ASSESMENT OF VIRAL SHEDDING AND LOCAL IMMUNE RESPONSES TO HIV IN THE FEMALE REPRODUCTIVE TRACT USING SHIV/BABOON MODEL

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This thesis is submitted in partial fulfillment of the requirements for the award of Masters of Science (MSc) degree in Reproductive Biology at the University of Nairobi.

2002

DECLARATION

This is my original work and has not been submitted for a degree in any other University.

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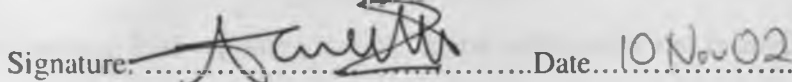

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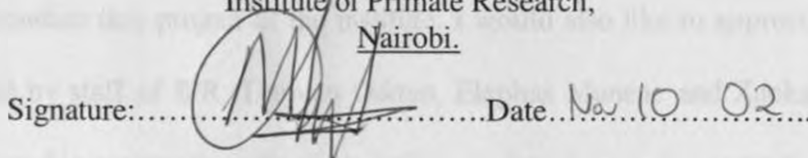

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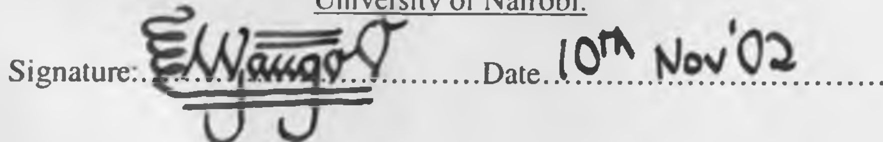

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DEDICATION

This thesis is dedicated to my late parents, John and Regina for their excellent parental guidance and inspiration.

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ABSTRACT

Heterosexual transmission accounts for majority of human immunodeficiency virus (HIV) infection among adults in developing countries. The risk of female to male transmission of HIV is likely to be determined by concentration of virus and the local immune response to the virus in the cervicovaginal secretions (CVS) of the infected women. A better understanding of the correlates of genital shedding of HIV may significantly aid preventive measures aimed at reducing this transmission. Recent studies suggest a possible role of female genital immune response in preventing HIV infection. Both local immune response and the viral load in genital secretions may be influenced by the stage of the menstrual cycle. This may determine when the female is most vulnerable to HIV infection or can transmit the virus most efficiently. These issues can only be best investigated in a suitable animal model. Currently, simian immunodeficiency virus (SIV) infection of macaques is regarded as the best model since it mimics HIV infection in various ways. However, existing differences in envelope glycoproteins between the two viruses limit the use of the SIV/macaque model in HIV-1 vaccine trials and for molecular studies assessing envelope determinants of HIV-1 pathogenicity. Simian/Human immmunodeficiency virus (SHIV) infection of baboons provides suitable alternative for such studies. This project assessed the relationship of the menstrual cycle to genital mucosal and systemic immunity, and to viral shedding within cervicovaginal secretions of female olive baboons (*Papio anubis*) experimentally inoculated with SHIV-89.6P.

Cervicovaginal lavage (CVL) and blood from two SHIV-89.6P infected (n=2) and two uninfected (n=2) adult female baboons were sampled at three stages namely; menstruation (men), mid-follicular (mfl) and luteal (lut) stages of two successive cycles. Isolation of cell-free

virus was attempted from plasma and CVL by limiting culture dilution method. The presence of virus was confirmed by SIV p27 core antigen assay. SHIV-specific immnoglobulins A (IgA) and immunoglobulin G (IgG) were detected in both CVL and plasma by ELISA and confirmed by HIV-1 and HIV-2 Western blots. Radioimmunoassay (RIA) for estradiol (E_2) and progesterone (P_4) in plasma was performed to corroborate the perineal skin swelling with the menstrual cycle stage.

The cyclical changes in blood levels of E_2 and P_4 correlated positively with the menstrual cycle stage as determined by perineal skin swelling. Infected animals were able to secrete SHIV-specific antibodies in their genital fluids. Detection of SHIV-specific IgA and IgG in CVL appeared to be influenced by the stage of the menstrual cycle. Similarly, anti-SHIV IgG titers in CVL varied with the menstrual cycle, being highest during menses, lower in luteal stage and not detectable in mid-follicular stage of the two successive menstrual cycles. On the other hand, a menstrual cycle-dependent variation in IgG titers in plasma was not observed, probably indicating a lack of influence of the menstrual cycle on the presence and titers of anti-SHIV antibodies in systemic circulation of the baboon model. Anti-SHIV IgG titers were higher in plasma than in CVL of infected animals. Virus was isolated from and detected in both plasma and CVL of SHIV inoculated baboons. However, no consistent cyclic pattern was observed with respect to the isolation of this virus from either plasma or CVL. The negative control animals remained virus isolation and SHIV-specific antibody negative throughout the study period.

This project has shown that SHIV infected female baboons shed virus in their genital secretions. A strong local humoral response to the virus, dependent on the phase of the cycle, is present

within the genital tract. The observed hormonal regulation of SHIV-specific secretory immunity may have important implications for preventive measurers aimed at reducing female to male transmission of HIV. In order to be more effective such approaches need to consider the menstrual cycle phase of women. Given the number of animals used in this study the results presented here are limited. If developed further, SHIV infection of baboon can provide an alternative model for investigating unresolved issues in heterosexual transmission of HIV.

CHAPTER ONE

1.0 Introduction And Literature Review

1.1 Introduction

Human immunodeficiency virus (HIV) infection and the resultant disease acquired immunodeficiency syndrome (AIDS), has emerged as a global epidemic since the first reported cases in 1981 (UNAIDS., 2000). It has caused considerable mortality and morbidity among adults and children (UNAIDS., 2000). The epidemic has also impacted negatively on the reproductive health of men and women, as well as global demographic trends (Coggins and Segal., 1997). These devastating effects of HIV infection have been more pronounced in Sub-Saharan Africa in particular and developing countries in general where the infection rates are considerably higher than in developed countries (UNAIDS., 2000).

Effective control strategies of this pandemic will depend, to some extent, on information regarding the biology and transmission of HIV. In spite of recent advances in these areas, a cure for those already infected has remained elusive. Various antiretroviral agents have been formulated and shown to prolong the survival of HIV-infected individuals (Piketty., 1999). However, the drugs are unaffordable for the majority of HIV-infected people, especially in developing countries. A safe, efficacious and affordable preventive vaccine against HIV remains the most effective way of controlling the pandemic (UNAIDS., 2000). Different HIV immunization strategies have been tried (Clements., 1995). Since HIV is mainly transmitted by sexual contact involving multiple exposures of mucosal surfaces to cell-free and cell-associated virus, followed by dissemination to local mucosal lymphoid tissues resulting in systemic

infection (Alexander., 1990), the best vaccine strategy to prevent transmission would be to induce long lasting genital mucosal and systemic protective immunity.

In this respect, a more detailed understanding of the virology and immunology of HIV within the female genital tract is critical. It has been documented that HIV-infected women actively shed virus into their genital secretions (Shaheen et al., 1999) and HIV-specific humoral immunity is demonstrable in such secretions (Lu et al., 1993). The influence of this response on the rate of female-to-male transmission of HIV needs to be elucidated. It can be hypothesized that local immunity to HIV and HIV viral load in the genital tract are likely to be influenced by the menstrual cycle stage. This may determine when a woman is most vulnerable to acquiring HIV infection or can transmit the virus most efficiently.

For practical and ethical reasons, these studies need to be pursued in an appropriate animal model of HIV infection. SIV infection of macaque monkeys is one such model and has been previously used because of its similarities with HIV infection in man (Kindlt et al., 1992). However, differences exist in envelope antigens between HIV-1 and SIV/macaque system, limiting its further use in vaccine trials. To overcome this, chimera virus simian human immunodeficiency virus (SHIV) has been constructed using HIV *env*, *tat*, *rev* and *vpu* genes on an SIV backbone (Shibata and Adachi., 1992; Reihmann et al., 1996a). SHIV infection of baboons provides a suitable alternative for studying pathogenesis as well as prophylaxis of HIV (Allan et al., 1995). This project assessed the relationship of the menstrual cycle to genital mucosal and systemic immunity, and to viral shedding within the CVS of female olive baboons experimentally inoculated with SHIV $_{89.6P}$.

1.2 Literature Review

1.2.1 Biology of human and simian immunodeficiency viruses

Human and simian immunodeficiency viruses belong to the retrovirus family (Abimiku and Gallo., 1995). These viruses are unique in that they posses the enzyme reverse transcriptase (RT) enabling them to use single-stranded RNA (ssRNA) to code for double-stranded DNA (dsDNA) which then becomes integrated into the host cell genome (Abimiku and Gallo., 1995). Retroviruses have been classified into the following subfamilies on the basis of the *in vivo* and *in vitro* consequences of their infection (Cullen., 1993; Abimuku and Gallo., 1995): (i) the lentivirus subfamily consist of viruses whose infection is associated with long incubation period, often culminating into immunodeficiency and sometimes with neurologic complications. Examples include HIV, SIV and feline immunodeficiency virus (FIV); (ii) the oncovirus subfamily is composed of viruses which give rise to a number of malignancies (Cullen., 1993; Abimuku and Gallo., 1995). Such viruses include Human T-cell lymphotropic virus (HTLV) and Simian T-cell lymphotropic virus (STLV); (iii) the spuma (foamy) virus subfamily is made up of viruses causing a distinct cytopathic effect *in vitro* hence the term foamy (Cullen., 1993; Abimuku and Gallo., 1995). The role of these viruses in disease causation is unknown. Other criteria for classification of retroviruses are based on the pattern of viral gene regulation, and viral morphology (Cullen., 1993).

AIDS was first reported in 1981 as an outbreak of severe opportunistic infections among a group of previously healthy homosexual males (Gottlieb et al., 1981; Mansur et al., 1981). The initial viral isolates from patients with AIDS, now established to be closely related members of the same virus, were initially variably designated; lymphadenopathy associated virus (LAV), HTLV-

III, immunodeficiency associated virus (IDAV) or AIDS-related virus (ARV) (Schupbach., 1989). In 1986 the International Committee on the Taxonomy of Viruses recommended the renaming of AIDS-causing virus as human immunodeficiency virus (HIV) (Coffin et al., 1986).

Based on sequence variations two types of HIV are now recognized namely HIV type 1 (HIV-1), the cause of the current epidemic in most parts of the world (UNAIDS., 2000), and HIV type 2 (HIV-2). The two types differ with regards to serological and molecular characteristics (Clavel et al., 1986). HIV-2 was first identified in West Africa, and has been shown to be less pathogenic than HIV-1. Infected subjects often have longer incubation periods between infection and AIDS-defining conditions than those infected with HIV-1 (Pepin et al, 1991; Whittle et al., 1994).

Two years after the first reported cases of AIDS, Letvin and colleagues (1983) reported an outbreak of a similar disease to human AIDS in a colony of macaque monkeys. A simian retrovirus related to HIV, SIV_{mac}, was subsequently isolated from these animals and shown to induce simian AIDS (SAIDS) in rhesus macaques (Daniel et al., 1985; Letvin et al., 1985). A number of SIV strains have since been isolated and characterized from several non-human primates (Hayami et al., 1994). The various isolates and respective hosts from which they have been isolated are: SIV_{agm} (African green monkey; *Cercopithecus aethiops*); SIV_{smm} (Sooty mangabeys; *Cercocebus atys*); SIV_{mnd} (Mandrils; Mandillus sphynx); SIV_{syk} (Sykes monkey; *Cercopithecus mitis*) and SIV_{cpz} (Chimpanzee; *Pan troglodytes*) (Hayami et al., 1994). Infection of some non-human primates may result in disease progression and AIDS. However, others remain asymptomatic (Kurth et al., 1997). SIV infection of macaque monkeys mimics AIDS in human. Thus, the model may be analogous (Kindlt et al., 1992).

1.2.2 Structure and genomic organization of HIV and SIV

The structural organization of HIV, which has been well described (Abimiku and Gallo., 1995; IARC., 1996) is depicted in figure 1. The virion measures approximately 100 nm in diameter with a dense cylindrical nucleoid surrounded by a lipid membrane. The viral core is made up of RT (p55/p66), endonuclease or integrase, protease (p32) and nucleocapsid proteins (p6 and p7), and two copies of positive strand viral RNA, all within an icosahedral capsid protein (p24) (Abimiku and Gallo., 1995; IARC., 1996). The myristoylated matrix protein (p17) lies just below the lipid bilayer surrounding the virion. Embedded within the lipid bilayer are the viral glycoproteins, gp120 and gp41. The genome of HIV and SIV, like that of other retroviruses, are organized similarly, being composed of three structural genes, *gag*, *pol* and *env* integrated between two long term repeat (LTR) elements (Schupbach., 1989; Cullen., 1993; Hayami et al., 1994; Abimiku and Gallo., 1995). The genomic organization of primate lentiviruses is illustrated in figure 2. The LTRs contain sequences required for polyadenylation of viral transcripts within the 3'LTR. LTRs also contain enhancer and promoter elements that act as targets for cellular and viral regulatory proteins, which influence viral replication (Cullen., 1993). The *gag* gene encodes proteins that constitute the core of the virus. The HIV *gag* gene is transcribed as a polyprotein precursor molecule, and undergoes proteolytic cleavage to give rise to a number of smaller core proteins (p18, p24, p7 and p9) (Abimiku and Gallo., 1995; IARC., 1996). The *pol gene* codes for various retroviral enzymes namely protease, RT and endonuclease while env gene encodes the various envelope glycoproteins. The HIV *env* is transcribed as a glycosylated protein precursor (gp160). It is processed to form the exterior glycoprotein (gp120) and the transmembrane glycoprotein (gp41). These viral glycoproteins are involved in viral attachment and fusion of the virus to a susceptible cell (Siliciano., 1996). Two other regulatory genes, *tat*

and *rev*, are unique for the primate lentiviruses, and are located within the *env* open reading frame (ORF) (Hayami et al., 1994). In addition HIV and SIV have three or four auxiliary genes namely *vif, nef, vpu, vpr* and *vpx* (Gibbs and Desrosiers., 1993). Little is known about their function. However, the observed conservation of these genes, particularly over large phylogenetic distances, implies that they may play a role in viral replication, persistence, and transmission (Gibbs and Desrosiers., 1993). Based on the combination of three auxiliary genes, five groups of primate lentiviruses are now recognized (Hayami et al., 1994): the HIV-1 group (HIV-1/SIV_{cpz}), HIV-2 group (HIV-2/SIV_{smm}/SIV_{nme}/SIV/SIV_{mac}), SIV agm group (SIV_{agm}/SIV_{wcm}), SIV_{mnd} and SIV_{syk} (Figure 2). *Vpu* is unique for HIV-1 group, *vpr* is seen in HIV-1, HIV-2, SIV_{mnd}, and SIV_{syk} groups, and *vpx* is present only in the HIV-2 and SIV_{agm} groups.

1.2.3 Replication cycle of HIV

The life cycle of HIV is initiated by the high affinity attachment of the virion to the CD4+ receptor on a target cell by means of the envelope glycoprotein, gp120 (Siliciano., 1996) as illustrated in figure 3. Further studies have shown that this attachment alone is not sufficient for fusion to occur. The presence of β chemokine receptors is essential for viral entry into cells (Lyster., 1998). Such two co-receptors have been identified and their roles in viral entry, tropism and disease progression assessed (Berger et al., 1999) (Figure 4). Macrophage-tropic strains of HIV-1 utilize the chemokine receptor CCR-5 while lymphotropic strains use CXCR4 (Lyster., 1998). The binding of CD4 induces conformational change(s) in the gp120 that exposes, creates, or stabilizes the co-receptor–binding determinants. The gp 120 interaction with co-receptor then induces a further conformational change (s) in the *env* that results in activation of gp41, presumably by exposing and extending its fusion peptide so that it can insert into the plasma

membrane of the target cell (Berger et al., 1999). The above model is illustrated in figure four. The subsequent events in the replication cycle of HIV-1 have been described (Abimiku and Gallo., 1995; Connor and Ho., 1995; IARC., 1996). Once internalised, the virus rapidly uncoats its genomic ssRNA, which is reverse transcribed to a double stranded proviral DNA copy through the enzymatic activities of polymerase and ribonuclease H part of the RT enzyme. The pre-integration complex is then translocated into the nucleus and becomes randomly integrated in host cellular DNA by the viral integrase enzyme. Proviral DNA acts as a template for the production of viral progeny.

Integrated virus can remain in a latent form, not replicating but persisting in the host cell. In quiescent cells, HIV replication is arrested during reverse transcription, resulting in generation of an incomplete replicative intermediate that is labile within the cell (Zack et al., 1990). Activation of the host cell, mediated by exogenous antigens, mitogens, or select cytokines, is required for successful completion of HIV integration and expression of proviral DNA (Zack et al., 1990). In activated cells, the proviral DNA undergoes transcription into viral RNA molecules. These serve both as genomic RNA and mRNA to code new viral proteins including regulatory proteins that in turn control viral replication. Synthesized viral proteins undergo post-translational processing before assembly and release. Budding through the cell membrane allows acquisition of an outer lipid bilayer containing the envelope gycoproteins, gp120 and gp41 and the eventual formation of mature virions.

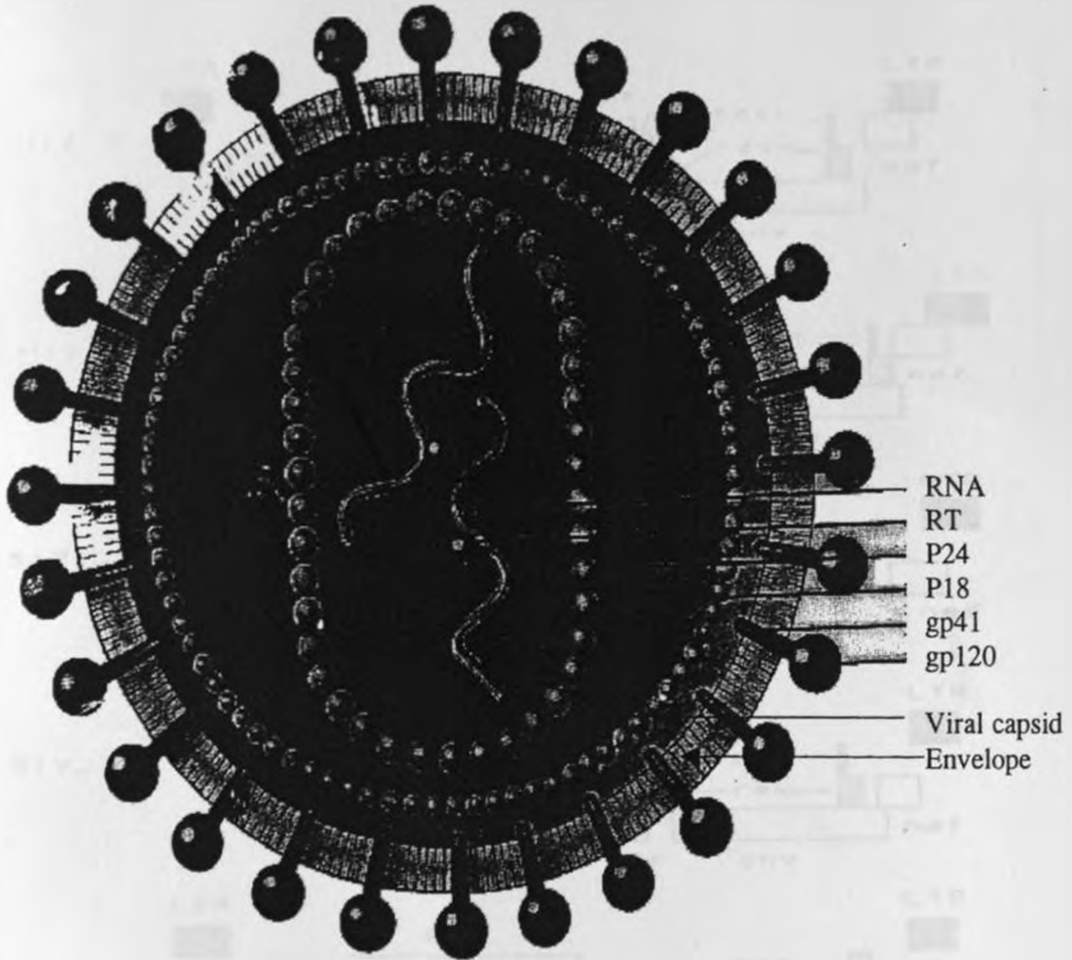
1.2.4 Mechanism of HIV-induced disease

HIV and SIV show selective tropism for cells expressing CD4 molecules on their surface (Kindlt et al., 1992; Siliciano., 1996). The distribution of HIV-infected cells in the body is, therefore, primarily determined by the distribution of $CD4^+$ cells. HIV has been shown to infect $CD4^+$ lymphocytes and monocyte-derived macrophages in peripheral blood and lymphnodes, as well as a variety of tissues and organs (Planells et al., 1993). The pathologic manifestations associated with AIDS are believed to result from primary HIV infection in the corresponding tissues (Planelles et al., 1993). The immune dysfunction characteristic of HIV infection is mainly due to a progressive decline in $CD4^+$ lymphocytes (Siliciano., 1996). The mechanisms underlying depletion of $CD4^+$ cells are not known but are believed to involve the following; i) the high level of retroviral DNA found in the cytoplasm of infected cells may impair cell viability or function, ii) the binding of gp120 to the CD4 molecule of non infected cells results in formation of multinucleated giant cells or syncytia, iii) $CD4^+$ lymphocytes present in the vicinity of productively infected cells may absorb sufficient amounts of excess gp120 rendering them susceptible to destruction by cytotoxic T cells or antibody-dependent cytotoxic effector cells ($CD16^+$), iv) failure to compensate for infected mature T cells due to infection of T-cell precursors, v) cytokine dysregulation, e.g. high levels of α-interferon, may impair T cell proliferation, vi) autoreactive and anti-idiotypic antibodies in the sera of HIV-infected subjects (possibly indicating cross-reactivity between HIV antigens and self proteins), may lead to blockage of communication needed for protective immune responses, vii) non-infected cells may bind previously shed gp 120 priming them for programmed cell death (apoptosis) (Abimiku and Gallo., 1995; Siliciano., 1996).

The CD4-mediated depletion of T lymphocytes impairs the immune system substantially rendering the body susceptible to opportunistic infections (including *Mycobacterium tuberculosis*, *Cryptococcus neoformans*, Cryptosporidium, Cytomegalovirus, *Candida albicans*, *Toxoplasma gondii*, *Pneumocytis carinii*) and malignancies (e.g. Kaposis sarcoma and non-Hodgkins lymphoma) culminating in AIDS (Levy., 1993). Neurological manifestations in HIV-1 infected subjects can occur as a result of infection of astrocytes (Brack-Werner., 1999). Gastrointestinal disturbances associated with HIV infection may be secondary to HIV infection of mononuclear phagocytes in the gut stroma, or as a result of direct infection of gut cells (Rodgers and Kagnoff., 1987).

Figure 1

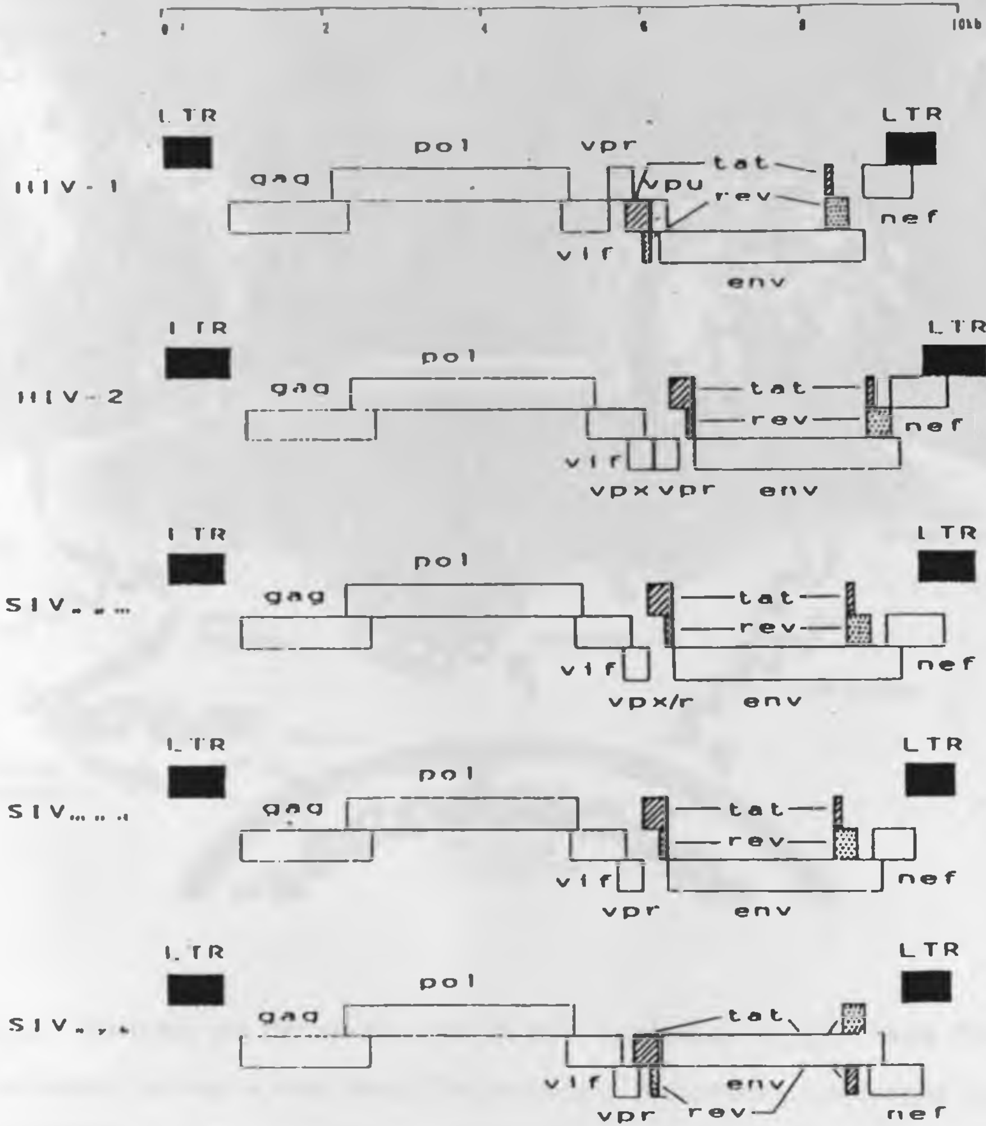
Structure of Human immunodeficiency virus (HIV)



The figure illustrates structural organization of HIV. The virus is bound by part of the host plasma membrane acquired during budding and modified by insertion of gp41 and gp120. The membrane encases the viral capsid made of p24 and p18 within which are two RNA strands and the enzyme reverse transcriptase (RT) (adapted from Muesinget al., 1985).

Figure 2

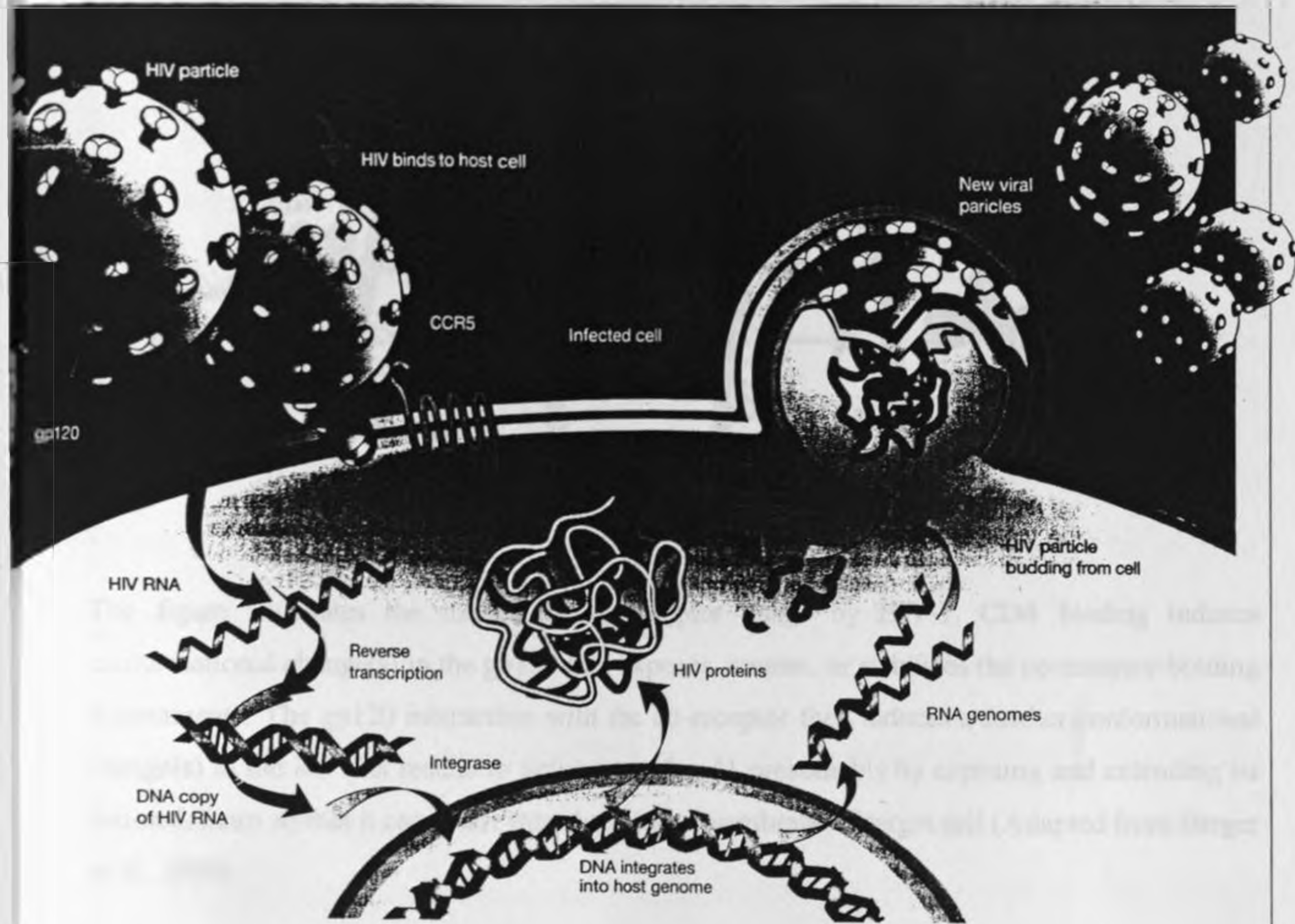
Genomic organization of primate lentiviruses



Genome organizations of the five major groups of primate lentiviruses. (Adapted from Hayami et al., 1994).

Figure 3

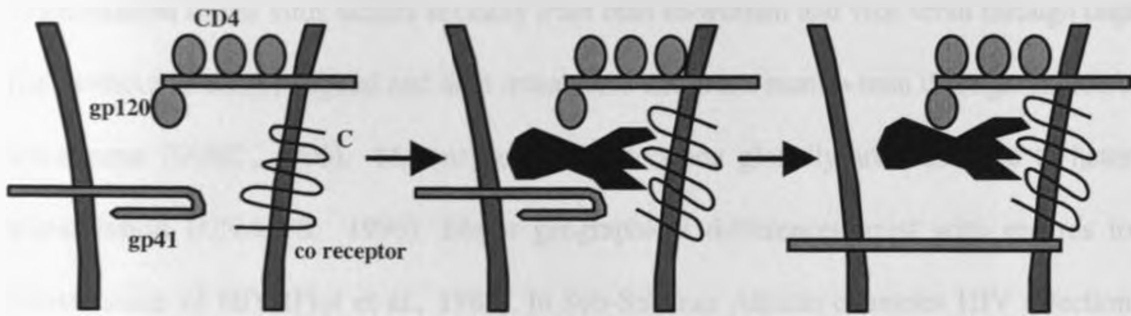
Replication cycle of HIV



The figure illustrates the replication cycle of HIV. Interaction of gp120 with CD4 triggers a series of events leading to viral entry. The presence of β chemokine co-receptor is required for efficient infection of susceptible target cells. Viral entry is followed by the synthesis of cDNA and double stranded DNA (dsDNA) that is integrated into the host DNA using the viral encoded integrase enzyme and expressed into viral RNA and proteins during the host transcription and translation. (Weiss., 2001).

Figure 4

Model for co-receptor usage by HIV-1



The figure illustrates the model for co-receptor usage by HIV-1. CD4 binding induces conformational change(s) in the gp120 that exposes, creates, or stabilizes the co-receptor-binding determinants. The gp120 interaction with the co-receptor then induces a further conformational change(s) in the *env* that results in activation of gp41 presumably by exposing and extending its fusion domain so that it can insert into the plasma membrane of target cell (Adapted from Berger et al., 1999)

1.2.5 HIV transmission

HIV is mainly transmitted by any of the three modes below:

(i) Sexual transmission

Transmission of the virus occurs sexually from man-to-woman and vice versa through unprotected (i.e. without condom) vaginal and anal intercourse and from man-to-man through unprotected anal intercourse (IARC., 1996). Majority of HIV infections globally are attributed to heterosexual transmission (UNAIDS., 1996). Major geographical differences exist with regards to sexual transmission of HIV (Piot et al., 1988). In Sub-Saharan African countries HIV infection mainly spreads through heterosexual means while in America, Europe and Australia spread has occurred predominantly among men and injecting drug users (IDU).

Various studies conducted have established that male-to-female transmission is significantly more efficient than female-to-male transmission (Alexander., 1990; Pedian et al., 1991; Pedian et al., 1997). As compared to other sexually transmitted diseases (STDs) HIV is, however, less efficiently transmitted by sexual contact (Alexander., 1990). Infectivity for a single sexual contact is variable and is estimated to be as low as 0.0009 to 0.3% (Lawrence et al., 1989; Pedian et al., 1997). Some individuals become infected after a single or a few contacts (Alexander., 1990) while others remain uninfected despite several contacts (Alexander., 1990). Increased infectivity or susceptibility of an individual is dependent on the presence of co-factors. These include concomitant STDs that enhance transmission and promote viral replication (Cohen., 1998). This may result in increased secretion of HIV in genital secretion in both men and women (Ghys et al., 1997; Dyer et al., 1998; Plummer et al., 1998). Infected individuals with advanced infection, as determined by clinical and laboratory parameters, are more likely to transmit HIV to

their sexual partners than asymptomatic individuals (Mayer and Anderson., 1995). This is consistent with an increasing viral burden (Clark et al., 1991). Thus, HIV-1 is more frequently isolated in semen of chronically infected individuals (Anderson et al., 1992). Similarly, shedding of HIV-1 in cervicovaginal secretions is influenced by the stage of the infection (John et al., 1997; Cohen., 1998). The currently identified risk factors for transmission are not precise predictors of transmission (Pedian et al., 1997). There is need, therefore, to investigate the role of other factors including immunological, genetic and viral factors likely to influence transmission. It has been shown, for instance, that during sexual transmission selection of viral genotype occurs (Miller., 1998a). Viral isolates showing tropism for macrophages are more readily transmitted than those showing tropism for T lymphocytes only (van't Wout et al., 1994; Miller., 1998a).

(ii) Mother- to-child (Perinatal) transmission

The highest proportion of paediatric HIV infections occur through perinatal transmission (Newell., 2000). The estimated rates of transmission range between 15 to 35% and it is associated with advanced maternal disease, increased maternal viral load, prematurity, prolonged rupture of membranes, length of labour and vaginal delivery (Newell., 2000). Transmission may occur *in utero* via transplacental route, or during labour and delivery or postnatally from infected breast milk during lactation (Peckham and Gibbs., 1995). Intrapartum transmission accounts for the highest proportion of perinatally acquired HIV infection (Peckham and Gibbs., 1995).

(iii) Transmission through direct exposure to blood

Direct blood-to-blood contact, occasioned by transfusions or use of contaminated needles is the most efficient mode of HIV transmission (IARC., 1996). In retrospective studies of people transfused with HIV-infected blood, transmission rates were essentially 100% (Donegan et al., 1990). Effective measures to ensure a safe blood supply and to reduce use of contaminated needle, including needle exchange programme for IDU minimizes this route of transmission (Dias-Ferrao et al., 1999).

1.2.6 Diagnosis

The diagnosis of HIV infection is conventionally made on the basis of a repeatedly reactive enzyme immunoassay, which is then confirmed with a more specific test; most notably the Western blot (Gurtler., 1996). Non serological tests such as PCR, viral culture, immunohistochemistry and hybridization, which directly test the presence of the virus or its products in clinical samples have the advantage of being very sensitive (Davey and Lane., 1990).

1.2.7 Anti-HIV therapy

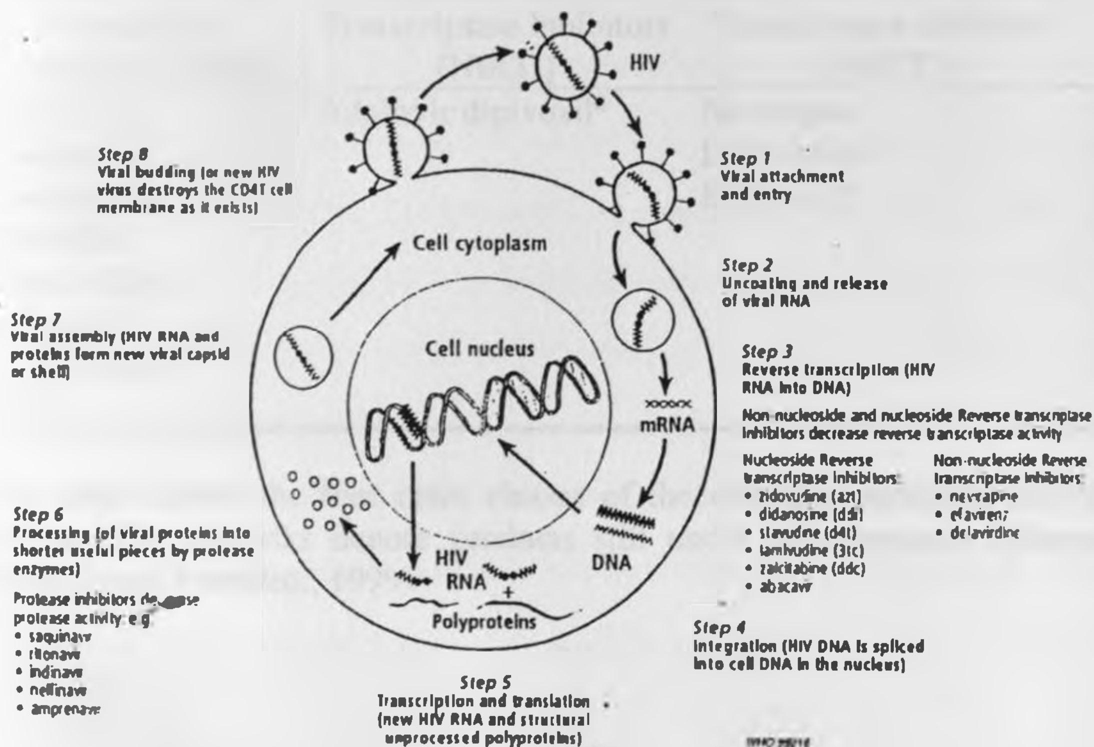
No known cure for HIV infection exists but a number of potent anti-retroviral drugs have been developed (Rizzard and Pentaleo., 1999) as shown in table 1. Plasma viral load quantitation has been used to monitor disease progression, to evaluate the efficacy of anti-retroviral therapy, and to assess the risk of heterosexual and vertical transmission of the virus. These agents inhibit the viral replication cycle using different targets (Piketty., 1999) (Figure 5). Nucleoside analogues competitively inhibit the utilization of cellular deoxythymidine triphosphate by viral RT, blocking the replicative cycle before the integration of the viral genome into the host cell DNA.

Non-nucleoside RT (NNRTI) inhibitors act on the same target as NRTIs but in a non-competitive manner, by attaching to the catalytic site of viral RT. Protease inhibitors inhibit HIV-specific protease. This enzyme cleaves viral polyproteins during the maturation phase of the replicative cycle.

In order to circumvent the development of drug resistance, combination therapy or highly active anti-retroviral therapy (HAART) instead of monotherapy is now widely recommended in most countries (Piketty., 1999). Regimens most often include the use of two NRTIs plus either a protease inhibitor or a NNRTI. Introduction of HAART as the standard of care has led to a possible decline in HIV-related morbidity and mortality (Vittinghoff et al., 1999).

Figure 5

Anti-retroviral agents inhibiting HIV replication at different targets



(Adapted from Piketty., 1999)

Table 1

Different classes of antiretroviral agents

Nucleoside Reverse Transcriptase Inhibitors (NRTI)	Nucleotide Reverse Transcriptase Inhibitors (NtRTI)	Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI)	Protease Inhibitors)
Zidovudine	Adefovir dipivoxil*	Nevirapine	Saquinavir
Didanosine		Delavirdine	Ritonavir
Zalcitabine		Efavirenz*	Indinavir
Stavudine			Nelfinavir
Lamivudine			Amprenavir*
Abacavir*			Tipranavir*
Lodenosine*			ABT-378*
FTC*			

The table shows the four main classes of the currently available anti-retroviral agents. The asterisks denote products still under development. (Adapted from Rizzard and Pentaleo., 1999)

1.2.8 Mucosal immunity to HIV

1.2.8.1 Overview of mucosal immune mechanisms

The body has an extensive mucosal surface. A complex interaction between the local adaptive immune system and physical innate mechanisms protect this surface against pathogen invasion (Cohen., 1992; Brandzaeg., 1997). Physical innate protection is conferred by a hydrophilic layer of surface glycolipids and glycoproteins, referred to as glycocalyx, generated by epithelial cells (Cohen., 1992). Non-specific factors present in mucus such as lactoferrin, peroxidases and lysozyme also play an important role in the prevention of colonization of mucosal surfaces (Cohen., 1992). Specific mucosal defense mechanisms include humoral and cellular immunity. The principal humoral mediators of mucosal immunity are secretory immunoglobulin A and immunoglobulin M (SIgA and SIgM) synthesised locally and from serum transudation. These are transcytosed to the epithelial surface by polymeric immunoglobulin receptor (pIgR)-mediated mechanisms (Kraenbuhl and Neura., 1992; Miller et al., 1992; Hocini et al., 1995; Brandzaeg., 1997).

Systemic and/or locally produced immunoglobulin G (IgG) may also aid in protection against infections in the external environment by contributing to immune exclusion, particularly at mucosal surfaces where it is less degraded (Braendzaeg., 1997). IgA is synthesized at all mucosal sites mainly as dimers and large polymers collectively referred to as polymeric IgA (pIgA). These are actively translocated onto the epithelial surface (Brandzaeg., 1997). Polymeric SIgA in mucosal secretions are generally more effective at neutralizing pathogens than monomeric isotypes such as IgG because of increased valency and proteolytic stability (Childers et al., 1989). Suggested roles of SIgA in mucosal secretions include virus neutralization,

antibody-dependent cell-mediated cytotoxicity (ADCC), enhancement of phagocytosis, increased adherence of bacteria to mucus and interference with growth factors and enzymes required by pathogens (Miller et al., 1992)

Induction of antigen specific immune response is believed to occur mainly in the Payers patches of the small intestines (Brandzaeg., 1997). Following induction, antigen-specific T and B cells leave the patches via efferent lymphatics and gain access to the systemic circulation through the thoracic duct. The lymphocytes ultimately enter mucosal effector sites such as lamina propria (LP) of respiratory, gastrointestinal and reproductive tracts as well as other sites. This dissemination of antigen-specific cells has lead to the concept of the ‘common mucosal immunologic system (CMIS)’ whereby mucosal immunization at one anatomical site induces detectable mucosal immune response at the site of immunization and also at distant mucosal effector tissues (McDermott and Bienenstock., 1979).

1.2.8.2 Secretory immunity in the female reproductive tract

Local cervicovaginal immunocompetence has been demonstrated by the induction of specific IgA (and IgG) after intravaginal inoculation of inactivated polivaccine (Ogra and Ogra., 1973). Generally, IgG is the predominant antibody in the genital secretions of women and female macaques (Lu et al., 1998; Lu et al., 1999). A recent study employing different methods for collection and quantitation of antibodies in genital secretions showed that IgA predominates in endocervical mucus while IgG is the main antibody in vaginal fluid (Quensnel et al., 1999). IgG has also been demonstrated in cervical samples (Kutteh et al., 1996). IgG in genital secretions may be derived from both serum transudation and from local synthesis (Hocini et al., 1995). On the other hand, IgA is mainly locally synthesized within the female reproductive tract (Kutteh

and Mestecky., 1994; Brandzaeg et al., 1997). The endocervix contains the largest number of immunocytes with a predominance of IgA producing cells. Ectocervix, vagina and fallopian tubes contain significant numbers of immunoglobulin-producing cells which mostly produce IgA (Kutteh and Mestecky., 1994). The synthesis and secretion of immunoglobulins in the female genital tract are influenced by cyclical variations in the levels of sex hormones during menstrual cycle (Wira and Sullivan., 1985; Lu et al., 1999). The precise mechanisms by which the hormones exert this influence is not well understood but may involve the action of estradiol (E_2) and progesterone (P_4) on the following: (a) transudation of immunoglobulins from blood into tissues; (b) the synthesis and expression of the polymeric immunoglobulin receptor by cervical and uterine cells; and (c) the migration of lymphocytes and macrophages into the reproductive tract (Wira and Rossoll., 1995; Lu et al., 1999). The implication of these findings is that secretory immunity to infectious pathogens is likely to vary with the phase of the menstrual cycle.

The genital tract is colonized by a varied population of microbial flora (Larsen and Monif., 2001), the regulation of which has not been fully elucidated. Evidence seems to indicate that the composition of the genital microbial flora may be regulated by the menstrual cycle (Johnson et al., 1985; Eschenbach et al., 2000). These observations may have important implications in as far as causation of disease in the genital mucosa is concerned since the microbiological environment may influence production of disease by a specific agent in local mucosal sites (Larsen and Monif).

1.2.8.3 Local immune response to HIV in the female genital tract

Given that HIV-1 is mainly transmitted though sexual contact, a proper understanding of the immune system in the genital tract can provide useful information regarding the biology of this transmission (Yeaman et al., 1998). In HIV-infected women, a secretory immune response to the virus has been demonstrated (Lu et al., 1993). IgG as opposed to IgA predominates in genital secretions of infected women (Lu et al., 1993; Haimovici et al., 1997). IgA is the predominant antibody in the endocervical secretions whereas IgM is the least detectable in CVL samples (Lu et al., 1993; Artenstein et al., 1997; Haimovici et al., 1997). Both local synthesis and serum transudation contribute to the genital pool of HIV-specific antibodies (Lu et al., 1993; Belec et al., 1995a; Belec et al., 1995b). Increased serum transudation of the immunoglobulins, particularly IgG, may occur due to HIV-induced alteration in the genital mucosal barrier in infected women (Belec et al., 1996). A marked deficiency of IgA producing plasma cells has been reported in the genital tissues of SIV infected macaques (Miller et al., 1992). This may be responsible for the abnormal mucosal immunity found in HIV-infected women characterized by an impaired IgA response to HIV (Miller et al., 1992; Belec et al., 1995c).

The host immune response to HIV may determine the risk of heterosexual transmission. Non-transmitting HIV-infected individuals have a broad neutralizing systemic antibody activity against virus (Fiore et al., 1997). Systemic immunity alone is insufficient to interrupt transmission. A complex interplay between systemic and local immunity to the virus may influence susceptibility or infectivity. A high virus specific antibody titer in serum is associated with low plasma viral load (Fiore et al., 1997). Since there is an association between plasma viraemia and viral load in CVS (Goulston et al., 1998), it is possible that the nature of systemic

and local secretory immunity to HIV determines the risk of female to male transmission by influencing genital shedding of HIV. Although HIV-specific antibodies have been demonstrated in the genital secretions of infected women, their influence on transmission of HIV is not well understood. Evidence is now emerging indicating that an HIV-specific IgA response within the female genital tract may confer protection against sexual transmission of HIV among highly exposed women (Kaul et al., 1999). Other workers have reported that resistance against HIV can occur even in the absence of specific antibodies to the virus within the genital tract of infected women (Dorell et al., 2000). HIV-specific cytotoxic T cell (CTL) activity has also been reported in the reproductive tissue of HIV-1 infected women (Musey et al., 1997). Further studies are needed to establish the influence of local immunity (both humoral and cellular) on shedding and infectivity of HIV in genital secretions.

In this regard, the baboon is suitable for studies aimed at understanding the role of mucosal immunity to human pathogens. In terms of humoral responses, the baboon has a similar immunoglobulin profile as humans (Damian et al., 1971; Shear et al., 1999). Recently it has been shown the distribution of immune cells in the reproductive tract of female baboons was comparable to that in human, offering the potential of this primate to be used as a model for the study of human reproductive immunology (D'Hooghe et al., 2001).

1.2.9 HIV infection in the reproductive tract of women

1.2.9.1 Genital shedding of HIV in women

Genital shedding of HIV-1 is known to occur in both semen and CVS (Anderson et al., 1990; Clemetson et al., 1993). In HIV-infected women, various studies have demonstrated the presence of both cell-free and cell-associated virus genital secretions (Vogt et al., 1987; Clemetson et al.,

1993; Henin et al., 1993). However, the detection rates of both HIV RNA and/or DNA in such secretions have been low (Nielsen et al., 1996; Ghys et al., 1997; Uvin et al., 1997). This is probably due to the techniques applied for collection and processing. Recently, O'Shea and colleagues (1997) have reported a 75% detection rate of HIV-1 in cervicovaginal secretions collected with tampons left *in situ* overnight in the genital tract of infected women. The HIV virus within the genital tract of women is mainly a macrophage-tropic variant, which uses CCR-5 as its co-receptor (Shaheen et al., 1999).

The presence of HIV within the genital secretions and factors that influence shedding of HIV in the female genitalia are likely to determine the risk of female to male transmission of this virus (Bernstein., 1990). Genital shedding of HIV in women positively correlates with plasma HIV-1 load (Goulston et al., 1996;Uvin and Caliendo., 1997; Goulston et al., 1998). This association may have implications for reduction of sexual transmission of HIV. Intervention strategies such as administration of anti-retroviral agents result in a reduction in plasma viral load and also lower the quantity of virus in CVS (Uvin and Caliendo., 1997). A positive association of concurrent STDs and genital shedding of HIV in women has been established (Ghys et al., 1997; Mostad et al., 1997). The presence of ulcerative and non-ulcerative STDs increases susceptibility of individuals to HIV-1 infection by several fold (Plummer., 1998). This may occur as a result of increased up regulation in plasma viral load leading to an increase in viral levels in CVL (Cohen., 1998; Plummer., 1998). Concurrent STDs also cause an increase in the population of $CD4^+$ bearing lymphocytes in the genital tract (Al-Harthi and Landay., 2001).

Increased viral shedding in CVS has also been reported among oral contraceptive users suggesting that hormonal levels may regulate viral replication and/or infectivity (Clemetson et al., 1993; Mostad et al., 1997; Cohen., 1998). The severity of vitamin A deficiency correlates positively with cervicovaginal shedding of HIV-1 (John et al., 1997; Mostad et al., 1997). It is not yet understood whether pregnancy or menstrual cycle, both being characterized with profound alterations in sex steroid hormonal levels, exerts effect on the secretion of HIV CVS.

1.2.9.2 Localization of HIV in the reproductive tract

Although the presence of HIV in the genital secretions has been demonstrated, its source has not been well defined (Critchlow and Kiviat., 1997). Analysis of cervicovaginal biopsy specimens from HIV-infected women has revealed that the predominant cell types infected with HIV are monocyte-macrophages, endothelial cells within the submucosa and T lymphocytes within the mucosa of the lower reproductive tract (Pomerantz et al., 1988). HIV-1 is capable of infecting uterine cells (Gosselin et al., 1995). More recently, *in vitro* experiment demonstrated that HIV-1 infects multiple cell types within the reproductive tract of women (Howell et al., 1997). The cell types infected include epithelial, stromal, and dendritic cells and cells with CD14+ CD4+, CD14- CD4-, and CD14+ CD4- phenotypes. Once infected, these cells may serve as a source of dissemination of the virus throughout the reproductive tract before systemic infection. Studies conducted in the SIV/macaque system appear to support the hypothesis that dendritic/Langerhans' cells are the reservoirs of HIV/SIV replication *in vivo* in the female genital tract (Hu et al., 1998; Miller et al., 1998b). Further research is required to determine the source of HIV in the reproductive tract.

1.2.10 Animal models of HIV infection and AIDS

Ethical and practical reasons limit the study of HIV and AIDS in human beings. Suitable animal models have, therefore, been sought. Infection of chimpanzees with HIV can serve as an ideal model for HIV (Kindlt et al., 1992). This is because of similarity of the CD4 receptor in humans and chimpanzees. Similarities also exist between their respective immune systems. It is only recently, however, that signs of disease analogous to AIDS in man was reported in two chimpanzees experimentally infected with HIV (Fultz et al., 1991). This apparent lack of disease in this animal coupled with its status as an endangered species has prevented further characterization of HIV-1 in this primate model.

SIV infection of macaques is a more appropriate model (Kindlt et al., 1992). SIV mimics HIV in genomic organisation, genetic variability, transmission, pathogenesis and clinical manifestation of infection (Kindlt et al., 1992). Table 2 shows similarities between SIV infection of macaques and HIV infection of man. Differences between the envelope glycoproteins of SIV and HIV-1 limit the use of SIV/macaque model in molecular studies of envelope determinants of HIV-1 pathogenicity (Reihmann et al., 1996 a). Antibodies raised against the two viruses neither cross-react nor cross-neutralize (Javaherian et al., 1992; Murphy-Corb et al., 1996).

To overcome this setback, a genetically engineered chimeric virus, Simian/Human Immunodeficiency virus (SHIV), composed of HIV-1 *env*, *tat*, *rev* and *vpu* genes in a SIV backbone has been developed (Shibata and Adachi., 1992; Reihmann et al., 1996a). Experimental inoculation of macaques with various constructs of SHIV has failed to show an association between pathogenic effect and persistence of infection or viral load during primary

infection (Li et al., 1992). Moreover, virulent strains have only been obtained through serial *in vivo* passage of SHIVs in macaques (Joag et al., 1996; Reihmann et al., 1996b; Joag et al., 1998). The baboon animal model is increasingly being explored as an alternative model to the SIV/Macaque system (Locher et al., 2001). Previous studies have shown that experimental inoculation of baboons with SHIV chimeric virus results in infection with high viral titres, and infection of baboon peripheral blood mononuclear cells (PBMCs) (Allan et al., 1995; Klinger et al., 1998). Besides, baboons are more closely related to humans genetically (Rogers and Hixson., 1997) and physiologically (Vagtborg., 1963) making them suitable for studying pathogenesis of human diseases as well as testing various candidate vaccines and drugs.

This project used female olive baboons systemically inoculated with SHIV $_{89.6P}$ strain. The construct is a recombinant virus composed of the *env* gene of a cytopathic primary isolate of HIV-1 $_{89.6}$ within an $SIV_{mac}239$ backbone (Reihmann et al., 1996a) HIV-1 $_{89.6}$ was isolated and cloned from peripheral blood of an AIDS patient (Collman et al., 1992). The isolate is a macrophage-tropic variant with efficient replication in macrophages and syncytium-inducing cytopathic characteristics (Collman et al., 1992). The envelope glycoproteins of HIV-1 $_{89.6}$ have been shown to confer a high level of early replication of SHIV chimeras *in vivo* as compared to envelope glycoproteins derived from the laboratory-adapted strain of HIV-1 (HXBc2) (Reihmann et al., 1996 a). The structure of the chimera virus used in this study is shown in figure 6.

Table 2

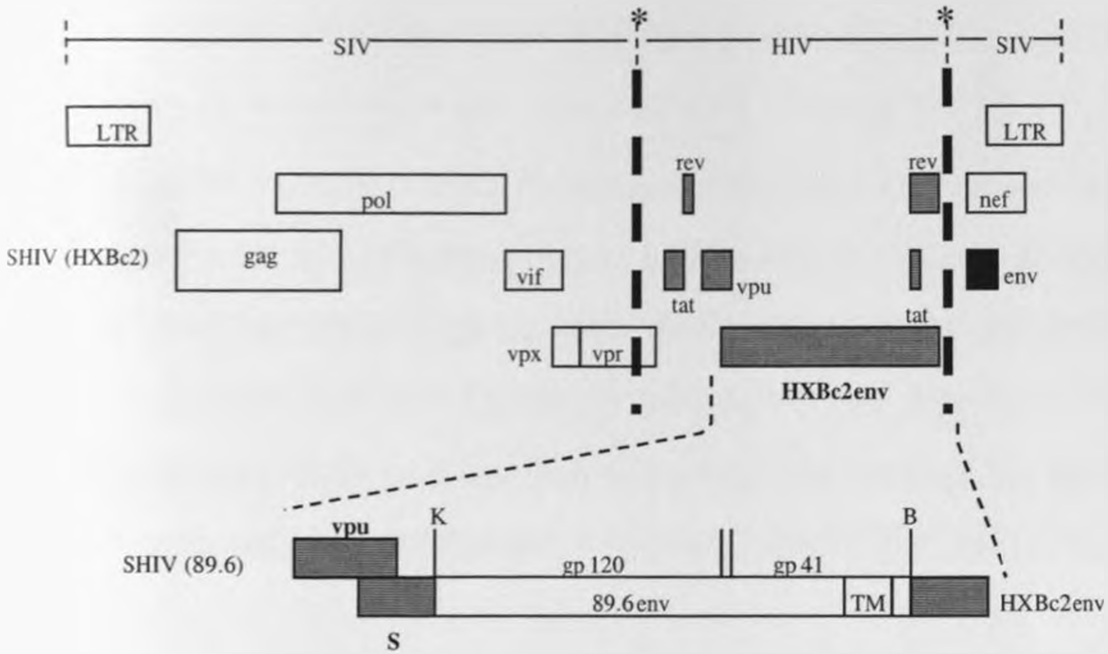
Simian Immunodeficiency Virus (SIV) infection of macaques for human AIDS

Characteristics of virus and disease	SIV/Macaques	HIV-1/Humans
Tropism for CD4	Yes	Yes
Macrophage tropic variants	Yes	Yes
Syncytium formation	Yes	Yes
Decline in CD4 cells with onset of disease	Yes	Yes
Antigenemia	Yes	Yes
gag antibodies decline with disease	Yes	Yes
High virus load terminally	Yes	Yes
V3 as neutralizing epitope	No	Yes
CD8 viral suppression	Yes	Yes
Rapid generation of variants (viral swam)	Yes	Yes
High proportion of unintegrated viral DNA	Yes	Yes
Opportunistic infections		
Cytomegalovirus	Yes	Yes
Candida	Yes	Yes
Pneumocytis	Yes	Yes
Mycobacterium	Yes	Yes
Lymphoma	Yes	Yes
Kaposi's sarcoma	No	Yes
Central nervous system disease	Yes	Yes
Wasting	Yes	Yes
Variable clinical progression	Yes	Yes
Perinatal maternal transmission	Probable	Yes
Sexual transmission	Unknown	Yes

(Adapted from Kindlt et al., 1992)

Figure 6

The structure of the chimeric virus, $SHIV_{89.6P}$



The structure of SHIV89.6P which was used in this study.The SHIV-HXBc2, which has an intact gene is shown at the top. Asterisks denote the junctions of HIV-1 and SIVmac sequences. The residual 3' end of the SIV *env* gene is shown in solid black. The structure of $SHIV_{89.6P}$ chimera is shown at the bottom. Shaded sequences were derived from the HXBc2 isolate, and unshaded sequences were derived from the 89.6 isolate. The Kpn1 (K) and BamH1 (B) restriction sites used for insertion of 89.6 sequences into the SHIV-HXBc2 construct are shown. The signal peptides (S) and transmembrane region (TM) of the envelope glycoproteins are also shown (Reihmann et al., 1996 a).

1.2.11. Physiology of the menstrual cycle of the baboon

The baboon is a suitable animal model for various studies including reproductive biology [illegible] to it's close phylogenetic relationship to humans, its large size, hardy constitution [illegible] adaptability to life in captivity (Eley and Bambra., 1993).

The physiological events that underlie the menstrual cycle of this animal have been well [illegible] (Hendrickx and Kraemer., 1971; Wildt et al., 1977; Kling and Westfahl., 1978). The cycle [illegible] is 31 to 34 days with a wide range both within and among individuals, as compared [illegible] classical 28 days for humans (Stevens., 1997). The length of the cycle can be determined [illegible] ways (Eley and Bambra., 1993). One way of doing this is by counting the day from the [illegible] observation of menstruation in one cycle (day 1) to the day of the next menstruation.

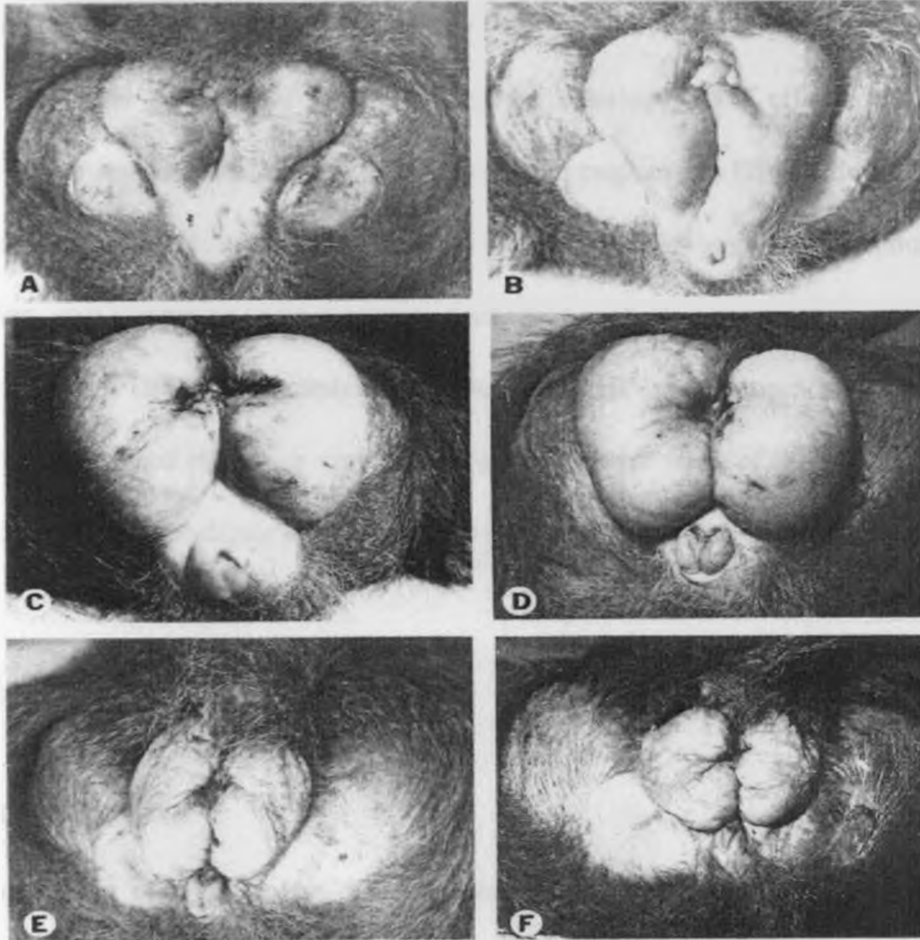
The length of the menstrual cycle can also be determined by the perineal skin cycle (Hend[illegible] and Kraemer., 1971; Eley and Bambra., 1993) (Figure 7 and appendix 8). The onset of the [illegible] day of rapid detumescence of the swollen sex skin divides the cycle into two distinct phas[illegible] tumescence phase and a detumescence phase. The detumescence phase extends from the ons[illegible] rapid detumescence and continues into the menstruation. The tumescence phase correspond[illegible] the period of follicular activity in the ovarian cycle, while the detumescence phase corresp[illegible] to the luteal portion of the ovarian cycle (Hausfater., 1975).

Studies have shown a positive correlation between the cyclical perineal skin fluctuations [illegible] changes in blood E_2 and P4 levels (Stevens., 1970; Wildt et al., 1977; Kling and Westfahl., 19[illegible] Shaikh et al., 1982). Tumescence occurs during the follicular phase of the menstrual cycle [illegible]

is characterized by E_2 dominance while detumescence occurs during the luteal phase when E_2 levels are reduced and progesterone is elevated (Kling and Westfahl., 1978; Stevens., 1997). A similar hormonal pattern has been observed during the menstrual cycles of baboons and humans (Kling and Westfahl., 1978; Eley and Bambra., 1993; Stevens., 1997). Increasing concentrations of circulating E_2, which peaks at over 300 pg/ml before luteinizing hormone (LH) surge, and subsequent ovulation marks the follicular phase of the baboon's cycle. Circulating P_4 levels remain low throughout the follicular phase. An increase in P_4 concentration occurs during the first few days of the postovulatory period (Kling and Westfahl., 1978; Eley and Bambra., 1993) The major difference observed in the hormonal patterns between women and baboons is in E_2 secretion (Kling and Westfahl., 1978; Eley and Bambra., 1993; Stevens., 1997). The sex steroid hormonal levels are also reportedly lower in the baboons (Kling and Westfahl., 1978).

Figure 7

Perineal skin cycle of the baboon



Normal cyclic changes in the perineum. A. early initial turgescent stage, day 2 of cycle. B. late initial turgescent stage, 5th day of cycle. C. maximum turgescent stage, 16th day of cycle, 1 day after the time of anticipated ovulation. D. early initial detugescent stage, 18th day of cycle. Note the marked decrease in size of clitorial lobe. E. late initial deturgescent stage, 20th day of cycle. F. quiescent stage, 22nd day of cycle (adapted from Hendrickx and Kraemer., 1971)

1.3 Justification of the study

The risk of female-to-male transmission of HIV is likely to be determined by the concentration of the virus in CVS of infected women (Critchlow and Kaviat., 1997). A better understanding of determinants of genital shedding of HIV is critical for development of effective strategies for prevention of this transmission. The role of local immune response to HIV in the genital tract is not clear. Despite demonstration of HIV-specific humoral immunity in genital secretions of HIV-infected women, still it remains to be established whether such immune responses play a role in sexual transmission of HIV. Identification of the source of HIV in the reproductive tract is also important for efforts aimed to reduce vertical and sexual transmission of the virus. This present study was undertaken to investigate these questions in the SHIV/baboon model of HIV infection

1.4 Objectives

1.4.1 General objective

The overall objective of this project was to achieve a better understand the virology and immunology of HIV in the female reproductive tract using the baboon-SHIV-89.6P model.

1.4.2 Specific aims

- To isolate and detect the presence of SHIV-89.6P in the CVS of SHIV-infected female baboons

- To determine and quantitate anti-SHIV89.6P antibody responses within the reproductive tract of SHIV-infected female baboons.

CHAPTER TWO

2.0 Materials and methods

2.1 Materials

2.1.1 Animals

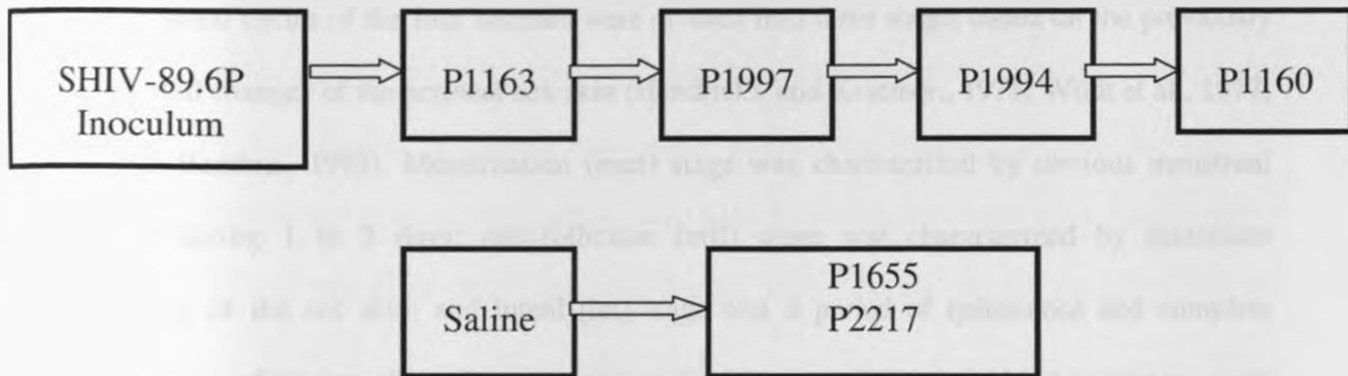
Four female olive baboons (*Papio anubis*), maintained at the Institute of Primate Research (IPR), Kenya were used for this study. The animals were treated in accordance with the internationally accepted guidelines for the humane treatment of animals for research, and the Institutional Scientific and Ethical Research Committee of IPR approved this protocol. Daily monitoring of the perineal skin was done to determine the stage of the menstrual cycle of each animal in concordance with the established pattern (Hendrickx and Kraemer., 1971; Wildt et al., 1977; Eley and Bambra., 1993). Before sample collection, the animals were sedated by an intramuscular injection of a combination of ketamine hydrochloride (Ketaset®, Fort Dodge Laboratories Inc, Fort Dodge, IW) and xylazine (Chanazine®, Chanelle Pharmaceuticals Manufacturing Ltd, Galway, Ireland) at a ratio of 5:3 at a dosage of 100mg/kg body weight ketamine and 60-mg/kg body weight xylazine.

2.1.2 Experimental design

The four animals used in the present study were part of a previous study in our laboratory aimed at determining *in vivo* infectivity and pathogenesis of $SHIV_{89.6P}$ infection in adult baboons (Otsyula et al. Unpublished work). The animals were shown to be SIV and STLV-I negative prior to being used in the study. In brief, the $SHIV_{89.6P}$ stock virus used to infect the baboons in the earlier study was in the form of cell culture supernatant derived from an experimentally infected rhesus macaque that had developed simian AIDS (a donation from Dr. Norman Letvin,

Harvard University, USA). The $SHIV_{89.6P}$ had been prepared as earlier described (Reihmann et al., 1996a). Infectious virus for animal inoculation was obtained through short-term co-culture of the stock virus with phytohemaglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs) derived from a healthy adult baboon. Upon development of cytopathic effect (CPE), 1ml aliquot of culture supernatant fluid was then intravenously inoculated into the first baboon (P1163) to begin the serial *in vivo* passage (Figure 8). Two weeks post-inoculation, 1ml of bone marrow was drawn from P1163 and then intravenously inoculated into the second baboon (P1997). Two similar serial passages involving intravenous inoculation (i.v) of 1ml bone marrow from the donor to the recipient baboon were carried out (Figure 8), and the interval between each passage was two weeks. Two adult baboons (P1655 and P2217) were injected with i.v with phosphate buffered saline (PBS) (Sigma Chemical Co., St. Louis, MO) to serve as negative controls. The present study utilized two of the previously SHIV-infected (P1160 and P1994) and two negative controls (p1655 and P2217) baboons. The period between infection and commencement of sample collection from the animals for use in the present study was approximately one and a half a year.

Figure 8



The *in vivo* serial passage of $SHIV_{89.6P}$ in adult female baboons. The original virus stock was obtained from culture supernatant of rhesus macaque that had died of simian AIDS (a gift from Dr. Norman Letvin, Harvard, USA). Infectious virus for animal inoculations was obtained through short-term co-culture of the stock virus with phytohemaglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs) derived from a healthy adult baboon. Upon development of cytopathic effect (CPE), culture supernatant was harvested, and an aliquot of 5ml administered intravenously into P1163 to begin the in vivo passage. The virus was passaged four times by intravenous (i.v) inoculation of 5ml bone marrow from the donor to the recipient baboon. The interval between each in vivo passage was two weeks. P1655 and P2217 were injected i.v with phosphate buffered saline (PBS) to serve as negative (Otsyula et al., Unpublished work).

2.2 Methods

2.2.1 Sample collection

The menstrual cycles of the four animals were divided into three stages based on the previously documented changes of the perineal sex skin (Hendrickx and Kraemer., 1971; Wildt et al., 1977; Eley and Bambra., 1993). Menstruation (men) stage was characterized by obvious menstrual bleeding lasting 1 to 3 days; mid-follicular (mfl) stage was characterized by maximum tumescence of the sex skin; and luteal (lut) stage was a period of quiescence and complete detumescence of the sex skin. Paired cervicovaginal lavage (CVL) and blood specimens were collected from $SHIV_{89.6P}$ infected and uninfected adult female baboons during these three stages of two successive menstrual cycles.

Whole blood was collected into 10-ml sterile vacutainers (Becton Dickinson, Co. NJ) containing ethylene diamine tetra acetic acid (EDTA) through venipuncture of the femoral vein. An aliquot of whole blood was taken immediately for complete blood cell count. To obtain plasma, the blood was centrifuged at 1,200x g for 15 minutes. Peripheral blood mononuclear cells (PBMCs) were obtained by separation on lymphocyte separation medium (LSM) (Organo Technika, Westchester, PA). CVL samples were collected by vigorous infusion of 5 ml of sterile PBS (Sigma) into the cervical opening and then aspirating as much of the infused saline as possible. The CVL samples were centrifuged at 1,000x g for 10 minutes, and the supernatant filtered using 0.2 μm sterile syringe filters (Nalge® Co., NY). All samples were stored at -70° C until analysis.

2.2.2 Hormonal assays

To correlate perineal skin swelling with the stage of the menstrual cycle, concentrations of estradiol and progesterone in plasma of each baboon was determined as previous described (Sufi et al., 1997). These assays have been validated for baboon samples. In brief, 500 µl and 200 µl of plasma were extracted with 10 times volume of diethyl ether for E_2 and P_4 respectively. Separation of the mixture was achieved by freezing the aqueous solution and decanting off the ether fraction into pre-labeled clean LP_3 tubes. The extract was dried by placing the tubes in a vacuum drier. Reconstitution was then done with 2 ml steroid assay buffer (buffer S) containing gelatin and sodium azide at pH 7.4. To 0.5 ml of this, 100µl (10,000 counts per minute) of the tracer, and 100µl of antibody was added. The mixture was then incubated overnight at 4 °C. Bound antigens were separated from the free using pre-chilled dextran activated charcoal. Subsequently, this was centrifuged at 1,500 g for 15 minutes. The bound hormone was decanted off into clean vials and the unbound disposed. The bound hormone was then scintillated with 4 ml Toluene /PPO mixture and counted in β-scintillation counter (Tri-Carb® 2000, Packard Instruments Co., USA). Standard samples were used to obtain standard curves from which the values of the unknown were determined. The results are reported as mean concentration of the hormones at each stage of the two menstrual cycles.

2.2.3 Serology

To determine the presence of anti-SHIV antibody responses in CVL, two ELISA systems were employed. The first protocol was a modification of a previously described technique (Otsyula et al., 1996). The following alterations were made to the protocol: A 20-amino acid synthetic peptide derived from a conserved immunodominant region of HIV-1 transmembrane glycoprotein (gp41) with a sequence of H_3N-GIWGCSGKLICTTAVPWNAS-COOH (Research Genetics Inc.,

Huntsville, AL) was used to coat microtiter plates (Dynatech, Chantilly, VA). TEST buffer (5mM Tris base, 0.1 mM EDTA, 0.15 M Nacl, 0.05% Tween 20, pH 7.4) was used for dilution and wash procedures in this assay. Following coating with the peptide antigen, the plates were incubated overnight at 4°C. The plates were then blocked with blocking buffer consisting of 0.1% Bovine Serum Albumin (BSA) and 5% non-fat skimmed milk in TEST buffer. After washing, 1:4 dilution of either test or control CVL was added in duplicate wells followed by overnight incubation at 4°C. Reactivity was detected with either 1:500 dilution of goat anti-monkey IgG or IgA conjugated to horseradish peroxidase (HRP-IgG, HRP-IgA; Nordic Lab, Capristrano Beach CA) at 37°C for 1 hour followed by incubation at room temperature with *o*-phenylene-diamine tetrahydrochloride (OPD) as chromogen and hydrogen peroxide (Sigma) as substrate. The reaction was stopped after 30 minutes with 4N H_2SO_4 and then read spectrophotometrically at a wavelength of 490 nm. The presence of anti-SHIV IgA in plasma was determined in 1:10 dilution of control and test specimens in duplicate wells analysed with the synthetic gp41 ELISA as already described for IgA in CVL. To determine the presence of anti-SHIV IgG in plasma, the following alterations were made to synthetic gp41 ELISA protocol used for detection of IgG in CVL. Antigen coated plates were incubated with blocking buffer for 30 minutes at 37°C. Then 100μl of 1:100 dilution of control and test plasma were dispensed into the respective wells. Plates were then washed and incubated with 1:2000 dilution of HRP-conjugated goat anti-monkey IgG (Fc) (Nordic Labs). Plasma or CVL samples were considered to be positive for anti-SHIV IgG and IgA antibodies, in the synthetic gp41 ELISA, if the mean OD of the sample wells was greater than the mean OD of the negative control plus three standard deviations (3SD) from the mean of the negative control samples (Otsyula et al., 1996)). In order to determine anti-SHIV IgG titers, control and test plasma samples were diluted serially from 1:100 while CVL were diluted serially from 1:4 of the sample collected. Titration was

performed with 100 μl of each dilution of specimen in duplicate wells, and the end-point titer defined as the reciprocal of the last dilution giving a positive reaction.

The second ELISA protocol utilized to detect anti-SHIV antibody responses in CVL was a combined HIV-1 and HIV-2 commercial ELISA kit (Genelavia Mixt®, Sanofi Diagnostics Pasteur, Inc. Chaska, MN) performed as per the manufacturer's specifications. The presence of SHIV-specific antibodies in CVL were confirmed by HIV-1 (Cambridge Biotech, Rockville, MD) and HIV-2 (New LAV Blot II®, Sanofi Diagnostics Pasteur, Marness-La-Coquette, France) Western blot assays, performed as per the manufacturers' instructions, to detect antibodies to envelope glycoproteins of HIV-1 and core proteins of SIV_{mac} 239 which constitute the SHIV construct used in this study.

2.2.4 Virus isolation and detection

Frozen plasma and CVL samples were thawed prior to performing cell culture experiments. Either 1:1 or 1:2 dilutions of CVL and plasma respectively were co-cultured with 10^6 Molt-4-clone–8 cells in T25 tissue culture flasks while 1:10 to 1:50 of either CVL or plasma dilutions were cultured with the same number of cells in replicas of two in 24-well microtiter plates. Cultures were maintained in complete RPMI 1640 medium at 37 °C in humid CO_2 incubator (with 5% CO_2 and 95% O_2). They were monitored regularly for cytopathic effect (CPE) and fresh culture medium replenished after every 3 days. All cultures were terminated after 30 days and culture supernatants obtained for p27 core antigen ELISA. The presence of p27 Core antigen in plasma and CVL culture supernatants was determined by the use of a commercial antigen capture ELISA (Coulter., USA). A detailed protocol is contained in appendix 7

CHAPTER THREE

3.0 Results

3.1 Hormonal assays

Concentrations of estradiol were consistently higher in blood during the mid-follicular stage (range: 319 pmol/L to 636 pmol/L) compared to lower levels at menstruation and luteal stages (range: 78pmol/L to 195pmol/L, and 59 pmol/L to 214 pmol/L respectively)(Figure 9). On the other hand, as expected, highest levels of progesterone in plasma of the animals occurred in luteal stage samples (range: 12 nmol/L to 44 nmol/L) (Figure 9). The concentration of this hormone remained below 4 nmol/L in samples collected during menstruation and mid follicular stages (range: 1.1 nmol/L to 2.9 nmol/L, and 1.0 nmol/L to 3.6 nmol/L respectively. Recoveries for both estradiol and progesterone were 95%. Intra-assay coefficient of variation (C.V) for estradiol was 3.9% and 2.6% for progesterone. The assay sensitivity (i.e. the minimum detectable dose) was 93% (76 pmol/L) at 1SD and 95% (0.086 nmol/L) at 1SD for estradiol and progesterone respectively.

3.2 Serology

Results on detection of anti-SHIV antibody responses in plasma and genital secretions are presented in table 3 and table 4. The pattern of detection of anti-SHIV IgG responses in CVL was similar by the two different methods i.e. the synthetic gp41 in-house ELISA and the commercial combined HIV-1/HIV-2 ELISA. Anti-SHIV IgG responses were detected in CVL of P1160 and P1994 during menstruation stage of cycle 1 and cycle 2, and in CVL of P1160 during luteal stage of cycle 1 and cycle2. P1994 had detectable anti-SHIV IgG in CVL samples collected during luteal stage of cycle 2, and not in luteal stage of cycle 1. The antibody was consistently undetectable in CVL samples of SHIV infected animals during mid-follicular stage

of both cycles. With regards to mucosal anti-SHIV IgA responses, CVL of P1160 had detectable levels of the antibody at all the three stages of the first cycle (Table 3). In the same cycle, samples of P1994 were reactive for anti-SHIV IgA at menstruation and luteal stages (Table 3). In the second cycle, an IgA response was demonstrable in CVL of P1160 and P1994 during menstruation and luteal stages only (Table 3). Plasma samples of SHIV-inoculated animals showed anti-SHIV IgA and IgG reactivity in all the three stages of both cycles (Table 3). Neither SHIV-specific IgA nor IgG responses were detected in plasma and CVL of non-infected baboons during the study period. Western blot assays confirmed the antibodies in CVL to be SHIV-specific as they reacted to HIV-1 envelope glycoproteins, gp160, gp120 and gp41, and SIV_{mac} core antigen (p26) (Figure 10 and Figure 11 respectively).

Comparatively higher titers of anti-SHIV IgG were detected in plasma (range: 1:800-1:6400) samples than in CVL samples (range: 1:32-1:128) (Figure 12). The highest titers of the antibody in CVL occurred during menstruation stage as compared to lower and undetectable levels during luteal and mid-follicular stages respectively (Figure 12). The titers of anti-SHIV IgA in CVL was not determined due to limited sample volume. With regards to SHIV-specific IgG titers in plasma, P1160 and P1994 had titers ranging from 1:800 to 1:6400 as illustrated in figure 12. In both cycles, P1160 had higher antibody titers as compared to P1994. Within a given menstrual cycle of both infected baboons, no apparent menstrual cycle dependent variations were observed. However, IgG titers were comparably higher in cycle one than in the second cycle of both SHIV-infected baboons.

3.3 Virus isolation and detection

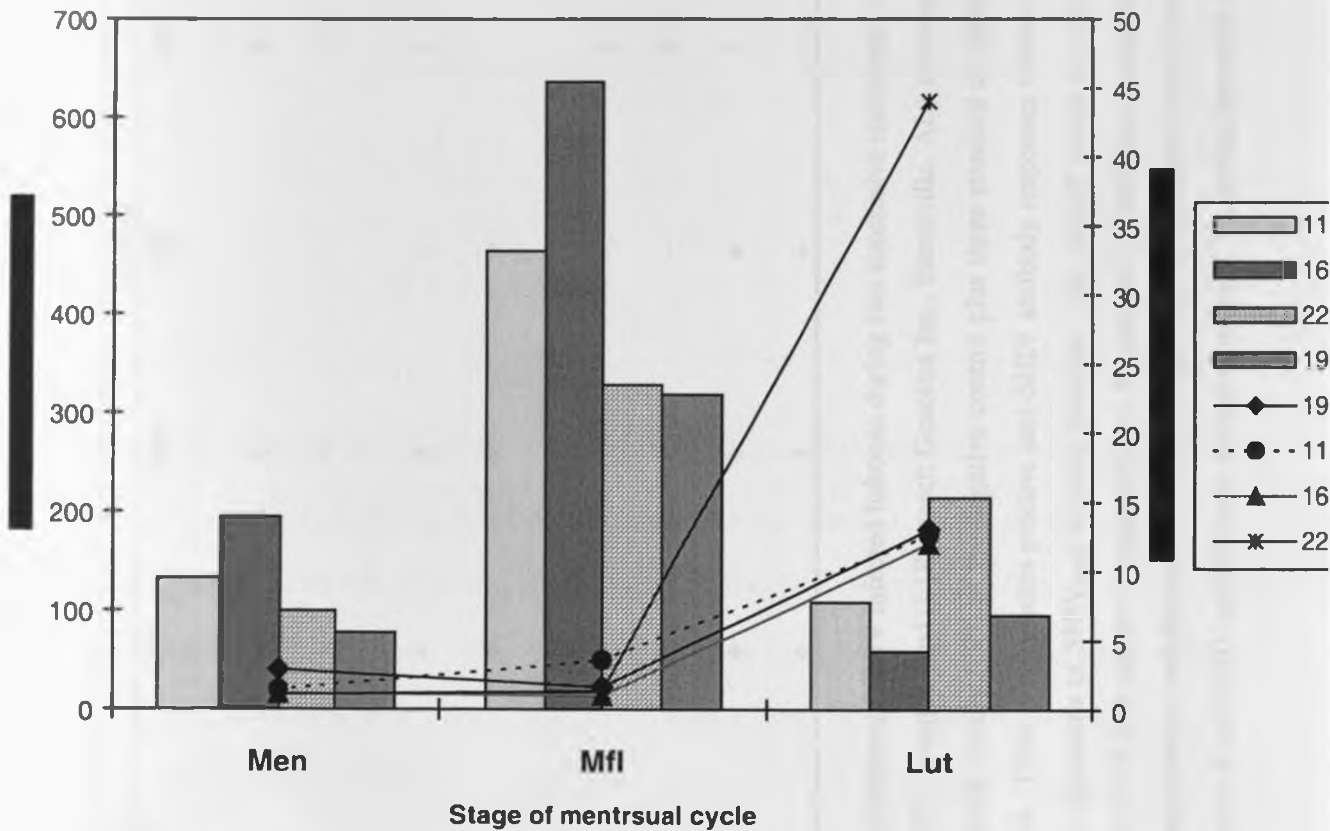
Table 5 shows results for the isolation and detection of SHIV-89.6P in CVL and plasma of SHIV-infected baboons. The virus was not detectable in plasma of P1160 during the first cycle while in the second cycle of this animal virus was detectable in plasma samples collected during mid-follicular and luteal stages. On the other hand CVL cultures of P1160 were positive for the presence of the virus during luteal stage of cycle one and mid-follicular stage of cycle two. Plasma cultures of P1994 were positive for the presence of p27 antigen only in specimens that were collected during menstruation and luteal stages of both cycles. The antigen was detected in CVL of P1994 during menstruation and luteal stages of this cycle.

3.4 Clinical status of the animals

All the animals remained clinically normal throughout the study period as determined by physical examination during sample collection and by laboratory evaluation of the total and differential blood cell count.

Figure 9

Concentration of Estradiol and Progesterone in plasma of female baboons



The figure illustrates mean concentration of estradiol (pmol/L) and progesterone (nmol/L) in plasma of the four baboons used in the study. The sensitivity of the radioimmunoassay (RIA) used was 93% (76 pmol/L) at 1SD and 95% (0.086 nmol/L) at 1 SD for estradiol and progesterone respectively.

Tables 3

Anti-SHIV antibody responses in plasma and CVL of female baboons

Animal No./ Antibody	Cycle 1			Cycle 2		
	Men	Mfl	Lut	Men	Mfl	Lut
P1160						
Anti-SHIV IgG in CVL	+	-	+	+	-	+
Anti-SHIV IgA in CVL	+	+	+	+	-	+
Anti-SHIV IgG in plasma	+	+	+	+	+	+
Anti-SHIV IgA in plasma	+	+	+	+	+	+
P1994						
Anti-SHIV IgG in CVL	+	-	-	+	-	+
Anti-SHIV IgA in CVL	+	-	+	+*	-	+
Anti-SHIV IgG in plasma	+	+	+	+	+	+
Anti-SHIV IgA in plasma	+	+	+	+	+	+

The table illustrates antibody responses to SHIV in CVL and plasma of SHIV infected baboons during two successive menstrual cycles. The responses were detected using a 20-amino acid synthetic HIV-1 peptide (gp41) {Research Genetics Inc., Huntsville, AL} antibody ELISA. The cut-off value in this assay was determines as mean optical density (OD) of the negative control plus three standard deviations (3SD) from the mean of the negative control samples (Otsyula et al., 1996). (+) denotes positive anti-SHIV antibody responses while (-) denotes negative anti-SHIV antibody responses in CVL and plasma specimens of $SHIV_{89.6P}$ infected baboons. The cut-off values for IgG in CVL, IgG in plasma, IgA in CVL and IgA in plasma are 0.024, 0.247, 0.384 and 0.471 respectively. An asterisk (*) indicates values marginally above cut-off while men, mfl and lut respectively denote menstruation, mid-follicular and luteal stages of the menstrual cycle. Non-infected negative control animals remained non-reactive for the presence of anti-SHIV antibodies in both plasma and CVL during the study period.

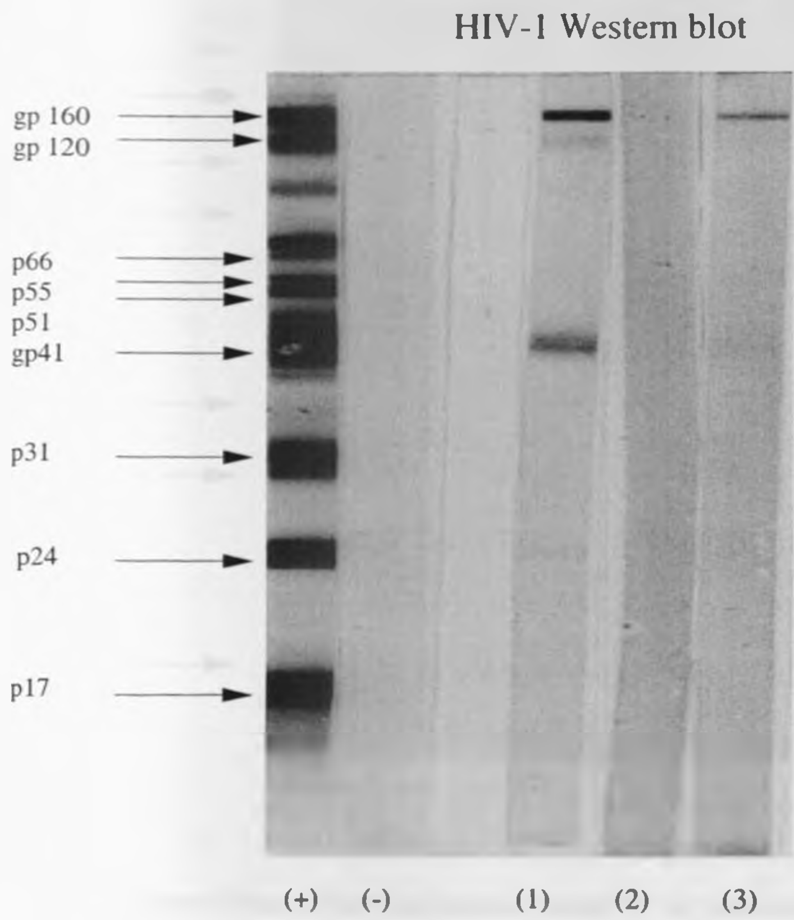
Table 4

Detection of Anti-SHIV antibody response in CVL with the Genelavia Mixt® ELISA

	Cycle 1			Cycle 2		
	Men	Mfl	Lut	Men	Mfl	Lut
P1160	+	-	+	+	-	+
P1994	+	-	-	+	-	+

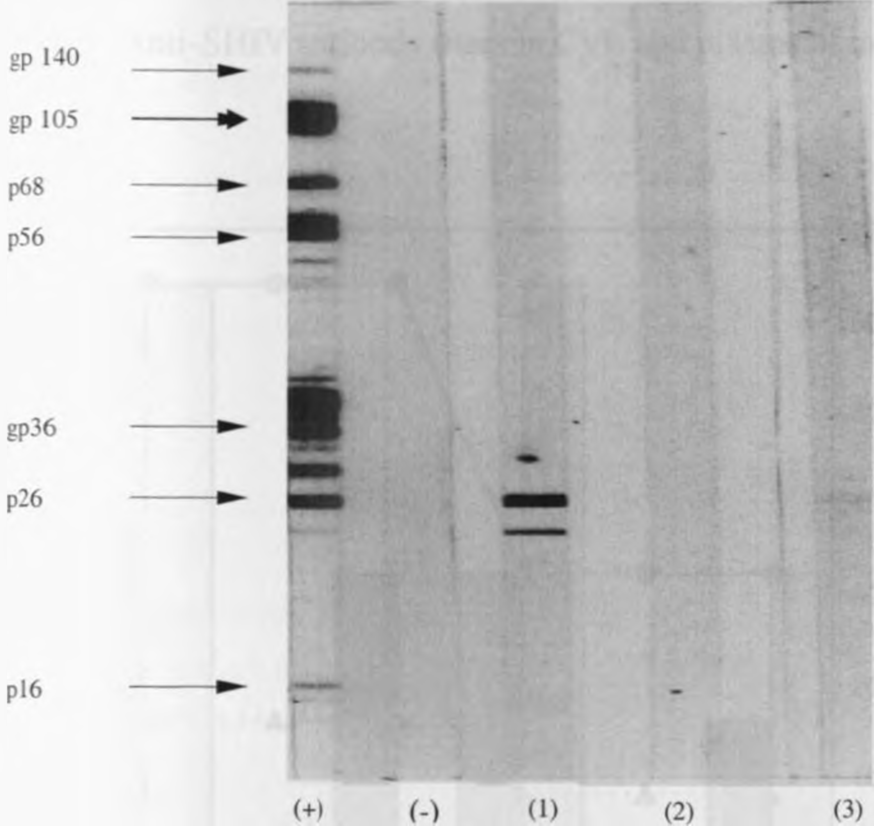
Detection of anti-SHIV antibody responses in CVL with a combined HIV-1/HIV-2 commercial ELISA kit (Genelavia Mixt®, Sanofi Diagnostics Pasteur, Inc. Chaska, MN). The assay was performed as per the manufacturer's specifications. The cut-off value for this assay was 0.202, defined as one-tenth the mean OD of cut-off control sera. The assay met the quality control criteria as specified by the manufacturers. (+) denotes positive antibody responses while (-) denote negative anti-SHIV antibody responses in CVL while men, mfl and lut respectively denote menstruation, mid-follicular and luteal stages of the menstrual cycle. All CVL specimens from the negative control baboons were non-reactive on this assay.

Figure 10



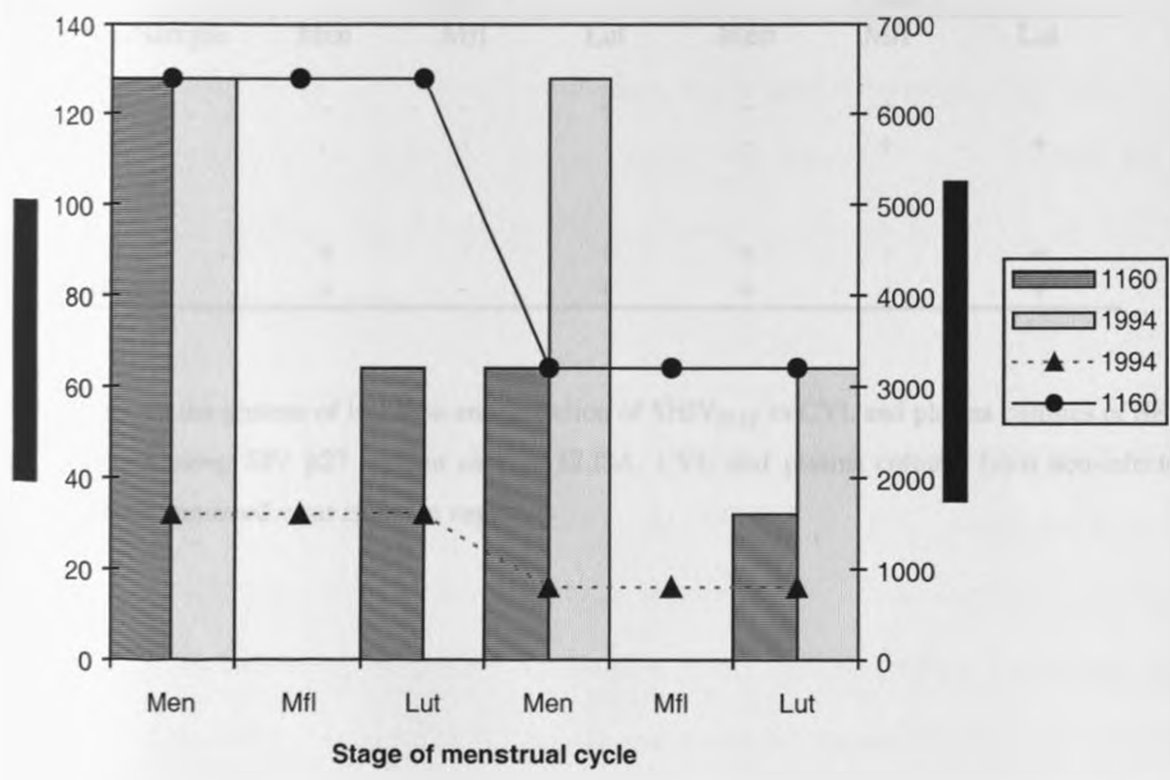
The figure illustrates Western blot confirmatory results for the presence of anti-HIV-1 antibodies in CVL of P1160 during three stages of the first cycle. (+) denotes positive control (provided in the kit), (-) denote negative control (provided in the kit). Nitrocellulose strip incubated with CVL collected during menstruation (Lane 1) showed reactivity to envelope glycoproteins (gp160, gp120 and gp41) and core protein (p24) of HIV-1. CVL collected during luteal stage showed strong reactivity to gp160 and moderate reactivity to gp120 and gp41 (Lane 3) while sample collected during mid-follicular stage did not show reactivity to any HIV-1 antigens (Lane 2)

Figure 11



The figure illustrates Western blot confirmatory results for the presence of anti-HIV-2 antibodies in CVL of P1160 during three stages of the first cycle. (+) denote positive control (provided in the kit). (-) denote negative control (provided in the kit). CVL collected at menstruation and luteal stages (Lane 1 and Lane 3 respectively) showed reactivity to the core antigen of HIV-2 (p26). No HIV-2 specific bands appeared on the strip incubated with CVL collected during mid-follicular stage (Lane 2).

Figure 12



Illustrates anti-gp41 IgG titers in CVL and plasma of SHIV-inoculated animals. The bar graphs represent indicate anti-SHIV IgG titers in CVL and the lines graphs indicate anti-SHIV IgG titers in plasma. Antibody titers were defined as the reciprocal of the last dilution of the sample giving a positive reaction. Men, mfl and lut respectively denote menstruation, mid-follicular and luteal stages of the cycle.

Table 5

Isolation and detection of SHIV-89.6P in CVL and plasma

	Cycle 1			Cycle 2		
Animal No./Sample	Men	Mfl	Lut	Men	Mfl	Lut
P1160						
CVL	-	-	+	-	+	-
Plasma	-	-	-	-	+	+
P1994						
CVL	+	-	+	+	-	+
Plasma	+	-	+	+	-	+

The figure shows the pattern of isolation and detection of $SHIV_{89.6P}$ in CVL and plasma cultures of SHIV-infected female baboons using SIV p27 antigen capture ELISA. CVL and plasma cultures from non-infected negative control baboons remained virus isolation negative.

CHAPTER FOUR

4.0 Discussion and conclusion

4.1 Discussion

This study has shown that cyclic changes in the levels of sex steroids correlate positively with the perineal skin cycle of the baboon. Antibody responses to the virus were detected in both plasma and in CVL. The presence and the levels of SHIV-specific antibodies in the genital secretions observed in this study, seemed to be influenced by endocrine changes during the menstrual cycle. Further, the study has demonstrated that $SHIV_{89.6}$ can be isolated and detected in plasma and CVL of female baboons experimentally inoculated with the virus.

The observed correlation of the cyclic variation in the levels of estradiol and progesterone in the present study is consistent with previously documented work (Wildt et al., 1977; Kling and Westfahl., 1978; Shaikh et al., 1982). The concentrations of the two hormones were within normal ranges for the female baboon (Kling and Westfahl., 1978; Eley and Bambra., 1993; Stevens., 1997). This study, however, did not assess the association between SHIV infection and levels of reproductive hormones in plasma of the female baboons. Recent studies have indicated lack of HIV-induced menstrual cycle disturbances in HIV infected women suggesting normal ovulatory cycles and intact hypothalamic-pituitary-ovarian axis (Chirgwin et al., 1996; Ellerbrock et al., 1996). It is still not established yet whether HIV infection results in alterations in levels of female sex hormones in blood. This can be investigated in the SHIV/baboon model.

The results on SHIV-specific antibody detection are indicative of menstrual cycle dependent variations in both the presence and concentration of these antibodies in the cervicovaginal mucus of infected animals.

Anti-SHIV IgA and IgG were more frequently detected in CVL samples during menstruation and luteal stages of the cycle, with higher titers of IgG occurring during menstruation stage as compared to lower levels during luteal stage. The CVL samples collected during mid-follicular stage were largely negative for the presence of both SHIV-specific IgA and IgG. The bands obtained with western blot were specific for HIV-1 envelope and HIV-2 core proteins consistent with the structure of the virus used in the study (Reihmannn et al., 1996a). The band corresponding to p24 of HIV-1 on lane 1 (Figure 10) was due to cross-reactivity with p27, the major core protein of SIV in the SHIV-89.6P construct (Clavel et al., 1986). No clear association was observed between the titers of anti-SHIV IgG in plasma and hormonal levels during different stages of the menstrual cycle, probably indicating lack of influence of female sex steroid hormones on systemic antibody responses in this animal model.

The influence of female sex steroid hormones on antibody responses in the genital tract has been documented (Wira and Kaushic., 1996; Wira et al., 1999). As is the case in women, the concentration of antigen-specific antibodies and immunoglobulins in the reproductive tract of rhesus macaques is determined by the menstrual cycle stage, being highest during menses, and lowest during periovulatory period (Lu et al., 1999). By influencing the nature of antibody responses within the female reproductive tract, variations in levels of sex steroid hormones during the course of the menstrual cycle could determine sexual transmission by probably influencing viral dynamics in this site. Recent studies have shown that both progesterone and estrogen influence vaginal transmission of SIV (Marx et al., 1996; Smith et al., 1997). The precise mechanism(s) by which these hormones exert their influence is not well established. Virus-specific antibodies in CVL of infected baboons in the present study are likely to have arisen from peripheral blood transudation, as evident by detection of antibodies in CVL at menstruation stage when samples are contaminated with menstrual blood, and also from local synthesis within the

reproductive tract, which is consistent with previous reports (Kutteh and Mestecky., 1994; Wira et al., 1999; Lu et al., 2000). On the other hand, anti-SHIV antibodies demonstrated in CVL during luteal stage are probably due to sex steroid hormone(s) dependent active secretion within the genital tract. In the present study, lower and/or undetectable titers of SHIV-specific IgG during mid-follicular and luteal stages is likely due to the observed higher plasma concentrations of estradiol and progesterone characterizing the two stages. Furthermore, failure to detect both IgG and IgA during mid-follicular stage in CVL of infected animals might have been due to the effect of dilution of samples with increased volume of cervical mucus around this period (Kutteh et al., 1996). Suppression of local antibody immunity in the female genital tract during the periovulatory period has been suggested to be essential for conception (Kutteh et al., 1996). Detection of anti-SHIV antibodies in genital secretions might also have been influenced by other factors such as viral load within systemic circulation and/or in genital tract compartments, and local immune regulating mechanisms such as cytokine profile within the genital secretions. Recent evidence indicates that differential regulation of cytokines occurs within the course of ovulatory cycle in HIV seropositive women, and this can influence the nature of HIV specific antibody responses within the genital tract (Al-Harthi et al., 2000).

In this study, titers of anti-SHIV IgG were higher in plasma compared to CVL. The lower concentration of the antibody in CVL could partly be explained by the sampling procedure used, resulting in dilution of collected samples with the lavage fluid (PBS). It has been shown that samples collected by lavage of the female reproductive tract are generally several times lower in immunoglobulins and protein concentration as compared to secretions captured directly from mucosal surfaces with either Sno-strips or wicks (Quesnel et al., 1997). Despite demonstration of HIV-speficic antibody responses in genital secretions of HIV infected women, the role of such antibodies on virus transmission is not known. A recent study has

indicated that mucosal immunity to HIV-1 is not able to prevent viral shedding in the female reproductive tract and thus cannot modulate infectivity of genital secretions (Fiore et al., 2000). It is, however, probable that interaction of local humoral and cellular immunity within the female genital tract may determine virus shedding and infectivity. HIV-1-specific CTL activity has been reported in the reproductive tract tissues of HIV infected women (Musey et al., 1997; White et al., 2001). Further studies are needed to establish the influence of local immune responses to HIV in the genital tract on virus infectivity and shedding.

The present study has shown that $SHIV_{89.6P}$ can be recovered from plasma and genital secretions of baboons infected experimentally with the virus. Successful culturing of SHIV from blood of infected baboons has been documented (Allan et al., 1995; Otsyula et al personal communication). However, no other study has reported the secretion of SHIV in genital fluids of baboons. In the present study, CVL samples of P1994 were positive for the viral antigen (p27) when corresponding plasma samples were also positive. Further, virus detection in CVL of this animal correlated well with antibody detection, with samples positive for anti-SHIV IgG also showing reactivity for p27 antigen. In P1160, virus detection did not correlate with anti-SHIV antibody detection and that it occurred intermittently during the cycle. Based on the limited data presented here, its difficult to conclusively determine whether there is any cyclic pattern in the isolation and detection of $SHIV_{89.6P}$ in plasma and CVL of infected baboons. These results seem to suggest that genital shedding of $SHIV_{89.6P}$ occurs intermittently in female olive baboons experimentally infected with the virus. In this regard, this study had the following limitations. It did not assess the shedding of cell-associated virus, believed to be more abundant in genital secretions of HIV-infected women (Alexander., 1990). Secondly, the study had a small sample size coupled with a short observation period.

Few studies have so far been conducted to determine the pattern of genital secretion of HIV in infected women using tissue culture technique. These studies have largely been inconclusive in showing whether genital shedding of HIV is cyclic (Vogt et al., 1986; Wolfsy et al., 1986; Vogt et al., 1987). The use of more sensitive techniques such as gene amplification by PCR is now providing useful information about HIV dynamics in peripheral circulation and within the genital tract. Even with these sensitive methods the association of menstrual cycle with shedding of virus in genital secretions is still controversial. A study has reported a decline in HIV genital shedding during follicular phase and an increase during luteal phase (Reichelderfer et al., 2000). Others have been unable to detect a menstrual cycle pattern of genital shedding of HIV (Paxton et al., 1997; Goulston et al., 1998; Villanueva et al., 2002). The observed disparities could partly be explained by variations in assay methods and small number of women sampled in previous studies. It has been shown, for example, that comparatively detection of HIV RNA by PCR is more sensitive than virus isolation by cell culture technique (Sarcino et al., 2000). Furthermore, endocervical swab samples are more sensitive than cervicovaginal lavage for HIV-1 RNA PCR (Baron et al., 2000). However, various workers have shown that HIV-1 RNA levels in plasma correlates positively with cervicovaginal shedding of the virus and that virus titers are much higher in plasma than in CVS (Uvin and Caliendo., 1997; Hart et al., 1999). It is now well established that hormonal changes during the menstrual cycle exert an influence on both antigen-specific antibody responses and cell-mediated immunity within the female genital tract (White et al., 1997; Lu et al., 1999). By influencing the immunological marker to HIV such as HIV-specific antibodies (Lu et al., 1993), CTL activity to HIV (Musey et al., 1997; White et al., 1997), and the cytokine profile (Crowley-Nowick et al., 2000) in the genital secretions, menstrual cycle dependent variations in female reproductive hormones are likely to determine virus quantity and infectivity in the reproductive tract of HIV-infected women. Recently sex

based differences in circulating HIV-1 RNA levels and lymphocyte subsets have been reported (Katzeinstein et al., 1996; Evans et al., 1997; Farzadegan et al., 1998). This suggests that the fluctuations in reproductive hormone levels during menstrual cycle influence HIV-1 RNA in plasma. Such an influence has been implied from the reported increased HIV-1 RNA shedding in CVS among HIV-1 infected oral contraceptive users (Clemetson et al., 1993; Mostad et al., 1997; Cohen et al., 1998). Evidence is now emerging regarding the possible impact of ovulatory cycles on virologic and immunologic markers in HIV-infected women (Greenblatt et al., 2000). Plasma HIV-1 viral load correlates positively with cervicovaginal shedding of the virus (Uvin and Caliendo., 1997; Goulston et al., 1998; Hart et al., 1999). Thus, it is possible that the reported impact of menstrual cycle on circulating levels of HIV could concomitantly influence the levels of HIV female genital secretions. A recent study has shown that progesterone could exert an inhibitory on HIV-1 infection of target cells though chemokine- and chemokine-receptor mediated mechanisms on viral entry and infectivity (Vassiliadou et al., 1999). If it is clearly established that sex steroid hormones could influence genital shedding of HIV in infected women, then this is likely to have important implications for strategies such as mucosal vaccination and mucosal administration of anti-retroviral agents to prevent sexual transmission of HIV. Such approaches will, therefore, need to take into consideration the menstrual cycle stage of the woman in order to achieve high efficacy.

4.2 Conclusion

This project has shown that SHIV infected female baboons shed virus in their genital secretions. A strong local antibody response to the virus, dependent on the phase of the cycle, is present within the genital tract. The observed pattern of detection of anti-SHIV antibody responses in the genital secretions of the baboon during the course of the menstrual cycle has important implications for preventive measures aimed at reducing female to male transmission of HIV. In order to be more effective, such approaches

need to take into consideration the menstrual cycle phase of women. Given the limited number of animals used in this study the results presented here are just preliminary. If developed further, SHIV infection of baboon can provide an alternative model for investigating the unresolved issues in heterosexual transmission of HIV such as (a) the source of HIV within the genital tract; (b) dissemination of the virus within various tissues of susceptible host following sexual exposure; (c) the role of local humoral and cellular immunity to the virus in the genital tract, and lastly (d) trials of HIV candidate vaccines and anti-HIV drugs.

Future studies utilizing this model to understand the biology of heterosexual transmission of HIV should address the following issues:

- Determine the neutralizing capacity of SHIV-specific antibodies in genital secretions of SHIV infected baboons.
- Determine the presence and role of CTL activity to SHIV in the genital tract of SHIV infected baboons.
- Determine the cytokine profile in the genital tract of SHIV infected baboons and assess whether it affects the quantity and infectivity of the virus in the genital tract.
- Determine the impact of ovulatory cycles on virus dynamics in the peripheral circulation and in the genital tract secretions of SHIV infected baboons.
- Localize SHIV within the reproductive tract tissues of SHIV infected baboons to determine the source of the virus in genital secretions, and lastly
- Characterize the genotype and phenotype of the virus shed in genital secretion of SHIV infected baboons.

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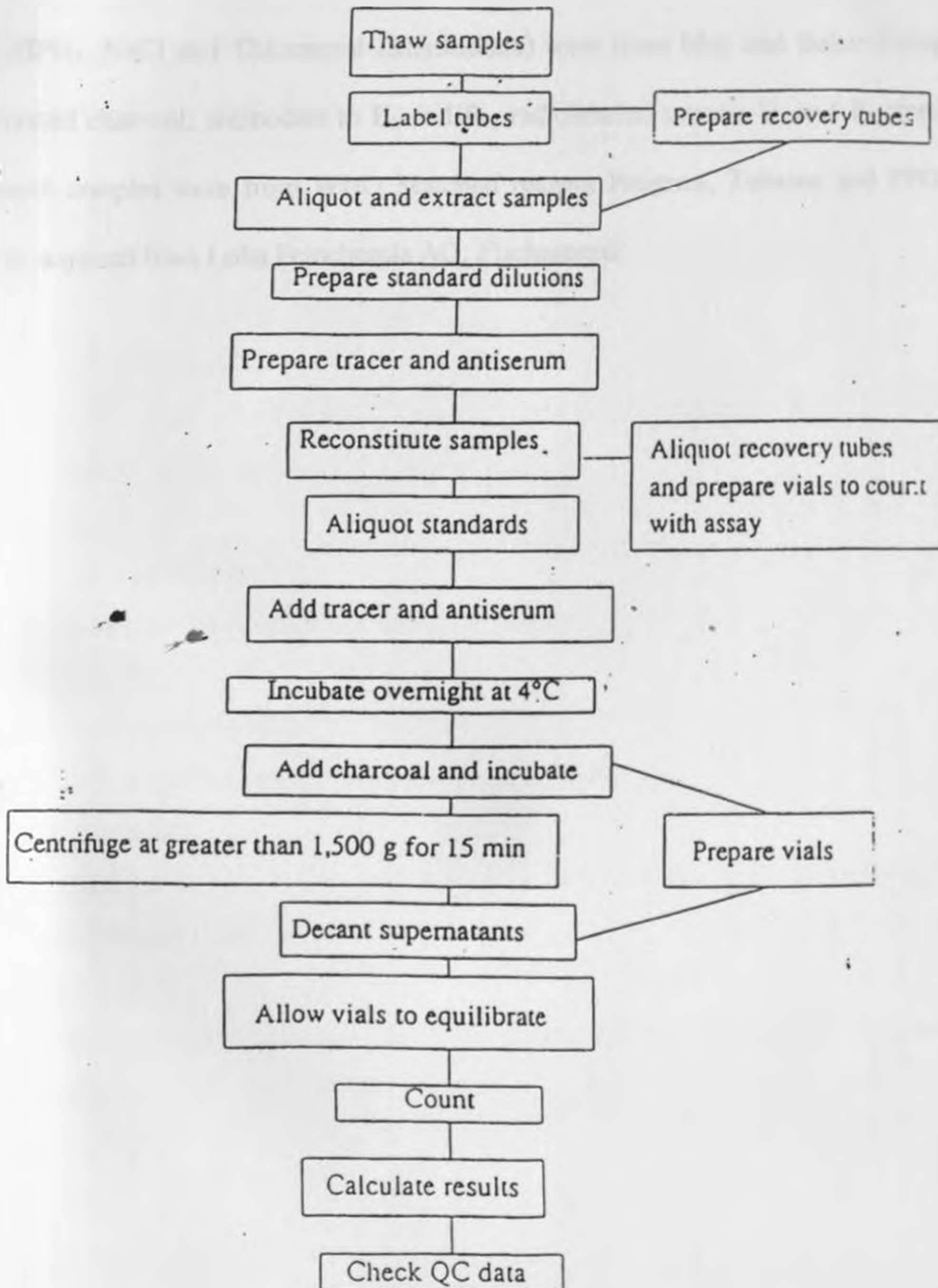
6.0 Appendices

Appendix 1

Flow chart of Radioimmunoassay protocol



Appendix 2

Radioimmunoassay reagents

The following reagents from various sources were used in RIA experiments for E_2 and P_4: $Na_2H_2PO_4$, Na_2HPO_4, NaCl and Thiomersal (Merthiolate) were from May and Baker Company, England. Dextran activated charcoal, antibodies to E_2 and P_4, radiolabeled tracers, E_2 and P_4 standards as well as quality control samples were from WHO Matched reagent Program. Toluene and PPO (2,5-Diphenyloxazole) were acquired from Loba Feinchemie AG, Fischamend

Appendix 3

HIV-1 in house ELISA reagents

<u>0.1M CAPS coating buffer</u> Shelf life: 2 months.

22.13g (3- {cyclohexylamino} – 1- Propane Sulfonic acid). FW = 221.3

Dissolve in 500ml double distilled water (dd H_2O).

Dissolve CAPS completely before adjusting to pH 11.0, using NaOH, then bring volume to 1 liter using dd H_2O.

<u>Wash buffer</u>

8.77g Nacl (0.15m), 0.34g EDTA (0.001m), 6.05g Tris base (0.05m).

Dissolve in 900ml ddH_2O

Adjust PH to 7.4

Add 0.5 ml (0.05%) Tween 20, then bring volume to 1 liter.

Usually make 10 liters (or 1 liter of 10X).

<u>Diluent buffer</u>

Same as wash buffer.

Add 0.1% BSA (Bovine Serum Albumin).

<u>Blocking buffer</u>

Same as wash buffer

Add 0.1% BSA and 5 % non-fat skimmed milk.

<u>Conjugate solution</u>

20ml of diluent buffer, add 7 µl goat anti-monkey Ig G conjugate to horseradish peroxidase. (Dilution factor = 1:2857).

<u>Substrate solution</u>

In 25ml ddH_2O, dissolve 1 big tablet (sigma buffer), let it dissolve, and then dissolve 1 small tablet. Sigma-OPD).

<u>Stopping reagent</u>

4N H_2SO_4

Appendix 4

HIV-1 Western blot procedure

An HIV-1 Western blot kit (Cambridge Biotech, USA) was used for confirmation of the presence of HIV-specific antibodies in CVL samples. The kit has the following contents: nitrocellulose strips in incubation trays, strong and weak positive control sera, negative control sera, wash buffer, blotting buffer, alkaline-phosphatase conjugated goat anti-human IgG, substrate 1(contains nitroblue tetrazolium and dimethyl formamide), substrate 2 (contains bromo-chloro-indolyl phosphate and dimethyl formamide) and blotting powder. The procedures outlined below were all performed at room temperature.

- Nitrocellulose strips were incubated for five minutes under gentle agitation with 2ml/well of prepared 1X blotting buffer.
- 20 μl of each undiluted sample (CVL) or control serum was added into each well with its assigned strip immersed in 1X blotting buffer (final dilution 1:100)
- The strips were washed thrice with 1X wash buffer and incubated with alkaline phosphatase-conjugated goat anti-human IgG (working dilution 1:200) for thirty minutes under gentle agitation.
- After washing as described above, the strips were incubated with 2 ml working substrate solution for 10 minutes and colour development monitored.
- When all the bands corresponding to the viral proteins had been displayed on the strong positive control strip, the reaction was then stopped by rinsing the strips with distilled water. The strips were dried between two pieces of absorbent paper for two hours.

CVL samples were considered positive for antibodies to HIV-1 if any two or more of the following bands were observed: p24, gp41, gp120 and gp160. The samples were regarded as being negative if they displayed no bands. Because SHIV-89.6P is a chimera of HIV-1 envelope glycoproteins (gp 41, gp 120 and gp 160) in an SIV backbone, reactions to HIV-1 core proteins such as p24 were not expected.

Appendix 5

HIV-2 Western blot

The following Western blot protocol was performed according to the manufacturer's specifications.

The kit was supplied with the following nitrocellulose strips in trays, positive and negative control serum, alkaline phosphatase-labelled goat anti-human IgG antibodies BCIP/NBT colour development solution and washing solution/diluent. The test procedure was carried out as follows:

- The nitrocellulose strips were incubated for five minutes under slow shaking with 2 ml/well of the reconstituted washing solution.
- CVL or control sera were added to each well (20 µl of sample/control serum, final dilution of 1:100) and incubated for 2 hours at room temperature under slow shaking.
- The strips were washed 3 times and incubated with alkaline phosphatase-labelled goat anti-human IgG conjugate for 1 hour at room temperature under slow shaking.
- The strips were washed again as described above, incubated with BCIP/NBT solution (provided in the kit as ready-to-use) and appearance of colour monitored.
- When all the bands corresponding to the virus proteins have been displayed on the positive control strip, the reaction was stopped by rinsing the strips with distilled water.
- The strips were then dried by placing them between two sheets of absorbent paper at room temperature. Bands were classified as *env* (gp 140, gp 105, and gp 36) *gag* (p 56, p26, p16) and *pol* (p68).

A sample was regarded as being positive if it displayed at least one HIV-2 specific band. A sample was defined negative if no band developed or if the bands appearing could not be classified as either *env*, *gag* or *pol*. According to the manufacturer's instruction, a test sample is defined positive if it developed at least one band in all the three regions (i.e. *env*, *gag* and *pol*). However, SHIV-89.6 P contains HIV-1 *env* instead of the SIV_{mac251}. Thus the anti-SHIV gp160, gp120 and gp41 antibodies may not cross-react with gp140, gp105 and gp36 proteins of HIV-2. The presence or absence of bands in the *env* region was therefore justifiably ignored.

Appendix 6

Tissue culture reagents

Complete RPMI 1640 medium s used for all tissue culture experiments consisted of RPMI 1640 (Atlant Biologicalsa, Norcross, Georgia) supplemented with 10% FBS (Intergen Company Purchase, New York) 1% L-glutamine (Sigma Chemical Company, St. Louis, MO), and antibiotics; Penicillin and Streptomycin (Sigma Chemical Company, St.Louis, MO).

Appendix 7

SIV p27 core antigen capture ELISA protocol

- The microtitre wells were removed from the cold-room and allowed to warm to room temperature.
- Except the blank wells, 20 µL of Lysis Buffer (provided with kit) was added into each antibody-coated wells.
- Then 200 µL of control sample was pipetted into 5 control antibody-coated wells. Out of the 5 control wells, 3 were negative control into which negative control serum (Normal Human Plasma, NHP, provided in the kit) was added. The remaining two wells were positive control, containing SIV_{mac} reagent (provided in the kit).
- 200 µL of each sample to be tested was added into the antibody- coated well, the microtitre plate sealed with a plate cover and incubated at room temperature (20-30° C) for 16-20 hours.
- After the incubation period, the plates were washed six times by use of wash solution (provide in kit) and the microtitre plate tapped gently on paper towels to remove any remaining liquid.
- Then 200 µl of anti-SIV biotinylated antibody solution (provided in the kit) was added to each well except the blanks. The plate was then incubated for 1 hour at 37° C after sealing it.
- The plate was removed from the incubator and washed six times as described previously.
- 200 µL of streptavidin conjugated to horseradish peroxidase solution was added into each well (provided in the kit) except the blanks, the plate sealed and incubated for 30 minutes at 37°C. The plate was removed from incubator and washed six times as previously described.
- 200 µL of TMB substrate solution (provided in the kit) was added to all wells (including the blanks) and incubated for 30 minutes at RT (without sealing). For the colour to develop (or until color develops to desired intensity).
- When the colour of positive controls developed to desired intensity, the reactions were stopped by addition of 2M H_2SO_4 (50 µL per well).
- The absorbance of the wells was read at 450 nm with a reference wavelength of 570 nm.

Samples with absorbance values greater than the cut-off value were considered positive for SIV antigen. The cut-off value was defined as the sum of mean negative control and a predetermined factor of 0.030 as per the manufacture's instructions.

Appendix 8

Perineal sex skin evaluation

The following changes in the sex skin were used for determination of the menstrual cycle stages of the baboons:

Stage 0: The perineal skin is flat, pale pink in colour, and heavy deep wrinkles of sex skin, especially around the rectum, are visible. There is no swelling of the vulval lips. This is the quiescent stage, which lasts 8 to 10 days before merging into stage 1.

Stage 1: The vagina begins to enlarge with slight paravaginal swelling. The Wrinkles of the skin begin to unfold stretching the skin slightly. The colour is dull pink. If the animal is viewed from the side, no swelling of the vaginal area is evident when the tail is raised. The stage lasts 2 to 3 days.

Stage 2: The vaginal swelling begins to approach the ischial callosities and the expansion is downward. Any wrinkles, if present, are not as deep as in stage 0 and the colour is a brighter pink than in stage 1. If the female is viewed from the side slight swelling of the vulva may be seen. This stage lasts about 3 to 4 days.

Stage 3: There is continued expansion and stretching of the skin downwards and outwards. The colour changes to a bright pink and then red. When viewed from the side there is a distinct swelling backwards of the vulval lips and the perineum. Duration is 3 to 4 days.

Stage 4: Wrinkles disappear completely and in some animals swelling is at a maximum, reaching as far outwards as the ischial callosities and downwards. From the sides swelling is prominent and the colour is red. The duration of this stage is 4 to 6 days.

Stage 5: This stage is not attained in some animals; for them maximum turgesence has the characteristics of stage 4. In stage 5 the color of the skin is deep red and shiny and there is an absolute absence of wrinkles. The sex skin is large, and gives the

impression of over inflated balloon. When viewed from the side the whole rear end of the animal appears inflated. Often if the animal has been cycling frequently the tautness of the skin is so extreme that cuts appear which bleed. The stage can last up to 10 days and as noted above may not be present in all animals.

Stage 6: This is the stage of detumescence and lasts up to 10 days. Wrinkles begin to form on the sex skin and the expanded area begins to deflate. At the same time there is a noticeable change from bright red to dull red. If the animal has been mated successfully this then may turn to crimson. If not, then stage 0 resumes.

Stage 7: This stage lasts 1 to 3 days is when menstrual bleeding is obvious. The amount of bleeding varies from a few drops, which can hardly be seen to heavy bleeding. The latter parts of this stage usually coincide with stage 1 i.e. start of inflation.

Stage 8: This is the stage of pregnancy and is denoted by a completely flat perineum of crimson color. The sex skin is often observed to be flaking off. The stage lasts approximately six months.

Stage 9: The period postpartum and prior to resumption of cycling is referred to as stage 9 and is the period of lactational amenorrhoea.

(Adapted from Eley and Bambra, 1993)

Appendix 9

Abbreviations

AIDS	Acquired immunodeficiency syndrome
CAPS	Cyclohexylamino-1-propane sulphonic acid
CD	Cluster differentiation antigen
CVL	Cervicovaginal Lavage
CVS	Cervicovaginal Secretions
CPE	Cytopathic effect
EDTA	Ethylenediamine tetraacetate
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
E_2	Estradiol
P_4	Progesterone
gp	Glycoprotein
HIV-1	Human immunodeficiency type 1
HIV-2	Human immunodeficiency type 2
IgA	Immunoglobulin A
IgG	Immunoglobulin G
LSM	Lymphocyte separating media
LTRs	Long terminal repeats
Lut	Luteal
Men	Mentruation
Mfl	Mid-follicular
OPD	O-phenylenediamine
ORF	Open Reading Frame
PCR	Polymerase chain reaction
PBMC	Peripheral blood mononuclear cells
rev	Regulator of expression of virion proteins
rpm	Revolutions per minute
RT	Reverse transcriptase
SDS	Sodium dodecylsulphate

SHIV	Simian Human Immunodeficiency Virus
SIV	Simian immunodeficiency virus
SIVmac	Simian immunodeficiency virus from Rhesus macaques
SIVsmm	Simian immunodeficiency virus from sooty mangabeys
SIVagm	Simian immunodeficiency virus from African green monkeys
SIVcpz	Simian immunodeficiency virus from chimpanzee
SIVsyk	Simian immunodeficiency virus from sykes
SIVmnd	Simian immunodeficiency virus from mandrils
SIV_{nme}	Simian immunodeficiency virus from macaque nemestrina
SIV_{Deb}	Simian immunodeficiency virus from De Brazza's monkey
ssRNA	Single stranded RNA
tat	Transactivator
$TCID_{50}$	Tissue culture infectious dose 50%
vip	Virus infectivity factor
vpr	Virus protein R
vpu	Virus protein U
vpx	Virus protein X

UNITS

μL	Microlitre
μM	Micromolar
g	Gram
mM	Millimolar
M	Molar
bp	Base pairs
OD	Optical density
SD	Standard deviation
°C	Degree Celsius/Centigrade
ng	Nanogram
%	Per cent
pH	Hydrogen ion concentration
nm	Nanometre
hr	Hour
min	Minute