

**DEVELOPMENT OF A HETEROSEXUAL
TRANSMISSION ANIMAL MODEL OF HIV USING
THE EAST AFRICAN BABOON**

THIS THESIS HAS BEEN ACCEPTED FOR
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**This thesis is submitted in partial fulfillment of the requirements for the Award of
Master of Science degree in Reproductive Biology at the Department of Animal
Physiology of the University of Nairobi.**

November, 1999.

DECLARATION

I declare that this thesis is my original work and has not been submitted for a degree in any University.

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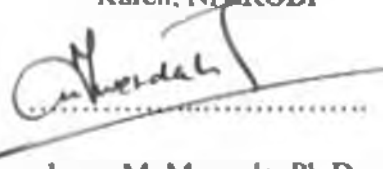
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ABSTRACT

Human immunodeficiency virus, (HIV), is transmitted sexually by both homosexual and heterosexual contact. Heterosexual transmission is the major route of HIV spread in Africa, accounting for over 70% of all transmissions, and is still the mode of transmission that is least understood. The development of an animal model for heterosexual transmission is therefore of great value in understanding this mechanism of transmission and more importantly, for testing candidate anti-retroviral and immuno-modulating drugs as well as vaccines. In the attempt to develop the baboon as a heterosexual transmission animal model for HIV, six adult baboons were inoculated with HIV-2 and two with Simian/Human Immunodeficiency Virus, (SHIV). Of the six exposed to HIV-2, two were inoculated via the penile urethra, two vaginally and two intravenously. Of the two exposed to SHIV, one was inoculated vaginally and the other intravenously. Two SIV negative baboons were also inoculated intravenously with saline and served as controls. Blood samples were obtained for culture and serology from all animals at regular time-points and biopsies of inguinal lymph node, spleen, liver, bone marrow and cerebral spinal fluid were obtained for culture from three animals, each representing one group. Virus was recovered from both blood and inguinal lymph node of all SHIV-infected and one of six HIV-2-infected baboons. In addition, all animals inoculated with SHIV were antibody positive by ELISA and one of six HIV-2-infected animals was weakly positive. These results show that the baboon can be developed further as an animal model for HIV. If developed further, the baboon will be a valuable and practical animal model for studying heterosexual transmission, assessing the role of cofactors in heterosexual transmission of HIV and testing the effectiveness of spermicides, pharmacological agents and vaccines in preventing the heterosexual transmission of HIV.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.0 General Introduction

Only two decades ago, it was widely believed that infectious diseases were no longer much of a threat in the developed world and that it was only a matter of time for developing countries to acquire a similar status. The remaining challenge to public health therefore was viewed as stemming from non-infectious conditions such as cancer, heart disease and degenerative diseases (Haseltine and Wong-Staal, 1988). That confidence was shattered in early 1980's by the advent of Acquired Immunodeficiency Syndrome, (AIDS). Around this time, emerged a highly devastating disease caused by a class of infectious agents called retroviruses. In view of the startling nature of the epidemic, scientists responded quickly and in 2 years time, between 1982-84, the nature of the epidemic was clarified, a new virus - the human immunodeficiency virus (HIV) - was isolated and shown to cause the disease, a blood test was formulated and the virus targets in the body were established. Yet the virus has outpaced science because no cure or vaccine is yet available, as the epidemic continues to spread.

Currently, over 30 million people worldwide are living with HIV/AIDS, with over, 11 million AIDS deaths since the began of the pandemic which includes 2.3 million deaths in 1997 alone (Carael *et al.*, 1998). Unlike other diseases, HIV usually infects adults in their most productive years, often causing a wide range of psychosocial and economic trauma at the community and national level as well as undermining decades of development progress (Stover and Way, 1998). Sub-Saharan Africa has been hardest hit by HIV/AIDS and United Nations Population Division (United Nations, 1996) reported a reduction of the projected populations of 24 countries in Sub-Saharan Africa by 13 (3.9%) million people

by the year 2005 and by 30 (5.5%) million people by the year 2025. Controlling the HIV epidemic is therefore of utmost importance and an uphill task. Although behavioural intervention is a critical component, vaccination is the only effective and economical way of controlling an epidemic of such magnitude. An understanding of HIV structure, mechanisms of transmission, immune responses, and good animal model systems are what is needed to develop an effective vaccine.

1.1 Biology of the Virus

Human immunodeficiency viruses fall into two types: HIV-1 and HIV-2. Both HIV-1 and HIV-2 have similar biological properties though they differ significantly in some regions of their genomes and serological reactivity. HIV-1 is distributed throughout the world whereas, HIV-2 seem to be confined to West Africa (Munn *et al.*, 1988). Also HIV-2 has been found to be less pathogenic than HIV-1 (Gody *et al.* 1988; Clavel *et al.* 1986). Both HIV-1 and HIV-2 share many features with other members of non-transforming cytopathic lentivirus family of retroviruses. Of particular note are its morphological, biological and molecular similarities to the Visna virus of sheep, equine infectious anaemia virus of horses and the recently described feline immunodeficiency virus of cats (Narayan and Clement, 1990). These viruses, including HIV in humans, cause slowly progressive and inevitably fatal disease in their hosts. HIV is also related to other non-human primate lentiviruses known as Simian Immunodeficiency Viruses, (SIV). (Fukasawa *et al.* 1988). Simian immunodeficiency viruses have been isolated from captive Asian macaque, (*Macaca mulatta*), African Mangabey monkey, (*Cercocebus ans*), African green monkey, (*Cercopithecus aethiops*), West African mandril monkey, (*Papio mandrillus*), Sykes monkey, (*Cercopithecus mitis*), and the chimpanzee (Daniel *et al.* 1985; Fultz *et al.* 1986; Ohta *et al.* 1988; Tsujimoto *et al.* 1988; Emau *et al.* 1991). In Asian

macaque. SIV causes an AIDS-like syndrome similar to AIDS in human (Benveniste *et al.*, 1989; Daniel *et al.*, 1985; Letvin *et al.*, 1985). In their African non-human primates, SIV infection is non-pathogenic (Ohta *et al.*, 1988; Tsujimoto *et al.*, 1988).

1.1.1 Structure of HIV

The structure of HIV has been described in detail by Hockley *et al.* (1988); Haseltine and Wong-Staal (1988); Gelderblom *et al.* (1989); and Levy (1993). The virus is composed of a dense cylindrical core surrounded by a lipid envelope (Fig. 1). Viral glycoproteins emerge from the core and jut through the lipid envelope. The core is made of structural viral proteins and it encases the two identical strands of RNA (genome) and viral enzymes

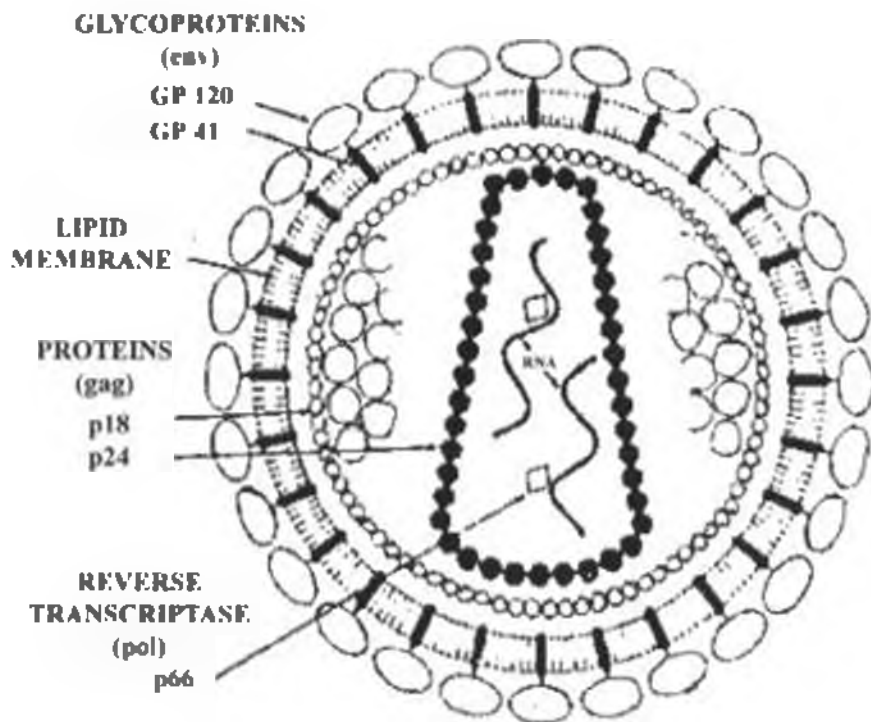


Figure 1: The Structure of HIV-1 Virion.

(Source: Adapted from Schild and Minor, 1990)

The HIV genome has been well characterised (Fig.2). It is approximately 10 kb in length. Both HIV-1 and 2 genomes are generally similar but differ in finer details (Haseltine and Wong-Staal, 1988; Levy, 1993). Both comprise the flanking long terminal repeat (LTR) sequences that contain regulatory segment for replication and three structural genes designated: *gag*, *env* and *pol* that encode the viral core proteins (e.g. p24, p17, p9, p7, of HIV-1 and p26, p16, p34 of HIV-2), the envelope glycoproteins (gp160, gp120 gp41 of HIV-1 and gp140, gp105, gp36 of HIV-2) and nonstructural proteins (e.g. reverse transcriptase, protease and integrase), respectively. Each of these genes encodes a large polyprotein precursor, which is then cleaved to render the final gene proteins active. In addition to the *gag*, *env* and *pol*, both HIV-1 and 2 genomes include at least six essential additional genes; virion infectivity factor (*vif*), viral protein R (*vpr*), transactivator (*tat*), regulator of expression of virion proteins (*rev*), negative infectivity factor (*nef*), and either viral protein U (*vpu*) in HIV-1 or viral protein X (*vpx*) in HIV-2. Two of these genes, *tat* and *rev* are split genes which are read in different reading frames on the polyribosome to yield the *tat* or *rev* gene product. The *tat*, *rev* and *nef* genes encode regulatory proteins that control the expression of the structural genes *gag*, *pol* and *env*. The proteins encoded by *tat* up-regulate transcription and those encoded by *nef* have a down-regulatory effect. The proteins encoded by *rev* promote transcription of the structural viral proteins that are necessary for viral assembly in the lytic stage. The *vif*, *vpu* and *vpx* genes encode proteins required for virion maturation. The remaining HIV gene *vpr* encodes a regulatory protein that is a weak transcriptional activator (Haseltine and Wong-Staal, 1988).

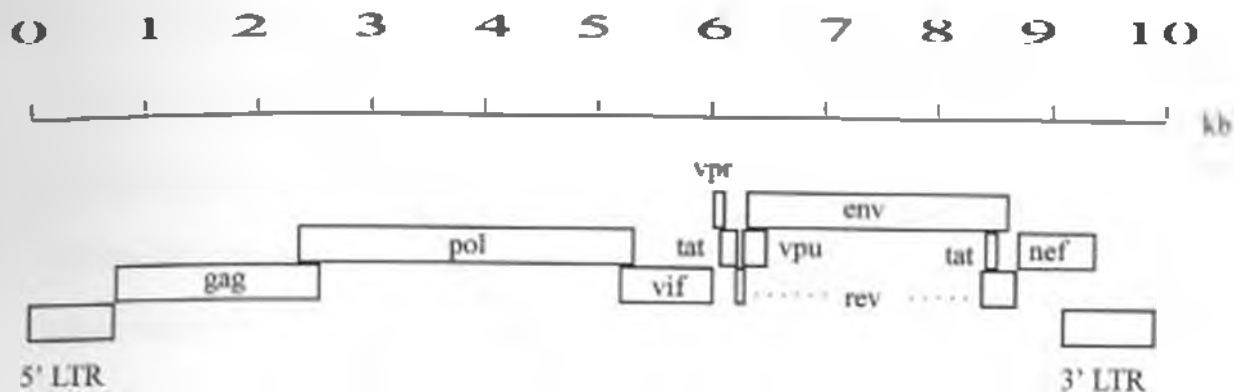


Figure 2: Schematic representation of the HIV-1 genome. The various reading frames are based on the complete nucleotide sequence. Also note that sequences constituting some genes overlap or are discontinuous.

Source: Adapted from M. Essex and P.J. Kanki, 1988.

1.1.2 Replication of HIV

The HIV life cycle begins when it binds to the outside of host cell and injects its core into the cell. This process is facilitated through the interaction of its gp120 and gp41 envelope glycoproteins with CD4 receptor molecules (Klatzmann *et al.*, 1984; McDougal *et al.*, 1986) in conjunction with members of chemokine receptor family mainly CXCR4 and CCR5 (Feng *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Alkhatib *et al.*, 1996; Littman, 1998). Some HIV-1 and HIV-2 isolates in cultured laboratory and several primary SIV isolates do not depend on CD4 receptor for efficient entry, and bind to chemokine receptors (CXCR4 and CCR5) without prior CD4 interaction (Clapham *et al.*, 1992; Endres *et al.*, 1996; Chan and Kim, 1998). The viral reverse transcriptase enzyme converts viral RNA into DNA. The synthesized DNA migrates to the cell nucleus and become integrated into host's DNA to form a provirus. The provirus duplicates together with the cell's own genes every time the cell divides. Thus, the infection is permanent. The second phase of the viral life cycle, (i.e. production of new viral particles), takes place only sporadically, and only in

some infected cells. The process involves copying the integrated viral DNA into RNA. Some RNA provide the genetic material for a new generation of virus, while certain other RNA strands serve as the mRNA that guide cellular machinery into producing the structural proteins and enzymes of the new virus. The genetic material (RNA) and the enzymes are assembled inside structural proteins. The completed virion is then enclosed in a patch of host's cell membrane as it buds from the cell. This second phase of HIV life cycle is regulated by an elaborate set of viral genes and a wide range of HIV-inducing and HIV-suppressing host factors. The balance between these factors determine the net level of viral replication (Haseltine and Wong-Staal, 1988; Levy, 1993; Fauci, 1996; Chan and Kim, 1998; Liu *et al*, 1999).

1.2 Infectivity and Mechanism of Pathogenesis

Human immunodeficiency virus has selective tropism for cells which express a selective marker on the surface of the cells, called CD4 molecules (Klatzmann *et al*, 1984; McDougal *et al*, 1986; Fauci, 1988; Wyatt and Sodroski, 1998). The distribution of HIV-infected cells in the body is primarily determined by the distribution of cells bearing the CD4 molecules. Such cells are found in the lymphoid tissues (T4 cells), brain (macrophages and glial cells), lungs (alveolar macrophages), blood (lymphocytes), reproductive tract (lymphocytes in semen and vaginal fluids, and macrophages and dendritic cells lining the mucous membrane), skin (Langerhans cells), and in bone marrow (Weber and Weiss, 1988; Levy, 1993). Chromaffin cells in the intestines are also susceptible although they lack the CD4 molecules (Weber and Weiss, 1988; Levy *et al*, 1989).

The pathogenesis of HIV/AIDS can be divided into two broad categories:- (i) a direct cytopathic effect of HIV infection on CD4+ T cells (viral pathogenesis), and (ii)

indirect effects (immunopathogenesis). Both effects result into depletion of the helper inducer subset of T-lymphocytes (Fauci, 1988; Shearer, 1998). The direct viral pathogenesis occur when the CD4⁺ T cells are infected by the HIV directly or via infected Antigen Presenting Cells (APC) or follicular dendritic cells (Heath *et al*, 1995). Indirect effects or immunopathogenesis are thought to include; killing of HIV-infected CD4⁺ cells by HIV-specific CD8⁺ Cytotoxic T-lymphocytes (CTL), destruction of immune system by autoantibodies arising from HIV infection, immune suppression induced by HIV proteins e.g. gp120, activation of APC and/or T-cells (a characteristic observation in AIDS), and apoptotic T-cell death (Zinkemagel and Hengartner, 1994; Ameisen and Capron, 1991; Ho *et al*, 1993; Pantaleo and Fauci, 1995; Oyaizu and Pahwa, 1995; Chougnet *et al*, 1998).

Since the T-helper lymphocytes are critical cells of the immune system involved directly or indirectly in the immunological functions, this depletion and/or functional defect of T-helper cells may result in a profound immunosuppression that renders the body susceptible to opportunistic infections (such as those resulting from *Mycobacterium tuberculosis*, *Cryptococcus neoformans*, *Cryptosporidium*, *Cytomegalovirus*, *Candida albicans*, *Toxoplasma gondii*) and cancers (eg. Kaposi sarcoma) culminating in AIDS. Infection of the brain cells results in neuropsychiatric abnormalities such as behavioural changes (eg. apathy, mental confusion and impaired mental concentration), ophthalmic disorders and cerebral atrophy in children (Fauci, 1988; Levy, 1993).

1.3 Modes of Transmission

The major factors that facilitate the spread of the virus are mechanisms that lead to the exchange of body fluids between infected and uninfected individual. This may occur through any of the following means:-

- a) Unprotected sexual contact (i.e. sex without use of condoms) when semen and

vaginal secretions are exchanged. This is the major mode of transmission and accounts for at least 75% of HIV infections among adults (Johnson and Laga, 1989; Piot and Tezzo, 1990). In Kenya and most developing nations, especially in sub-Saharan Africa, this transmission is mainly through heterosexual contact as opposed to homosexual contact in North America and Western Europe (Piot *et al.*, 1988; Merson, 1993; National AIDS Control Programme of Kenya, 1993).

- b) Perinatal transmission, occurs when an infected mother infects her baby during pregnancy, parturition or shortly after birth. In Kenya, this mode of transmission accounts for about 21% of all transmissions (National AIDS Control Programme of Kenya, 1993).
- c) Breast transmission, occurs when an infected mother infects her child through breastfeeding.
- d) Blood transfusion when HIV-infected blood or blood products are transfused to uninfected patients.
- e) Other modes of transmission occur through the use of HIV contaminated equipment like needles, sharp instruments, razor blades, tooth brushes, dental and surgical equipment.

1.4 Clinical Presentation of AIDS

Infection with HIV normally leads to a progressive disease process known as acquired immunodeficiency syndrome, (AIDS). First, the virus often replicates abundantly in target cells and free virus appears in blood and later in the cerebrospinal fluid (Fauci *et al.*, 1996). This may be asymptomatic or symptomatic. When symptomatic, the host has fever, rashes, flu-like symptoms and sometimes neurological complaints. It is often dismissed as common cold or flu. Then within a few days or weeks, the amount of virus in

circulation and cerebrospinal fluid drops precipitously and the initial symptoms disappear. After this, the infection may remain asymptomatic for many months or years before the appearance of AIDS symptoms (Redfield *et al.*, 1986; Redfield and Burke, 1988; Price *et al.*, 1988; National AIDS Control Programme of Kenya, 1993).

The progression of symptoms extend from the initial acute stage of HIV infection to asymptomatic HIV infection to symptomatic HIV infection and finally to full clinical syndrome, AIDS. Symptoms and signs associated with the symptomatic HIV infection include fatigue, poor appetite, unexplained weight loss, generalized lymphadenopathy, persistent diarrhoea, fever and night sweats. Full clinical AIDS is characterized by opportunistic infections and carcinomas (Redfield and Burke, 1988). The clinical presentation is specific for each opportunistic infection involved. Tuberculosis is the most common opportunistic infection in African patients who die from AIDS (DeCock *et al.*, 1992; Mukadi *et al.*, 1997; Garin *et al.*, 1997; Boerma *et al.*, 1998).

Evidence from longitudinal studies on mortality amongst HIV-infected persons indicates that the median survival time of adults is 8-10 years in Africa, which is similar to the estimates of survival times from developed countries in earlier years of epidemic when anti-viral and prophylactic drugs use was limited (Boerma *et al.*, 1998). However, data on the impact of HIV on child survival time is about 2 years in Africa, which is shorter than in developed countries (Boerma *et al.*, 1998; Tinuicus, 1998). Poorer health care and delayed utilization of health care have been suggested as playing a role in the shorter survival period in developing countries (Lucas *et al.*, 1993; Boerma *et al.*, 1998)

1.5 Diagnosis

A diagnosis of HIV infection is usually made on the basis of a repeated reactive enzyme linked immunosorbent assay, ELISA, test and confirmed by a more specific assay,

e.g. western blot. (Redfield *et al.*, 1986). Virus isolation from the body fluids is the most highly specific means to make a definitive diagnosis whereas the polymerase chain reaction (PCR) is the most highly sensitive test which can detect the infection during the early stage (Young *et al.*, 1990).

AIDS may be diagnosed with or without laboratory evidence of HIV infection. Specific clinical criteria must be present, for example, as determined using the WHO staging criteria (WHO, 1990) or Walter Reed staging classification for HTLV-III / LAV infection (Redfield *et al.*, 1986).

1.6 Therapy

At present there is no cure or vaccine against HIV infection. However a number of vaccines, antiviral agents (eg. protease inhibitors), immunomodulators and other agents are in the experimental stages. For persons with HIV infection, zidovudine may be effective in delaying the clinical condition of the disease (Glatt *et al.*, 1988). However, the so called 'triple therapy' is nowadays recommended for treating persons with HIV infection (Montaner *et al.*, 1998). Triple therapy is a regimen consisting of two nucleoside reverse transcriptase inhibitors (eg. zidovudine, stavudine, didanosine, lamivudine) plus a potent protease inhibitor (eg. indinavir, ritonavir, nelfinavir) or two nucleoside reverse transcriptase inhibitors plus one non-nucleoside reverse transcriptase inhibitor (eg. nevirapine)

For persons in whom AIDS has been diagnosed, standard therapy consists of antiviral therapy, opportunistic infection prophylaxis and treating opportunistic diseases aggressively as they occur.

1.7 Heterosexual Transmission of HIV

Heterosexual transmission is the major route of HIV spread in Africa and most developing countries (Piot *et al.*, 1988; Quinn, 1996). "Safe sex" education and use of condoms can reduce the risk of heterosexual transmission but will not eliminate the need for an HIV vaccine. The majority of healthy HIV-infected individuals are unaware of their infected status. Because the mean incubation period from seroconversion to AIDS is at 7-8 years (National AIDS Control Programme of Kenya, 1993), there is a high likelihood for these individuals to transmit HIV to their sexual partners before they become aware of their positive HIV status. Thus, HIV will almost certainly continue to spread. Epidemiological studies indicate that HIV is not efficiently transmitted by sexual contact (May *et al.*, 1989). For example, 22-25% of individuals are infected by one exposure to *Neisseria gonorrhoea* (Holmes *et al.*, 1970) and hepatitis B virus is transmitted in 20-23% of exposures (Molsey, 1975; Judson, 1981). In contrast, less than 15% of those monogamous individuals repeatedly exposed to an infected sexual partner become infected with HIV (Holmberg *et al.*, 1988). It is estimated that for a single sexual contact, the infectivity is 0.3% (Peterman *et al.*, 1988). Thus, sexual transmission appear to be considerably less efficient and highly variable (Peterman *et al.*, 1988; Royce *et al.*, 1997).

Potential explanations for this variability in sexual transmission include biological differences in infectiousness of inoculating virus dose (Vernazza *et al.*, 1999), susceptibility of the person exposed to the virus, differences in mode of sexual contact (Friedland and Klein, 1987; Lazzarin *et al.*, 1991; Seidlin *et al.*, 1993) or a combination of them. These biologic factors can be divided broadly as host and viral factors.

One of the major host factor associated with increased susceptibility and excretion of HIV in the genital secretions is the occurrence of sexually transmitted diseases (STD). Sexually transmitted diseases have been shown to play an important role in sexual

transmission of HIV (Wasserheit, 1992). A number of studies have shown that the prevalence and incidence of HIV is considerably greater in patients who present in STD clinics with genital ulcers and mucosal inflammatory diseases (Plummer, *et al*, 1991; Schoenbach *et al*, 1993; Hayes *et al*, 1995; Torian *et al*, 1995; Deschamps *et al*, 1996; Frankel *et al*, 1997) and in patients with a history of STD (Lazzarin *et al*, 1991; Mastro *et al*, 1994). Sexually transmitted diseases appear to facilitate HIV transmission by disrupting the integrity of the genital epithelium, increasing the number of cells in genital secretions that are receptive to HIV infection, as well as increasing the genital shedding (infectivity) of HIV into the semen and genital secretions (Eron *et al*, 1996; Ghys *et al*, 1997; Vemazza *et al*, 1999).

Other host factors associated with increased excretion of HIV in genital secretions are systemic factors such as systemic infections. A number of studies have shown a weak but significant correlation between the plasma viral load and HIV in genital secretions (Dyer, *et al*, 1996; Vemazza *et al*, 1997; Goulston *et al*, 1998; Evans *et al*, 1998), indicating that HIV concentration in the genital secretions is a function of systemic factors. It has also been reported that HIV-DNA correlates with CD4 lymphocyte count in semen and is inversely correlated with peripheral CD4 count (Xu, *et al*, 1997). However, it is not clear whether this association between plasma viral load with HIV concentration in the genital secretions arises as a result of blood and genital compartments being connected, the two compartments experiencing similar environmental pressures, or systemic factors influencing the functional or anatomical integrity of the genital mucosa and hence, the excretion of the virus in semen and genital fluids.

Both progesterone and oestrogen have important physiological effects on female genital tract that could plausibly influence HIV transmission. Since hormonal contraceptives (including oral, injectable, and implantable forms) are in widespread use, it

is important to know whether they play any significant role in the vaginal transmission of HIV. This query has been addressed by several investigators (Piot *et al.*, 1987; Howe *et al.*, 1994; Stephenson, 1998), but very little is still known. Stephenson has analysed the quality of 32 studies for their ability to investigate the relationship between hormonal contraception and risk of HIV transmission and concluded that they are poor and inappropriate for statistical meta-analysis. She found that quantitative findings were inconsistent across studies and the studies reporting increased HIV risk with hormonal contraception had residual confounding factors. However, evidence from animal models showed a clear relationship between progesterone treatment and risk of SIV infection (Marx *et al.*, 1996). In that particular study (Marx *et al.*, 1996), 14 out of 18 macaques pre-treated with subcutaneous progesterone pellets and then vaginally inoculated with SIV became infected, compared with one out of 10 macaques pre-treated with placebo pellets before inoculation. The eight-fold increase in infection in the progesterone-treated animals was associated with marked thinning of the vaginal epithelium. Although this animal study hypothesize that progesterone reduces the efficacy of the vaginal barrier, some information is lacking on local defence mechanisms in the female genital tract and physiological changes during menstrual cycle, mechanisms by which viruses cross the vaginal epithelium, and hormonal effects on the mucosal immunology of the vagina and cervix.

A variety of viral factors have been suspected to play a role in the infectiousness of HIV and hence influencing the sexual transmission. Some HIV strains form syncytia (syncytia inducing) and others do not (non-syncytia inducing) form syncytia in lymphocyte culture as a result of sequence variation in the V3 envelope region. The non-syncytia inducing isolates of HIV are preferentially transmitted (Roos *et al.*, 1992) and in some AIDS patient, differences in recovery of syncytia inducing and non-syncytia inducing isolates in semen and blood has been noted (Vernazza *et al.*, 1994).

Envelope sequences also can be used to define geographically distinct HIV subspecies, called clades. The predominant subspecies, and which is responsible for more than half of all HIV infections is the African variant, clade C, while the epidemic in the U.S. and Western Europe has resulted from clade B. A segregation of clade B in homosexual and clade C in heterosexual contacts was found in South Africa (van Hanneken, *et al.*, 1997), suggesting that some clades are transmitted with greater efficiency than others, especially from men to female partners.

It is clear that HIV and SIV infection can occur via an intact genital mucosa without breaching the epithelial barrier (Miller *et al.*, 1989; Alexander, 1990; Kraehenbuhl and Wain-Hobson, 1996). However, the mechanisms involved are poorly understood. Human immunodeficiency virus is present in semen and cervico-vaginal secretions in both cell-free and cell-associated form and can be isolated from asymptomatic individuals and AIDS patients (Wofsy *et al.*, 1986; Anderson *et al.*, 1990). Whether HIV is predominantly transmitted as a cell-free virus or in a cell-associated form is not known. In the macaque model, cell-free SIV is transmitted vaginally more efficiently than the cell-associated virus (Miller *et al.*, 1989; Sodora *et al.*, 1998). *In vitro* cell culture experiments with human cervical epithelium and HIV suggest a potential cell-associated mechanism of transmission in human (Tan *et al.*, 1993; Tan and Phillips, 1996). In this model, the HIV infected monocytes adhere to the monolayer and viral particles were internalized by pinocytosis into the epithelial cells. Whether dendritic cells in the sub-epithelial tissue can serve as direct target for HIV or are infected after a passage of the virus through the epithelial cell layer remain unknown. The relative contribution made by cell-free and cell-associated HIV in sexual transmission is still under-investigated.

Information on the biology of female-to-male transmission is limited. HIV can be detected in endocervical swabs specimens, cervicovaginal lavage samples and CD4⁺ cells

but little is known about potential target cells in the male genital tract (Vernazza *et al*, 1999), but Miller *et al* (1989) have been able to infect male macaque after placing cell-free SIV on the penile urethra.

The cellular fraction of semen contains spermatozoa, immature germ cells, leukocytes (lymphocyte, granulocytes and macrophages), and epithelial cells. HIV can be detected in lymphocytes/monocytes and cell-free seminal plasma. Also, HIV-1 proviral DNA has been detected in sperm from HIV-1-infected men by electron microscopy and *in situ* polymerase chain reaction (Bagasra *et al*, 1994), but Quayle *et al* (1997) did not detect HIV in motile sperms. The latter finding is supported by the fact that, while unprocessed semen from HIV-infected men artificially inseminated to their women partners resulted in HIV transmission, over 1400 artificial insemination procedures with processed sperm from HIV-infected men failed to result in transmission of HIV suggesting that the motile sperm fraction from semen is not likely to transmit the virus (Semprini, 1993; Vernazza *et al*, 1999). The processing of sperm used by Semprini and others separates motile spermatozoa from contaminating leukocytes by density-gradient centrifugation and swim-up technique.

The fate of HIV-infected cells in the vagina is unknown. It is unlikely that infected cells can cross an intact genital mucosa, but following lysis of the seminal lymphocytes, virions are released and could bind to target cells in the genital mucosa. The difficulty in transmitting cell-associated virus in semen, may be related to limited lifespan of seminal lymphocytes in the vagina due to the presence of bacterial proteases, lysozymes and due to the fact that the cells are washed from the vagina by action of vaginal secretions. This issue need to be addressed using an animal model system.

In cell-free mechanism of transmission, virions can directly bind to HIV target cells and cause the infection. Whether latently infected cells in semen can survive long enough in the vagina to produce infectious virions, is not clear. Also the identity and location of

the cellular targets are yet to be determined in human. In the non-human primate model, Miller *et al* (1992) found only a few CD4+ T cells present in the submucosa of the vagina. Their findings indicate that the most likely target cells in the vagina mucosa are the macrophages and Langerhans cells. There is some evidence that vaginal epithelial cells (non-CD4+ cell types) can be infected with via a CD4 independent mechanism (Futura *et al*, 1994). However, Langerhans cells may have a major role as target cells in sexual transmission of HIV. These antigen-presenting cells are potentially efficient disseminators of these viruses from the genital mucosa to draining lymph nodes. These same cells are known to transport antigens from the stratified squamous epithelium to the draining lymph nodes. Langerhans cells of the skin have been shown to be infected with HIV in AIDS patients (Tschachler *et al*, 1987; Zambruno *et al*, 1991). Thus elucidation of interactions between HIV and the Langerhans cells of the vaginal and penile urethra mucosa seem to be the key in understanding the mechanism of heterosexual transmission.

1.8 Use of Animal Models

The only animals susceptible to HIV-1 are the chimpanzee (Alter *et al*, 1984), gibbon ape (Markam *et al*, 1987), the rabbit (Gardner and Luciw, 1989) and the pigtail macaque (Agy *et al*, 1992), but AIDS -like disease has not been reported in these species. The macaques (Castro *et al*, 1991(a)) and the baboon can be experimentally infected with HIV-2 with resultant AIDS-like condition in the baboon (Castro *et al*, 1991(a); Barnett *et al*, 1994).

1.8.1 The HIV-2 Baboon Model

Initially, infection of yellow baboons with a virulent strain of HIV-2 resulted in 100% seroconversion, 83% persistent infection and 67% development of lymphadenopathy

and CD4 T-cell loss at 18 to 24 months post infection (Barnett *et al.*, 1994). The baboon may therefore be the long-sought animal model for HIV infection and AIDS. If the model is developed further, the baboon model has more advantages than either the macaque or chimpanzee. It is less costly and readily available in the wild than either the macaque or chimpanzee. Also, on the basis of its suitability as a reproductive animal model (Eley and Bamba, 1993), the baboon could be a good model for studying heterosexual transmission of HIV. However, prior to this study reported in this thesis, there had been no reported work aimed at determining whether HIV-2 could be transmitted, either experimentally or naturally, via the reproductive mucosa.

1.8.2 The SHIV_{89.6} Baboon Model

Even though the SIV macaque model has proven to be of enormous value in studying AIDS pathogenesis and evaluation of vaccine strategies, a number of weaknesses remain inherent. The major limitation of this model arises from the divergent nature of the envelope glycoproteins of HIV type 1 and SIV_{mac}, resulting into limited cross-reactivity of antibodies directed against the envelope of these two viruses (Kanki *et al.*, 1985; Murphy-Corb *et al.*, 1996). The antibodies also, do not cross-neutralize (Javaherian *et al.*, 1992). This feature has led to lack of meaningful correlates for immunity against infection with HIV-1. This prompted Arthur *et al.*, (1992) to raise the question of whether a successful vaccine against SIV_{mac} can easily be modified for use against HIV. The obvious conclusion is that the use of SIV-macaque model is limited for evaluating envelope-based vaccine strategies to prevent HIV infection.

While the HIV-1 chimpanzee model has been used almost exclusively for testing HIV-1 based vaccines, the scarcity of this species (because it is highly endangered in the wild) and the resulting cost associated with its use have precluded detailed analysis and

testing of vaccines that might be efficacious in humans. In this respect, alternative models are desperately needed.

Today, most vaccines strategies incorporate HIV-1 envelope glycoprotein. Thus a suitable animal model would require the use of a challenge virus which contains an HIV-1 envelope gene. Researchers have therefore recognised the recombinant viruses containing the envelope of HIV-1 as the model of choice for use in non-human primates (Shibata *et al.*, 1991; Li *et al.*, 1992; Sukuragi *et al.*, 1992; Allan *et al.*, 1995). Following this cue, several constructs consisting of SIV_{mac 239} clone chimerized to contain the HIV-1 *env*, *tat*, *rev*, and *vpu* genes (so-called Simian/human immunodeficiency viruses, SHIVs) have been made through genetic engineering and shown to replicate to high titres in macaque (Sukuragi *et al.*, 1992; Shibata *et al.*, 1992; Li *et al.*, 1992; Reimann *et al.*, 1996 (a), Reimann *et al.*, 1996 (b); Joag *et al.*, 1996; Joag *et al.*, 1998; Harouse *et al.*, 1998) and baboon (Allan *et al.*, 1995, Otsyula *et al.*, 1998 – unpublished data) lymphocytes both *in vitro* and *in vivo*. Taken together, these studies have shown that recombinant SHIVs might be very useful as challenge viruses when testing candidate HIV-1 envelope-based vaccines.

It has been known for quite sometime that primary isolates of HIV-1 differ substantially from the laboratory adapted viruses in their cell tropism, replication kinetics in PBMC, and sensitivity to neutralizing antibodies and soluble CD4. These properties are determined primarily by differences in the envelope glycoproteins, (Cheng-Mayer, *et al.*, 1990; Shioda *et al.*, 1991; Sullivan *et al.*, 1995). Moreover the envelope region has been found to contain major determinants for macrophage tropism, (Hwang *et al.*, 1991), fibroblast-like CD4⁺ cell tropism (Takeuchi *et al.*, 1991) and cytopathogenicity (Cheng-Mayer *et al.*, 1990; York-Higgins *et al.*, 1990). Development of a chimeric SHIV that expresses HIV-1 glycoproteins from a patient isolate for studies of AIDS pathogenesis and vaccine protection would be of paramount importance. Previous studies indicated that the

envelope glycoproteins of a primary HIV-1 isolate, HIV-1_{89.6}, conferred a high level of early replication on SHIV chimeras *in vivo* compared to envelope glycoproteins derived from the laboratory adapted HIV-1 isolate, HXBc2 (Reimann *et al*, 1996 (a)). All the three HIVs (ie SHIV_{HXBc2}, SHIV_{SF7} and SHIV_{89.6}) were chimerized with SIV_{mac 239} clone (LTR, gag, pol, vif, vpx, vpr and nef). In both studies, the baboons were inoculated via the intravascular route.

The aim of the present study was to establish if the SHIV_{89.6} could infect the baboon via the genital mucosa. The genome of SHIV_{89.6}, which was used in this study, is depicted in Fig. 3 below. The study was undertaken in order to generate some preliminary information and lay a foundation for an elaborate study (as one described in later sections of this manuscript) in an endeavour to develop the baboon as an animal model for heterosexual transmission of HIV as well as a model for testing candidate vaccines, which are based on envelope glycoproteins of HIV-1.

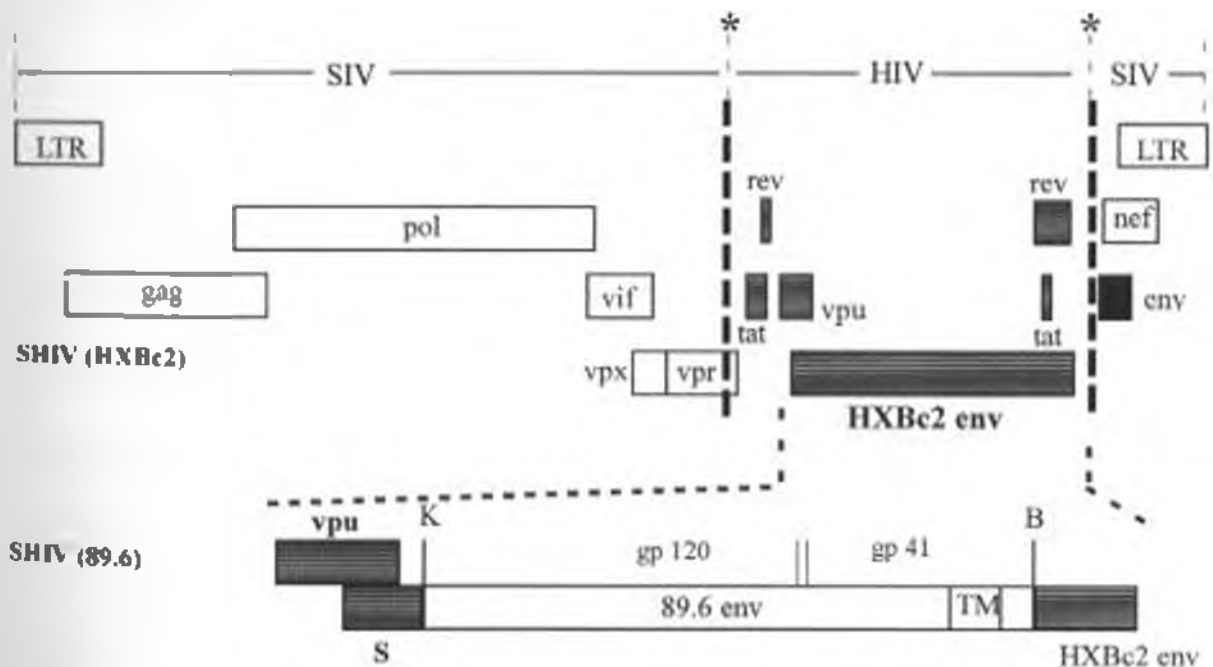


Figure 3. The structure of the chimeric virus, SHIV_{89.6}, which was used in this study. The SHIV-HXBc2, which has an intact *vpu* gene is shown at the top. The junctions of HIV-1 and SIV_{mac} sequences are denoted by asterisks. The residual 3' end of the SIV *env* gene is shown in solid black. The structure of SHIV-89.6 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 *KpnI* (K) and *BamHI* (B) restriction sites used for insertion of the 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.

Source: Reimann *et al*, 1996(a)

1.9 Study Justification

1.9.1 Objective and Aims of the Study

The general objective of the experiments described in this study was to develop the baboon as a model for heterosexual transmission of HIV. The specific aims of the study were therefore :-

1. To induce HIV-2 infection in adult baboon by intravenous inoculation of HIV-2.
2. To induce HIV-2 infection in adult baboon by inoculation of HIV-2 onto the genital mucosa (i.e. vagina mucosa of females, and urethral mucosa of males).
3. To determine whether exposure of genital mucosa of baboon to SHIV_{89.6p} could produce persistent infection.
4. To determine whether HIV-2 infected and SHIV_{89.6p} infected baboons develop a disease condition comparable to human AIDS.

1.9.2 Rationale

Heterosexual transmission is a major route of HIV spread and its mechanisms of transmission are poorly understood. The elucidation of interaction between HIV and the cells lining the reproductive mucosa are key in understanding the mechanism of heterosexual transmission. There is also need of assessing the role of biological co-factors in HIV transmission, and more importantly, testing the effectiveness of spermicides, candidate therapeutics agents and vaccines in preventing heterosexual transmission of HIV.

These issues can only be addressed in an animal model.

HIV-2 and SHIV_{89.6} have been reported to infect the baboon (Barnett, *et al*, 1994; Allan *et al*, 1995; Otsyula *et al*, 1998- unpublished data). In these studies, the baboons were inoculated intravenously. The baboon has also been developed as a practical reproductive animal model (Eley and Bambra, 1993) due to its suitability over the macaque

and the chimpanzee. On the basis on the above facts, there was need to develop the baboon further as an animal model for heterosexual transmission of HIV. The experiments described in this study, were aimed at determining whether HIV-2 or SHIV_{89.6p} can be transmitted via the reproductive mucosa of the baboon. If the results could demonstrate a comparable mode of sexual transmission of HIV-2 and SHIV_{89.6p}, between the baboon and human, this would make the baboon a valuable model system for studying the mechanisms involved in sexual transmission of HIV, and provide a system for testing pharmacologic and biologic cofactors that may affect HIV transmission. In addition, the model system would also be useful in testing the capacity of candidate vaccines to produce immune responses capable of preventing systemic infection after exposure of the genital mucosa to the virus.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals

Adult olive baboons (*Papio anubis*) and yellow baboons (*Papio cynocephalus*) kept in a colony at the Institute of Primate Research, (IPR), were used for these studies. A total of 10 animals were used. All the animals were screened for SIV/HIV antibodies by ELISA to ensure that they were unexposed to these lentiviruses prior to the commencement of these studies. All the animals were singly caged during the entire period of the experiment.

2.1.2 Inoculum

(a) The HIV-2 Inoculum

The HIV-2 virus inoculum (designated HIV-2 UC-2) was kindly donated to us, by Dr. Jay Levy from University of California, San Francisco, USA. The virus stock was expanded by growing in SupT1 cell line with RPMI 1640 medium (Atlanta Biologicals, Norcross, Georgia) supplemented with 10% FBS (Intergen Company, Purchase, New York), 1% L-glutamine (Sigma Chemical Company, St. Louis, MO) and 1% antibiotics (Penicillin-Streptomycin, Sigma Chemical Company, St. Louis MO).

(b) The SHIV_{89.6p} Inoculum

The SHIV inoculum stock (kindly donated by Dr. Norman Letvin of Harvard University via Dr. B.S. Graham of Vanderbilt University Medical Center, Nashville, TN) that was used in this study was derived from the original construct, SHIV_{89.6}. This chimeric virus was composed of SIV_{mac239} expressing HIV-1 *env* and associated auxillary genes *tat*, *vpu* and *rev* (Fig. 3) as previously described (Reimman *et al*, 1996 (a); 1996 (b)). The *env* sequences were derived from a cytopathic macrophage-tropic clone of a patient isolate of HIV-1 (89.6) after transfection into CEMx174 cells and the virus expanded on

lectin-activated rhesus monkey PBMC. This parent virus was passaged via the rhesus monkey once to produce the stock SHIV_{89.6P} and then serially (four times) passaged through the baboon. The inoculum used to infect the two baboons in this study was the bone marrow aspirate obtained from the fourth baboon passage.

2.1.3 Tissue Culture Reagents

For propagation of PBMC and cell lines in cultures, RPMI 1640 was utilized. It was supplemented with 10% FBS, 1% L-glutamine and 1% antibiotics (100 U/ml penicillin, 10 µg/ml streptomycin) as previously reported in section 2.1.2(a). Recombinant IL-2 (Biosource International, Camarillo, California) and Concanavalin A (Sigma Chemical Company, St. Louis, MO) were added for activation of PBMC when found necessary.

2.1.4 Antigen Capture ELISA Reagents

To detect viral antigen in tissue culture fluid, an SIV Core (p27) Antigen Assay commercial kit from Coulter (Coulter Corporation, Miami, Florida) and one kit donated by NIH AIDS Reagent Programme were used. These kits utilize an anti-SIV_{mac} core antigen p27 monoclonal antibody. (See a detailed method in 2.4.3).

2.1.5 Antibody ELISA Reagents

Two ELISA systems were used. System I utilized a 21-amino acid SIV_{mac251} synthetic peptide derived from the conserved immunodominant region of the transmembrane glycoprotein. The peptide sequence was as follows: H-NAWGCAFRQVCHTTVPWPNAS-OH. (Research Genetics, Huntsville, AL). The other reagents which were used are shown in Appendix 5. A detailed method is given in 2.4.4(a).

ELISA system II was a Genclavia® Mixt commercial kit (Sanofi Diagnostics Pasteur Inc., Chaska, MN). A detailed method is given in 2.4.4(b).

2.2 METHODS

2.2.1 Handling of Animals

The animals were handled under moderate to heavy anaesthesia during experimental manipulations (i.e. HTV-2 inoculations and bleeding). Anaesthesia was induced by intramuscular injection of a mixture of ketamine hydrochloride (Ketaset[®], Fort Dodge Laboratories Inc., Fort Dodge, Iowa) and xylazine (Chanazine[®], Chancell Pharmaceuticals Manufacturing Ltd., Galway, Ireland) at a ratio of 5:3 at a dosage of 100 mg/kg body weight ketamine and 60 mg/kg body weight xylazine.

2.2.2 Animal Husbandry

Throughout the study, the experimental animals were taken care of, according to the standard operating procedures (Chai, 1993), which are in place at IPR. These studies were approved by the Institutional Scientific Evaluation and Review Committee, ISERC, and the Animal Care and Use Committee, ICUC, of the Institute of Primate Research.

2.2.3 Pre-screening Experiment – *In Vitro* infection of Baboon PBMC

To determine whether this parent virus stock could infect the East African baboons (olive baboon and yellow baboon) housed at the Institute of Primate Research, the following *in vitro* test was carried out.

Two baboons, a yellow baboon and an olive baboon, were bled to obtain PBMC and plasma. Both animals tested negative upon screening for SIV antibodies using an ELISA test (both System I and System II) as described latter. PBMC were isolated from blood by gradient centrifugation, using lymphocyte separation medium (Organon Teknika Corporation, Durham, NC). The PBMC were cultured in stimulation medium (i.e. 5 µg/mL

Con A and 50 units/ml recombinant IL-2 in complete RPMI 1640 medium) for 3 days and the medium washed off. Each cell pellet was resuspended in 1ml HIV-2 UC-2 supernatant and incubated on ice for 2 hours. The cell suspension was transferred to T25 culture flask (Falcon, Becton Dickinson and Company, Lincoln Park, NJ), 1ml medium added and incubated for 24 hours in CO₂ incubator (5% CO₂ and 95% O₂) at 37°C. The culture cells suspension was then transferred to a centrifuge tube (Becton Dickinson and Company, Lincoln Park, NJ), the cells washed 3 times and then put into a new T25 flask and medium containing rIL-2 added. The cells were incubated at 37°C in CO₂ incubator for 5 days, and some culture supernatant taken for p27 antigen capture ELISA (Coulter™ STV core Antigen Assay) as described below.

2.2.4 Inoculation

(a) Preparation of HIV-2 Inoculum

UC-2 inoculum was prepared from tissue culture fluid of Molt 4 clone 8 cells infected with HIV-2 UC-2. Cell-free inoculum was prepared by centrifugation at 500g of HIV-2 UC-2-infected Molt 4 tissue culture suspension to pellet the cells. The resultant supernatant was aliquoted into 1 ml. doses. Five stocks of such supernatant of cultures infected at different times were prepared in the similar manner as above. All of them tested strongly positive on p27 antigen capture ELISA. To select the most potent one, serial dilutions were performed to final dilutions of 1:2, 1:10, 1:50, 1:150 and 1:1250. These dilutions were used to inoculate fresh Molt 4 cells. Two of the stocks showed cytopathogenic effects in all the dilutions. One of them was picked at random and was used as UC-2 inoculum.

(b) Preparation of 'Saline'

'Saline' was prepared from tissue culture fluid of uninfected Molt 4 clone 8 cells. Cell-free inoculum was prepared by centrifugation of Molt 4 clone 8 tissue culture suspension to pellet the cells. The supernatant was aliquoted in 1ml. aliquots. A randomly picked aliquot tested negative in p27 antigen capture ELISA.

2.2.5 Estimation of Virus Infectivity

The infectivity of the inoculum stock was estimated at the time of inoculation using the Reed and Muench (1938) method. A detailed protocol is given in section 2.4.2 and Appendix 2).

2.3 EXPERIMENTAL DESIGN

2.3.1 Animal Groupings

Six olive baboons, code-named P1803, P2021, P2031, P2070, P2073, P2078, P2079 and P2082 and two yellow baboons, code-named P182 and P183 were placed into four groups as follows:

- Group 1 :** Comprised of 4 adult olive baboons (2 females and 2 males). This was the control group. A negative control group (P2070 and P2079) was made up of a male and a female and positive control group (P1803 and P2078) of one male and one female
- Group 2 :** Comprised of 2 adult male olive baboons, P2021 and P2031.
- Group 3 :** Comprised of 2 adult female olive baboons, P2073 and P2082.
- Group 4:** Comprised of 2 adult female yellow baboons, P182 and P183.

2.3.2 Inoculation Plan

- Group 1:** (a) P2070 and P2079 (Negative Control Group) – each animal was given once, 1 mL of 'Saline' via the femoral vein.
- (b) P1803 and P2078 (Positive Control Group) – each animal was given once, 1 mL of UC-2 inoculum via the femoral vein.
- Group 2:** P2021 and P2031 (Urethral Group) - each animal was given 1 mL of UC-2 inoculum, a total of six (6), twice per week by depositing the inoculum in

the penile urethra using thin, non-pyrogenic plastic tubing improvised from 21 G $\frac{1}{4}$ Vacutainer[®] Brand Blood Collection set (Becton Dickinson, Franklin Lakes, New Jersey, USA) infusion set. KY jelly was used to lubricate the tubing before insertion into penile urethra. The first day of inoculation was taken to be day 0 post inoculation.

Group 3: P2073 and P2082 (Vaginal Group) - each animal was given 1 ml. of UC-2 inoculum, a total of six (6), twice per week by depositing the inoculum in the vagina using thin, non-pyrogenic plastic tubing improvised from HELM[®] intravenous set (Helm Pharmaceutical GMBH, Hamburg, Germany). KY jelly was used to lubricate the tubing before insertion into the vagina. The first day of inoculation was taken to be day 0 post inoculation.

Group 4: One female baboon, P183, was inoculated via the intravenous route by infusing 1ml. of bone marrow aspirate of the inoculum from SHIV_{89.6P} infected baboon. The other female was inoculated via the perivaginal route using the same inoculum. This technique consisted of positioning the tip of a 25-gauge hypodermic needle beneath the vaginal epithelium so that the tip was in the submucosal connective tissue of the vagina as previously described (Lohman *et al.*, 1994). Using this inoculation procedure, 1mL of bone marrow inoculum was placed into the vaginal submucosal at three different sites (approximate 0.3 mL per site). Both animals were at follicular phase (stage 3) of their reproductive cycle.

Table 1: Animal Grouping and Inoculation Plan

Group No.	Group Name	Animal No.	Sex	Inoculum	Route
1 (a)	Negative	P2070	F	'Saline'	i.v.
		P2079	M	'Saline'	i.v.
1 (b)	Positive	P1803	F	HIV-2	i.v.
		P2078	M	HIV-2	i.v.
2	Urethral	P2021	M	HIV-2	Urethral
		P2031	M	HIV-2	Urethral
3	Vaginal	P2073	F	HIV-2	Vaginal
		P2082	F	HIV-2	Vaginal
4 (a)	Intravenous	P183	F	SHIV	i.v.
(b)	Submucosal	P182	F	SHIV	Vaginal submucosa

2.3.3 Sample Collection

(i) **Blood:** Blood samples used to obtain plasma and PBMC were obtained from all the animals via venipuncture of femoral vein at 0, 1, 2, 3, and 4 weeks post inoculation except P2070 and P2079 (Negative Control group), in which they were obtained at 0, 1 and 4 weeks post inoculation. Blood was also obtained at week 5 (P1803 only), week 6 (P2078 only), week 8 and 12 (P182 and P183) week 15 (P2021, P2031, P2073, P2078, and P2082), week 16 (P182 and P183) week 17 (P2021 only), week 18 (P2082 only) week 20 (P182 and P183) and week 25 (P2031, P2070, P2073 P2078, P2079) post inoculation (Table 2). Blood was centrifuged at 500 g for 25 minutes and serum/plasma collected. PBMC were isolated from heparinised blood by

gradient centrifugation, using lymphocyte separation medium (Organon Teknika Corporation, Durham, NC). Serum/plasma were stored at -30°C and PBMC were used freshly in culture and the remainder stored in liquid nitrogen.

- (ii) **Lymph node:** An inguinal lymph node biopsy to be used for tissue culture was obtained aseptically from each animal at week 3 post inoculation. More lymph node biopsies were obtained for culturing from a representative of three groups, (P1803, P2021 and P2082 at week 5, 17 and 18 respectively).
- (iii) **Other tissues:** Biopsies of spleen, liver, lymphnode bone marrow aspirate, and cerebral spinal fluid were also obtained for culturing from P1803, P2021, and P2082 at week 5, 17 and 18 respectively (each animal representing one group).

2.3.4 Monitoring Health

During sampling, all animals were weighed and physically examined to monitor their health parameters.

Table 2: Animal Grouping and Sampling Schedule

Group Number	Group Name	Identity	Sampling Time (in weeks)											
			0	1	2	3	4	5	6	15	17	18	25	
1(a)	Negative	P2070, P2079	X X	X X		X _n X _n								X X
1(b)	Positive	P1803, P2078	X X	X X	X X	X _n X _n	X X	X _e [*]		X	X			X
2	Urethral	P2021 P2031	X X	X X	X X	X _n X _n	X X				X X	X _e [*]		X
3	Vaginal	P2073 P2082	X X	X X	X X	X _n X _n	X X				X X		X _e [*]	X

KEY TO TABLE 2 ABOVE:

- X** Blood sample
- Xn** Blood sample and inguinal lymph node biopsy
- Xc*** Blood sample, spleen, liver, lymph node, bone marrow aspirate and cerebral spinal fluid.

Notes: P182 and P183 were sampled at week 0, 1, 2, 3, 4, 6, 8, 12, 16 and 20

2.4 LABORATORY PROCEDURES

All the laboratory procedures were performed at IPR using facilities available in the Virology laboratories. The following laboratory procedures were performed.

2.4.1 Virus Isolation

Tissue culture work for expansion of HIV-2_{UC-2} virus stock, virus isolation and maintenance of cell-lines utilized RPMI 1640 culture medium, which was prepared as previously described in section 2.1.3. All the cell cultures were incubated at 37°C in humidified, CO₂ incubator, (with 5% CO₂ and 95% O₂). The RPMI 1640 medium in the cultures was replenished twice a week.

2.4.2 Estimation of HIV-2 Inoculum Infectivity

This was performed in 24-well flat-bottomed tissue culture plates (Falcon[®], Becton Dickinson and Company, Lincoln Park, NJ). The cultures were incubated at 37°C in humidified CO₂ incubator as previously described and then assayed for p27 antigenemia by antigen capture ELISA. To determine the TCID₅₀, the Reed and Muench (1938) method was utilised.

This method entailed performing a diminishing serial dilution of the virus stock in

duplicates in a 24-well flat bottomed tissue culture plate. Molt 4 Clone 8 cells were added into each well and the plate incubated at 37° C in humidified CO₂ incubator for 14 days with daily assessment of observable CPE. The endpoints were determined and the TCID₅₀ calculated accordingly. An illustration of the method is shown in Appendix 2.

2.4.3 Antigen Capture ELISA Test

This test was performed by use of Coulter® SIV Core Antigen Assay Commercial Kit (Coulter Corporation, Hiialeah, FL). The Coulter SIV Core Antigen Assay is an enzyme Immunoassay (EIA) which uses a murine monoclonal antibody (anti-SIV core antigen p27) coated onto microwell strips, to detect SIV (or cross-reactive) antigens in plasma, serum or tissue culture media. If present, the antigen (p27) would bind to the antibody-coated microtitre wells. The bound SIV antigen was detected by biotinylated human antibodies which recognized SIV (or HIV-2) and reacted with conjugated streptavidin horseradish peroxidase. The breakdown of the hydrogen peroxide in the presence of the enzyme, horseradish peroxidase, causes tetramethylbenzidine (TMB) chromogen (which is colourless) to produced a coloured product, whose optical density was measured in a photometer, (see an illustration in Appendix 3). The intensity of colour developed is directly proportional to the amount of SIV (or HIV-2) antigen present in the plasma, serum or tissue culture media. The assay was performed according to the protocol provided together with the kit and the absorbance read on a spectrophotometer at 450 nm. A detailed protocol is shown in Appendix 4).

Samples with absorbance values greater than the cut-off value were considered positive for SIV (or HV-2) antigen. (The cut-off value was defined by kit manufacturers as the sum of mean negative control and a predetermined factor of 0.030.

2.4.4 Enzyme-linked Immunosorbent Assay (ELISA)

To demonstrate the presence or absence of anti-HIV antibodies in the monkey sera/plasma, two assay systems were employed:

(a) ELISA System I

The ELISA protocol used was a modification of a previously described technique (Gallarda *et al.*, 1992). ELISA microtitre plates (Falcon 3912, Becton Dickinson and Company, Lincoln Park, NJ) were coated with a 21 - amino acid SIV synthetic peptide derived from the conserved immunodominant region of the SIV_{mac251} transmembrane glycoprotein (Research Genetics, Huntsville, AL.). The peptide sequence was H- N A W A C A I' R Q V C H T T V P W P N A S - OH, diluted to a final concentration of 400 ng/well in 0.1 M CAPS (3-(cyclohexylamino) - 1- propane Sulfonic acid) coating buffer (Sigma Chemical, Co. St. Louis, MO). The plates were then sealed, incubated at room temperature for one hour, then stored at 4 ° C overnight. The plates were subsequently washed twice, incubated with blocking buffer (see Appendix 5) at 37° C for 1 hour. The plates were washed three times and control and test sera dispensed into the wells, in duplicates, in 1:100 dilutions. The control and test samples were diluted in diluent buffer (see Appendix 5). The plates were sealed, and incubated at 37° C for 3 hours. The plates were washed (three times) and incubated at 37° C for two hours with a 1:2800 dilution of goat anti-monkey IgG conjugated to horseradish peroxidase (Nordic Lab, Capistrano Beach, CA). The plates were washed five times, incubated for 30 minutes at room temperature with O-phenylene-diamine (Sigma Chemical Co., St. Louis, MO) as the chromogen and hydrogen peroxide (Sigma Chemical Co., St. Louis, MO) as the substrate and the absorbance read on a spectrophotometer at 450nm. The reaction was stopped by adding 50 µl. of stopping reagent. The cut-off value was defined as the sum of mean negative control and a predetermined factor of 0.100. Samples were considered positive if

the sample/cut-off ratio was greater than 1.0.

(b) ELISA System II

This procedure utilized a Genelavia Mixt[®] commercial kit (Sanofi Diagnostics Pasteur, Inc. Chaska, MN). Genelavia Mixt[®] is an indirect enzyme immunoassay for the detection of various HIV-1 and HIV-2 antibodies. It is based upon the use of a solid phase coated with purified antigens (gp160 recombinant protein and peptides mimicking the immunodominant epitopes of the HIV-1 & HIV-2 envelop glycoproteins) and of peroxidase labeled anti-human IgG and IgM goat antibodies purified by affinity chromatography.

The serum samples to be assayed and control sera were diluted 1:5 with diluent buffer provided with the kit, and 100 μ l pipetted into the microplate wells. HIV-1 and/or HIV-2 cross-reactive antibodies, if present, bind to antigens immobilized on the solid phase. The wells were washed, then peroxidase labelled goat anti-human IgG and IgM antibodies were added. They bind in turn to the IgG and IgM captured on the solid phase. The presence of the enzyme immobilized on the complexes was shown by incubation, in the presence of hydrogen peroxide as the substrate and OPD as the chromogen, after the excess conjugate fraction had been removed by washing. The reaction was stopped and the absorbances read spectrophotometrically. The absorbance measured on a sample allowed the presence or absence of HIV-1 and /or HIV-2 antibodies to be determined.

A detailed procedure was provided together with the Kit. Briefly, the protocol was as follows:-

The serum/plasma samples to be assayed and control sera (provided with the kit) were pipetted into the microplate wells (100 μ L/well) in a dilution of 1:5. Samples and controls were diluted in sample diluent provided with the kit. The plates were sealed, incubated at 40° C for 30 minutes and thereafter washed three times. The plates were incubated at 40° C for 30 minutes with peroxidase labeled anti-human IgG + IgM goat

antibodies after sealing, and washed after the incubation as described above. The plates were then incubated (without sealing) in the dark for 30 minutes at room temperature with hydrogen peroxide as substrate and O-phenylene-diamine as chromogen. The reaction was stopped by adding 4N Sulphuric acid, (50 μ L/well) and absorbance read at 450 nm with a reference wavelength at 630 nm.

Samples with absorbance values equal or greater than the cut-off value were considered to be positive. (The cut-off value was defined as one tenth of mean absorbance of the cut-off control serum provided with the kit).

2.4.5 Western Blot

To detect and confirm anti-SHIV antibodies in SHIV-infected animals, a New Lav – Blot II [®] (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) HIV-2 Western blot was performed according to the manufacturer's instructions (see detailed protocol in Appendix 6).

The test is based on the principle of indirect ELISA on a nitrocellulose support containing all the proteins which constitute the HIV-2 virus. Inactivated HIV-2 viral proteins were separated according to their molecular weights by polyacrylamide gel electrophoresis in dissociating and reducing medium and subsequently transferred onto a nitrocellulose membrane sheet. The nitrocellulose membrane sheet was cut into several strips, with each strip for running a single test sample/control. These strips were rehydrated, and incubated with the samples to be confirmed or the control serum. If anti-HIV-2 antibodies or cross-reactive antibodies (e.g. anti-SHIV) were present, they would bind to the virus proteins recognised, present on the strip. After washing off the excess and unbound antibodies, the strips were incubated with alkaline phosphatase-labelled anti-human IgG antibodies. The conjugate would bind to the anti-HIV-2 or cross-

reactive antibodies captured on the solid support (strip). After washing off the unbound and excess conjugate, addition of colour development solution (eg. 5-Bromo-4-Chloro-3-Indolyl phosphate, BCIP, in NitroBlue tetrazolium, NBT, buffer) allowed the enzymatic activity of the complex compounds bound to nitrocellulose strips to take place. The appearance of specific coloured bands allowed the presence of anti-HIV-2 or cross-reactive antibodies in the serum to be detected.

The bands were classified as *env* (gp 140, gp 105, & gp 36) *gag* (p 56, p26, p16) and *pol* (p68). A sample was defined positive if it displayed at least 2 *gag* bands together with a *pol* band. According to the manufacturer's instruction, a test sample is defined positive if it developed at least one band in all the three regions (ie *env*, *gag* & *pol*). However, the SHIV₂₄₆ contains the 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 is therefore justifiably ignored.

A sample was defined as negative if no band developed or if the bands appearing could not be classified, as either *env*, *gag* or *pol*.

CHAPTER THREE

RESULTS

3.1 Animal Health Status

During the 25-week period of this study, all the animals maintained good health status except a transient enlargement of inguinal lymph nodes, which was observed between the second and fourth week post inoculation in the two SHIV inoculated baboons. The transient enlargement was probably due to initial SHIV viraemia, which was observed in the two baboons during this period as was demonstrated by SHIV isolation (see section 3.3(b) and Fig. 5).

3.2 *In Vitro* Inoculation of Baboon PBMC

Five-day tissue culture supernatants taken for p27 antigen capture ELISA showed that HIV-2 replicated in PBMC from both species of baboon. (see Table 3 below). The O.D. values, suggested that the virus replicated better in PBMC of yellow baboon (*P. cynocephalus*) than olive baboon (*P. anubis*). CPE and syncytial formation was observed in cultures of PBMCs of both species, and it was further amplified, when SupT1. Cells were added into the PBMC cultures.

Table 3: Optical density (O.D.) values of *in vitro* assay (p27 antigen capture ELISA).

Sample	Mean O.D. Value
Negative Control (kit)	0.078
Positive Control (kit)	0.474
HIV-2 _{UC2} in Sup11 (Positive Control)	0.810
HIV-2 _{UC2} in *PCY PBMC	0.372
HIV-2 _{UC2} in *PAN PBMC	0.294
Cut-off value =	$0.078 + 0.030 = 0.108$

KEY *PCY *P. cynocephalus* (yellow baboon)

* PAN *P. anubis*. (olive baboon)

3.3 Estimation of HIV-2 Inoculum Infectivity

The HIV-2 inoculum given to the baboons was estimated to $10^{3.6}$ TCID₅₀ /ml. according to Reed and Muench (1938) method.

3.4 Virus Isolation

(a) HIV-2 -inoculated Baboons

Formation of CPE and syncytia as indicators of replication of virus in the cultured cells was observed only in two post inoculation cultures (Fig. 4 below) of the same animal but different bleed dates and sources. These were in P2078 lymph node biopsy taken at week 3 PI. and P2078 PBMC (stimulated) bled at week 4 P.I. Antigen capture ELISA was performed on the cultures from the animals and a positive result was obtained in the two cultures in which CPE and/or syncytial formation was observed (Table 5). Similar attempts to isolate the virus from various organs/tissue obtained from one animal in groups 1, 2 and 3 just before euthanasia at different periods post inoculation were unsuccessful, with regard

to CPE/syncytial observation and p27 antigen capture ELISA at the termination of cultures between 30-40 days. The organs from which co-cultures were examined are inguinal and auxiliary lymph nodes, spleen, liver, bone marrow aspirate and cerebro-spinal fluid.

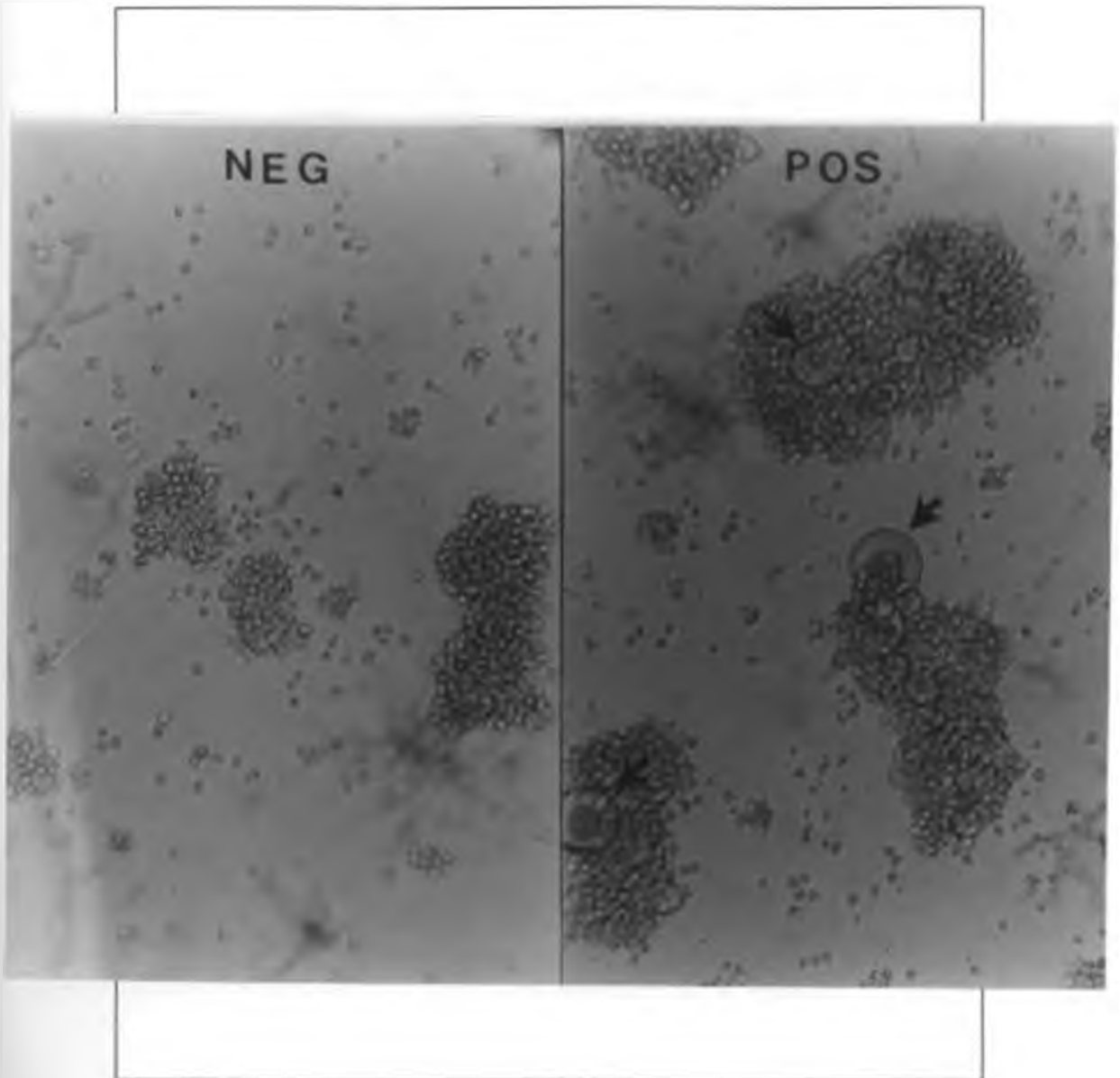


Figure 4: A microphotograph showing two sections labeled NEG and POS. NEG shows a culture of SIV/HIV-uninfected Molt 4 cells (negative control) and POS shows CPE of a lymph node culture of P2078. The lymph node biopsy was taken at 3 weeks post inoculation. Cells showing giant cell formation are indicated by arrows. (magnification: X200)

(b) SHIV -inoculated Baboons

Infectious virus was recovered from peripheral blood from the animals in weeks 1, 2, 3 and 4 following i.v. inoculation (P183) and in weeks 2, 3 and 6 following perivaginal inoculation (P182). The results are shown in Fig. 5 and Table 6 below).

Fig. 5 shows an example of giant cell formation which was characteristic for cultures in which SHIV replicated. Unlike HIV-2, SHIV replication in PM-1 cell-line showed a characteristic bulb-shaped ballooning of cells.

Antigen Capture ELISA:

All tissue culture fluids, in whose cultures CPE was observed, tested positive in Antigen Capture ELISA as shown in Table 6.

Table 6 shows that SHIV₈₉₆ was recovered as early as first and second week post inoculation in the i.v.-inoculated and vaginally-inoculated baboons respectively. This was demonstrated by CPE observation and confirmed by p27 antigen capture ELISA. The virus was consistently recovered at week 2, 3, 4 and 6 post inoculation thereafter. However due to high costs involved in performing tissue cultures, virus recovery was not attempted at week 4 (P182 only), week 6 (P183 only) and weeks 8, 12, 16 and 20. The table also shows that anti-SHIV antibodies were first detected at week 3 post inoculation in both animals and consistently thereafter, up to week 20.

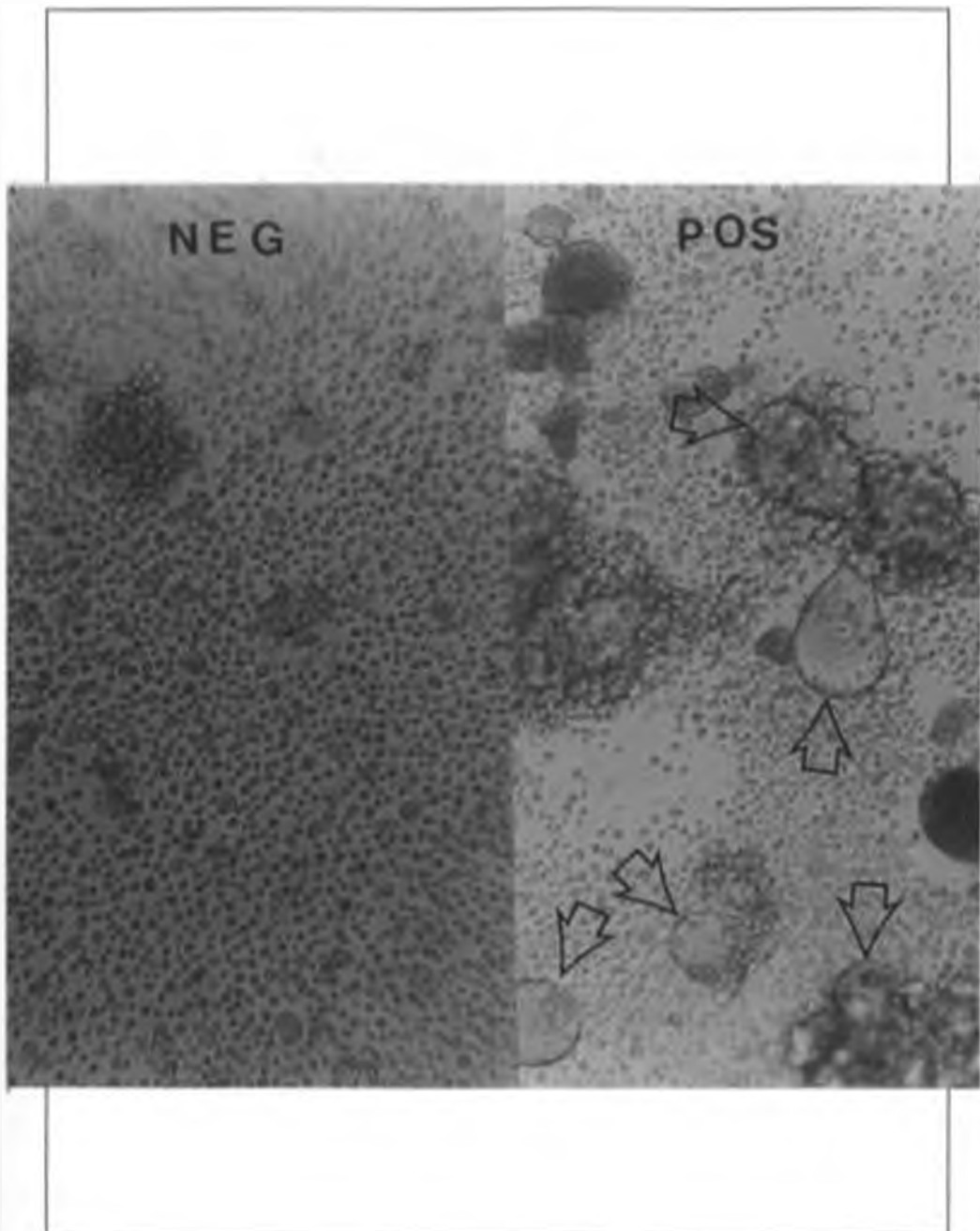


Figure 5: A microphotograph showing two sections labeled NEG and POS. NEG is a negative control culture (uninfected PM-1 cell-line) and POS is a culture of PM-1 cell-line infected with SHIV_{89.61} which was recovered from the PBMC of P183 at week 2 post inoculation. Cells showing giant cell formation are indicated by arrows. (Magnification: X200)

3.5 Antibody ELISA

(a) Detection of anti-HIV-2 Antibodies

Anti-HIV-2 antibodies were undetectable in all serum/plasma tested with exception of plasma obtained from P2078 at week 6 and week 15, which was detected by ELISA system II and not system I. However, the O.D. values (Table 4(a), which were just above the cut-off values, suggested that the level of antibody detected was very low.

Table 4(a) shows the optical densities values of HIV-2 antibody ELISA. It was noted that, with the exception of weeks 6 and 15 in the one of the i.v.-inoculated baboons (P2078), all the O.D. values were around the level of that of the negative control sera (0.012), indicating that anti-HIV-2 antibodies, if any, were completely undetectable.

Table 4(a): Mean optical density (O.D.) values of antibody ELISA – HIV-2

Weeks PI	0	1	2	3	4	5	6	15	17	18	25
P1803	0.012	0.009	0.005	0.011	0.012	0.007					
P2078	0.018	0.012	0.012	0.010	0.012		0.068*	0.065*			0.016
P2021	0.011	0.009	0.008	0.008	0.007			0.009	0.010		
P2031	0.014	0.010	0.009	0.006	0.009			0.011			0.013
P2073	0.015	0.012	0.011	0.006	0.006			0.008			0.010
P2082	0.012	0.008	0.009	0.013	0.009			0.007		0.002	
P2070	0.010	0.010			0.011						0.009
P2079	0.010	0.008			0.011						0.008

Table 4(b): Mean optical density (O.D.) values of antibody ELISA – SIV

Weeks PI	0	1	2	3	4	6	8	12	16	20
P182	0.007	0.012	0.008	0.401*	0.366*	0.401*	0.319*	0.319*	0.388*	0.462*
P183	0.006	0.011	0.010	0.358*	0.401*	0.559*	0.388*	0.441*	0.423*	0.569*

Key: (Tables 4 (a) and 4 (b))

- * O.D. values above the cut-off value of 0.040
(cut-off value was defined as one tenth of mean O.D. of the Cut-off Control Sample, which is provided with the kit, provided that 0.7 of the calculated cut-off value is greater than the O.D. of the negative control sample, also provided with the kit.

Notes for Tables 4(a) and (b)

O.D. values of Control Samples (provided with the kit) were as follows:-

- Negative Control: 0.012
- Cut-off Control Sample: 0.438, 0.396, 0.376 (mean = 0.403, thus the cut-off value is 0.040)
($0.403 \times 0.7 = 0.2821$, which is greater than O.D. of Negative Control Sample [0.012])

Positive Control: 0.488

O.D. values of other control samples were as follows:-

- Serum of SIV_{Deb}-infected DeBrazza's monkey: 0.422
- Serum of SIV_{agm}-infected vervet: 0.408

Table 5: Summary of culture observations, antigen capture ELISA and antibody ELISA.

ID		P1803	P2078	P2021	P2031	P2073	P2082	P2070	P2079
Route		i.v.	i.v.	Urethra	Urethra	Vagina	Vagina	Saline	Saline
Wk.0	Cpe	-	-	-	-	-	-	-	-
	P27	-	-	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	-
Wk.1	Cpe	-	-	-	-	-	-	-	-
	P27	-	-	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	-
Wk.2	Cpe	-	-	-	-	-	-	NT	NI
	P27	-	-	-	-	-	-	NI	NT
	ELISA	-	-	-	-	-	-	NT	NI
Wk.3	Cpe	-	+	-	-	-	-	NI	NT
	P27	-	+	-	-	-	-	NT	NI
	ELISA	-	-	-	-	-	-	NI	NT
Wk.4	Cpe	-	+	-	-	-	-	-	-
	P27	-	+	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	-
Wk.5	Cpe	-	-	-	-	-	-	NI	NT
	P27	-	-	-	-	-	-	NT	NI
	ELISA	-	-	-	-	-	-	NI	NT
Wk.6	Cpe	NI	-	NT	NI	NI	NT	NI	NI
	P27	NT	-	NI	NI	NI	NI	NI	NT
	ELISA	NI	+	NT	NI	NI	NT	NI	NI
Wk.15	Cpe	NT	-	-	-	-	-	NT	NT
	P27	NI	-	-	-	-	-	NI	NT
	ELISA	NT	+	-	-	-	-	NT	NI
Wk. 17	Cpe	NI	NT	-	NI	NT	NT	NI	NT
	P27	NT	NI	-	NT	NI	NI	NT	NI
	ELISA	NI	NT	-	NI	NT	NT	NI	NT
Wk. 18	Cpe	NT	NI	NT	NT	NT	-	NT	NI
	P27	NI	NT	NT	NI	NI	-	NT	NT
	ELISA	NT	NI	NT	NT	NI	-	NT	NI
Wk. 25	Cpe	NI	-	NT	-	-	NT	-	-
	P27	NT	-	NT	-	-	NT	-	-
	ELISA	NT	-	NI	-	-	NI	-	-

KEY:

- Negative outcome

NT Not tested

+ Positive outcome

i.v. intravenous

Table 5 shows that the CPE was observed and confirmed by core antigen detection only in one i.v.-inoculated baboon (P2078) at only two occasions – weeks 3 and 4 post inoculation. Anti-HIV-2 antibodies were demonstrated in the same animal only twice at weeks 6 and 15 post inoculation. All the other samples tested repeatedly negative by both ELISA systems, which were employed. (Results of repeat assays are not shown in the table.)

(b) Detection of Anti-SHIV antibodies

Antibodies in plasma were first detected at week 3 post inoculation and persistently thereafter (Tables 4(b) and 6).

Table 6: Results of CPE observation, p27 antigen capture and antibody ELISA of P183 and P182.

Week		0	1	2	3	4	6	8	12	16	20
P182	CPE	-	-	+	+	NT	+	NT	NT	NT	NT
	P27	-	-	+	+	NT	+	NT	NT	NT	NT
	ELISA	-	-	-	+	+	+	+	+	+	+
P183	CPE	-	+	+	+	+	NT	NT	NT	NT	NY
	P27	-	+	+	+	+	NT	NT	NT	NT	NT
	ELISA	-	-	-	+	+	+	+	+	+	+

KEY:

- Negative outcome

+

Positive outcome

NT Not tested

Table 4(b) shows that, while the O.D. values of pre-inoculation and weeks 1 and 2 post inoculation sera were as low as that of the negative control serum (0.012), there was a dramatic rise in O.D. values at week 3 post inoculation, without passing through an intermediate level. The O.D. values in the sera of both animals remained high throughout the study period without waning off. The O.D. values also suggested that there was no difference between the i.v.-inoculated and the vaginally-inoculated baboons in terms of time of onset of antibody production and their titres.

Table 6 shows that SHIV replication was first detected at week 1 and week 2 post inoculation in the i.v.-inoculated and the vaginally- inoculated animals, respectively. SHIV replication was demonstrated consistently up to week 6 post inoculation. The table also shows that the observation of CPE corresponded well with detection of the core antigen, suggesting that CPE observation in cultures is an accurate indicator of SHIV replication. The table shows that anti-SHIV antibodies were first detected at week 3 post inoculation in both animals irrespective of the route of inoculation. The animals remained persistently antibody positive throughout the study period (20 weeks post inoculation).

3.6 Anti-SHIV Antibodies Confirmation by Western blot

Pre-inoculation sera did not show any cross-reactive bands as was expected. However, plasma obtained at week 8 (P182) and week 16 (P183) cross-reacted with the following HIV-2 antigen proteins as shown in Table 7 below (see also Fig. 6).

Table 7: Summary of western blot analysis.

I.D.	Gp140	Gp105	P68	P56	Gp36	P34	P26	P16
P182	-	-	++	+	-	+	+++	+++
P183	-	-	++	++	-	++	+++	+

KEY:

- no band
- + weak band
- ++ Strong band
- +++ Very strong band

Table 7 gives an assessment score of the strength of bands in the western blot analysis. It shows that antibodies raised against p68, p56, p34, p26 and p16 were all detected with various strengths in both animals. The strengths of the bands may however not be compared directly between the vaginally- and the intravenously- inoculated baboons since the sera used for western blot analysis were taken at different time points (P182- at week 8, P183- at week 16 post inoculation). As was expected, anti- gp140, gp105 and gp36 antibodies were not detected. The portion of SHIV genome that corresponds with these segments is from HIV-1_{89.6} and therefore antibodies raised against these glycoproteins do not cross-react with HIV-2 glycoproteins (gp140, gp105 and gp36). The absence of this cross-reaction in the western blot analysis shown in this study therefore demonstrated that the antibodies that were detected, were anti-SHIV and not anti-SIV.

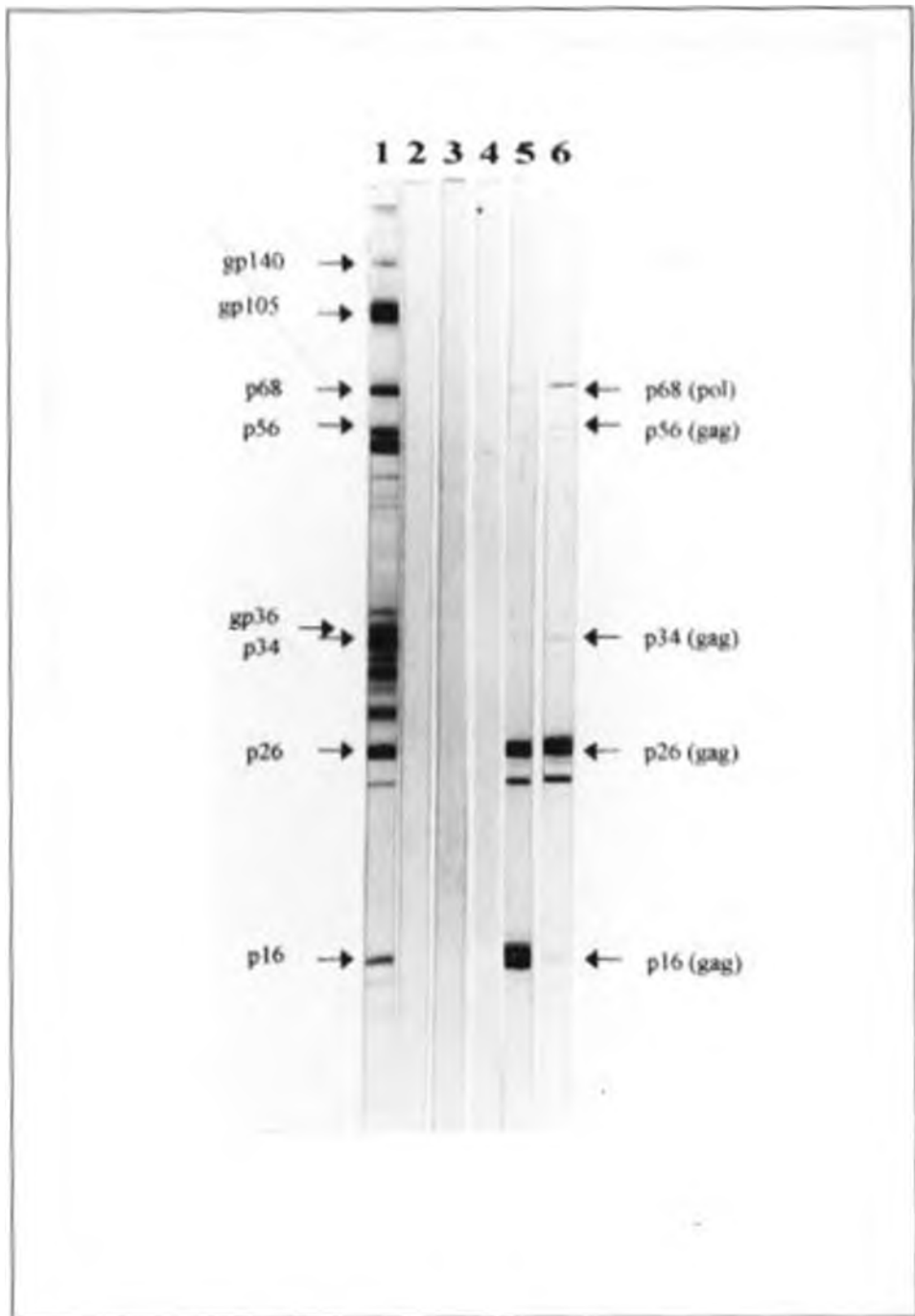


Figure 6: Western blot bands obtained in the test described above. Lane 1 is the positive control (kit), Lane 2: negative control (kit); Lanes 3 and 4: pre-inoculation sera of P182 and P183 respectively; Lanes 5 and 6: post inoculation sera of P182 (week 8 PI) and P183 (week 16 PI) respectively.

Fig. 6 shows that there were no anti-SHIV antibodies in pre-inoculation sera as was expected (lanes 3 and 4). Lanes 5 and 6, which represent 8 and 16 weeks post inoculation sera respectively, show that one anti-*pol* protein (p68) and four anti-*gag* proteins (p16, p26, p34 and p56) antibodies were present in both SHIV inoculated baboons. Lanes 5 and 6 also show that no anti-*env* protein (gp140, gp105 or gp36) antibody was detected in any of the SHIV-infected baboons. This was the expected observation because SHIV_{89.6p} has a HIV-1 *env* gene instead of the SIV *env* and also because antibodies raised against HIV-1 *env* proteins do not cross-react with HIV-2 *env* proteins and vice versa. These results therefore, confirm that the test was specific for anti-SHIV antibodies. Fig. 6 also shows that the SHIV infection in the baboons was persistent as the antibodies could be demonstrated at weeks 8 and 16 post inoculation.

APPENDIX 5**ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)****0.1M CAPS COATING BUFFER** Shelf-life: 2 months.

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

dissolved in 500ml double distilled water (dd H₂O).

Dissolve CAPS completely before adjusting to PH 11.0, using NaOH. then bring volume to 1 litre using dd H₂O.

WASH BUFFER

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

Dissolve in 900ml ddH₂O

Adjust PH to 7.4

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

Usually make 10 litres (or 1 litre of 10x).

DILUENT BUFFER

Same as wash buffers.

Add 0.1% BSA (Bovine Serum Albumin).

BLOCKING BUFFER

Same as wash buffer

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

CONJUGATE SOLUTION

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

SUBSTRATE SOLUTION

In 25ml ddH₂O, dissolve 1 big tablet (sigma buffer), let it dissolve, and then

dissolve 1 small tablet. Sigma-OPD).

STOPPING REAGENT

4N H₂SO₄

APPENDIX 6

PROTOCOL OF NEW LAV-BLOT II

The following Western blot protocol was performed according to the manufacturers instructions.

The kit was supplied with 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 5 minutes under slow shaking with 2 ml/well of the reconstituted washing solution.
- Sample or control serum was added to each well (20 μ l 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 anti-human IgG goat antibodies (provided in the kit as ready-to-use) for 1 hour at room temperature under slow shaking.
- The strips were washed again as previously 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 and bands were classified as *env* (gp 140, gp 105, & gp 36) *gag* (p 56, p26, p16) and *pol* (p68). A sample was determined positive if it displayed at least 2 *gag* bands together with a *pol* band.

NB: According to the manufacturer's instruction, a test sample is defined positive if it

developed at least one bands in all the three regions (ie *env*, *gag* & *pol*). However, the SHIV contain the 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 is therefore justifiably ignored.

A sample was defined negative if no band developed or if the bands appearing could not be classified, as either *env*, *gag* or *pol*.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 HIV-2 INFECTION OF BABOON

In view of the high TCID₅₀ inoculum used (10^{10} TCID₅₀/mL), the results obtained from these experimentations were unexpected. Animals in group I were expected to show persistent infection and virus recovery was expected to be successful. Virus was recovered on two occasions only from one of the animals at week 3 and 4 post inoculation. It is noteworthy that at week 3, virus was recovered from the inguinal lymph node cells (biopsy) and not the peripheral blood mononuclear cells from blood obtained at the same time point. Even at week 3 post inoculation, the PBMC from which the virus was recovered, was co-cultivated for one week with normal baboon PBMC (which were previously stimulated with con A for 3 days), before adding the Molt 4 cells. Surprisingly, no virus was recovered from P1803, which was inoculated i.v. P1803 was euthanised at week 5 post inoculation in an attempt to elucidate the dissemination of the virus in early stage of infection. As it turned out, virus was not recovered from the PBMC, lymphocytes of the liver, spleen, inguinal and auxiliary lymph nodes and bone marrow aspirate, plasma and cerebrospinal fluid. The same tissues were obtained from P2021 (urethral group) at week 17 PI and P2082 (vaginal group) at week 18 and co-cultured in the same way with Molt 4 cells and again no virus was recovered. These results indicate that although the virus was cytopathic with high TCID₅₀ in the cell-lines (SupTi and Molt), the virus was not pathogenic in baboon. The fact that no virus was recovered from the spleen and bone marrow of all the sacrificed animals, and especially P1803, which was inoculated I.V., shows that the infectivity of the inoculum was very low *in vivo* despite the high infectivity obtained *in vitro* inoculation. One of the explanation is that growing of the virus in

continuous cell-lines may have attenuated the virus to the extent that it was no longer infective *in vivo*. It is also possible that the virus was subjected to selective pressures *in vivo*, such that only the macrophage-tropic viruses did infect the animals and resulting into re-isolation difficulties using the T4-rich Molt 4 and SupT1 cell-lines.

Another postulation is that the East African baboons, may be possessing a system of clearing the virus. This may explain the low prevalence of SIV antibodies observed in the East African baboons (Otsyula *et al.*, 1996), both in captivity and in the wild. The same or a similar system may also suppress the virus to the extent that the virus load is so low that the virus is not readily recovered from the peripheral blood lymphocytes by use of the conventional methods. This view is supported by the results reported by Castro *et al.* (1991(a)). In their study, they noted that a baboon that was inoculated with HIV-2_{UC-2} started yielding the virus (on co-cultivation) at week 38 onwards, but in all the virus isolation attempts, they either stimulated the PBMC with PHA (3 µg/ml) for 3 days, followed 1 week later by co-cultivation with PHA-stimulated human PBMC from seronegative donors or the untreated PBMCs were co-cultivated with PHA-stimulated human PBMC. Both methods worked well for baboon infected with HIV-2_{UC-2}. This second method correlates with the findings reported in this study in which, the virus was recovered from P2078 when its PBMC were co-cultivated with Con A-stimulated baboon normal PBMC for one week, followed by addition of Molts 4 cells. These data suggest that if all virus isolations were attempted using mitogen-stimulated cells, the efficiency of virus recovery especially from P1803 and P2078, would have improved somehow.

Further work by Blackburn *et al.* (1997) in the HIV-2-infected baboons attributed the suppression of virus replication to CD8+ cells. In their study, they found that CD8+ cells of HIV-2-infected baboons developed substantial anti-HIV-2 activity following HIV-2 infection, which could have accounted for intermittent or transient virus isolation from the

PBMC of some experimentally infected baboons. Also, they found that CD8⁺ cells from uninfected baboons showed antiviral activity with acutely infected autologous CD4⁺ cells, suggesting that some HIV-naïve baboons have an inherent capacity to inhibit HIV replication. In this regard, baboons may resemble chimpanzees since CD8⁺ cells from some non-infected chimpanzees also possess a constitutive immune mechanism for control of HIV replication (Castro *et al.*, 1991(b)). The anti-viral activity of CD8⁺ cells from HIV-naïve baboons could be due to a cross-reactive CD8⁺ cell immune response which has been induced by either an exogenous or endogenous virus other than HIV (van der Kuyl *et al.*, 1996).

The absence of detectable antibodies in sera/plasma from all animals (except very weak responses in P2078 at week 6 and week 15 PI) is rather surprising. Two antibody ELISA systems were used, one based on purified recombinant proteins and peptides mimicking immuno-dominant epitopes of both HIV-1 and HIV-2 and the other system on a synthetic peptide derived from a conserved region of SIV_{mac251} as the solid phase antigen. Two of the positive control samples in both systems were plasma from SIV-infected De Brazza monkey and an SIV-infected African green monkey. Both these control samples reacted strongly positive, indicating that the ELISA systems were capable of picking cross-reactive antibodies to HIV-2. From these observations, therefore, it can be inferred that the anti-HIV-2 antibodies were of very low titres and below the detectable levels (except in P2078) of the two ELISA systems employed. This may be explained by the fact that the inoculum did not result into productive infection, except in P2078, in which transient infection occurred. This means that with the absence of successful virus recovery and non-detection of anti-HIV-2 antibodies in the urethrally- and vaginally-exposed groups, and failure of these animals to develop the clinical symptoms of Simian AIDS, leave doubts as to whether the HIV-2_{UC2} infection of baboon is a suitable animal model for HIV. To

remove these doubts and to achieve the goal of developing the baboon as a HIV heterosexual animal model, I recommend further studies in the following direction:-

- (a) Production of a pathogenic strain of HIV-2 that would readily replicate in baboon cells both *in vitro* and *in vivo*. This may require to passage intravenously the lymph node-isolated HIV-2 of P2078 severally, until, a pathogenic strain is produced.
- (b) Inoculation with the baboon pathogenic HIV-2 strain to HIV/SIV-naïve adult baboons, via the genital mucosa in an animal grouping similar to the one studied in this experiment.
- (c) Use of the submucosal route of inoculation in order to shorten the time required to produce infection and to increase the efficiency of producing infection in the baboons. This technique involve the deposition of the inoculum directly below the epithelium by use of special needles. Lohman *et al* (1994) have reported successful mucosal immunization of female rhesus macaques with SIV, using this immunization technique. The accuracy of this technique in delivering inoculum to the vaginal submucosa has been assessed in control animals by histologic examination of the vagina following infusion of India ink by Miller (personal communication, cited by Lohman *et al*, 1994).

4.2 SHIV INFECTION OF BABOON

The results of this study are very encouraging. All the tests: virus isolation, CPE observation, antigen capture ELISA, antibody ELISA and western blot confirm that the baboons were successfully infected with SHIV_{296P}. The results obtained after I.V. inoculation of the baboon corresponded well with results obtained by Otsyula *et al* - (unpublished data) with virus recovery being positive within the first week post inoculation. However, unlike their results, in the study reported here, virus was recovered in the first and second week post inoculation. In the vaginally inoculated

baboon, there was a delay in virus recovery by one week as compared with the intravenous inoculation. This delay was to be expected since the virus in the inoculum is first picked by the Langerhans cells and macrophages present in the vaginal submucosa, which would then sequester it in the genital lymph nodes before being released into the blood stream.

The antibody responses were strong and persistent in both animals as demonstrated by high optical density values in the ELISA test (Table 4(b)) and moderate to strong bands on western blot (Fig. 6). The antibodies also appear as early as 3 weeks post inoculation and remain strong as late as 20 weeks post inoculation (P183). The fact that there was no significance difference in the strength of the antibody response (see Table 7.) between the intravenously- and vaginally- inoculated animals demonstrates that both routes of inoculation are equally effective in inducing infection.

The results reported in this study illustrate the usefulness of perivaginal technique of inoculation in terms of effectiveness and shortening of the period required to induce infection vaginally. In human, and presumably in non-human primates, in the absence of co-factors, sexual transmission of HIV (SIV in non-human primates) is not efficient (May *et al*, 1989), requiring an unpredictable number of multiple exposures of mucosal surfaces to cell-free and cell-associated virus, followed by viral dissemination to local mucosal lymphoid tissue before finally achieving systemic infection (Alexander, 1990). This also suggests that the best vaccine strategy to prevent transmission of AIDS would be to induce both genital and system protective immunity which is long lasting.

During the whole period of this study, the SHIV-infected animals did not develop any signs of simian AIDS. The explanation could be that these baboons were not infected long enough to determine whether SHIV_{89.6P} would induce an immunodeficient state. However, it is interesting that their inguinal lymph nodes enlarged considerably between

second and fourth week post inoculation probably due to initial viremia. It would be of interest to let these baboons stay for at least three years to investigate whether they would develop any clinical disease associated with immunodeficiency condition. It would be interesting to regularly monitor their viral loads, total blood count, absolute CD4 count and CD4 / CD8 ratios. However, this kind of study go beyond the mandate and time frame of this thesis.

This study shows that the baboon can be developed as an animal model for studying heterosexual transmission of HIV, assessing the role of cofactors in heterosexual transmission of HIV and for testing the effectiveness of spermicides, candidate therapeutic agents and vaccines in preventing heterosexual transmission of HIV. To achieve this goal, I would recommend further studies to:-

- (a) Monitor the clinical condition of both intravenously- and vaginally-SHIV-infected animals for at least three years.
- (b) Elucidate the viral dissemination in the intravenously- and vaginally-infected animals during early and late stages of SHIV infection.
- (c) Develop in the male baboons an inoculation technique comparable to perivaginal inoculation in terms of efficiency and shortening of the period required to achieve infection.

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APPENDIX 1
ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
CAPS	Cyclohexylamino-1-propane sulphonic acid
CD	Cluster differentiation antigen
CPE	Cytopathic effect
EDTA	Ethylenediamine tetraacetate
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
FTV	Feline immunodeficiency virus
gp	Glycoprotein
HIV-1	Human immunodeficiency type 1
HIV-2	Human immunodeficiency type 2
IgG	Immunoglobulin G
LSM	Lymphocyte separating media
LTRs	Long terminal repeats
OPD	O-phenylenediamine
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
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
SIVnucl	Simian immunodeficiency virus from mandrills
SIV_{Deb}	Simian immunodeficiency virus from De Brazza's monkey
ssRNA	Single stranded RNA

tat	Transactivator
TCID₅₀	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

APPENDIX 2

ILLUSTRATION OF REED AND MUENCH METHOD

The following example illustrates the Reed - Muench Method of calculating the TCID₅₀.

The table show the outcome of CPE observations of a serial dilution of a virus stock.

Virus Dilution	Tissue Culture CPE Data			Accumulated Values			
	CPE	No CPE	CPE ratio	CPE	No CPE	CPE	
						Ratio	Percent
10 ⁻¹	8	0	8/8	-	-	-	-
10 ⁻²	8	0	8/8	17	0	17/17	100
10 ⁻³	7	1	7/8	9	1	9/10	90
10 ⁻⁴	2	6	2/8	2	7	2/9	22
10 ⁻⁵	0	8	0/8	0	15	0/15	0
10 ⁻⁶	1	7	1/8	-	-	-	-

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The positive (CPE) and negative (no CPE) outcomes were accumulated in the direction of their respective arrows. Note that the deviant outcomes (false positive) in the 10⁻⁶ dilution was neglected, as was the 10⁻⁴ dilution in the abridged accumulated data. The proportionate distance from the dilution just above the 50% endpoint to the endpoint itself is calculated as follows:

Proportionate distance (PD) = $\frac{\%CPE_a - 50\%}{\%CPE_a - \%CPE_b}$ X (log dilution factor)

$\%CPE_a - \%CPE_b$

= $\frac{90 - 50}{90 - 22}$ X (log 10)

= 0.6

Where, $\%CPE_a = \%CPE$ at dilution above 50%

$\%CPE_b = \%CPE$ at dilution below 50%

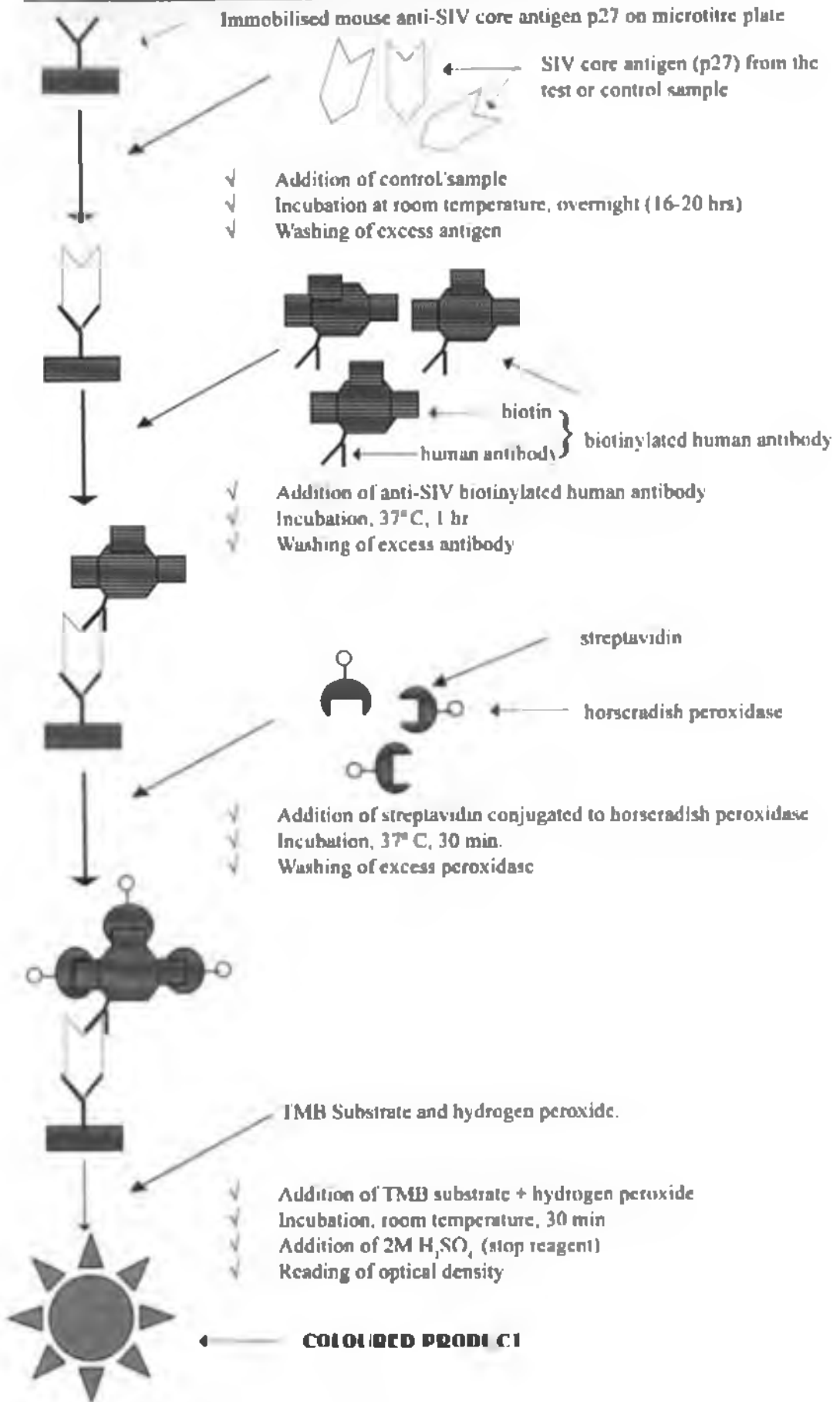
The $TCID_{50}$ titre is calculated using the following formula:

$$\begin{aligned} \log TCID_{50} \text{ titre} &= \log \text{dilution above } 50\% - PD \\ &= (-5) - 0.6 \\ &= -5.6 \end{aligned}$$

$$\text{Therefore, } TCID_{50} \text{ titre} = 10^{5.6}$$

APPENDIX 3

SKETCHMATIC ILLUSTRATION OF ANTIGEN CAPTURE ELISA



APPENDIX 4

SIV p27 ANTIGEN CAPTURE ELISA

- The microtitre wells were removed from the cold-room and allow 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 in which the control sample was tissue culture medium obtained from SIV/HIV non-infected cultures. The remaining two wells were positive control, which was tissue culture medium obtained from HIV-2(UC-2) or SHIV 89.6 infected cultures.
- 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 with 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 with 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 colour 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 (kit manufacturers).