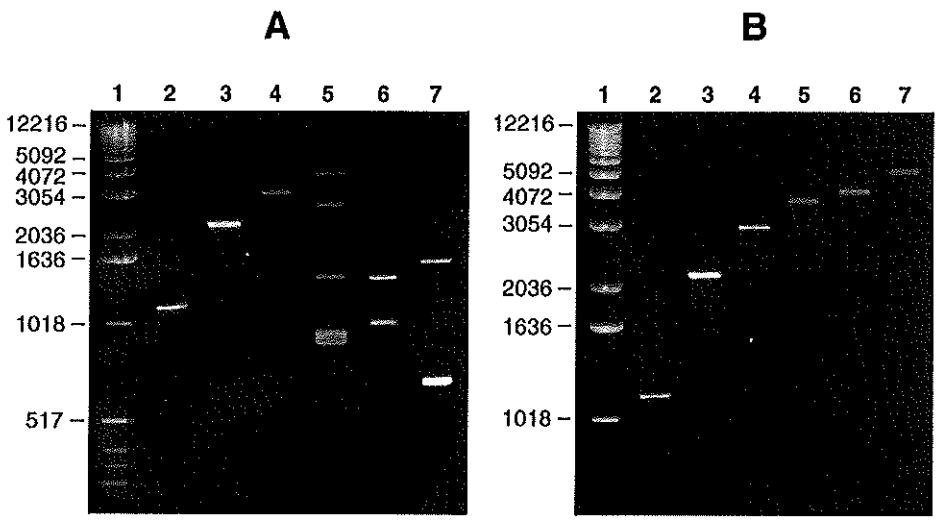

Figure 2. Analysis of RT-PCR products generated by the *standard* method (2A) and by the *modified primer extension* method (2B) on Rhesus monkey serum.
1-kb DNA Ladder (Bochringer) (lane 1). Agarose (1%) gel electrophoresis of amplification products generated by primer pairs : 10077-9051 (lane 2), 10077-8048 (lane 3), 10077-7278 (lane 4), 10077-6541 (lane 5), 10077-6060 (lane 6), 10077-5106 (lane 7). In each lane 20 µl of a RT-PCR (100 µl) reaction is loaded, except for the lanes 6 and 7 in figure 2b where 75 µl was loaded.

However, PCR performed using *Taq/Pfu* mixtures or just *Taq* alone was successful under similar conditions. This suggests that *Pfu* needs a higher template concentration or has a much slower progression rate, both compared to *Taq*. Utilising *Taq/Pfu* mixtures rather than *Taq* alone has been reported to allow the production of larger fragments with less PCR mistakes (7). Various *Taq/Pfu* ratios were tested with the primer Nos. 10077 and 5108 on pBK28 (24), generating 5-kb amplimers (data not shown). The ratio of *Taq/Pfu* (1U/1U) gave the highest yield in full-length product and was used in amplifications on SIV_{8980} cDNA templates.

The final PCR protocol for the amplification of full-length cDNA consisted of two steps. The first PCR (10 cycles), using *Taq/Pfu* mixtures, served only to generate sufficient template for the second PCR. The second PCR (35 cycles), using *Pfu* only, was performed on 10 µl of the first PCR amplification. The number of cycles we used in the first PCR was determined by a RT-PCR performed on virus RNA from serum with *Taq/Pfu*. Thereafter, 10 µl samples were taken following 5,10,15,20 and 30 cycles. These aliquots served as templates for the second amplification (35 cycles), in which only *Pfu* polymerase was used. Ten cycles in the first amplification were enough to yield detectable products after re-amplification. The exonuclease activity of enzymes with 3'--> 5' exonuclease-mediated proofreading has also been reported to degrade primers for DNA synthesis, thus resulting in diminished primer specificity or no PCR product at all (36). As primers with 3'-terminal phosphorothiolate linkages are resistant to this exonuclease activity (31), we consequently used phosphorothiolated primers in all amplifications.

Hot start in PCRs is a commonly used method which increases low-copy-number specific amplification but at the expense of side reactions due to mispriming. In hot start PCR, reagent addition to the reaction tube is designed so that all reactants do not mix until reaching a temperature high enough to suppress primer annealing to non-target sequences (80-90°C). In all experiments described here we used the AmpliWax™ PCR Gem-mediated hot start technique (3). PCR reactions performed under these conditions showed considerably less background and much higher yields of full-length products compared to amplifications without hotstarts. In addition, this method reduces cross-contamination which is considered to be a potential disadvantage of the hot start technique, requiring the manual addition of the polymerase at 90°C.

Results obtained with the modified RT-PCR method are depicted in figure 2B. Under optimised reaction conditions, no non-specific products were observed, and amplification products up to 5-kb were consistently seen (Figure 2B). The optimised RT-PCR method was tested three times and amplification products up to 5-kb was seen, but not smaller non-specific products. Twenty clones of SIV containing the 3' genome half were obtained using this technique. Primer specificities and the presence of highly conserved restriction sites in the cloned fragments, demonstrated that the cloned 5-kb products were of SIV origin (Figure 1C). This was also confirmed by dot blot hybridisation of the amplified subgenomic fragments with viral RNA (Figure 1D). Hybridisation as well as washes was carried out under such stringent conditions that positive signals due to PCR-primer (25-mer) homology alone were not likely to occur.

Finally, to reduce the instability that occurs during the propagation of molecular clones of HIV in bacteria (34), separate cloning of 5' and 3' halves of viral RNA genomes should be considered. By using this cloning technique it is possible to consider more routine isolation of full-length molecular clones (ca. 10-kb), which can be reconstituted either in vivo (37, 42) or in vitro (14). After further optimisation, the modified RT-PCR method described here, may be of interest in the construction of molecular clones of lentiviruses directly from sera enabling the cloning and characterisation of biologically relevant virus populations. To avoid the accumulation of smaller products, which may occur because of the presence of two identical LTRs in retrovirus genomes, primers could be designed that specifically anneal to the 5' cap sequence and to the 3' poly (A) tail. These particular sequences are only present in encapsidated RNA and not in proviral DNA. Last, this technique has the potential to be applied to the cloning and characterisation of other RNA viruses directly from bodily fluids.

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CHAPTER 4

DEVELOPMENT OF A MOLECULAR CLONE OF SIV DIRECTLY FROM SERUM

CHAPTER 4.2

A STRATEGY FOR CLONING INFECTIOUS MOLECULAR CLONES OF RETROVIRUSES FROM SERUM OR PLASMA

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4.2.1 ABSTRACT

To enable biological characterisation of lentiviral variants which emerge during infection and development of AIDS, we developed a method to construct molecular clones from circulating simian immunodeficiency virus (SIV) particles present in as little as 20 µl of serum from infected rhesus monkeys. This technique uses a long distance RT-PCR method optimised for the amplification of partly overlapping 5-kb SIV (half genome) amplimers. Ligation of the genome halves resulted in the construction of full-length clones which, after transfection, were able to replicate well in rhesus peripheral blood mononuclear cells (PBMC) and in various human T-cell lines inducing syncytia. In addition to the study of molecular cloned virus quasispecies emerging in circulation as a result of immune escape, this method may also be applied to obtain entire genes or full-length molecular clones. These clones may be present in other relevant extra-cellular body fluids such as, urine, saliva, tears, lymph, and bronchial or cerebral spinal fluid. Genes amplified in this way can be quickly inserted in new recombinant expression vectors and may then be applied in DNA vaccination approaches.

4.2.2 INTRODUCTION

Human immunodeficiency virus (HIV), discovered in the eighties as the etiological agent causing AIDS (1), still continues to spread worldwide (17, 27). A vaccine for HIV is now an urgent public health priority. The first and most extensively studied HIV-1 vaccine candidates were based on the recombinant envelope glycoproteins (rgp 120 and rgp 160 (16, 20) of HIV-1_{LAI}, HIV-1_{SF-2} and HIV-1_{MN}. These vaccine experiments resulted in poor protection of chimpanzees immunised with envelope-based immunogens after intravenous challenges with heterologous viruses (4-6, 14, 15, 30). Some papers have already dealt with immunological and virological analysis of persons which became HIV-1 infected while participating in recombinant gp120 subunit vaccines trials (3, 10) as currently conducted in the United States (16, 20). Genetic characterisation of immune escape mutants was in general restricted to attempts to identify eventual patterns in the envelope protein variability, that could be considered indicative of vaccine-induced selective pressure among the infected vaccine recipients (10).

Immune escape of HIV, following challenge with heterologous virus strains after immunisation with regulatory proteins, may therefore best be studied by characterising the biological properties (co-receptor use, cell tropism, replication rate), of the molecular clones which are most abundant in circulation. As a consequence of the fact that most regulatory proteins act in trans, escape variants may contain mutations in both the regulatory gene itself as well as in the protein-binding site. The combined effect of these mutations may result in the production of a biologically modified virus that is able to escape host immunity. Detailed analysis of the biological features of such individual molecularly cloned escape variants may be more informative than analysing relevant but limited numbers of sequences obtained from the entire pool of circulating quasispecies. For these purposes the study of molecular clones

derived from the predominant circulating extracellular viral population in serum or other body fluids is of particular interest.

Clinical trials of first generation HIV-1 vaccine candidates are in progress. As the number of trials increases information to guide the design and improvement of future generations of HIV-1 vaccines will be needed. Molecular clones will be invaluable for gaining insight in several aspects of viral immune escape. Our goal was to investigate if a method could be developed for the routine construction of replication-competent lentiviral clones from circulating virus particles. We report on the optimised RT-PCR method that enabled us to generate full-length SIV clones from small numbers of viral RNA templates using serum from SIV-infected macaques as starting material.

4.2.3 MATERIALS AND METHODS

4.2.3.1 Viral, RNA isolation and cDNA synthesis

SIV_{8980} was derived from SIV_{B670} (2, 25) by five subsequent *in vivo* passages in Indian rhesus macaques (Holterman et al., submitted). Serum from animal 8980 was used as starting material for construction of molecular clones of SIV. Serum of animal 8980 contained $6x10^8$ SIV-RNA copies per ml at the time of death. In this method serum aliquots equal to 3.10^5 copies were used to synthesise cDNA, the 5-kb amplimers were produced and cloned from 1000-fold diluted cDNA samples (=300 SIV-RNA copies). A restriction map of the SIV_{8980} genome was generated to facilitate cloning. Overlapping fragments ranging from 130 bp to 2571 bp, spanning the entire genome, were synthesised using a standard RT-PCR technique. cDNA synthesis was performed on viral RNA extracted by the guanidium thiocyanate-phenol-chlorophorm extraction method (7). First strand cDNA molecules were produced using SuperScript™II Rnase H reverse transcriptase and performed according to the instructions of the manufacturer (Gibco-BRL). PCR amplification was carried out on 5 µl of cDNA after addition of 10 pmol of both sense- and anti-sense primers and a mixture of 0.5 unit of both *Taq*-((28, 31) Perkin-Elmer, Gouda, The Netherlands) and *Pfu* (23); Stratagene, La Jolla, CA, USA) DNA polymerases. The PCR reaction mixtures (50 µl) were overlaid with mineral oil and incubated as follows: 35 cycles of denaturation for 60 seconds at 94°C, annealing for 45 seconds at 58°C and elongation for 0.5, 1 or 2 minutes depending on the length of the fragment. All amplifications were performed in a Perkin-Elmer DNA Thermal Cycler 480. Amplification products were then subjected to restriction endonuclease digestions and analysed on 1.5 % agarose gels. Based on this restriction analysis a cloning strategy for both genome halves was developed. The exact positions of all PCR primers (RD1 / RD18), relative to the $SIV_{smmF236\text{-}smh4}$ genome (Los Alamos accession number: X14307) (18) were as follows: RD1 (nt 10138 to 10161) and RD2 (nt 9025 to 9049); RD3 (antisense primer) complementary to RD2 and RD4 (nt 8022 to 8048); RD5 (antisense primer) complementary to RD4 and RD6 (nt 7253 to 7277); RD7 (antisense primer) complementary to RD6 and RD8 (nt 6550 to 6575); RD9 (antisense primer) complementary to RD8 and RD10 (nt 5187 to 5211); RD11 (anti-sense primer) complementary to RD10 and RD12 (nt 5082 to 5106); RD13 (antisense

primer) complementary to RD12 and RD14 (nt 2536 to 2559); RD15 (antisense primer) complementary to RD14 and RD16 (nt 1042 to 1065); RD17 (antisense primer) complementary to RD16 and RD18 (nt 75 to 102). Antisense primers were used to synthesise first strand cDNA's.

4.2.3.2 Long range RT-PCR and cloning of amplified SIV genome fragments

cDNA molecules containing either the 5' (*LTR, gag, pol*) or the 3' (*pol, env, LTR*) SIV genome halves (5-kb) were generated using an optimised RT-PCR method (19). Two cDNA synthesis primers (SP-1, SP-2) and seven amplification (phosphorothioated) primers (L3-1, P3-1, P3-2, P5-1, P5-2, L5-1 and L5-2) were used in nested PCR reactions for producing long amplimers. Control experiments were performed without reverse transcriptase. To reduce the amount of nucleotide misincorporations during PCR a limited number of cycles were used for both genome halves (R/U5/*gag/pol* and *pol/env*/U3/R) in the first round (15 cycles) and 35 cycles in the nested amplification. To further increase fidelity the use of an optimised reaction buffer for cDNA synthesis was used. The mutation frequency of MMLV-RT was decreased substantially (33%) by lowering the dNTP and the $MgCl_2$ concentration. To minimise the chance of creating artefacts due to template interactions during RT-PCR on serum, we generated all 5-kb products from low numbers of RNA- and DNA templates. Reverse transcriptase reactions were therefore carried out on diluted RNA templates. The cDNA's synthesised were used as templates in separate first round PCR amplifications. The second round of PCR was performed on a 1:10 diluted reaction mixture generated in each of the first round PCRs. Samples from the second amplifications were analysed by gel electrophoresis. The highest dilution, which still yielded detectable amounts of 5-kb products on agarose gels was used for cloning and restriction analysis. The sequences and exact positions of all primers, relative to the SIV_{smH4} genome (Los Alamos database), are shown in figure 1. Synthesis of LTRs was performed on DNA from peripheral blood mononuclear cells (PBMC) of rhesus monkey no. 8980 and used as template for PCR to generate complete 5' and 3' LTR sequences. The 5' LTR was amplified with primer pair: DL1-5 and DL2-5. The 3' LTR was generated using primer pair: DL3-3 and DL4-3 (Figure 1). Both LTRs were synthesised by PCR at: 35 cycles of 1 minutes denaturation at 94°C, 30 seconds of annealing at 58°C and elongation periods of 1 minutes at 72°C by using (2 U) *Pfu* DNA-polymerase and with phosphorothioated PCR primers.

4.2.3.3 Plasmids

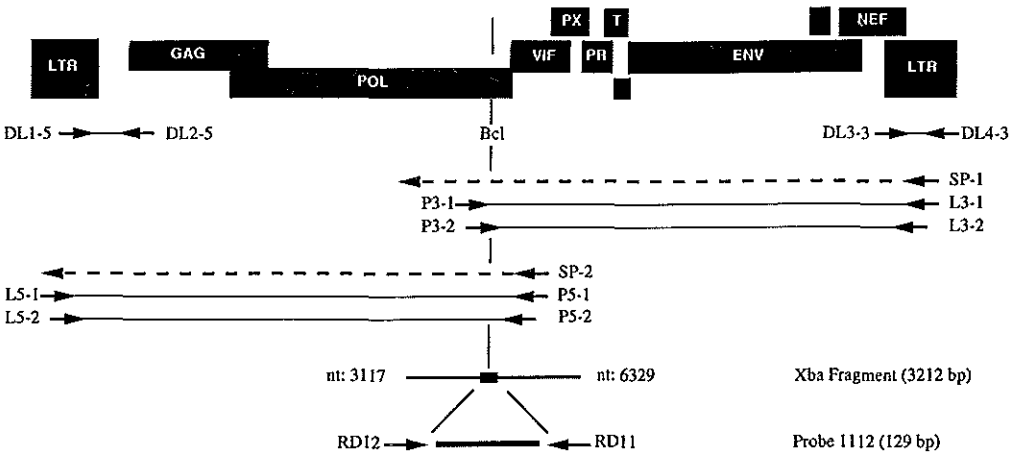
To allow cloning of the PCR products multiple cloning sites of commercially available plasmids were modified by adapter insertions. pScript AB12 was derived from pBluescript KS^+ after insertion of both adapters (AB and 12). Adapter AB was formed after annealing of oligomers A: 5'-GGCCAAGTCGGCCGCGGCCGCC-3' and B: 5'-TCGAGGCGGCCG-CGGCCGACTTGGCCGGTAC-3'. Adapter AB contained *Not*I and *Sfi*I restriction sites and could be ligated into the *Kpn*I / *Xho*I site. Adapter 12 originated after annealing of the oligomers: 1: 5'-GAGCTCTGATCAGGGCCGCATAGCT-3' and 2: 5'-ATGCGGCCG-

CGGCCGACTTGGCCTGATCAGAGTTCTGCA-3' contained *Sst*I, *Bcl*I, *Sfi*I, *Not*I sites and could be cloned into a *Pst*I / *Sst*I site (deleting the *Sst*I site). pScript CD56 was derived from pBluescript KS$^+$ after insertion of the two adapters (CD and 56). Adapter CD was formed after annealing of the oligomers: C: 5'-CGGCCAAGTCGGCCGCA-TGCGATATCGATCCTGCA-3' and D: 5'-GGATCGATATCGCATGCGGCCGACTTG-GCCGAGCT -3'. Adapter CD contained *Mbo*I, *Eco*R5, *Sph*I, *Sfi*I, restriction sites and could be inserted into a *Pst*I / *Sst*I site (inactivating the *Sst*I site). Adapter 56 was formed after annealing of the oligomers: 5: 5'-GTGATCAACTAGTATGCATGATCGTACGCGG-CCGCGGTAC-3' and 6: 5'-CCGCGGCCGCGTACGATCATGCATACTAGTTGATCA-CTGCA-3'. Adapter 56 contained *Kpn*I, *Not*I, *Nsi*I, *Spe*I, *Bcl*I restriction sites and was inserted into the *Kpn*I / *Pst*I site (deleting the *Kpn*I site). All cloned (RT)-PCR fragments were transfected into MAXefficiency STBL2^{TM} competent cells (Life Technologies, Roosendaal, The Netherlands) and cultured at room temperature for 8 hours.

4.2.3.4 Transfection of C8166 cells, infection of rhesus PBMC.

Two approaches were followed to reconstitute a full-length molecular clone from the two separate genome halves. Strategy A) the *in vitro* ligation approach. The recombinant plasmids containing the 5' or 3' SIV genome halves were linearised by *Bcl*I digestion, precipitated and washed twice with 70% ethanol. Mixtures of 10 µg from each of the linear plasmids were ligated (5 units T4 ligase) in 80 µl at 18°C overnight. Ligation products were precipitated, washed and dissolved in 100 µl RPMI (Gibco, Roosendaal, The Netherlands). Human T-cell line C8166 cells ($5x10^6$) were resuspended in 150 µl of RPMI supplemented with 10% fetal calf serum (FCS) and mixed with 100 µl of the ligated DNA. Ligation mixtures, of different 5' and 3' genome combinations, were electroporated (230 Volts and 960 µF) in a 4 mm gap cuvette. After the pulse (Biorad Gene Pulser), the cells were diluted in 8 ml RPMI (10% FCS) and divided over 4 wells of a 24-wells plate (Greiner Labortechnik, The Netherlands) and cultured for fourteen days in RPMI supplemented with 10% FCS.

Strategy B) the *in vivo* ligation approach made use of the ability of eukariotic cells to join (non)-homologous sequences *in vivo* (33). Five million cells were mixed with 10 µg of both circular plasmids (pS.SIV5'-3 and pS.SIV3'-59) in 250ul RPMI supplemented with 10% FCS in a 4 mm electroporation cuvette. After electroporation the cells were transferred to culture disks with the same medium composition. One-day post transfection medium was changed and cells were cultured for two more weeks with medium changes every two days. Southern blotting was used to determine if proper recombination had occurred. Seven days after transfection, chromosomal DNA was isolated from transfected C8166 cells and digested to completion with XbaI. Digested DNA was transferred to a nylon membrane and probed with the 129 bp PCR-fragment generated by primer pair RD11 and RD12 (probe 1112). The blot was washed at a stringency of 0.1 SSPE at 80^0 C for two hours. The recombinant plasmid (15 µg) containing the full-length SIV genome ($pS.SIV_{F359}$), which was constructed later, was transfected using the electroporation conditions as described above.



Primer	Sequence (5'-3')	Genome position (nt)
	Amplification of the 3' SIV genome half	
SP1	GAGTGGCTCCACGCTTGCTTGCTTAAAGACCTCTTCAATAAAGCTGCCAATTAGAAGTAAGC	10120-10058
L3-1	GCAGCTTTATTGAAGAGGTCTTTAAGC	10139-10112
L3-2	CAGAGTGGCTCCACGCTTGCTTGCTT	10077-10051
P3-1	GGAGTAGTGGAAGCAATGAACCATC	4960-4985
P3-2	GAAGGGGAGGAATAGGGATATGACTCC	5076-5103
	Amplification of the 5' SIV genome half	
SP2	GATGGGGCACATAACAAGCCATCTGTAGGTCTTTAGTGTTATATTTCAGGTGTTTGATGAGGC	5460-5397
P5-1	CCTTTCCACAATAGCTCACCGGGTCC	5250-5224
P5-2	GTCTCTGCCTTCTCTGTAATAGACCCG	5210-5183
L5-1	GGAGAGGCTGGCAGATTGATGCCC	526-550
L5-2	CCAGCACTAGCAGGTAGAGCCTGGG	562-587
	Amplification of the 5' LTR	
DL1-5	CAGATTGGCAAAATTACACATCAGG	75-100
DL2-5	CTGCCTTCACTCAGCCGTACTCAG	881-857
	Amplification of the 3' LTR	
DL3-3	ATAGCCTACCTACAATATGGGTGGCG	9026-9051
DL4-3	CCGAGATGACCAGGCGGCGACTAG	10199-10175
	Amplification of the probe	
RD12	GAGGAATAGGGGATATGACTCCAGC	5082-5106
RD11	TCTCTGCCTTCTCTGTAATAGACCC	5187-5211

Figure 1. Schematic representation of the complete SIV genome with the locations of all (nested) primer pairs used for : DNA-PCR (DL1-5 / DL2-5 and DL3-3 / DL4-3), cDNA synthesis (SP-1 and SP-2) and RT-PCR (L5-1 / P5-2, L5-2 / P5-2, P3-1 / L3-1 and P3-2 / L3-2). Names and sequences of all PCR primers are shown below. The nucleotide (nt) positions correspond to the SIV_{smH4} genome (acc.no.X14307). The XbaI restriction fragment and the probe that were used to analyse the *in vivo* recombination event of pS.SIV5'-3 and pS.SIV3'-59 after electroporation of T-cells are indicated by the solid bars.

In all three transfection experiments cultures were maintained for two weeks and used to prepare rhesus stocks. Five million Rhesus PBMC were isolated and infected with 100 TCID 50 of the SIV_{F359} virus stock for four hours. Cell cultured were washed three times with RPMI supplemented with 10% FCS to remove the free unbound virus and cultured for fourteen days.

4.2.3.5 Immunocytological analysis of virus (p27) antigen production

Primary rhesus PBMC cultures were observed regularly for cytopathic effect (CPE). Infection with SIV_{F359} was confirmed by immunocytochemistry for the expression of SIV*gag* antigen. Single-cell preparations for immunocytochemistry were prepared on acetone cleaned glass slides, which had been air dried for 30 minutes. Cells were fixed in acetone/methanol (1:1) and in ethanol (70%) for 15 and 30 minutes, respectively. Slides were washed in 0.05 M Tris-HCl pH7.6, 0.1 M NaCl for 5 minutes and incubated with 20 µl (1:25 dilution) of anti-*gag* monoclonal antibody (26). Cells were washed for 5 minutes and incubated with goat anti-Mouse IgG antibody for 30 minutes at room temperature. To amplify the signal the cells were washed and incubated with mouse-anti-alkaline phosphatase (APAAP-complex) (Boehringer). Cells were washed for 5 minutes and incubated with 20 µl substrate solution: (10:1:1) 0.1 M Tris-HCl pH 9.5, NBT (nitro-blue tetrazolium), BCIP (5-bromo-4-chloro-3-indolylphosphate), 5 mM levamisole for 30 minutes at room temperature. Cells were washed for 5 minutes in tap water, one drop of (50%) glycerol / PBS was added per slide, and the cells was covered with a cover slip. The preparations were microscopically examined at a 40x magnification and CPE was quantified. In cell culture supernatants virus was quantified by measuring p27 concentrations (SIV p27 antigen capture ELISA, Coulter Corp., Hialeah, FL).

4.2.4 RESULTS AND DISCUSSION

4.2.4.1 PCR amplification and cloning of LTR and (5' and 3') genome SIV fragments

The replication cycle of retroviruses consist of a integrated DNA (provirus) stage and a RNA (extracellular) stage. The two LTR's flanking the proviral genome each contain the complete U3RU5 motif while in the extracellular form only incomplete forms (RU3 and RU5); motifs which are present in the 5 'and 3' LTR respectively. Since only complete LTR sequences are able to aid in proviral integration DNA fragments from proviral 5' and 3' LTR regions, they were linked to the 5' and 3' genome halves respectively. Serum and lymphocytes were separated from the same blood sample collected from rhesus monkey no. 8980 as the sources for extracellular viral RNA and proviral DNA, respectively. For subcloning purposes a restriction map of this particular SIV genome was made by synthesising overlapping RT-PCR fragments, which covered the entire genome and ranged from 129 bp to 2571 bp. In addition two new recombinant plasmids pScript AB12 and pScript CD56 were also constructed. Utilisation of the *Sst*I and *Pst*I restriction sites just outside the 5' and 3' LTR aided in the exchange of the incomplete (virion) LTR's for complete (proviral) ones. The positions of all primers used in the synthesis of cDNA, the amplification of LTR's and of viral genomic sequences are shown in figure 1. The cDNA synthesis started at synthesis-primer SP-1 located in the R region of the 3' LTR and SP-2 which was located in the 3' part of the *pol* gene (synthesis represented by the dashed lines in figure 1. Complete 5' and 3' LTRs were generated with a single round of PCR amplification of proviral DNA using primer pairs DL1-5 / DL2-5 and DL3-3 / DL4-3 respectively.

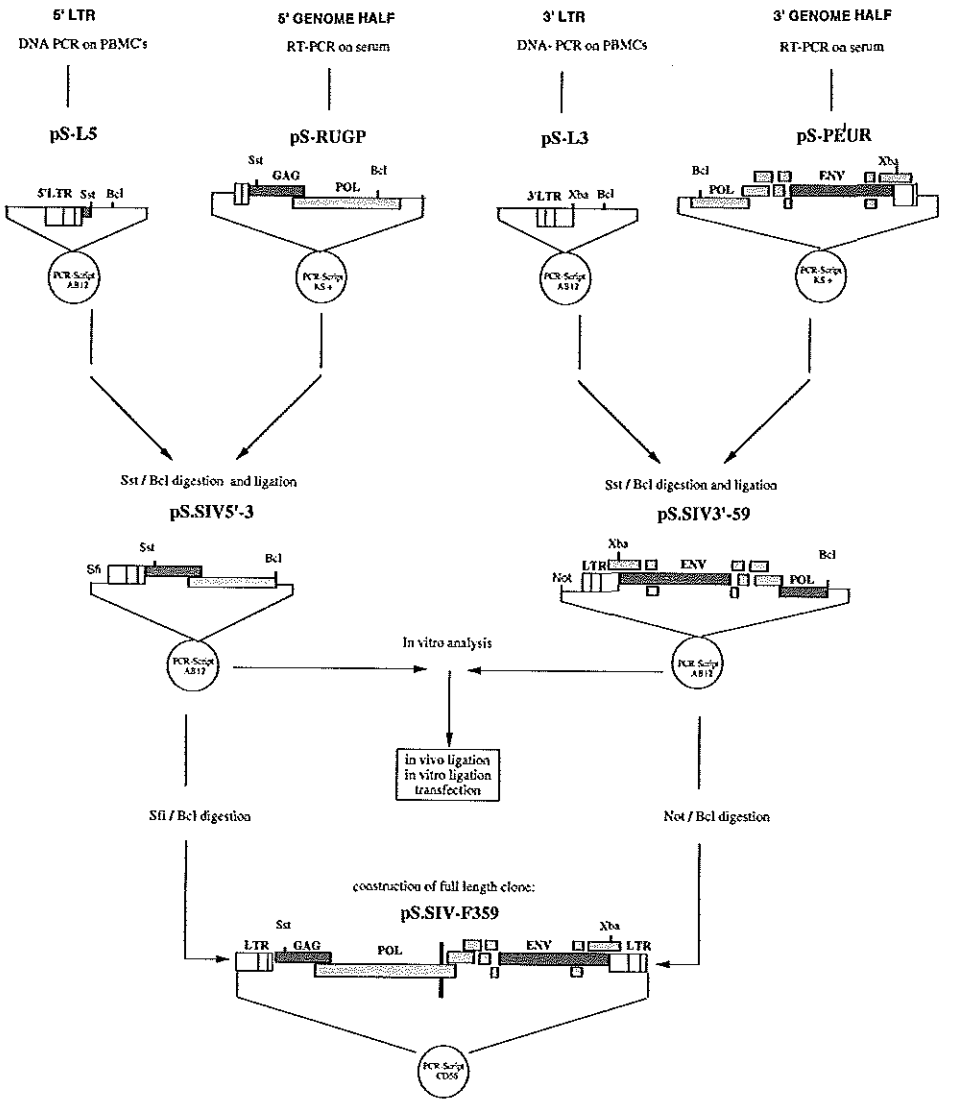


Figure 2. Cloning strategy of full-length SIV molecular clones. Both 5' and 3' LTR's were amplified from provirus DNA which was present in SIV_{8980} infected rhesus PBMC 's. The 5' SIV genome half and 3' SIV genome half were generatedfrom serum using a modified RT-PCR method. All amplimers were cloned and sub-clonedin orderto fuse the 5' LTR in front of the gag/pol-region and the 3' LTR behind the regulatory genes / ENV- region. After linearisation and ligation of the genome halves at the uniquely present Bcl restriction site the entire SIV genome was reconstituted.

Double stranded 3' cDNA molecules containing *pol/env/* U3/R sequences were generated by a nested PCR amplification. The same anti-sense primer L3-1, was used with sense primer P3-1 in the first amplificationand with sense primer P3-2 in the second PCR. Both sense primers were located 90 bp apart. The 5' genome fragment was synthesised by a

nested PCR using primer pair L5-1 / P5-1 in the first round and L5-2 / P5-2 in the second amplification round, generating amplimers of approximately 5-kb (Figure 1). Sequences derived from the U3-R region present in RNA were similar to the corresponding region present in proviral DNA. The $SIV_{pbj\ 1.14}$ clone was used as a characterised template for quality control of fidelity of the PCR conditions. A 5-kb amplimer of these clones was checked. Secondly, to control the fidelity of the RT-PCR reaction five of our own 8980 clones were partly sequenced and compared. To check for possible errors during amplification, one cloned 5'SIV-genome half with defined sequence was amplified and sequenced. One (silent) mutation was detected between the original insert (5000 bp) and the amplified product. We calculated that the error rate of these amplifications was around the 0.02% and comparable to previous results obtained with thermostable enzymes (12, 24). All four amplimers (5'-LTR and 3'-LTR, and R/U5/*gag/pol* and *pol/env*/U3/R fragments) were separately cloned as blunt-ended fragments into the *Srf*I restriction site of PCR Script KS^+ (yielding the plasmids pS-L5 and pS-L3 and pS-RUGP and pS-PEUR) (Figure 2). The 5' and 3' LTR's were then subcloned respectively as a *Hind*III / *Sst*I and *Hind*III / *Xba*I fragments into pScript AB12. *Gag/pol* (4.3-kb) and *pol/env* (3.8-kb) fragments were then retrieved from pS-RUGP and pS-PEUR as *Sst*I / *Bcl*I and *Xba*I / *Bcl*I fragments and fused downstream to the cloned 5' and 3' LTR (yielding the plasmids pS.SIV5' and pS.SIV3') (Figure 2).

4.2.4.2 *In vivo* and *in vitro* reconstitution of functional full-length clones

Three clones containing the 5' genome half (U5RU3/*gag/pol*) and three clones containing the 3'genome half (*pol/env*/U5RU3) were linearised at the *Bcl*I site and ligated *in vitro* (Figure 1). Combinations of ligated genome halves were functionally tested for replication competence by transfecting full-length genomes in human C8166 T-cells. Virus production was assayed by screening for syncytia formation, the presence of p27 antigen in the supernatants and by immunohistochemistry. A total of nine ligation mixtures (three 5' halves combined with three 3' halves) were tested *in vitro*. Cytopathic effect (CPE) caused by viral replication could be observed for all cells transfected with the combination of pS.SIV5'-3 and pS.SIV3'-59.

Rhesus PBMC were used for preparation of virus stocks. Characteristic CPE with typical syncytium formation was observed 14 days post infection. All stocks were screened for virus production and a $TCID_{50}$ was determined on (C8166) T-cells. Plasmids containing the functional 5' and 3' genome halves were analysed by gel electrophoresis and sequenced completely (Figure 3).

In contrast, the nature of rearrangements that occur during *in vivo* ligation is complicated and quite inefficient. Southern blot analysis was needed to convincingly show that the observed CPE and p27 production was produced by the (*in vivo*) recombined full-length SIV genome rather than from one of the separate plasmids as a result of some unknown integration artefact. The positive signal on the southern blot had a similar relative migration mobility as a marker fragment of about 3200 bp. A *Xba*I fragment of 3212 bp that hybridises to probe 1112 was in agreement with a correct SIV genome recombination.

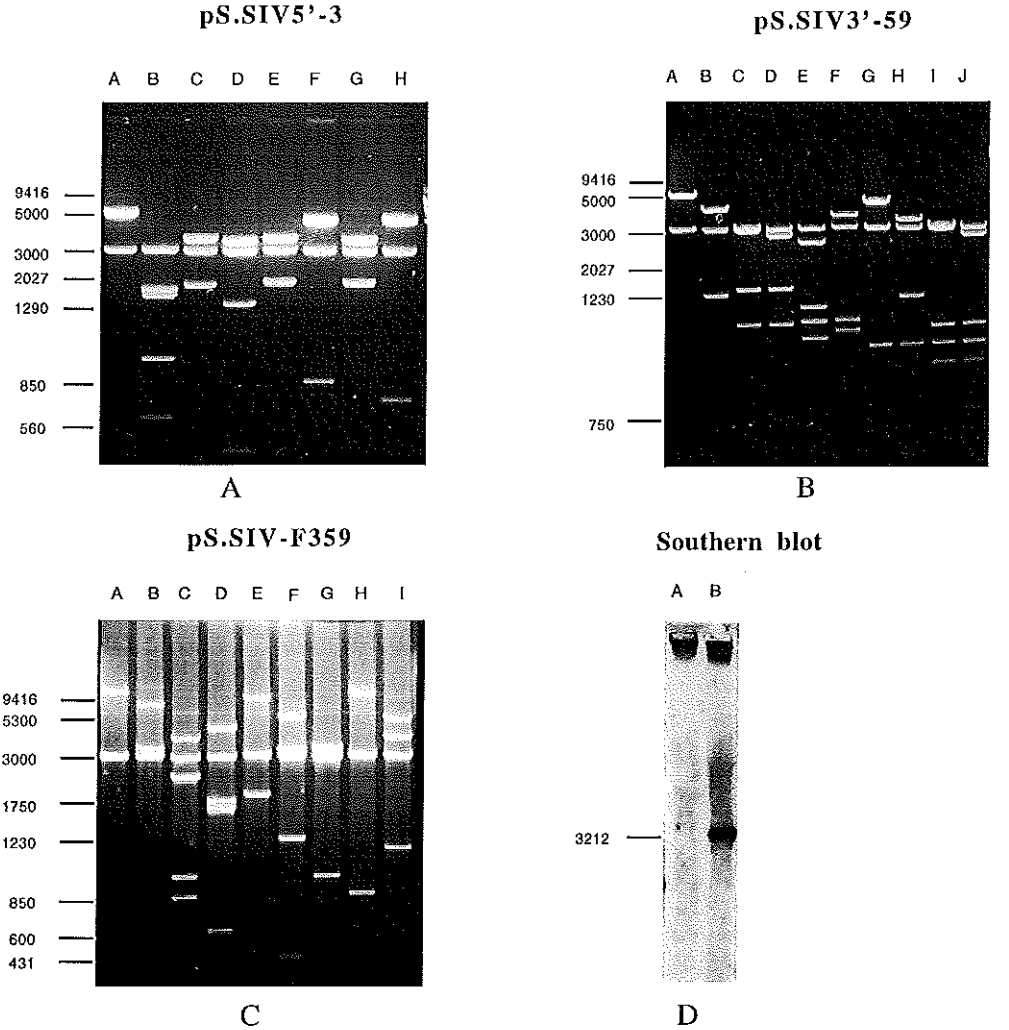


Figure 3. A. Restriction pattern of the cloned 5' SIV genome half (pS.SIV5'-3). DNA was digested with: Not-I (lane A), Not-BamHI (lane B), Not-EcoRI (lane C), Not-Hind III (lane D), Not-Pst (lane E), Not-Sst (lane F), Not-Xba (lane G) and Not-Xho (lane H). **B.** Restriction pattern of the cloned 3' SIV genome half (pS.SIV3'-59). DNA was digested with: Not-I (lane A), Not-Pst (lane B), Not-Xba (lane C), Not-Pst-Xba (lane D), Not-Nco (lane E), Not-Nsi (lane F), Not-BamHI (lane G), Not-BamHI-Pst (lane H), Not-BamHI-Xba (lane I) and Not-BamHI-Xba-Pst (lane J). **C.** Restriction pattern of the cloned full-length SIV genome (pS.SIV-F359). DNA was digested with: Not-Sfi (lane A), Not-EcoRI-Sfi (lane B), Not-Nco-Sfi (lane C), Not-BamHI-Sfi (lane D), Not-Hind III-Sfi (lane E), Not-Pst-Sfi (lane F), Not-Xba-Sfi (lane G), Not-Sst-Sfi (lane H) and Not-EcoRV-Sfi (lane I). **D.** Southern blot analysis of (XbaI) digested chromosomal DNA of: non-transfected C8166 T-cells (lane A) and co-transfected (p.SIV5-3 and p.SIV3-59) C8166 T-cell line. The oligo-labelled PCR- fragment generated by primers RD11 and RD12 was used as probe. Molecular weight marker is indicated on the left of each (A, B, C, and D).

The nine combinations of 5' and 3' genome halves were evaluated three times *in vitro* ligation and transfection experiments. The combination of pS-SIV 5'-3 and pS-SIV3'-59 yielded in all three cases infectious virus. Using the in *in vivo* ligation technique infectious virus production were yielded in two cases.

Virus production by *in vivo* ligation of the genome halves required a longer time span to produce the same virus titre than the *in vitro* method (20 days versus 14 days respectively).

No further study has been conducted to elucidate the failure of the other combinations to produce infectious viruses. However, we did prepare detailed restriction maps in order to determine if (large) deletions or insertions might have been the reason for the observed results. We know that this is a rather insensitive method but since no differences were observed on polyacrylamide gels of different percentages we (carefully) believe that point mutation(s) may be considered as the cause of the biological inactivity of the other clones.

4.2.4.3 Construction of a biological active molecular clone

Subsequently, a plasmid containing the entire SIV genome was generated. The 5' genome half was excised from pS.SIV5'-3 as a *BclI / Sfi* fragment and cloned into *BclI* / Sfi digested pScript CD56 yielding pScript CD56-5). The 3' genome half was excised from pS.SIV3'-59 as a *BclI / NotI* fragment and cloned into pScript CD56-5, yielding pS.SIV-F359. Recombinant clones were analysed by agarose gel electrophoresis and sequenced completely (Figure 3). Following electroporation pS.SIV-F359 induced CPE in the C8166 T-cell line (Figure 4A). Virus stocks were prepared and titrated in rhesus PBMC (Figure 4B).

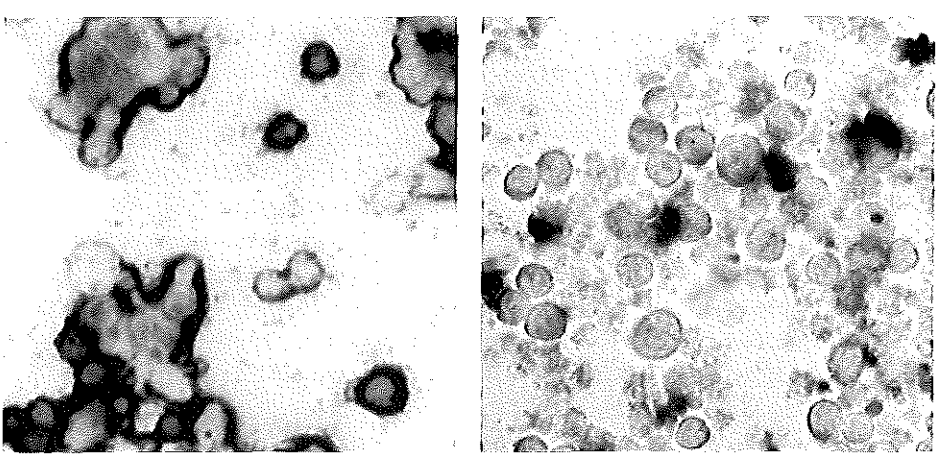


Figure 4. Cytopathic effect of the SIV_{F359} on human T-cell line (C8166) (A); and on rh-PBMC (B) immunocytologically stained for p27 antigen production. Cells were stained 12 days after infection with SIV_{F359} (stock propagated on rhesus PBMC). Photomicrographs 660 x magnification.

In summary, we describe an optimised long distance RT-PCR method used to derive molecular clones from circulating viral RNA in serum. Restriction fragment analysis and sequence analysis of both the genome halves and the full-length clone proofed the absence of (small) deletions, inserts or point mutations. The cumulative PCR error rate of less than 0.02 percent indicated that by using the (highest) reliable pfu (13, 23) in combination with highly controlled reaction conditions functional clones could be generated from small amounts of SIV particles. The result of the transfection experiment that was obtained by using the *in vivo* ligation potency of eucaryotic cells further extended the application of this technique to those cases in which no unique restriction site (such as *BclI*) was available. The expression of SIV (p27 antigen) in transfected human T-cell lines and the increase in p27 concentration after inoculation of rhesus PBMC, with small volumes of (C8166) supernatant, revealed the replication competence as well as the infectious nature of this molecularly cloned virus. More detailed studies in a panel of rhesus macaques are being conducted with the SIV_{F359} molecular clone in order to characterise and compare the pathogenic potential of this clone with those previously described from proviral DNA.

Using infectious molecular clones of HIV or SIV obtained from serum or plasma viral RNA has been considered more representative in AIDS pathogenicity (29), studies and have some important advantages over clones derived from provirus DNA. Firstly, the RNA-derived clones are more representative of the replicating virus pool than the proviral equivalents (8, 9). Secondly, the HIV- or SIV-proviruses, present as DNA in infected lymphocytes contain high levels of replication defective proviruses containing deleted or otherwise mutated sequences (32). Finally, using circulating viral RNA as template for molecular clones (29) also circumvents the need to culture infected cells *in vitro* and hence diminishing the adaptations, observed in both individual viral genomes and in the quasi-species composition of the viral isolate (22). Notably pathogenic molecular clones of HIV have been generated by PCR from proviral DNA (11), we report here the first to be derived from extracellular viral RNA. The main importance of using clones directly obtained from circulation is that they represent the most abundant virus variant population present *in vivo* at a certain time in disease. These clones may become even more valuable now. Kimata et al has convincingly shown that particular viral variants emerge and become dominant during the development of AIDS (21). These virus variants resulted from escape of the immune response within the host. In this manuscript we presented one full-length clone of SIV that was biological active displaying similar clinical signs as the parental strain. Sequence analyses of the V1 region of SIV_{F359} (the molecular clone) and SIV_{B670} (the parental strain) revealed that despite *in vivo* passage of SIV_{B670}, the predominant viral genotype becomes characterised by the sequences encoded by the SIV_{F359} molecular clone.

This observation proved that the efficiency of the method is sufficient to obtain these relevant SIV variants and that these may be valuable for gaining insight into viral escape and to further our understanding of AIDS pathogenesis. This strategy will allow the study of lentiviral variants in many different body fluids including CSF, lymph as well as peritoneal and thoracic transudates.

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CHAPTER 5

DISTINCT CHARACTERISTICS OF A PATHOGENIC MOLECULAR CLONE OF AN END-STAGE VARIANT OF SIMIAN IMMUNO-DEFICIENCY VIRUS (SIV_{F359})

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5.1 ABSTRACT

End-stage SIV isolates are suggested to be the most fit of the evolved virulent variants that precipitate the progression to AIDS. *In vivo* selection of a highly virulent SIV isolate was obtained by serial end-stage passage in rhesus monkeys (*Macaca mulatta*). To determine if there were common characteristics of end-stage variants which emerge from accelerated cases of AIDS, a molecular clone was derived directly from the last serum sample collected prior to acute onset of AIDS in a rhesus monkey. Sequence analysis confirmed that this clone (F359) was a dominant variant selected by serial end-stage *in vivo* passage and was related to but distinct from other molecular clones of the SIV_{sm}/SIV_{mac} group. It caused marked cytopathic effect (CPE) and replicated to very high levels in activated but not resting peripheral blood lymphocytes. This clone also infected, but did not replicate in rhesus monocyte derived macrophages (MDM). Interestingly, although at low doses this end-stage variant did not use any of the known co-receptors except CCR5, it was able to infect and replicate in human PBMC homozygous for the Δ32 deletion of CCR5, suggesting the use of a novel co-receptor. Mature rhesus macaques infected with SIV_{F359} developed high virus loads, loss of $CD4^+$ T-lymphocytes, anemia, weight loss and opportunistic infections characteristic of AIDS. It represents the first pathogenic molecular clone of SIV derived from viral RNA in serum and provides evidence that not only the genetic but also the biological characteristics acquired by highly fit late-stage disease variants are distinct from one another.

5.2 INTRODUCTION

Simian immunodeficiency virus (SIV) of sooty mangabeys causes AIDS in macaques providing an important animal model for Human Immunodeficiency Virus (HIV)-induced AIDS in humans (12, 18, 20, 29, 38). Molecular clones of HIV and SIV have been valuable for addressing specific questions in AIDS pathogenesis (27, 41, 49), vaccine development (5, 8, 9, 12, 28, 50, 52) and in the evaluation of antiviral drugs (2, 55). To date molecular clones of SIV have been derived from proviral DNA rather than viral RNA and most proviral clones have been obtained from cultured cells and frequently from infected human cell lines (13, 22, 29, 33, 41, 46, 48). It has been demonstrated that by *in vitro* propagation certain viral variants are selected (19, 57). In particular, growth of virus in human cell lines results in major changes in the SIV genome such as deletions leading to truncation of the transmembrane envelope protein (6, 23, 31, 32). This has resulted in important biological differences between the derived clones and the original pathogenic virus population in the host. In addition, proviral DNA frequently contains a high proportion of defective proviruses (34, 39, 40, 43, 58).

Recently it has been demonstrated that late-stage SIV variants are the most fit having acquired multiple mutations encoded at several genetic loci facilitating immune escape and increasing replication and cytopathic properties (30). These observations have been supported by a different line of experiments. A series of *in vivo* passage studies were

performed in which blood samples taken at the time of AIDS development were subsequently used to infect naive rhesus macaques. End-stage blood samples were taken from the most rapidly progressing animal and passaged *in vivo*. This *in vivo* passage of end-stage variants resulted in a progressively accelerated disease course with each successive passage until the fourth passage, by which time AIDS had developed in as little time as 2 weeks (26). Taken together the results of these two independent lines of investigation suggested that the passage of primate lentiviruses late in disease could result in the transmission of highly virulent variants capable of causing rapid progression to AIDS. Furthermore, data suggested that highly fit end or late-stage "fitness" variants had common biological properties (30).

The provirus population in mononuclear cells *in vivo* is generally considered to be a sanctuary of biological variants which have accumulated in such intracellular reservoirs as a consequence of previous host immune pressures and/or defective viral replication (58). In contrast we reasoned that extracellular virions represent a virus population which may be considered as dominant as they replicate to high titer, becoming most fit and predominant in the host at a particular point in disease development. SIV and HIV clones that are derived directly from extracellular virus populations in biological fluids such as serum have not been characterised or evaluated for virulence *in vivo*. A key feature of lentivirus pathogenesis is a persistent high level cell-free viremia. Since the predominance of certain extracellular lentiviruses after seroconversion is most likely the result of escape from immune surveillance or escape from drug therapy during treatment, such viral variants are of particular biological interest. Recently we developed a strategy to generate pathogenic clones directly from extracellular virions present in the circulation, in serum or plasma (24, 25). Using this strategy we derived a full-length infectious molecular clone of SIV_{8980}, an end-stage isolate from a macaque which had progressed rapidly to AIDS following serial end-stage passage of SIV_{B670} *in vivo* (26). Sequence analysis revealed a unique relationship placing this virus between the two groups of SIV_{sm} and SIV_{mac} primate lentiviruses. During *in vivo* passage the variability of V1 decreased as virulence increased. The F359 molecular clone represented the most dominant variant that had emerged during end-stage passage. This variant was highly cytopathic and replicated to high titers *in vivo*. It was predominantly T-cell-tropic infecting but not replicating in macrophages. Of all the known co-receptors SIV_{F359} was selective for CCR5, but could still replicate in human PBMC homozygous for the Δ32 deletion, suggesting the additional use of a novel co-receptor.

5.3 MATERIALS AND METHODS

5.3.1 Molecular clone derived from serum

SIV_{8980} was derived from SIV_{B670} by four subsequent *in vivo* passages in Indian rhesus macaques. Monkey 8980 rapidly progressed to AIDS following the fourth *in vivo* passage (26). Serum from this animal was, without culture, directly used to derive the F359 molecular clone of SIV (24). Since the synthesis of full-length (10-kb) SIV cDNA molecules from small amounts of RNA templates proved to be very difficult, a modified RT-

PCR technique was developed to separately generate 5' and 3' halves of the SIV genome (25). By ligating these two 5-kb fragments we were able to reconstitute the F359 infectious molecular clone directly from serum as we have previously described (24).

5.3.2 Characteristics *in vitro*.

Primary rhesus PBMC cultures were maintained with medium change every two days and were observed regularly for CPE. Infection was confirmed by immunocytochemistry for the expression of SIV*gag* antigen. Single-cell preparations for immunocytochemistry were prepared on acetone cleaned glass slides, which had been air dried for 30 minutes. Cells were fixed in acetone/methanol (1:1) and in ethanol (70%) for 15 and 30 minutes, respectively. Slides were washed in 0.05 M Tris-HCl pH7.6, 0.1 M NaCl for 5 minutes and incubated with 20 µl (1:25 dilution) of anti-*gag* monoclonal antibody (47). Cells were washed for 5 minutes and incubated with goat anti-Mouse IgG antibody for 30 minutes at room temperature. To amplify the signal the cells are then washed and incubated with mouse anti-alkaline phosphatase (APAAP-complex, Boehringer). Cells were washed for 5 minutes and were incubated with 20 µl substrate solution: (10:1:1) 0.1 M Tris-HCl pH 9.5, NBT (nitro-blue tetrazolium), BCIP (5-bromo-4-chloro-3-indolylphosphate), 5 mM levamisole for 30 minutes at room temperature. Cells were washed for 5 minutes in tap water, one drop of (50%) glycerol / PBS was added per slide, and the cells was covered with a cover slip. The preparations were examined at a 40x magnification and CPE was quantified. In cell culture supernatants virus was quantified by measuring p27 concentrations (SIV p27 antigen capture ELISA, Coulter Corp., Hialeah, FL).

5.3.3 In vivo infection

Adult rhesus macaques (Macaca mulatta) used in this study were housed at the animal facility of the Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands. Animals were negative for SIV, STLV and Simian D type retroviruses. Two outbred Indian rhesus macaques were inoculated intravenously with 50 $TCID_{50}$ of the SIV_{smF359} stock grown on rhesus PBMC. EDTA-treated blood samples were collected every two weeks post infection for quantitative virus isolations (QVI) from PBMC and for determination of SIV p27 antigen in plasma. Rhesus monkeys that developed clinical evidence of AIDS were euthanised and full pathological analysis was performed to confirm the diagnosis. For histological examination, tissues were formalin-fixed and paraplast-embedded. Four-micron-thick sections were stained with hematoxylin and eosin (H&E). For the detection of microsporidia, a gram staining was applied on gall bladder sections.

For QVI, PBMC were prepared from EDTA-treated blood by lymphocyte separation medium (LSM) density gradient centrifugation. Cells at the interface were collected and washed twice with RPMI. Two-fold dilutions of PBMC (starting with $1x10^6$ cells) were co-cultured with 2.5×10^5 cells of the human T-cell line C8166 in a 24-wells plate (Greiner Labortechnik, The Netherlands) in duplicate. Cell culture medium (RPMI with 10% FCS) was partly changed twice a week. The cell cultures were screened regularly for the presence of CPE.

The phenotype of rhesus PBMC was assessed by two-colour FACS analysis. Briefly, heparinised blood (100 µl) was incubated with 10 µl of monoclonal antibody mix at room temperature. After incubation, 2.5 ml lysing solution (Becton Dickinson, Etten-Leur, The Netherlands) was added followed by an incubation at room temperature for 10 minutes, then centrifugation for 10 minutes at 200g. Four ml PBS with 2% formaldehyde were added and the tubes were centrifuged for 10 minutes at 200g. The supernatant was aspirated and the cells were resuspended in 5 ml PBS with 2% formaldehyde and stored overnight at 4°C. Flow cytometry was performed on a FACScan using the CellQuest software (Becton Dickinson, The Netherlands) and 5,000 events analysed. To assess CD4 T-cell levels in peripheral blood the following mAbs were used; anti-CD3 monoclonal (FN18, BPRC-Rijswijk) antibody covalently coupled to fluorescent isothiocyanate-phycoerythrin; and anti-CD4 (SK3, Becton Dickinson) monoclonal antibody covalently linked to phycoerythrin conjugate.

5.3.4 Cell tropism and co-receptor studies

To assess susceptibility to infection of resting and activated lymphocytes, blood was taken from healthy rhesus monkeys, which were negative for SIV, STLV and type D retroviruses. PBMC were isolated by lymphocyte separation medium density gradient centrifugation and were washed twice with RPMI. Activated lymphocytes were prepared by ConA mitogen stimulation (5 µg/ml, 48 hours) and interleukin-2 (IL-2) (50 units/ml, starting after virus adsorption and throughout the experiment). Resting lymphocytes were cultured in RPMI (+ 10% FCS) without PHA and IL-2. Resting and stimulated lymphocytes were distinguished by double labelling by anti-CD3 antibody, specific for T-cells and anti-MIB-1 antibody specific for the cellular proliferation marker Ki-67 (3, 14, 37). Resting and stimulated cell cultures ($5x10^6$) were simultaneously infected with 100 $TCID_{50}$ of $SIV_{mac239/YEnef}$ (a molecular clone capable of proliferating in resting cells) (14) or of the SIV_{F359} clone at 37°C for 18 hours. Unbound virus particles were removed by washing the cell pellets five times with 5 ml of RPMI (+ 10% FCS) and the cells were cultured for twelve days in RPMI (+ 10% FCS) either with or without IL-2 for stimulated or resting PBMC respectively. Supernatants were monitored for the production of virus p27 by antigen capture ELISA. The absence of Ki-67 staining was used to confirm that resting lymphocyte cultures remained in a quiescent state.

To determine if SIV_{F359} was able to infect rhesus MDM cultures, LSM isolated PBMC were seeded at a concentration of $5x10^6$ cells per ml in 24 well plates in RPMI with 10% FCS. Adherence was allowed for 5 days. Prior to infection non-adherent cells were separated from adherent cells by rigorous washing with culture medium. Adherent cells were checked for purity (> 98%) and for being macrophages by demonstrating the presence of the cell surface marker CD68 and by microscopic examination of their characteristic morphology. After infection with SIV_{mac316} (macrophage-tropic molecular clone of SIV as positive control) (44) or SIV_{smF359}, unbound virus was removed by washing the cells twice. Macrophage cultures were maintained for 14 days in RPMI 1640 medium supplemented with 20% FCS, penicillin and streptomycin with medium change once per week. At day 14

samples were analysed for intracellular *gag* expression and the presence of SIV p27 *gag* antigen in supernatants.

SIV p27*gag* expression in MDM cultures was studied by double staining immunocytochemistry. Briefly, cells were incubated with a mixture of the mouse anti-*gag* mAb 2E4 (IgG2a, kindly provided by Dr. M. Niedrig (47)) and mouse-anti-CD68 mAb KP1 (IgG1, DAKO, Glostrup, Denmark), which was used to co-stain macrophages. Consequently, slides were incubated with alkaline phosphatase conjugated goat anti-Mouse IgG2a subclass specific antibody (Southern Biotechnology Inc., Birmingham, UK) and horseradish peroxidase conjugated goat anti-Mouse IgG1 subclass specific antibody (Southern Biotechnology). All incubation steps were performed at 20°C for 30 minutes. Endogenous peroxidases were blocked with 0.1% NaN_3 plus 0.3% H_2O_2 in PBS after the incubation with the first antibody. Alkaline phosphatase activity was detected with naphthol-AS-MX phosphate (Sigma chemical Co., St. Louis, MO), and Fast Blue BB (Sigma) in 0.1 M Tris-HCl, pH 8.5 (20 minutes in dark) yielding a blue colour. Horseradish peroxidase activity was detected using H_2O_2 (0.03%) and 3-amino-9-ethylcarbazole (AEC, Sigma) yielding a red colour.

The co-receptor usage of the molecularly cloned virus was determined by three different assays. The first assay involved the use of HOS/CD4$^+$ cell lines expressing either the macaque or the human CCR5 and was based on immunostaining of SIV infected cells. The HOS/CD4$^+$ cell lines were infected with SIV_{smF359} by adding $1x10^4$ $TCID_{50}$/ml virus to the adherent cells in 3 ml of medium. After 72 hours, cells were analysed for syncytia formation, washed once in serum free medium, fixed in methanol / acetone (50 / 50) for 2 minutes at -20°C and washed twice in PBS supplemented with 1% FCS. Anti-*gag* mouse monoclonal antibody (0.6 ml / well) was added, incubated for one hour at room temperature and washed three times in PBS supplemented with 1% FCS. Goat anti-Mouse ß-gal conjugated polyclonal antibodies (0.6 ml / well) were added, incubated for one hour at room temperature and washed three times in serum free PBS. X-Gal substrate (0.6 ml / well) was added and the preparation was incubated in a sealed box for 30 minutes at 37°C. For quantification the stained cells were washed three times in PBS.

The second assay used the astroglia cell line U87 stably expressing human CD4 and either one of the chemokine receptors CCR2b, CCR3, CCR5 or CXCR4. These cells were seeded in 24 well plates at 2 x 10^4 cells per well in 1 ml of medium. Infection was performed overnight at 37°C with ten-fold serial dilutions of virus (1 ml final volume) beginning with a 1:8 dilution of the SIV_{smF359} stock. After infection, the cultures were washed three times with DMEM and cultured for 13 days. Medium was changed twice a week. Cultures were examined microscopically for cytopathic effect and supernatants, collected at several time points after infection, were tested for p27 concentration.

In the third assay several CD4 transformed human osteosarcoma HOS cell lines were used, expressing the chemokine receptors CCR1, CCR2b, CCR3, CCR4, CCR5, CXCR4, BOB/GPR15, Bonzo/STRL33, CXCR1 (V28), CCR8, APJ, GPR1 and US28 (the reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAD, NIH from Dr. Vineet, N. KewalRamáni and Dr. D. R. Littman). The CD4

transformed (under neomycin selection) HOS parental cells containing HIV-2 LTR driving green-fluorescence protein (GFP) introduced via co-transfection with the CMV promotor driving a hygromycin-resistant construct, were maintained in Dulbecco's modified Eagle Medium (DMEM; Gibco) supplemented with 10% FCS under selection of neomycin (G418 0,5 mg/ml; Gibco) and hygromycin (100 µg/ml). Co-receptor genes were introduced via retroviral infection with the pBABE-puro vector (10, 36) under the selection of puromycin (1 µg/ml; Calbiochem, La Jolla, CA). For cell-free infection experiments HOS-CD4 cells expressing the different co-receptors were seeded in 2 x 10^4 cells per well (2 ml) in 12 well plates and cultured in DMEM with 10% FCS. The next day, infection with the virus stocks (500µl/well) was performed in the presence of polybrene (20 µg/ml) during overnight at 37°C. After infection the cultures were washed and cultured for another day. Forty-eight hours after infection cells were analysed for GFP fluorescence with FACS.

5.3.5 DNA sequencing and phylogenetic analyses

Double stranded plasmid DNA containing the 10-kb SIV_{smF359} insert was used as a template for sequencing. DNA sequencing reactions were carried out using dye-primer chemistry and were executed on a LiCor automated DNA sequencer. The entire insert was sequenced from both directions. Nucleotide sequences were aligned using ClustalW version 1.7 (53). Alignments were examined and adjusted as necessary using the GDE (Genetic Data Environment) program (51). Regions of sequences that could not be unambiguously aligned were removed from subsequent analyses. Neighbour-joining phylogenetic analyses were conducted using the DNADIST and NEIGHBOR programs from version 3.5c of the PHYLIP package (16). Maximum likelihood (ML) analyses of *env* sequences from selected SIVs were performed using the PAUP* program (version 4.0.0d60, Swofford) as follows: initial ML estimates for the *env* tree were produced using a 2 substitution-type model (HKY model) without rate variation among sites. The topology of this tree was used as a starting topology for subsequent analyses. The shape parameter (alpha) for the gamma distribution describing rate variation among sites was estimated using the maximum likelihood method to be 0.22966. This value was used for the estimation of the parameters for the 6 substitution-type model (general time reversible model). The values estimated (a=2.499, b=10.46, c=1.278, d=1.746, e=9.962, f=1) were then in turn used to refine estimates. Final estimates for the parameters were a=2.499, b=10.44, c=1.278, d=1.745, e=9.95, f=1, alpha=0.23156.

5.4 RESULTS

5.4.1 Cell tropism *in vitro*

To determine the replicative properties and cell tropism of SIV_{F359} a series of *in vitro* assays were performed to compare the properties of this molecular clone, with the well-established characteristics of two other well-defined SIV_{mac} molecular clones. Mitogen stimulated PBMC cultures were used to assess virus replication in rhesus lymphocytes. All viruses tested (SIV_{F359}, $SIV_{mac239/YEnef}$, SIV_{mac239}) grew well in stimulated rhesus lymphocyte

cultures as measured by p27 concentrations in the supernatants (Figure 1A). These data indicate that all viruses including SIV_{F359} were able to infect and replicate in stimulated rhesus lymphocytes. Subsequently we determined the ability of SIV_{F359} to replicate in resting rhesus lymphocytes, a capability that has been reported for SIV_{smmPBj} (17) and $SIV_{mac239/YEnef}$ (14). Virus production was only observed in resting cell cultures infected with $SIV_{mac239/YEnef}$ at day six post-infection producing 1.6 ng/ml of p27 in the supernatant, which increased to 12 ng/ml p27 / ml by day twelve post-infection (Figure 1B).

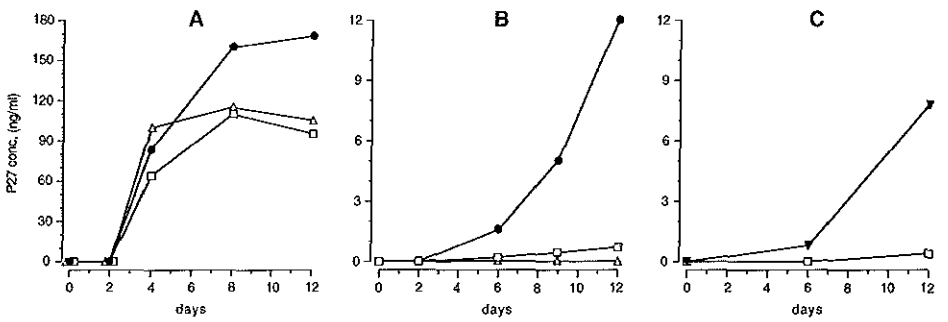

Figure 1. Production of SIV p27 antigen (ng/ml) in stimulated (A), resting (non-stimulated) rhesus lymphocyte cultures (B) and MDM cultures (C). Cultures were infected with equivalent amounts of either SIV_{F359} (□), $SIV_{mac239/YEnef}$ (●), SIV_{mac239} (Δ), or SIV_{mac316} (▲).

Infection of monocyte derived macrophage (MDM) cultures with SIV_{F359} or SIV_{mac316} yielded detectable virus production only upon infection with SIV_{mac316} with p27 concentrations in cell supernatants increasing from 0.8 to 7.8 ng p27/ml, from day six to day twelve respectively. During the entire experimental period virus production in supernatants could not be detected in cultures infected with SIV_{F359} (Figure 1C). The absence of SIV_{F359} in the supernatants of MDM cultures was concluded to be due to the inability to either, 1) infect, or 2) replicate efficiently in this cell type. MDM cultures were double stained immunocytochemically for the presence of both the macrophage marker CD68 and the viral antigen p27. Analysis of stained cells indicated that SIV_{F359}, although not able to produce detectable amounts of p27 in supernatant, did infect macrophages and resulted in the expression of *gag* protein (Figure 2A). In contrast SIV_{mac316} not only infected MDM (Figure 2B) but also replicated well producing substantial p27 concentrations in macrophage cultures (Figure 1C). In summary, these results were reminiscent of previous studies of late-stage variants in humans and macaques (30) indicating that the molecular clone SIV_{F359} was similar to cytopathic (syncytium inducing), rapidly replicating and predominantly T-cell-tropic variants but retained the ability to infect monocyte/macrophages.

5.4.2 *In vivo* infection

To investigate if the molecular clone SIV_{F359} was infectious and pathogenic *in vivo*, two rhesus macaques (L52 and WT) were intravenously infected with 100 $TCID_{50}$ of SIV_{F359} propagated in rhesus PBMC. $CD4^+$ T-lymphocytes were monitored by FACS analysis and fluctuations in virus loads were measured by Quantitative Virus Isolation (QVI) and are

depicted in Figure 3. For animal L52, these parameters were determined at two-week intervals. The same measurements were performed for WT at weekly intervals during the first month, subsequently at two week and at monthly intervals. Animal L52 developed a virus load of 1024 virus producing cells/10^6 PBMC two weeks after infection. Decreased values (524 virus producing cells/10^6 PBMC) were detected 4 and 6 weeks after infection and dropped further to 128 virus producing cells/10^6 PBMC at weeks 8, 10 and 14 post-infection A second peak of viremia, which reached 514 virus producing cells/10^6 PBMC, was observed 15 and 17 weeks after infection. At weeks 19 and 21 virus loads decreased again to 256 and 48 infected cells per 10^6 PBMC, respectively. A third peak, which was higher than the previous ones, was observed at the end of the experimental period, from week 23 till week 37: virus loads as high as 2024 infected cells per 10^6 PBMC at week 32 were detected (Figure 3A). The presence of this peak coincided with the development of severe anemia, diarrhea and the start of extensive weight loss. L52 was euthanised after 37 weeks of infection.

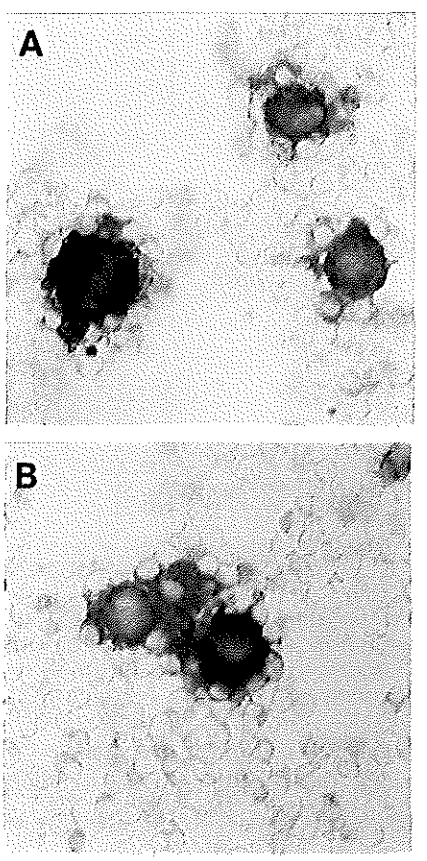

Figure 2. Photomicrographs of infected MDM cultures infected with SIV_{mac316} (A) and SIV_{F359} (B); infection is demonstrated by $CD68^+/p27^+$ double staining. Photomicrographs 660x magnification.

The first positive virus isolation from monkey WT was observed already one-week post infection (p.i): 512 virus producing cells per 10^6 PBMC were measured. This value increased to 1024 virus producing cells/10^6 PBMC at week two and three post-infection but dropped over time to 64 virus producing cells/10^6 PBMC as determined at week 8 after infection. Relatively low virus loads, fluctuating between 256, 64, 128 and 16 virus producing cells/10^6 PBMC, were found 10, 12, 14, 16 and 19 weeks post-infection. An increase in virus loads from 700 to 4048 virus-producing cells/10^6 PBMC was then detected from week 23 to week 41, respectively. During the last 22 weeks (till week 68) virus loads stayed at a constant level of 1012 virus producing cells/10^6 PBMC (Figure 3B). WT was euthanised after 68 weeks.

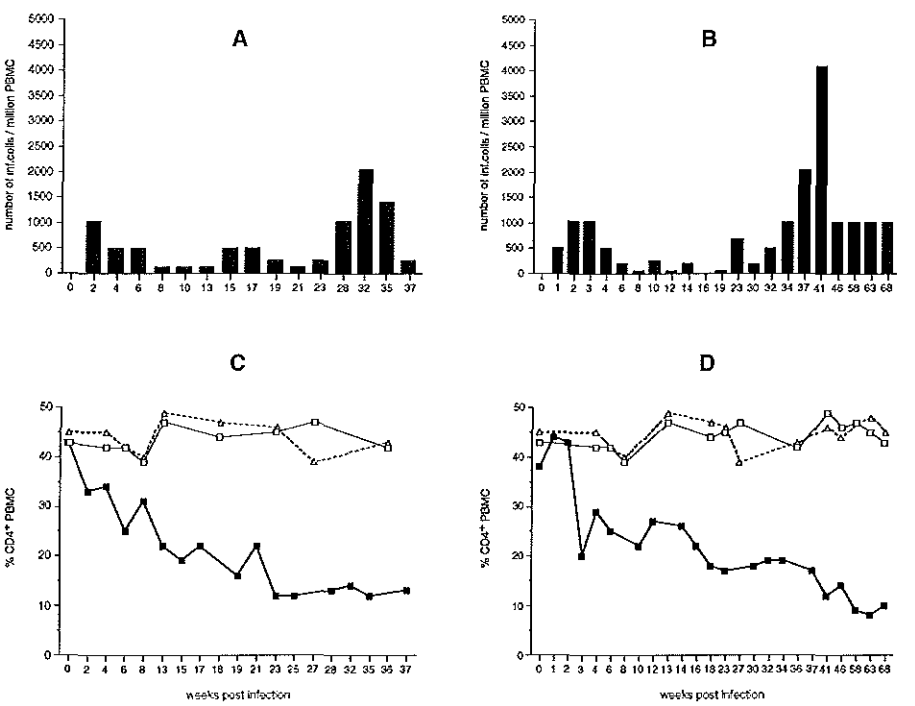

Figure 3. Virus load (A,B) and changes in CD4⁺ lymphocyte populations (C,D) of rhesus monkeys WT (A,C) and L52 (B,D) after infection with molecularly cloned SIV_{F359}. Progressive decline of CD4⁺ T-lymphocytes in infected rhesus monkeys (WT, L52) as compared to control monkeys (8637, 8711) represented by (Δ) and (□) respectively.

Histopathology revealed moderate catharrhalic enteritis, severe lymphoid hyperplasia in spleen and lymph nodes with moderate atrophy of splenic follicles. CD4 cell numbers of inoculated animals were compared to two uninfected rhesus monkeys of the same sex, age and body weight. The two infected animals showed a decrease in CD4⁺ T-cell lymphocytes during the course of the experiment (25% for L52 (Figure 3C) and 21% for WT (Figure

3D). $CD4^+$ T-lymphocytes cultured from these animals were positive by immunocytochemical staining for p27 antigen expression (data not shown). The uninfected animals had stable CD4 cell counts as expected.

In both animals histopathological findings were consistent with the diagnosis of AIDS. Cryptosporidial enteritis without remission as seen in WT is observed in advanced immunodeficiency (42) and is one of the criteria for the diagnosis of AIDS in man. In L52, a cholecystitis due to a microsporidial infection was identified. The etiologic organism has only recently been detected in SIV-infected rhesus monkeys and was classified as *Enterocytozoon bieneusi*-like microsporidial protozoan. *Enterocytozoon bieneusi* is a common opportunistic pathogen of AIDS patients causing significant morbidity.

5.4.3 Co-receptor usage

To further correlate the observations of cell tropism, the co-receptor use of SIV_{F359} was characterised. Infection experiments with SIV_{F359} virus were performed on HOS/$CD4^+$ cell lines expressing either the macaque or human CCR5 co-receptor. Both cell lines were highly susceptible to infection with SIV_{F359}, and almost 90% of the cells formed syncytia, which stained positive for SIV p27 antigen. To investigate the ability of SIV_{F359} to use additional co-receptors, the extent of its replication in GHOST cells and U87MG-CD4 expressing CCR1, CCR2b, CCR3, CCR4, CCR5, CXCR4, BOB/GPR15, Bonzo/STRL33, CX3Cr1 (V28), CCR8, APJ, GPR1 and US28 co-receptors (11, 21) was assayed in parallel with the well characterised SIV_{mac239} and SIV_{mac316} molecular clones. All three viruses were found to use CCR5 for infection (Table 1), as was determined by FACS analysis while SIV_{F359} proved to be unable to use any other co-receptor than CCR5 at standard doses. This observation taken together with the capability of SIV_{F359} to infect T-cells and macrophages corroborates previous findings that the CCR5 receptor is used by both M-tropic and T-tropic HIV-1 (7, 15). Interestingly, both SIV_{mac239} and SIV_{mac316} used multiple co-receptors including BOB/GPR15 and Bonzo/STPR in contrast to SIV_{F359}. Furthermore, the macrophage-tropic molecular clone SIV_{mac316} used as a fourth co-receptor CCR3 (Table 1). Finally, to determine if the SIV_{F359} clone was truly restricted to only CCR5 co-receptor use, we cultured SIV_{F359} on human PBMC homozygous for the 32 deletion that renders these cells resistant to viruses, which only use the ΔCCR5 co-receptor. Interestingly, although SIV_{F359} was unable to use any of the other known co-receptors it could replicate on these cells, while other CCR5 restricted isolates could not. At much higher doses there was evidence that SIV_{F359} could also infect cells bearing BOB, but it was unlikely that these were physiological concentrations of virus. These data suggested that the SIV_{F359} clone may additionally utilise a novel, previously undescribed co-receptor for entry.

Table 1. Comparison of co-receptor use and cell tropism of the well-defined T-cell-tropic and M-tropic SIV_{mac} strains compared to SIV_{F359}. Infection of SIV_{mac239}, SIV_{mac316} and SIV_{F359} in HOS/CD4$^+$, GHOST cells and U87 cell lines stably expressing the human co-receptors listed (partial list) in this table.

Co-receptor use:	Properties:	Virus clone		
		mac239	mac316	smF359
	M-tropism/infection	-	+	+
	M-tropism/productive	-	+	-
CCR1		-	-	-
CCR2B		-	-	-
CCR3		-	+	-
CCR4		-	-	-
CXCR4		-	-	-
CCR5		+	+	+
BOB/GPR15		+	+	-
Bonzo/STPR		+	+	-

5.4.4 Is SIV_{F359} representative of the predominant variant following *in vivo* passage?

The first hypervariable region (V1) represents the most variable region of the SIV genome and therefore it provides a sensitive indicator of the genotypic variation present within a quasi-species of an infected animal. Trichel et al identified twelve different genotypes in the SIV_{B670} inoculum based on sequence analyses of the V1 region (54). The V1 regions derived from the viral variants emerging during the *in vivo* passage (26) were determined (56) and compared with the V1 sequences of the viral variants that were present in the original SIV_{B670} inoculum (54). Homologies were quantified by using software that calculated the actual "alignment fit" as the fraction of the optimal fit. SIV_{B670}-CL12 emerged as the predominant variant from the original SIV_{B670} infection and represented the highest genome stability during the passage experiment (Figure 4). Clones obtained during the subsequent passages represented viral variants that were more closely related to SIV_{B670}-CL12 than to any other B670 clone (Figure 4). This indicated that SIV_{B670}-CL12 possessed the optimal fitness for replication in both B670 and the animals it was transmitted to. Indeed, based on our observations of high persistent virus loads in the most rapidly progressing animals (26) certain variants may have acquired mutations making them relatively resistant to neutralisation (30). V1 sequences derived from passage samples were compared to the SIV_{B670}-CL12, which had shown the highest homology with evolving variants during the passage study. Specifically, during the subsequent passages the number of different V1 clones decreased from four (out of four) to two (out of four) (Figure 4). These findings demonstrated that during *in vivo* passage there was a selection for the most fit variants (30). The molecular clone SIV_{F359} represented the dominant variant and had retained the identical V1 sequences observed in P4.1, P4.2 and P4.3 envelope clones (Figure 5).

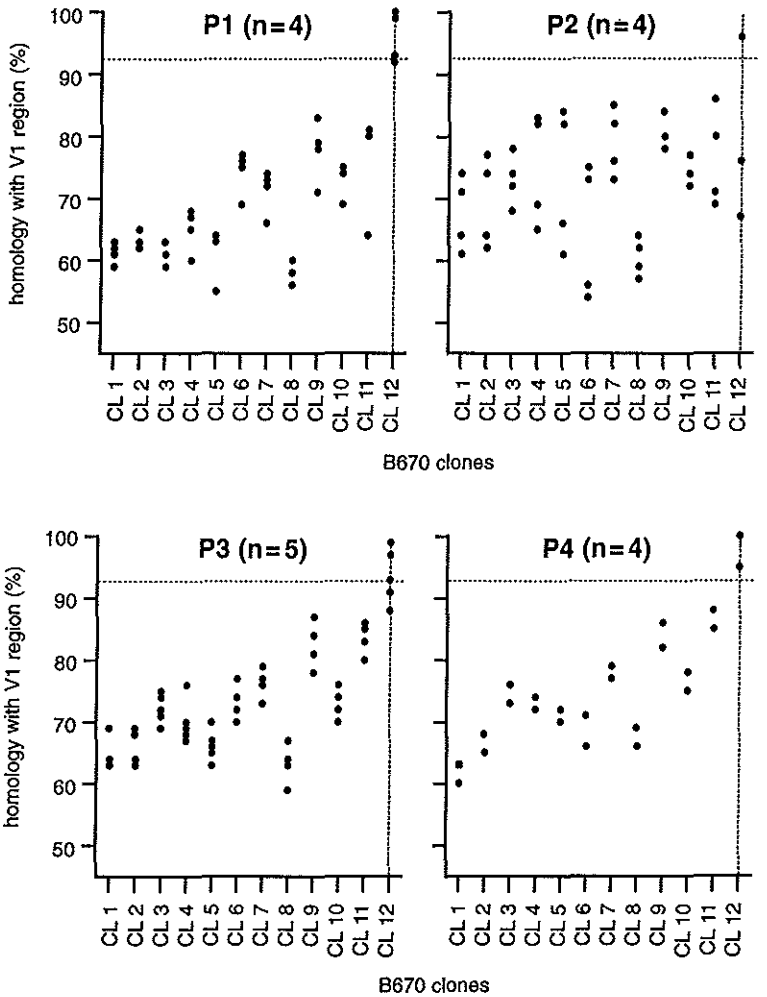

Figure 4. Comparison of V1 sequences of envelope clones that emerged during the *in vivo* passages (P1 to P4) to obtain SIV_{8980} from which the molecular clone SIV_{F359} was derived, and the V1 sequences of the viral variants (CL1 to CL12) which were present in the original SIV_{B670} inoculum (54, 56).

5.4.5 Phylogenetic analysis of SIV_{F359}

Phylogenetic analysis of *env* sequences using the neighbour-joining and GTR maximum likelihood method (16) confirmed that SIV_{F359} clustered together with SIV_{sm} clones such as SIV_{sm9} and $SIV_{smmPBj4.41}$ and was not closely related with SIV_{mac} clones such as SIV_{mac251}, SIV_{mac239} and $SIV_{mac1A11}$. As could be expected from the origin of SIV_{F359}, this clone was most closely related to but distinct from SIV_{B670} (envelope sequence only available) (Figure

6A). SIV_{B670} had been isolated from a rhesus macaque infected with material containing SIV from a sooty mangabey housed at the Tulane Primate Centre (45). These two viruses branch separately from other characterised SIV_{sm} viruses, which had also been derived from sooty mangabeys but originating from the Yerkes primate colony (17). Interestingly, these viruses formed a unique cluster of strains which branched separately from those macaque adapted isolates derived from the New England (mac251, mac239) and California (mac1A11) Regional Primate Centres (35). Neighbour-joining phylogenetic analysis performed on the entire sequence showed similar relationships within the SIV cluster (Figure 6B). The predicted amino acid sequences of SIV_{F359} proteins were more similar to those of SIV_{smH4} (87%) and $SIV_{smmPBj4.41}$ (88%) than to those of the SIV_{mac} cluster (83%) (Table 2). The greatest similarity with other SIVs was present in the *gag* (average 92%), *pol* (average 93%) and *vpx* (average 92%) genes, whereas the greatest divergence was seen in *nef* (average similarity 75%) and *tat* (average similarity 72%) genes (Table 2).

Table 2. Predicted amino acid similarity of SIV_{F359} compared to other SIV molecular clones.

F359	% Similarity								
protein	B670	SmH4	smmPBj4.41	mac239	mac1A11	SIVmac	Stm	Mne	mean
Gag	-	96	95	90	91	92	88	93	92
Pol	-	97	95	92	91	92	91	93	93
Vif	-	93	89	84	84	85	78	84	85
Vpx	-	94	92	94	94	93	88	88	92
Vpr	-	81	96	87	84	86	88	89	87
Tat	-	75	80	65	80	75	69	62	72
Rev	-	78	81	77	76	75	-	80	78
Env	95	88	86	83	80	78	83	83	84
Nef	-	81	75	78	74	66	77	75	75
mean		87	88	83	84	82	83	83	

5.4.6 Virulence of the SIV_{F359} molecular clone

To determine if the SIV_{F359} molecular clone had retained the same pathogenic potential as the parental SIV_{8980} isolate, an additional eight animals were infected with the same dose of the SIV_{F359} virus stock. As illustrated in figure 7, animals infected with the SIV_{F359} stock had very similar survival curves as compared with the SIV_{8980} stock from which it was derived, and was much more pathogenic than the original pre-passage SIV_{B670} isolate. The molecular clone was slightly less pathogenic, as could be seen by the slight shift of the survival curve to the right. This however could be a consequence of the genetically homogeneous nature of the SIV_{F359} inoculum as compared to the more heterogeneous SIV_{8980} inoculum, which represented a highly virulent quasispecies. Indeed, a heterogeneous quasispecies is more likely to escape from the immune responses of an outbred host.

```
P1  (n=4)
B670 CL12   WGLTGNAATTTTTTTTASTTTP.KGRADVVNETSSCVKNNNCTGLEQEP
P1.1        ..................A...S.........................
P1.2        ......................EE........P..............
P1.3        .........I.....................................
P1.4        ............I..................................

P2  (n=4)
B670 CL12   WGLTGNAA.TTTTTTTTASTTTPKGRADVVNETSSCVKNNNCTGLEQEP
P2.1        ......VEA.............S.EE.......................
P2.2        ......VEA.........-----.........................
P2.3        ..................-.............................
P2.4        ..............I.................................

P3 (n=5)
B670 CL12   WGLTGNAATTTTTTTTASTTTPKGRADVVNETSSCVKNNNCTGLEQEP
P3.1        ............I...-.....EE........................
P3.2        ..................-.............................
P3.3        ................--........N.....................
P3.4        ............I...........N.....................
P3.5        ..................-........N.....................

P4 (n=4)
B670 CL12   WGLTGNAATTTTTTTTASTTTPKGRADVVNETSSCVKNNNCTGLEQEP
P4.1        ..................-.............................
P4.2        ..................-.............................
P4.3        ..................-.............................
P4.4        ........................N.....................
```

Figure 5. Amino acid sequence alignments of the V1 envelope region of the predominate clone 12 (CL12) in the pre-passage SIV_{B670} inoculum. This sequence persisted through the passage as the diversity of the inoculum decline (figure 4). The CL12 V1 sequences are compared with the sequences of clones derived from the 4 different passages (P1 to P4). Clone P4.3 represents the same V1 sequence found in the molecular clone, F359, and which was originally found in CL12 within the quasispecies found in the pre-passage SIV_{B670} inoculum.

5.5 DISCUSSION

We have utilised a novel cloning strategy to derive an infectious pathogenic molecular clone of a primate lentivirus without cell culture passage directly from serum. This molecular clone from serum (SIV_{F359}) has unique properties. It is highly cytopathic *in vitro* and causes marked syncytia in lymphoid tissues *in vivo*. It readily infects activated but not resting rhesus lymphocytes. Infection of MDM cultures was demonstrated, however, in contrast to SIV_{mac316} no virus production in the supernatant was detectable. Co-receptor use of this clone was restricted to only CCR5 in contrast to that of other well-characterised SIV_{mac239} (T-cell-tropic) and SIV_{mac316} (macrophage-tropic) molecular clones studied for comparison. Suprisingly, although this virus did not use any of the know 13 primate lentivirus co-receptors it could replicate on human cells defective for CCR5.

The use of molecular clones derived from extracellular virions may provide additional insight into the evolution of the lentiviral pathogen. For instance, this cloning strategy may aid the analysis of the specific biological properties of viral variants in plasma or serum, which have escaped host immune responses at a specific point in time. Similarly, the study

of virus populations, which arise and become predominant in various extracellular compartments is feasible using this methodology. Studying the evolution of viruses using this strategy has a number of important advantages over conventional techniques that involve cloning proviruses from infected lymphocytes.

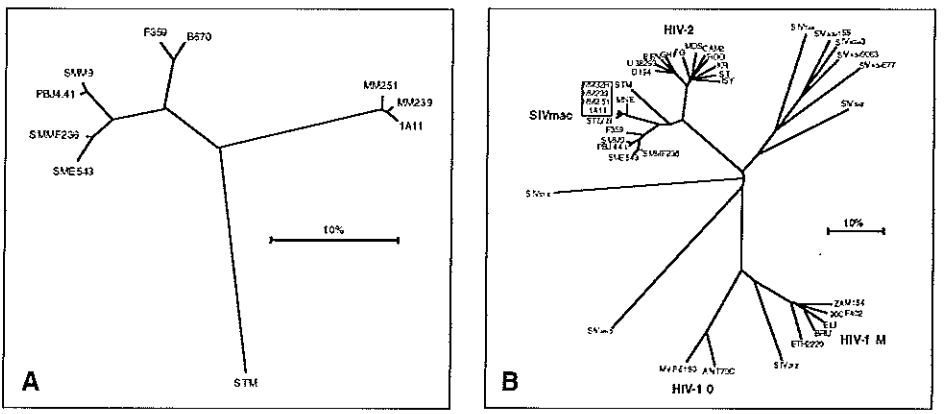

Figure 6. Phylogenetic trees based on the SIV_{smF359} sequence as compared with (A), envelope sequences from the parental SIV_{B670}, and as compared to less related SIV_{mac251}, SIV_{sm} and SIV_{stm} sequences. Tree was constructed using a GTR maximum likelihood analysis. Based on full-length sequences (B) phylogenetic analysis comparing the SIV_{smF359} molecular clone to other SIV, HIV-1 and HIV-2 clones. Phylogenetic trees were constructed by the neighbour-joining method using the PHYLIP program (version 3.5c).

The SIV_{F359} molecular clone was derived from a rhesus macaque, which had developed, AIDS following the fourth *in vivo* passage of virus derived from SIV_{B670}. The virus isolate SIV_{B670} originated from one of the first reports of simian AIDS in macaques, since has been used in a wide variety of studies (1, 4, 45). Phylogenetic analyses using available *env* genes or the entire SIV genomes revealed that SIV_{B670} and SIV_{smF359} branch off between two separate clusters of SIV_{sm} clones, represented by SIV_{smm9} and $SIV_{smmPBj4.41}$, derived from the Yerkes Primate Colony, and SIV_{mac} clones, such as SIV_{mac251} and SIV_{mac239}, derived from the New England Primate Colony (figure 6). As could be expected from the origin of SIV_{F359}, this clone was related to but distinct from SIV_{B670}. Originally SIV_{B670} had been isolated from a rhesus macaque (B670) infected with material containing SIV originally derived from a sooty mangabey from the Tulane Primate Centre (45). Using the entire sequence for phylogenetic analysis SIV_{F359} was found to branch separately between other characterised SIV isolates derived from sooty mangabeys or macaques (figure 6). The characteristics of the envelope evolution of SIV_{B670} during serial passage to animal 8980 from which SIV_{F359} was derived have recently been described (56).

In summary, we have utilised a new cloning strategy to generate infectious molecular clones of lentiviruses from extracellular viral RNA in body fluids. This has facilitated the isolation of a unique pathogenic molecular clone designated SIV_{F359}. Characterisation of SIV_{F359} *in vivo* and *in vitro* revealed that it was highly pathogenic causing marked syncytial

giant cell formation *in situ* in lymph nodes and CPE in rhesus $CD4^+$ T-cells *in vitro*. It was found to have limited and novel co-receptor usage (CCR5 and a yet undefined co-receptor) and could infect but not replicate in rhesus MDM. Nevertheless, it proved to be able to cause a rigorous infection and AIDS in rhesus macaques. It was genetically distinct from other molecular clones, mapping between two clusters of previously characterised clones (the sm and mac molecular clones respectively). The SIV_{F359} molecular clone may provide further insight into the pathogenesis of AIDS in the macaque model, and in particular to address specific questions such as the evolution of co-receptor use, cell tropism and disease progression.

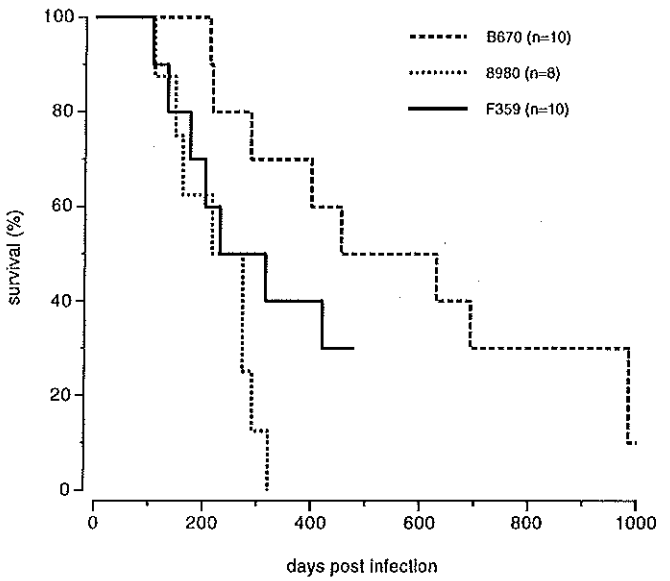

Figure 7. Comparison of survival of groups of animals infected with the original pre-passage SIV_{B670} isolate, the post-passage SIV_{8980} isolate and the subsequently derived F359 molecular clone as illustrated by the Kaplan-Meier plot.

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CHAPTER 6

THE PREDOMINANCE OF ATTENUATING SIV *NEF* VARIANTS CORRELATES WITH THE MAINTENANCE OF LOW VIRAL LOADS AND AN ASYMPTOMATIC DISEASE COURSE

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6.1 ABSTRACT

The *nef* gene has been demonstrated to be an important viral factor in the pathogenesis of lentivirus induced AIDS in primates. Several studies have revealed that in rhesus macaques infected with SIV_{mac} molecular clones with attenuating point mutations in *nef*, the virus quickly reverts to wild-type resulting in progression to disease. Furthermore, the repair of defective *nef* genes is often correlated with an increased ability to re-isolate virus. It remains unknown, however, if the mutated virus variants persist in the host and to what degree they may influence disease progression. A particularly interesting *nef* mutation resulted in a TAA stopcodon at amino acid position 107 in a novel molecular clone of SIV_{8980} designated F359. To determine the kinetics of reversion of *nef* and if reversion was associated with an increase in viral RNA in plasma, ten rhesus monkeys (5 juvenile and 5 mature) were simultaneously infected with the same dose of the molecular clone SIV_{F359}. Individual *nef* clones isolated from plasma of the various monkeys were used to analyse the correlation of *nef* reversion with virus load and disease progression. Half of the animals developed high virus loads and progressed rapidly to AIDS-like disease. The remaining five were either intermediate or non-progressors. An important correlation between the predominance and persistence of this specific *nef* variant in plasma and the subsequent course of disease was found.

6.2 INTRODUCTION

Unlike the genes *gag, pol, env, tat* and *rev* the accessory gene *nef* is dispensable for HIV and SIV replication *in vitro* (2, 8, 11, 15, 25). Functional *nef*, however, is an important virulence factor *in vivo* and thus facilitates the development of the full pathogenic potential of HIV-1 (3, 14) and $SIV_{sm/mac}$ (12).

Nef down-modulates the cell surface expression of the CD4 glycoprotein which is the receptor for both $SIV_{sm/mac}$ (1, 7) and HIV-1 (9, 17, 23). Experiments with cells over-expressing CD4 receptors indicated that an excess of CD4 receptor on the T-cell surface impairs virus release. Therefore, the observation that Nef has an enhancing effect on the rate of viral spread and infectivity may in part be explained by the specific down-regulation of the CD4 receptor. Several studies yielded evidence that Nef down-regulates also MHC class I genes. *In vivo* this may facilitate an increase in virus load by allowing infected cells to escape from CTL directed lysis and to persist while continuously producing virus.

The *nef* gene in both the HIV and SIV genome is located at the 3' end of the *env* gene. It extends into the U3 region of the 3' long terminal repeat (LTR) and encodes a 27- to 34-kDa myristoylated protein that is produced early after virus infection (13, 20). Myristoyation influences the intracellular localisation of Nef during the virus life cycle.

Nef of $SIV_{sm/mac}$ and HIV-1 binds to cellular kinases, tyrosine kinases (HCK and LCK) as well as serine/threonine kinases (16, 22). Since these enzymes are known to be involved in signal transduction and cell activation pathways, this strongly suggests that the various effects of Nef may in part be brought about by modulation of cellular regulatory processes.

The elucidation of the mechanisms of action by which Nef exerts its functions is complicated by the fact that the activities of different *nef* alleles vary considerably (17, 18). In fact, Nef proteins of various viral isolates differ significantly in their biological properties (10) and different domains of Nef mediate different functions. Several variants of Nef can exist in a viral quasispecies in a host at a particular point in time. In this study we wanted to obtain support for the hypothesis that the predominant variant, which is expressed by the most actively replicating virus in circulation, may have the most significant biological effect in the host and will determine the clinical outcome.

To address this question ten rhesus monkeys were infected with a novel molecular clone encoding a Nef protein being truncated due to a premature stop codon located after the PKVP motif. The time point of appearance and proportion of *nef*-open revertants of the attenuated *nef* variants in circulation (plasma) were found to be decisive factors for the subsequent disease course.

6.3 MATERIALS AND METHODS

6.3.1 Animals

A total of ten rhesus monkeys were used, one group consisting of five young (1.5 years of age) and a second group consisting of five mature animals (age > 4 years). All animals were from an outbred stock of Indian origin and were captive-bred at the Biomedical Primate Research Centre (BPRC). All animals were negative for STLV-1, Herpes B, CMV and Type D retroviruses. Animals were euthanized upon evidence of AIDS-like disease.

6.3.2 Infection and monitoring of macaques

Approximately $5x10^6$ C8166 T-cells were transfected with 10 µg of plasmid DNA containing the full-length molecular clone SIV_{smF359} by electroporation. After the pulse, cells were resuspended in RPMI supplemented with 10% FCS andwere incubated at 37 C. The supernatants of wells containing syncytia were harvested on day 12. The 50% tissue culture infectious dose ($TCID_{50}$) of stored aliquots (-80 °C) was determined on C8166 cells. The two groups of animals were inoculated intravenously with 100 $TCID_{50}$ of SIV_{F359}. From each inoculated macaque blood samples were obtained weekly through the first 8 weeks after inoculation and monthly thereafter. The health status of the macaques was carefully monitored for the duration of the experimental period by measuring hematology and clinical chemistry parameters, and by analysing their $CD4^+$ T lymphocyte levels by FACS as previously described (19). In order to monitor viral loads in PBMC, virus isolations were performed by co-culturing primary PBMC from each time point with C8166 indicator cells. Viral RNA levels in plasma were quantitatively measured by a RT-PCR assay (24). Each macaque was carefully monitored for clinical evidence of disease progression. The clinical diagnosis of simian AIDS was confirmed by autopsy for each monkey.

6.3.3 RT-PCR and sequencing of mutated regions of *nef*

Heparinized-blood samples were collected and viral RNA present in plasma was isolated using the Qiagen viral RNA isolation kit (Qiagen GmbH, Germany). First strand cDNA molecules were synthesised using primer *nef-1*: 5'-CTAAGATTCTATGTCTTCTATC-ACT-3'. PCR amplifications were carried out on 2 µl of the cDNA reaction as template in 100 µl PCR reaction mixtures containing 10 mM Tris-HCL (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each nucleoside triphosphate, 100 nM (each) oligo-primers *nef-1* and *nef*-2: 5'-CTGGTTCCAAGAAGCAG TCCAAG-3', and a mixture of 0.7 units of both the AmpliTaq and *Pfu* polymerases (DE Applied Biosystems and Stratagene, The Netherlands). Reaction mixtures were overlaid with 100 µl of mineral oil and subjected to 35 cycles of amplifications (95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute) followed by 72°C extensions for 10 minutes (first round). Five microliters from each reaction mixture were transferred to new reaction tubes containing PCR reaction mixtures plus 100 nM of primers *nef*-4: 5'-GTGGAGAGACTTATGGGAGAC-3' and *nef*-5: 5'-CTATAAAATATC-CCTTCCAGTCC-3'. The mixture was then reamplified for another 35 cycles under the same conditions. *Nef* amplimers were isolated from agarose gels and cloned into *Srf* pre-digested pScript SK+ (Stratagene, The Netherlands). Recombinant clones were propagated in the *E.coli* DH5 strain. Sequencing of the cloned insert was performed with pUC/M13 forward and reverse sequencing primers which bind on either site of the cloned inserts. Analysis was done on an ABI PRISM 310 Genetic Analyzer (DE Applied Biosystems, The Netherlands).

6.3.4 Determination of virus load

Quantitative RNA PCR was used to estimate the virus load in plasma as described recently (24). In brief, RNA was extracted from 200 µl EDTA plasma using guanidine isothiocyanate-mediated lysis, followed by propanol-2 precipitation. A known amount of internal standard RNA (IS-RNA) was added before the RNA extraction and was co-purified to monitor the efficiency of the purification. The RNA was reverse transcribed and amplified in a single reaction using *rTth* DNA polymerase and biotinylated primers. The IS-RNA was co-amplified to monitor the amplification efficiency. The amplified fragments were denatured and hybridized to an immobilised capture probe in a microwell plate. They were detected by an avidin-enzyme conjugate-mediated colorimetric reaction. The amplified internal standard was hybridized to a different capture probe in separate microwells. The lower detection limit of this RT-PCR method was 40 RNA equivalents per ml plasma.

6.3.5 Quantitative virus isolation

PBMC were isolated from EDTA blood samples by lymphocyte separation medium (LSM) density gradient centrifugation. Cells at the interface were collected and washed twice with RPMI. Two-fold dilutions of PBMC (starting with $1x10^6$ cells) were co-cultured with $2.5x10^5$ cells of the human T4 lymphocyte cell line C8166 in a 24-well plate in duplicate. Cell culture medium (RPMI with 10% FCS) was changed twice per week. The cells were scored weekly for the presence of cytopatic effects and the concentration of SIV p27 antigen was determined after 14 days of culture.

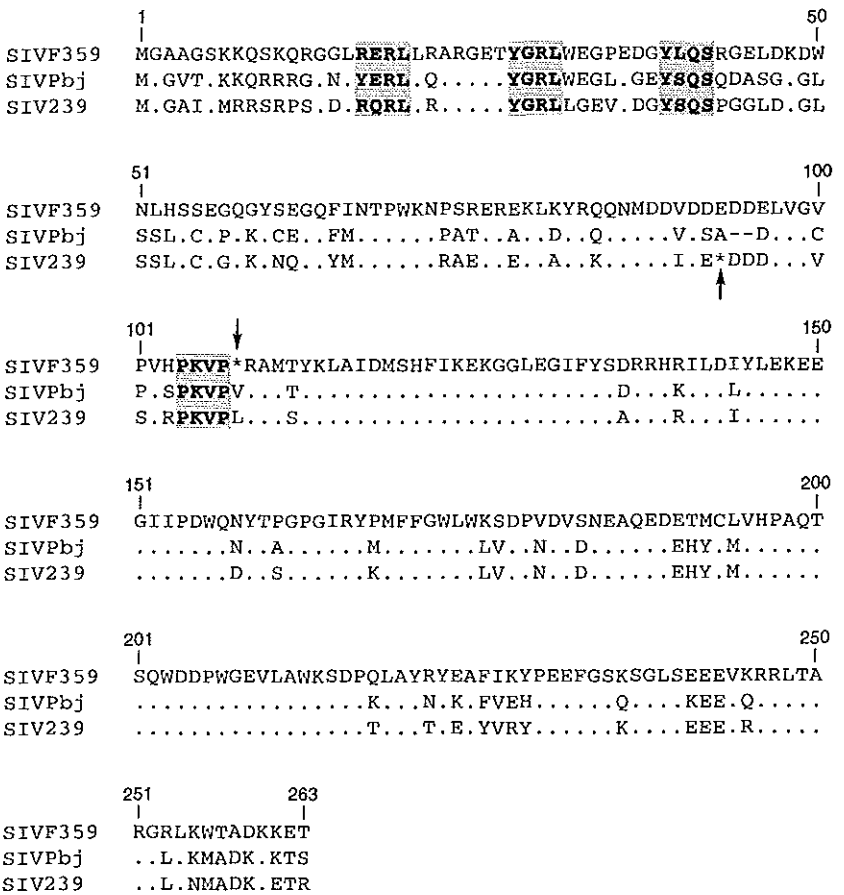

Figure 1. Comparison of amino acid sequences of the entire Nef protein from the molecular clones SIV_{F359}, $SIV_{smm\ PBJ14}$ and $SIV_{mac\ 239}$. A dot indicates amino acid identity; a dash indicates a gap in the sequence. The arrows indicate the presence of premature stop codons (*) in *nef* of SIV_{F359} and $SIV_{mac\ 239}$.

6.4 RESULTS

One of the infectious molecular clones derived from SIV_{89SO_4} designated SIV_{F359} encodes a truncated Nef protein due to a premature stop codon (TAA at codon 107) in the *nef* gene (Figure 1). A pilot experiment with two adult rhesus monkeys had demonstrated a rapid reversion of the stop codon to a leucine encoding one (TTA) and had revealed the pathogenic potential of reverted SIV_{F359} in rhesus monkeys. To determine, if the reversion of the *nef* mutation influenced virus load and if this was influenced by the age of the host, we infected a total of ten Indian rhesus monkeys with the SIV_{F359} molecular clone carrying the same *nef* mutation. Five young (age ≤ 1.5 years) (95012, 95021, 95032, 95038 and 95047) and five adult (age > 4 years) rhesus monkeys (14B, 40B, 58A, 88A and 97A) were

each infected with 100 $TCID_{50}$ of the SIV_{F359} *nef*-stop mutant. After infection the number of virus producing cells in circulation, plasma RNA viral load, $CD4^+$ T-cell levels and the frequency of reversion of the *nef* stop codon were determined. These findings were compared with the clinical and pathological outcome for each animal.

Infection resulted in AIDS-like disease of four (95047, 95012, 95021 and 95038) out of five juvenile monkeys and two (88A and 14B) out of five adult monkeys (Figure 2). This difference was, however, not significant according to Fisher's exact analytical test. Those five of the ten animals which developed a rapid disease course are subsequently referred to as rapid progressors (RP). A sixth animal, 95038, developed disease relatively slowly and was euthanized at week 64 because of signs of disease, and was designated as an intermediate progressor (IP). The four remaining animals, namely one (95032) out of the five young monkeys and three (40B, 58A and 97A) out of the five adult monkeys, did not show any clinical or pathological evidence of disease development when euthanized at the end of the experimental period (68 weeks). These four monkeys were designated as long-term survivors (LTS).

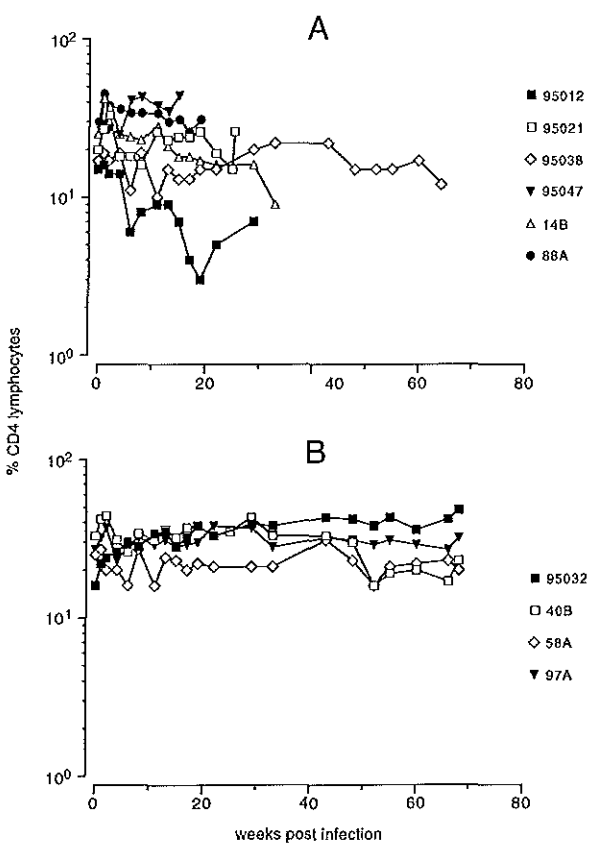

Figure 2. The kinetics of $CD4^+$ T-cells in the peripheral blood of SIV_{F359}-infected rhesus monkeys. A) rapid progressors, and B) slow or non-progressors.

Clinical evidence of disease progression was confirmed by the measurement of the $CD4^+$ T-cell levels in peripheral blood. In the group of rapid progressors there was in general a marked loss of $CD4^+$ T lymphocytes (Figure 2A) as compared to the LTS (Figure 2B). The two monkeys 95012 and 14B which were euthanized due to clinical signs of AIDS-like disease at weeks 29 and 33 post-infection, respectively, had a progressive decline in $CD4^+$ T-cells (Figure 2A), while in the very first two monkeys which developed disease (95047, 88A) percentages of $CD4^+$ T-lymphocytes, were not significantly reduced. The $CD4^+$ T lymphocyte levels in the LTS / non-progressors were relatively stable 68 weeks of the study. Only one of the LTS, animal 40B, showed a downward trend (Figure 2B).

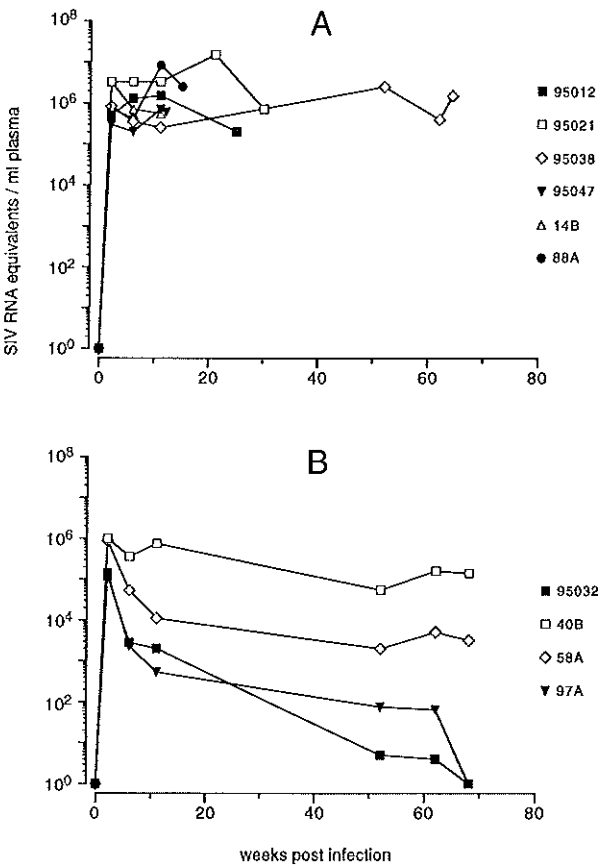

Figure 3. Plasma viral loads in A) rapid progressors and B) slow or non-progressors in weeks post-infection. Virus loads are expressed as RNA Equivalents per ml of plasma.

Cell-free viral loads estimated by SIV RNA present in plasma were determined at all available time points. All animals were positive in the quantitative SIV RT-PCR one week post-infection. All rapid progressors established stable high virus loads in the range of $2x10^5$ to $8x10^6$ RNA Eq/ml. (Figure 3A). At week 12 RNA concentrations of $1.5x10^6$, $7x10^5$, $8.2x10^6$, $3.3x10^6$, $2.5x10^6$ and $5.5x10^6$ Eq/ml were observed for 95012, 95047, 88A , 95021 , 95038 and 14B respectively. At the time of euthanasia in animals 95047 (week 15), 88A (week 19), 95021 (week 25), 95012 (week 29), 14B (week 33) and 95038 (week 64) viral loads of $6x10^5$, $2.5x10^6$, $7x10^5$, $2x10^5$, $2.5x10^6$ and $1.5x10^6$ RNA copies/ml plasma respectively were measured (Figure 3A).

The group of long-term survivors showed two types of responses. One group consisting of 95032 and 97A (Figure 3B) showed relatively low levels of viral RNA loads of $1.2x10^5$ and $1.4x10^5$ RNA copies /ml plasma at week two, respectively. At weeks six and eleven levels dropped from $2.8x10^3$ to $2x10^2$ for 95032 and from $2.3x10^3$ to $5.3x10^2$ for 97A. Later on in the experimental period the number of viral RNA copies decreased to below detection limit at weeks 52, 62 and 68 post infection for 95032. In the same period RNA levels for 97A declined from 76 to 65 and to undetectable levels ($\leq$ 40 RNA copies/ml plasma) (Figure 3B). The other two animals, 40B and 58A, showed considerably higher initial values of $1x10^6$ and $8.5x10^5$ at week two compared to the first group. From week two till week 68 for both animals RNA values declined to $1.4x10^5$ for 40B and to $3.2x10^3$ for 58A (Figure 3B).

Thus, all the rapid progressors showed viral loads that were higher than the pathogenic threshold (10^5 copies/ ml plasma) at time of euthanasia (Figure 3A). This in contrast to the group of long-term survivors that showed values equal or beneath this threshold (Figure 3B). Levels of cell-associated viremia as measured by QVI essentially paralleled those of plasma viremia (Figure 4A and B).

Next, we examined the frequency of reversion of the stopcodon in *nef* in the rapid progressors (95047, 88A, 95012, 95021, 14B and 95038) and the longterm non-progressors (95032, 97A, 58A and 40B) (Figure 5). We determined the relevant *nef* sequence containing the stopcodon of the SIV_{F359} inoculum and of virus isolated from all monkeys at week 2, week 6, week 15 and at time of euthanasia. From each animal and for every time point ten RT-PCR fragments of *nef* were cloned and sequenced. All virus from infected animals contained a premature stop codon in the *nef* gene at week 0, as did the inoculum. Our sequencing results demonstrate that reversion of the mutation had occurred as early as two weeks post-infection. In the group of the rapid progressors animals 95047, 88A and 95012 which developed disease first virus clones showed the fastest reversion (Figure 5A). In these cases, 10, 8 and 7 out of 10 10 *nef* clones, respectively, harboured a leucine instead of a stop codon as early as two weeks pi. and the proportion of revertants accumulated to 100% at week 15. Monkeys 95021 and 14B which were euthanized at weeks 29 and, respectively, showed initially a lower proportion of *nef* revertants, but week 15 all stopcodons had reverted. In the intermediate progressors, animal 95038, 4 out of 10 nef clones were identified as revertants until week 15 (Figure 5A). At euthanasia (week 64) the number of revertant clones had increased to 80%. The long-term non-progressors

showed a much lower proportion and a delayed occurrence of revertant viruses. These became first detectable only at week six (Figure 5B). At the time of euthanasia (week 68) all long-term survivors had a proportion of viruses in plasma that still contained the *nef* stop codon mutation equal or larger than 50%.

6.5 DISCUSSION

Four distinct regions in *nef* (1) RxxL (aa: 17-20), (2) YxxL (aa: 28-31), (3) YxxS (aa: 39-42) and (4) PxxP (aa: 104-107) have been described, based on sequence homology, conferring binding to specific cellular kinases. The importance of these regions became clear from studies in the SIV_{mac} model in which the amino acids RQ (region I, Figure 1) of SIV_{mac239}, were substituted for YE residues. These changes conferred the ability of the mutant to both activate and replicate in resting T-cells, a feature uniquely described for the acutely pathogenic SIV_{smmPBJ} isolate (5).

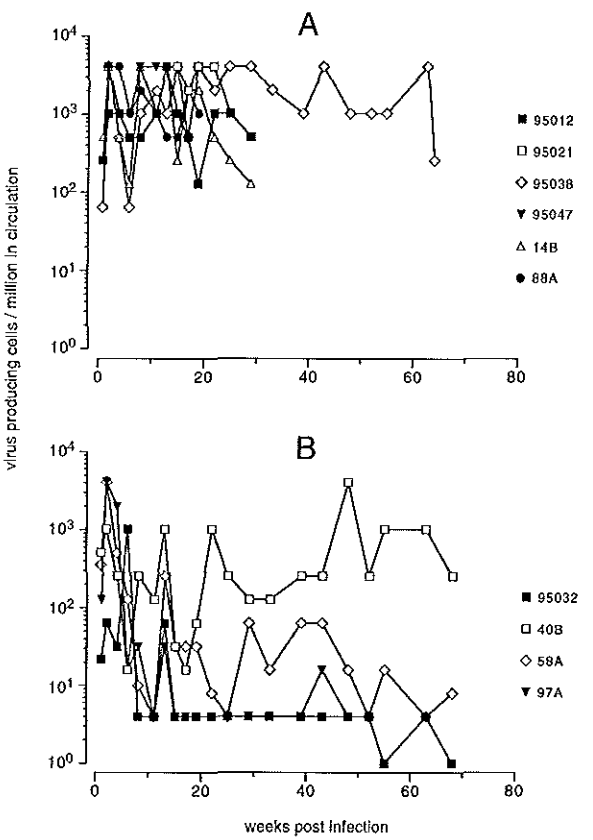

Figure 4. Cell-associated viral loads expressed as virus producing cells 10^5 PBMC in A) rapid progressors versus B) slow or non-progressors.

One of the initial studies which brought to light the relevance of the *nef* gene in viral virulence was the study of SIV_{mac239} with a premature stop codon at position 92 ($SIV_{mac239.stop}$) (4, 21). Infection of macaques with this attenuated *nef* mutatant resulted in an attenuated disease course which was characterised by the difficulty to re-isolate virus from infected animals (12). This Nef truncation resulted in lower numbers of infected PBMC per ml of plasma and a longer disease-free period (25). As a consequence sequences required for interaction of Nef with serine kinase are not present in this protein, a feature which was associated with the inability of the virus to replicate in macrophages (6).

Recently, we have characterised a novel molecular clone (F359), which is phylogenetically separate from SIV_{mac239} and SIV_{smPBJ} (Holterman et al., submitted). This clone has several unique features regarding *nef*. It has a RERL motif in contrast to the RQRL or YERL motifs found in SIV_{mac239} and SIV_{pbj} respectively at region one (Figure 1).

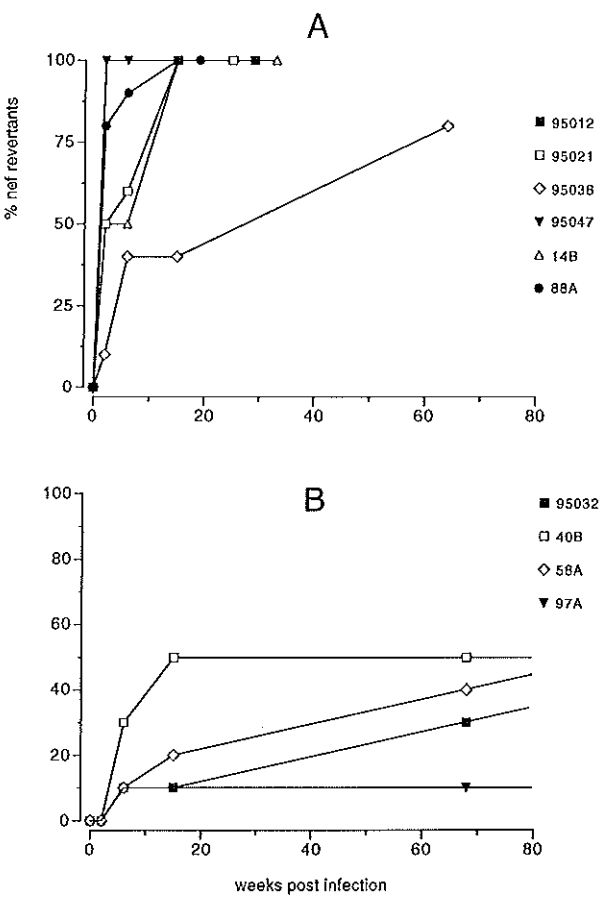

Figure 5. The percentage of viral clones in plasma reverting from TAA to TTA at *nef* codon 107. The percent revertants over time until death or euthanasia in rapid progressors (A), versus slow or non-progressors (B).

The YGRL motif (region two) of SIV_{F359} is identical to those of $SIV_{mac239.stop}$ and SIV_{pbj}. Region three (YxxS) differs just at one amino acid from the corresponding region in $SIV_{mac239-stop}$ also present in SIV_{F359} (Figure 1). An important difference to $SIV_{mac239-stop}$ was the presence of a stop codon in clone F359, which was located just downstream of the PKVP motif. The SIV_{F359} Nef contains the PKVP motif whereas $SIV_{mac239-stop}$ lacks this motif. SIV_{F359} can infect macrophages, but like $SIV_{mac239-stop}$, it lacks the ability to replicate in these cells to detectable levels (Holterman et al, submitted). Experiments performed by Flaherty *et al.* (16) showed that viruses expressing this $SIV_{mac239.stop}$ truncated Nef protein replicated inefficiently in primary macrophages. Based on these observations it was hypothesized that the conserved PKVP motif or other sequences downstream of this region contain critical elements necessary for yielding a productive infection in macrophages *in vivo* (6). However, SIV_{F359} which does contain the PKVP motif but showed similar biological characteristics in macrophages as $SIV_{mac239.stop}$ (efficient infection but no viral replication). Our new data suggest that sequences downstream the PKVP motif rather than this motif itself are involved in processes neccesary for viral replication in macrophages.

In this study we also investigated if infection of rhesus macaques with this unique mutant *nef* resulted in similar kinetics of infection and rapid reversion of the stop codon to wild-type *nef* as previously reported with the $SIV_{mac239-stop}$ clone (12). Infection of five mature and five juvenile macaques with SIV_{F359} resulted in two different patterns of infection irrespective of age. One group developed high steady state virus loads and proceeded to AIDS-like disease. Analysis of *nef* clones plasma of each of the ten animals revealed an important correlation between survival and the persistence of the stop codon mutation in virus circulating in the animals. Those animals which did not revert to the F359 *nef* wild-type viruses and which had persistently elevated levels of mutant *nef* viruses in plasma were long-term survivors. Conversely, rapid progression to AIDS correlated with a quick emergence of wild-type *nef* viruses. Notably, the proportion of revertants over time strongly correlated with RNA virus load and disease progression. Not only do these results support previous observations about the quick reversion of a truncated to a full–length nef it also reveal the timing and predominance of *nef* variants in infection and their influence on disease progression. Finally, these data strongly suggest that the predominant variants in circulation (plasma) may ultimately determine the course of the disease.

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CHAPTER 7

LIPOFECTIN-FACILITATED INFECTION OF RHESUS MACAQUE LYMPH NODES WITH DNA ENCODING FULL-LENGTH SIMIAN IMMUNODEFICIENCY VIRUS (SIV)

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7.1 ABSTRACT

We describe an efficient method of DNA inoculation to establish SIV infection in rhesus monkeys as a tool for studying the pathogenesis of AIDS. Four different strategies of administration of DNA of the F359 molecular clone of SIV were evaluated in four individual rhesus macaques. These included two different gene gun inoculations directly to exposed lymph nodes, and two different formulations of SIV-DNA-lipid complexes (lipofectin and DMRIE-C) inoculated directly into the lymph nodes. Gene gun inoculation or DMRIE-C-mediated delivery of DNA did not result in infection of animals. However, injection of lipofectin-DNA complexes resulted in the establishment of persistent SIV infection in rhesus monkeys. This strategy was much more efficient than the intramuscular injection of large volumes of naked proviral DNA previously reported. Lipofectin-mediated delivery will enable the *in vivo* characterisation of molecular clones of variant viruses in nonhuman primates at local sites such as lymph nodes and mucosal surfaces. This technique will help avoid the changes in the genetic and biological properties of primate lentiviruses, which occur during *in vitro* propagation of inocula. Most importantly it will facilitate the direct *in vivo* study of primate lentiviruses variants and help to elucidate their specific role in the pathogenesis of diseases such as AIDS.

7.2 INTRODUCTION

The induction of an AIDS-like disease in rhesus macaques by infection of sooty mangabeys (SIVsm) with simian immunodeficiency virus has provided a valuable non-human primate model for AIDS research (4, 7). The infidelity of retroviral reverse transcriptase causes a high mutation frequency (16, 19), resulting in a large number of closely-related but biologically different viral variants (quasispecies) within the host (11, 20). In order to study the contribution to disease of particular viral variants, infectious molecular clones are utilised (10). In general, transfection of susceptible cell lines with molecular clones *in vitro* is used to produce viral inocula. Unfortunately, *in vitro* replication frequently results in undesirable alterations of the genetic and the biological properties of the virus (8, 12, 13). Direct inoculation of an infectious molecular clone *in vivo* would avoid *in vitro* artefacts and will allow the direct study of viral variants at the molecular level *in vivo*. Intramuscular inoculation of large amounts of naked DNA has been routinely used to immunise mice with specific antigens and successfully used to induce FIV infection in domestic cats (14). SIV infection has been reported in a rhesus macaque but intramuscular inoculation and infection has not proved to be reproducable in this species (15). The direct delivery of viral NA to the target tissue has been a more successful approach in the case of hepatitis viruses. For instance liver-specific infections with genome-length RNA of hepatitis A and C viruses have been reported (5, 15).

For the purposes of immunisation with specific antigens delivery of DNA *in vivo* has been described using gene gun-mediated delivery (2, 3) by membrane fusion of cationic liposomes-DNA-complexes (1, 17), as well as using direct injection of naked DNA (6, 18).

The gene gun strategy utilises DNA-coated gold particles that are delivered by high velocity impact with the target tissue and has the advantage that relatively small amounts of DNA are required. These strategies have not been evaluated for their ability to deliver full-length viral genomes to target tissues *in vivo*.

Here we report a study in which we directly target the lymph nodes of four rhesus monkeys with DNA of the SIV_{F359} molecular clone (9) using different methods of DNA delivery.

7.3 RESULTS AND DISCUSSION

In order to select for optimal DNA-lipid mixtures for the delivery of molecularly-cloned SIV to cells we performed *in vitro* transfections of the human T-lymphoblastoid cell line C8166 using four different cationic lipid compounds (lipofectin, DMRIE-C, lipofectamine, and cellfectin) (Life Technologies, Breda, The Netherlands). After transfection, samples of cell culture supernatant were taken at regular intervals and analysed for SIV production using a Coulter SIV p27 antigen capture ELISA (Coulter Corp., Hialeah, Fl., USA). These pilot experiments revealed the superiority of the first two formulations, lipofectin and DMRIE-C, to induce SIV infection *in vitro*.

One approach to establish *in vivo* SIV infection consisted of gene gun administration directly to lymph node tissue of two rhesus monkeys. The second method used direct injection of the two different liposome-DNA formulations in lymph nodes of two other rhesus macaques. All animals were inoculated at four sites (two axillary and two inguinal lymph nodes). For the gene gun administration a small surgical incision was made to expose the axillary and inguinal lymph nodes. Plasmid DNA containing the full-length SIV_{F359} was bound to 2.6 um-diameter gold particles (Sigma-Aldrich Chemie, The Netherlands) to a concentration of 2 µg of DNA/mg of gold. Gene gun cartridges were prepared to a final payload of 1.0 µg of DNA bound to 0.5 mg of gold and administered directly to the lymph node tissues at 200 psi pressure. For the preparation of the lipofectin-DNA-complex and the DMRIE-C-DNA-complex, 5 µg of SIV_{F359}-DNA was diluted into 100 µl Opti-MEM I Reduced Serum Medium (Life technologies, Breda, The Netherlands). The liposome solution was prepared by dilution of 25 µl of lipofectin reagent or DMRIE-C reagent into 100 µl Opti-MEM medium. Both the DNA and liposome solutions were incubated at room temperature for 45 minutes before they were gently mixed and incubated at room temperature for another 20 minutes. Four lipofectin DNA-complex mixtures (200 µl each) and four DMRIE-C-DNA-complex mixtures (200 µl) were then inoculated directly into each of the four lymph nodes in separate animals.

Infection of macaques was monitored at regular time points. Evidence of infection was determined by the presence of SIV in serum qualitatively (RT-PCR) as well as quantitatively (QC-RT-PCR). For the RT-PCR assay, first strand cDNA molecules were synthesised using primer *nef-1* (CTAAGATTCTATGTCTTCTATCACT). PCR amplifications were carried out on 2 µl of the cDNA reaction using 100 nM (of each) primer *nef-1* and *nef-2* (CTGGTTCCAAGAAGCAGTCCAAG), and a mixture of 0.7 units of both the AmpliTaq

and *Pfu* polymerases (Perkin-Elmer Biosystems, The Netherlands and Stratagene Europe, The Netherlands). Reaction mixtures were overlaid with 100 µl of mineral oil and subjected to 35 amplification cycles (95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute) followed by a 72°C extension step for 10 minutes. Five microliters from the first reaction mixture were transferred to new reaction tubes containing a nested PCR reaction mixture containing 100 nM of primer *nef-4*: (GTGGAGAGACTTATGGGAGAC) and *nef-5* (CTATAAAATATCCCTTCCAGTCC) and amplified for another 35 cycles under the same conditions. *Nef* amplimers were analysed on agarose gels (Figure 1). The Q-RT-PCR method was performed as previously described (21).

PCR analysis revealed that neither the gene gun technique nor the SIV_{F359}-DNA-DMRIE-C-complexes were successful in facilitating SIV_{F359} infection of rhesus monkeys. Each of these animals remained negative by both RT-PCR and QRT-PCR. However, the inoculation of lipofectin-DNA-complexes directly into peripheral lymph nodes did result in systemic SIV infection which was detected four weeks post-infection (Figure 1) and remained persistent for the duration of the experimental period of four months (16 weeks).

We demonstrate the feasibility of establishing persistent SIV infection by direct injection of DNA-lipofectin-complexes to the lymph nodes of rhesus monkeys. This method of experimental SIV infection reveals the potential of the direct administration of DNA to different well-characterised tissues. This strategy provides a versatile tool for studying the pathogenesis of AIDS in selected target tissues with specific molecular clones representing distinct biological variants of lentiviruses.

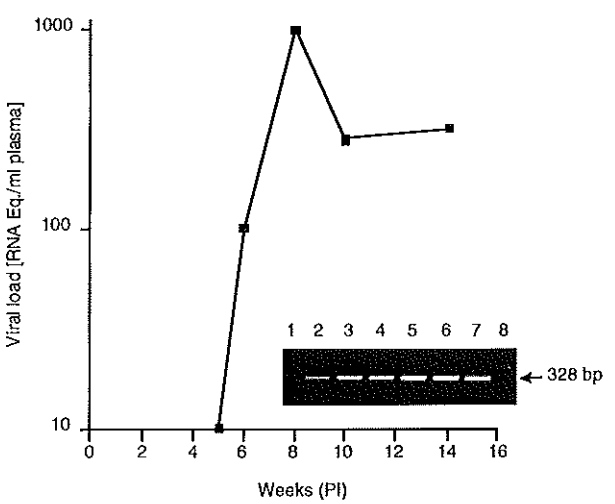

Figure 1. Evidence of viral infection in rhesus macaque 8903 following inoculation with SIV_{F359}-DNA-lipofectin complexes as determined by Q-RT-PCR (21). DNA-complexes (10 µg) were administered by injection into two axillary and inguinal lymph nodes. Serum samples were analysed at regular time points for SIV infection using Q-RT-PCR. The inset shows an agarose gel demonstrating the presence of *nef* amplimers generated by RT-PCR. Lane 1: negative control, lane 2: positive control, lane 3 to 8: amplimers derived from plasma samples obtained at 4, 6, 8, 10, 12, and 14 weeks post-infection.

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CHAPTER 8

GENERAL DISCUSSION

GENERAL DISCUSSION

An understanding of the pathogenesis of HIV infection will undoubtedly provide valuable information for the development of effective vaccines for the prevention of AIDS. Studies in humans are limited in practice since when HIV infection is eventually diagnosed valuable information is already lost. For instance, little or nothing is known with regard to the source, the viral characteristics of the inoculum, the route of infection, the exact time point of infection and the biological and molecular events which took place early in infection and which define the later disease course. There is enormous variation in survival time in people with HIV infection whose clinical course is defined as rapid, intermediate or long-term progression (14). This spectrum of disease progression is due to a complex interaction of viral and host factors (4, 15). To define these complex factors, prospective studies are needed in model systems to fill in the gaps of knowledge that otherwise cannot be obtained from retrospective human studies. The SIV model of AIDS macaques has proven to be particularly valuable for the study of the interactions between host and virus (13).

Little is known about viral virulence and its effect on rapid progression to AIDS. The research that was conducted using SIV_{smpbj} is in fact the only example of an acute disease syndrome (8), but which unfortunately represents an atypical rapid disease not characteristic of AIDS (11). It causes an acute haemorrhagic enteritis in infected pigtailed macaques and is considerably less pathogenic in rhesus monkeys which, if they survive the acute syndrome, a year later develop AIDS. For these reasons, SIV_{smpbj} is considered to be an atypical lentivirus and thus not an appropriate model to study rapid progression to AIDS (11).

It has been known for some time that an infectious viral inoculum consists of a quasispecies that evolves as the disease progresses. The limited evolution of a HIV-1 quasispecies observed during rapid progression to AIDS, is in contrast to that in long-term survivors, and reflects ineffective host-mediated immune pressures on replicating viral variants (7). When we began these studies several years ago our working hypothesis was that the most virulent viral variants would be found in rapid progressors and that they would be predominant at the end of the disease. Indeed when we transmitted blood from rapid progressors the disease course in age and sex matched rhesus macaques was found to accelerate (Holterman et al., 1999; Chapter 2). This observation was in agreement with later studies in the SIV model which demonstrated that the viral variants which emerge in late stage (AIDS) were neutralisation resistant in the host and also in subsequent hosts following *in vivo* passage (18). These end-stage variants were found to have optimal fitnesses for replication in these hosts and influenced the clinical outcome of the viral infection. Subsequently, we isolated this virulent SIV isolate (designated SIV_{8980}). This new SIV isolate caused an AIDS-like disease which was similar to the disease characteristics of the original SIV_{B670} strain, but disease developed more rapidly in SIV_{8980} (a minimum progression time to AIDS of 0.5 months versus 7 months or more). The significant change in pathogenic potential was convincingly shown by Kaplan-Meier plots comparing the parental (pre-passage) SIV_{B670} isolate with the post-passaged SIV_{8980} isolate.

In HIV-1 infection, the plasma level of viral RNA has proven to be the parameter with

the highest predictive value with regard to disease progression (22, 28). Recent studies in rhesus monkeys revealed that a pathogenic threshold of virus load could be defined which correlated with the virulence potential of a particular SIV isolate or SHIV chimeric to cause AIDS (34). Indeed viruses, which are more pathogenic, have increased concentrations of virus in plasma. One concern of our *in vivo* passage study was that we had inadvertently transmitted higher doses of virus by choosing end stage blood samples from rapid progressors even though in that experiment (Chapter 2) a consistent number of PBMC (2 x 10^6) were passaged. It was therefore critical for our interpretation to rule out that the accelerated disease observed with each transmission was not due to a dose effect. This question was addressed in Chapter 3 in which rhesus monkeys were inoculated with doses of SIV ranging from 100 to less than 1 ID_{50}. The study clearly revealed that there was a correlation with dose and infection but no correlation between the dose of the viral inoculum and viral loads *in vivo*. Furthermore, the results of that study demonstrated that differences in viral dose were not responsible for the accelerated disease progression observed during the SIV passage.

Several publications have suggested the possible role of other possible pathogens as co-factors in the pathogenesis of AIDS (5, 27, 29, 37). This possibility had also to be excluded. Two approaches were taken. The first approach was a retrospective screening of the viral inoculum and samples from infected animals in the *in vivo* passage study. Although no new seroconversions for potential pathogens were observed during the study we could not fully exclude the presence of some unknown agent or pathogenic variant in the inocula that might have some pathogenic effect which would influence our interpretation of the *in vivo* passage experiment.

In order to conclusively exclude the effects of any possible infectious co-factor we developed a strategy to derive a representative pathogenic molecular clone of SIV_{8980}. We reasoned that if a virus derived only from the genetic information encoded by SIV sequences in the passage monkeys could reproduce the disease observed this would help rule out other infectious co-factors. This clone should represent the dominant viral variant and should induce the same disease characteristics in approximately the same time frame as the uncloned virus (SIV_{8980}). Meeting these criteria dictated a novel approach different from previous strategies used for constructing earlier molecular clones of SIV or HIV-1 (Chapter 4).

The previous molecular clones of SIV were established using relatively insensitive molecular biological techniques such as the screening of genomic libraries. Since the number of infected cells *in vivo* were too low for these insensitive techniques, the *in vitro* propagation of the virus on human T-cell lines was needed to increase the number of virus infected cells (32). The artificial culture conditions and the forced replication of a monkey virus to adapt to human cell lines unknowingly caused genetic alterations in the SIV genome (16). *In vitro* culture caused the preferential replication of some virus variants over others resulting in quasiespecies that differed from the original *in vivo* material (23). Furthermore, viral sequences often proved unstable resulting deletions or atypical mutations in viral clones (6, 38). One may wonder in what respect these molecular clones represented the biological viral isolates they originated from. The application of the highly sensitive PCR technique

made it possible to use SIV infected PBMC as starting template and thus to circumvent the *in vitro* culture problems. The first heat stable DNA polymerases used in PCR lacked proofreading activity and were thus inaccurate (mutation rates of 10^{-4} and 10^{-5} per nucleotide per round of replication). These first enzymes also had limited synthetic capacity and could generate amplimers of a maximum of 5-kb with many mutational errors.

The next generation of DNA polymerases had proofreading activity and thus lower mutation rates. When we started the construction of the molecular clone only small aliquots of proofreading enzymes were available on request from suppliers. Later it was discovered that mixing two DNA polymerases had a synergistic effect on both the synthesising capacity as well as on the fidility of the PCR (2). The clone which we wished to construct had to be the dominant variant (the one which was most abundant in extracellular circulation). As a consequence and for our purpose the viral RNA present in plasma had to be used as RT-PCR template. It is known that SIV-DNA present as provirus in surviving blood mononuclear cells contains accumulated mutations of past viral variants. These genomes persist in host cell DNA but are replication defective. Hence, we were concerned that using proviral DNA as templates that we would frequently clone unwanted mutants or minor viral variants. We reasoned that many mutations would be self-limiting with reduced replication efficiency or so called fitness. In Chapter 4.2 an optimised RT-PCR strategy was developed which resulted in a molecular clone (F359) containing all the coding sequences derived from RNA obtained from rhesus monkey 8980 (P4) in the passage study described in Chapter 2. This development allowed us to characterise the disease potential as well as the biological and molecular characteristics of this particular clone. Recently, studies have shown that the variants, which replicate most rapidly, precipitate the development of AIDS. Based on similar reasoning we assumed that the biological characteristics of the dominant virus variant would be representative of the virulent variant influencing the acute disease course.

Comparison of the biological characteristics of the clone with the original SIV_{8980} isolate should demonstrate that we cloned the dominant virulent variant. For instance, co-receptor usage (25, 30), and the ability to replicate in resting versus activated $CD4^+$ T-cells (9) and macrophages (33, 39) are characteristics considered to influence pathogenicity. In this regard, no differences were observed between the cloned virus and the virus isolate in that both recognised only the CCR5 co-receptor and neither replicated in resting PBMC nor macrophages. The subsequent *in vivo* passages of SIV_{B670} resulting in SIV_{8980} were carefully monitored. The original SIV_{B670} inoculum was also used in studies performed by Tricher et al. (35) and Amadee et al. (1). They had determined the number of different genotypes in the SIV_{B670} inoculum based on the first hypervariable region (V1) sequence divergence (3, 24). Twelve different genotypes could be recognised in this isolate (B670-Cl.1 to Cl.12). The envelope sequence of B670 Cl.12 was one of the sequence variants present in the original SIV_{B670} stock. During our passage experiment the sequence variant Cl.12 had acquired an optimal fitness represented by its prevalence in the early SIV_{B670} isolate and in its maintenance during the passages. Indeed the SIV_{F359} molecular clone had the highest (96%) homology with B670-Cl.12 and therefore is representative of the dominant virus variant of SIV_{B670}.

The *in vivo* experiments in rhesus macaques demonstrated similar clinical and pathological characteristics of SIV_{F359} compared to SIV_{8980}. Several $SIV_{sm/mac}$ clones have been reported to cause different patterns of disease including attenuated virulence compared to the virus isolates from which they were derived. With specific regard to the first SIV molecular clones which were derived, a viral adaptation and attenuation often occurred as a result of *in vitro* propagation. This attenuation phenomenon may also be due to a failure to clone the dominant virus variant, possibly as a consequence of using proviral DNA as biological template or due to the use of biological material that contained still a "balanced" composition of early (M-tropic, slow-replicating, NSI-type) and late variants (T-tropic, SI-type and fast replicating). It may also reflect a certain synergistic effect of a quasiespecies that do not exist in case of a single molecularly cloned virus. A study demonstrating a regulatory effect on HIV replication mediated by defective proviruses has provided evidence in that direction. Convincing data has started to accumulate which explains the progression to AIDS in terms of the biological properties of virus variants which emerge during the course of infection. However, results from other studies suggest a more complex cascade of events in which variants in combination with other factors are involved in AIDS pathogenesis.

Studies of HIV-1 infected individuals revealed that viruses which appear to be cytopathogenic *in vitro* are found in about half of the individuals progressing to AIDS, but not in long-term asymptomatic individuals (31). The fact that syncytium-inducing variants are not found in all individuals progressing to AIDS raises the concern that they arise only following the failure of the immune system rather than being a cause of the failure. Furthermore, the relentless massive turnover of virus that occurs in HIV-1 infected individuals (17, 26) emphasises the fact that minor changes in the growth properties of the virus could have a major impact on the delicate balance between virus and the available target cell reservoir.

The diversity of the virus population present within an infected individual can be enormous. However, direct analysis of viral quasispecies in HIV-1 infected individuals reveals a correlation between outgrowth of a relatively uniform virus population in quickly progressing individuals and more rapid and greater diversification of the virus population in slowly progressing or nonprogressing individuals (20, 21, 40). The data of Edmonson et al. demonstrated that the formation of a diversified lentivirus population, while one of the more insidious properties thought to be crucial to escape from the immune system and fulminate persistence in the host, is not a prerequisite for the development of AIDS (10).

In vivo passage has been conducted in various occasions and resulted in virus isolates with properties distinct from those of the parental strain. For example, the acutely pathogenic $SIV_{smPBJ14}$ isolate was derived following a single *in vivo* passage of the minimally pathogenic SIB_{smH9}, (12) and molecular clones of that isolate have been shown to recapitulate the rapidly fatal phenotype (8). Interestingly this highly pathogenic property causing acute haemorrhagic enteritis appears to be in part conferred by a particular "YE" motif in the *nef* gene. Transfer of this motif to another molecular clone ($SIV_{mac289+}$ conferred the ability of that molecular clone to replicate in resting $CD4^+$ T-cells and the ability to cause

acute haemorrhagic enteritis. Interestingly, the SIV_{F359} had a number of similar and different *nef* motifs which may explain its particular pathogenic characteristics. For instance, instead of a YE motif it has a RE motif which may be responsible in part for its potential to cause rapid progression to AIDS. The occurrence of a mutation causing a premature stop codon in *nef* allowed us to design a study of the effect of this *nef* genotype upon reversion to a fully translated F359 *nef* variant.

The fate of the premature stop-codon that is present in SIV_{F359} *nef* was studied *in vivo* in rhesus monkeys. Infected animals (rapid progressors) which showed high virus loads and died of AIDS had the highest rate of stop-codon revertants (between 50 and 100%) within weeks of infection. In contrast, the non-progressors had very low viral loads, stayed healthy during the experimental period, had lower rates of reversion to the wild type *nef*, and the mutant *nef* genotypes remained dominant. The survival curves of the ten rhesus macaques infected with SIV_{F359} and SIV_{8980} showed a "biphasic" pattern. Kaplan-Meier survival plots revealed that the rapid progressors (*nef* revertants) coinciding with the curve of the parental SIV_{8980} infected group represents the contribution of the (non-revertants) non-progressors (Chapter 6, Figure 5). From both the nature of disease and the time of disease progression of SIV_{F359} compared to SIV_{8980} infected animals, we conclude that SIV_{F359} once the *nef* gene is repaired, represents the virus characteristics of the dominant variant present in SIV_{8980}. We showed that reversion to a functional *nef* protein can occur quickly *in vivo* (within two weeks), but occurred only in some animals a finding which is in contrast to other SIV *nef* studies.

Mutations arise in SIV molecular clones due to *in vitro* cultures. Indeed "to culture is to perturb" (36). By deriving the F359 molecular clone directly from serum, we had achieved the most representative molecular clone possible. Unfortunately, routine use and analyses require that virus stocks must be propagated by transfection of the molecular clone *in vitro* for short-term culture. Ideally, we wished to completely circumvent this step to avoid any possible atypical mutations which could occur. Liska et al. reported that direct injection of molecular clone DNA intramuscularly could result in SIV_{mac} infection of rhesus monkeys (19). This technique, however, proved to require tremendous doses of DNA and was not reproducible by other laboratories including our own. In Chapter 7, we describe a technique using DNA of the F359 molecular clone and lipofectin to induce SIV infection by directly delivering the virus to its target tissue, the lymph node. In this thesis we report on strategies to completely circumvent *in vitro* culture artefacts by deriving a molecular clone from *in vivo* material and delivering infectious molecular clones directly back to the host for study. In addition to increase insight into the role of viral virulence and rapid progression to AIDS, we also provide the tools for future studies to determine the molecular determinants of viral virulence in the SIV model.

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SUMMARY

AIDS (Acquired Immuno Deficiency Syndrome) is caused by the Human Immunodeficiency Virus (HIV). Most of the knowledge of both the virus and the disease we obtained from research performed on biological samples of infected individuals. However, a lot of scientific questions remain which cannot be studied in humans. For these studies relevant animal models are used. The SIV-rhesus macaque model has been used for studies described in this thesis. In general studies to the role of specific viral virulence as major factors in the rapid progression to AIDS has been described. Viral factors responsible for a delayed or very slow disease progression is known and are relatively well understood. Little is known, however, about viral virulence factors that may accelerate the disease causing a relatively acute AIDS syndrome. In chapter 1 an overview of the development and applications of the Simian Immunodeficiency Virus (SIV) macaque animal model for AIDS research in humans is presented. Rhesus macaques, when infected with SIV derived from sooty mangabeys, develop a disease which closely resembles AIDS in humans. HIV and SIV inocula contain many related but genotypically and phenotypically different viral variants (quasispecies). To address certain specific questions in AIDS research it is necessary to develop well-defined and representative molecular clones from specific viral variants. Current infectious molecular clones of SIV vary from highly pathogenic to non-pathogenic. They differ in the nature as well as the rate in which they induce the development of AIDS *in vivo*. In addition, differences with regard to replication, cell tropism, cytopathic effect and co-receptor recognition exists between specific molecular clones. The importance of these biological differences can be addressed in the macaque model of AIDS. The information gained has the potential to be applied to the development of new therapeutic and/or vaccine strategies. In chapter 2 a study is described in which we ask ourselves the question if viral virulence is a factor in cases of rapid progression to AIDS. An animal model for rapid disease progression was established by serial end stage passage of SIV_{B670} in rhesus macaques. Virus from the first animal to develop AIDS was transferred to recipients *in vivo* five subsequent passages. This reduced the survival of the subsequent recipients from a minimum progression time of 7 months to 2 weeks, from passage 1 to passage 4, respectively. The possibility remained that these observations were not due to increased virulence but alternatively were due to increased dose of SIV in the subsequent inocula or were caused by the (co-)passage of other pathogens present in the inocula. The study we describe in chapter 3 addresses the question whether virus virulence or viral dose influenced the rate of disease progression. Two experiments were performed (chapter 3.1 and 3.2). The first study involved the follow-up of rhesus macaques infected with different doses (ranging from more than 100 to less than 1 ID_{50}) of the cell free SIV_{8980} stock. The study revealed that there was no correlation between dose and the rate of disease progression. In the second experiment monkeys infected with an *in vitro* passaged attenuated SIV strain (SIV_{BK28}) were superinfected with another, more virulent strain (SIV_{8980}) to determine if the second pathogenic infection resulted in a more rapid disease course. The rate of disease remained constant and representative of the initial inoculum.

From these experiments we concluded that the rate of progression to AIDS was independent of the viral dose and was predominantly influenced by the viral characteristics of the initial infection. Having addressed the first question raised in chapter 2, in chapter 4 we describe a study in which we focussed on developing a method to obtain molecular clones, which represented the most abundant virus variant. The work of Kimato et al. (1999) demonstrated that SIV variants which emerge at the end of disease contribute most to the clinical outcome of SIV infection. We reasoned that the most dominant SIV variant in circulation could best be cloned from RNA from virions in plasma rather than accumulated proviral DNA, surviving from previous viral populations. Deriving molecular clones from end stage serum or plasma samples required an RT-PCR method that first needed to be optimised in order to obtain SIV amplimers of sufficient length. Such RT-PCR optimisation experiments are described in chapter 4.1. The construction of the full-length SIV molecular clone (designated SIV_{F359}) is described in chapter 4.2. Chapter 5 describes the *in vivo* characterisation of this new clone (SIV_{F359}). Phylogenetic analyses demonstrated that SIV_{F359} was related to but distinct from other SIVsm and SIVmac clones. Most SIV recognise multiple co-receptors (CCR5, BOB, Bonzo). In contrast, the parental strain of SIV_{8980} and the molecular clone SIV_{F359} were found to have the same restricted CCR5 co-receptor usage. SIV_{F359} was able to infect and induce syncytia in the human C8166 T-cell line, as well as in rhesus- and human peripheral blood mononuclear cells (PBMC). This clone was unable to replicate to detectable levels in unstimulated rhesus PBMC or in monocyte-derived macrophages, although it could *infect* the latter cell type. Pilot experiments conducted in two rhesus macaques revealed that the SIV_{F359} molecular clone was infectious as well as pathogenic *in vivo*. It was able to induce a similar (albeit slower) pattern of disease progression as observed with SIV_{8980}, the parental virus inoculum. In chapter 6 the study to the virulence factor *nef* by examining the effect of a mutant premature Nef stopcodon in SIV_{F359} is described. Ten rhesus monkeys were infected with this *nef* mutant of SIV_{F359}. The group that progressed to AIDS showed significantly higher virus loads and a higher prevalence of viruses which reverted to the wild type *nef*. In comparison, the group of non-progressors stayed healthy during the experimental period and contained much lower levels of the repaired *nef* stop codon. We concluded that the predominance of the attenuated SIV_{F359} *nef* variant facilitated the maintenance of low viral loads and an asymptomatic disease course. In chapter 7 we describe the refinements of the model in order to minimize the *in vitro* manipulations (which generate atypical mutations) by direct inoculation of rhesus macaques with SIV_{F359} DNA rather than using a virus stock requiring *in vitro* propagation. This approach proved that it was feasible to establish infection *in vivo* by direct injection of DNA of just one single viral genotype, enabling direct and detailed studies of the contribution of separate genes to viral pathogenicity.

These studies together now provide the basis for further studies to identify and characterise the molecular determinants of lentiviral virulence responsible for accelerating the progression to AIDS.

SAMENVATTING

De ziekte AIDS (Acquired Immuno Deficiency Syndrome) wordt veroorzaakt door het Humaan Immunodeficiëntie Virus (HIV). Veel kennis over het virus en de ziekte is verkregen door de bestudering van biologisch materiaal van zowel geïnfecteerde als zieke individuen. Er blijven echter wetenschappelijke vragen bestaan die niet bestudeerd kunnen worden door onderzoek van mensen. Daarom wordt tevens gebruik gemaakt van relevante diermodellen. De SIV-rhesus makaak is één van de diermodellen die het mogelijk maakt studies uit te voeren zoals beschreven in dit proefschrift. SIV (Simian Immunodeficiëntie Virus) is een virus dat zich handhaaft in rhesus makaken en is vergelijkbaar met HIV. Virale factoren die verantwoordelijk zijn voor een *vertraagde* ontwikkeling van AIDS worden in de literatuur relatief goed beschreven. Er is echter veel minder bekend over virale factoren die *versnelde* ziekteprogressie veroorzaken en welke resulteren in AIDS. Dit proefschrift beschrijft o.a. de rol die virulentiefactoren spelen in de snelle progressie naar AIDS. Een overzicht van de ontwikkeling en toepassing van het SIV-rhesus makaak model voor onderzoek naar AIDS in de mens is in hoofdstuk 1 beschreven. Wanneer rhesus makaken worden geïnfecteerd met SIV afkomstig uit sooty mangabey apen (SIVsm) ontwikkelt zich een ziekte die sterke overeenkomsten vertoont met AIDS bij de mens. HIV en SIV isolaten bevatten verwante, maar genotypisch en fenotypisch verschillende virale varianten (quasispecies). Voor het beantwoorden van specifieke vragen op het gebied van AIDS is het nodig om te beschikken over goed gekarakteriseerde en representatieve moleculaire klonen van specifieke virale varianten. Infectieuze moleculaire klonen van SIV kunnen sterk variëren in pathogeniciteit. Ze verschillen in de biologische aard en de snelheid waarmee ze de ontwikkeling van AIDS *in vivo* induceren. Het belang van deze verschillen kan in het SIV-rhesus makaakmodel bestudeerd worden. De zo verkregen informatie kan worden toegepast bij de ontwikkeling van nieuwe therapieën en/of vaccinatiestrategieën. In hoofdstuk 2 is beschreven welke rol virulentie speelt in gevallen waarin een snelle progressie naar AIDS plaatsvindt. Een diermodel voor deze snelle progressie is ontwikkeld door opeenvolgende passages van SIV_{B670} in rhesus makaken (SIV afkomstig van sooty mangabey B670). Hiervoor zijn bloedcellen uit het eerste dier met AIDS geïnjecteerd in een tweede dier, waarna op dezelfde wijze nog drie passages volgden. Hierdoor daalde de minimum overlevingstijd van 7 maanden in het eerste dier tot 2 weken in dieren na de vierde passage. De mogelijkheid bestond dat deze verandering niet een gevolg was van een toename in virulentie, maar van een toename van de hoeveelheid SIV in de opeenvolgende inocula, of veroorzaakt werd dat deze door mede-passage van andere pathogenen in de inocula. De rol van virulentie versus de hoeveelheid geïnjecteerd virus bij de progressie van AIDS is in hoofdstuk 3 beschreven. Twee studies werden uitgevoerd (hoofdstuk 3.1 en 3.2). In de eerste studie werden rhesus makaken geïnfecteerd met verschillende hoeveelheden (van minder dan 1 tot meer dan 100 ID_{50}) van de celvrije SIV_{8980} stock. Uit deze studie bleek dat er geen correlatie bestaat tussen de hoeveelheid geïnjecteerd virus en de snelheid van ziekteprogressie. In de tweede studie werden makaken, die geïnfecteerd waren met een *in vitro* gekweekte en verzwakte SIV stam (SIV_{BK28}), opnieuw geïnfecteerd met een andere, meer pathogene stam (SIV_{8980}) om te bepalen of deze tweede infectie resulteerde in een snellere ziekteprogressie. De snelheid van ziekteprogressie bleef constant en

representatief voor het eerste inoculum. Hieruit blijkt dat de progressiesnelheid naar AIDS onafhankelijk is van de hoeveelheid geïnjecteerd virus en voornamelijk beïnvloed wordt door de virale karakteristieken van de eerste infectie. In hoofdstuk 4 is een studie beschreven waarin een methode werd opgezet om moleculaire klonen te verkrijgen die representatief zijn voor de meest-voorkomende virale variant. Kimata et al. (1999) hebben aangetoond dat de dominante SIV-variant in het eindstadium van AIDS, verantwoordelijk was voor het uiteindelijk ontstaan van het ziektebeeld. We gingen ervan uit dat deze SIV-variant het beste verkregen kon worden uit viraal RNA aanwezig in plasma of serum, in plaats van via proviraal DNA afkomstig van cellen in de circulatie. Voor het verkrijgen van moleculaire klonen uit eindstadium plasma monsters moest een RT-PCR methode geoptimaliseerd worden. Deze is beschreven in hoofdstuk 4.1. De constructie van de volledige SIV moleculaire kloon (SIV_{F359}) wordt beschreven in hoofdstuk 4.2. Hoofdstuk 5 beschrijft de *in vivo* karakterisering van deze nieuwe kloon (SIV_{F359}). Stamboomanalyse toonde aan dat SIV_{F359} gerelateerd is aan zowel SIVsm klonen als SIVmac klonen. Fylogenetisch gezien zal SIV_{F359} tussen deze twee virusgroepen in geplaatst moeten worden. Doorgaans gebruikt SIV meerdere co-receptoren, o.a. CCR5, BOB, Bonzo. Echter, het in dit proefschrift gebruikte SIV_{8980} isolaat en de daarvan afgeleide moleculaire kloon SIV_{F359} gebruikten alleen CCR5. SIV_{F359} bleek in staat om zowel de humane T-cel lijn C8166, als rhesus en humane perifere bloed mononucleaire cellen (PBMC) te kunnen infecteren, en bleek tevens in staat om syncytia te vormen. Deze kloon vertoonde echter geen detecteerbare vermenigvuldiging in niet-gestimuleerde rhesus PBMC of in macrofagen, terwijl macrofagen wel door het virus geïnfecteerd werden. In een pilotexperiment in twee rhesus makaken bleek de SIV_{F359} moleculaire kloon zowel infectieus als pathogeen te zijn. De kloon vertoonde een vergelijkbaar, maar trager patroon van ziekte-ontwikkeling met dat van SIV_{8980}. In hoofdstuk 6 is het onderzoek beschreven naar een mutatie die resulteerde in een *nef* stopcodon in SIV_{F359}. Deze mutatie werd gebruikt om de rol van *nef* in de virulentie van SIV te bestuderen. Tien rhesus makaken werden met deze *nef* mutant van SIV_{F359} geïnfecteerd. De dieren die AIDS ontwikkelden, hadden een relatief hoge virusconcentratie en een hogere prevalentie naar het wildtype teruggemuteerd virus. De dieren zonder ziekteprogressie hadden een veel lagere virusconcentratie en een aanzienlijk lager aantal virusdeeltjes met een hersteld *nef* stopcodon. Hieruit hebben wij geconcludeerd dat de *nef* mutatie verantwoordelijk is voor de lage virusconcentratie en de afwezigheid van ziekteverschijnselen na infectie. Vervolgens is in hoofdstuk 7 de studie beschreven waarin het diermodel zodanig aangepast werd dat het aantal handelingen *in vitro* zoveel mogelijk beperkt kon worden. Dit werd bereikt door de rhesus makaken rechtstreeks met DNA van SIV_{F359} te infecteren en niet, zoals gebruikelijk, met een virus preparaat die veel tijdrovende manipulaties vereist. Het is gelukt om met het DNA van de moleculaire kloon van SIV een infectie te veroorzaken. Dit betekent dat het in principe mogelijk is om nauwkeuriger de invloed van aparte genen op het ziekteproces te onderzoeken.

Tezamen leggen de studies van dit proefschrift de basis voor verdere studies voor de identificering en karakterisering van de moleculaire determinanten die verantwoordelijk zijn voor de versnelde progressie naar AIDS.

CURRICULUM VITAE

Lennart Holterman werd op 10 september 1963 geboren in Amsterdam. Op scholengemeenschap "Duinzicht" te Oegstgeest werd in 1975 het MAVO-diploma behaald. In 1979 en 1983 werden respectievelijk het HAVO- en het VWO-diploma behaald aan "De Louise de Colligny" scholengemeenschap in Leiden. In 1983 werd begonnen met de studie Biologie aan de Universiteit Leiden. De doctoraalfase was samengesteld uit het hoofdvak Moleculaire Biologie (Faculteiten Biologie en Scheikunde) en het bijvak Biochemie (Faculteit Scheikunde). Het doctoraalexamen werd behaald in 1989. Na vervulling van de (vervangende) dienstplicht bij TNO-Rijswijk werd in oktober 1991 begonnen met het promotie-onderzoek aan de afdeling Immunologie van de Erasmus Universiteit Rotterdam. Dit onderzoek werd uitgevoerd binnen de afdeling Virologie van het Biomedical Primate Research Centre (BPRC) te Rijswijk, en stond onder leiding van Dr. J.L. Heeney en Prof. dr. J.J. Haaijman. Sinds oktober 1999 is hij werkzaam binnen de afdeling Parasitologie van het BPRC in Rijswijk.

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