ANALYSIS OF ENDOGENOUS RETROVIRUS-LIKE PARTICLE EXPRESSION IN NORMAL MALE OLIVE BABOON *(PAPIO ANUBIS)* REPRODUCTIVE TISSUES.

SICHANGI, W. MOSES

(B.Sc; Kenyatta University)

College of Agriculture and Veterinary Sciences, Department of Animal Physiology,

(Reproductive Biology Unit)

University of Nairobi.

This thesis is submitted in partial fulfillment of the requirements for the award of Master of Science Degree (Reproductive Biology) of the University of Nairobi.

(YEAR 2001)

DECLARATION

This is my original work and has not been submitted in any other institution for the award of a degree. I therefore submit this thesis for examination;

Sign _____

Date 29/08/02.

Sichangi, W. Moses

With the approval of my supervisors:

Sign _____

Date 30/8/02

Dr. Jason M. Mwenda,

Senior Research Scientist, Institute of Primate Research,

P.O. Box 24481,

Karen-Nairobi.

Sign _____

Date 30th Aug'02

Prof. E.O. Wango,

Coordinator, Reproductive Biology Unit,

Dept. of Animal Physiology,

University of Nairobi.

Sign _____

Date 30th Aug. 2002.

Prof. D. Oduor-Okello,

Professor of Anatomy, Dept. of Veterinary Anatomy,

University of Nairobi.

ACKNOWLEDGEMENT

I highly appreciate the financial support from WHO-Special Programme for Research, Development and Research Training in Human Reproduction. I also thank the Institute of Primate Research (IPR) for preparing me for this course during my attachment and for offering me access to facilities within the institute to carry out this research work. In addition, I enjoyed access to institute computer facilities while preparing this report. The close supervision by Dr. Jason Mwenda, my principal supervisor had immense contribution to the final shape of this work. Prof. Emmanuel Wango and Prof. Dominic Oduor-Okello are remembered for their wise advice and encouragement when undertaking this work. I am also grateful to IPR chief laboratory technologist Mr. Alloys Makokha, technicians Gerson Deya and Eric Omollo of the reproductive biology program for their technical support. Not forgetting the contributions by Mr. Richard Korir and Mr. Ibrahim Kwatsima of histopathology unit for assistance when preparing tissue sections.

The contribution by staff of animal resources in general is highly acknowledged, especially for performing the technical procedures required on the animals when obtaining samples. I am also indebted to Mr. Daudi Langat for his tireless advice regarding the computer software applied in organizing this thesis and for allowing access to his computer when preparing this report. Thank you, all those who contributed in one way or the other to the completion of this task.

DEDICATION

This work is dedicated to my parents Nelson and Rose for their foresight in introducing me to education. Also to all those who spend part of their time researching and searching knowledge regarding issues on reproductive health and contraceptive research and development.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND SYMBOLS

BrdUTP	5 Bromo-5-deoxyuridine Triphosphate
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra acetic Acid
EGTA	Ethylene Guanine Tetra acetic Acid
GTP	GuanidineTriphosphate
HEPES	N-2-Hydroxyethyl piperazine-N-2-Ethanesulfonic Acid
HIV-1 rRT	Recombinant reverse transcriptase of human immunodeficiency virus1
HLA	Human leukocyte antigen
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MmuLV-rRT	Recombinant reverse transcriptase of Moloney murine leukemia virus
Mn	Manganese
Pan	*Papio cynocephalus anubis*
RNA	Ribonucleic acid
RTase	Reverse Transcriptase
TBS	Tris Buffered Saline
TBS/T	Tris Buffered Saline with Tween-20
WHO	World Health Organization
µl	Microlitre
µM	Micromolar
ml	Milliliter
pg	Pico gram
mM	Millimolar

ABSTRACT

Endogenous retrovirus-like sequences (ERVs) represent a substantial component of most vertebrate genomes. Majority share morphological features with type-C retroviruses. To date, the significance of ERV-related sequences in primate reproductive processes is still unknown. In this study, testicular, epididymal and vasa deferentia tissues from sexually immature (n=2) and mature (n=4) male olive baboons were investigated for the expression of endogenous retrovirus-like particles. Immunohistochemical staining was done using antibodies raised against human immunodeficiency virus (HIV)-1/2, simian immunodeficiency virus (SIV) and ERVs. In addition sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot was done on homogenized sperm pellet and seminal plasma. Reverse transcriptase (RTase) activity in epididymal and ejaculated spermatozoa, and seminal fluid was evaluated. This study demonstrated ERV3 *env*-like antigens on early phases of spermatogenic cells in mature baboon testes (n=4) and on epididymal spermatozoa (n=4). Similarly, HIV-1 p24/25 *gag* and HIV-2 gp120-like antigens were expressed in mature and juvenile baboon testes. In addition, reverse transcriptase activity was detected in ejaculated spermatozoa, seminal fluid and epididymal spermatozoa. Proteins of approximately 49, 58 and 80 kDa were detected in seminal fluid, 58 and 32 kDa in sperm pellet from ejaculum and epididymis respectively. Antibodies reactive with the testes and epididymis did not cross-react with any of the proteins from semen samples including epididymal spermatozoa on immunoblot. These results indicate that retroviral-like antigens are expressed in normal male baboon testes and spermatozoa.

CHAPTER ONE

1. INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Endogenous retroviruses (ERVs) are viral elements transmitted vertically in the germ line and have been shown to comprise a significant proportion of the genome in most vertebrate species (Kalter *et al,* 1975; Coffin, 1984). Most of these viral sequences are defective due to multiple termination codons, preventing translation to functional proteins (Larsson *et al,* 1989; Leib-Mosch *et al,* 1990; Casau *et al,* 1999). However, there are some viral sequences that produce particles which may be infectious or in most cases non-infectious (Doolittle *et al,* 1990; Casau *et al,* 1999). Approximately 10% of the human genome have been shown to consist of retroviral sequences (Knossl *et al,* 1999; Casau *et al,* 1999) but their origin is still unknown. These sequences have genomic and structural analogies to exogenous retroviruses such as the human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), and are expressed in reproductive tissues, particularly the placenta (Larsson *et al,* 1989; Wilkinson *et al,* 1994; Langat *et al,* 1998; 1999) and testes (Larsson *et al,* 1994; Mi *et al,* 2000). Recent evidence suggests that ERVs could be involved in normal cellular processes like differentiation as well as in tumor formation and immunomodulation (Kato *et al,* 1987). The potential roles of ERVs in reproductive physiology (Lin *et al,* 1999; Lin *et al,* 2000; Mi *et al,* 2000), development and germ cell tumors (Casau *et al,* 1999) have been the area of recent research interest. Previous studies have demonstrated expression of intracisternal type A-like particles in mice epididymis (Kiessling *et al,* 1987) and ERV3 *env* transcripts in human testis

(Larsson *et al*, 1994). The role of these retroviral particles in normal male reproductive tissues requires further investigations. Previous studies have shown that the pattern of spermatogenesis in olive baboons (*Papio cynocephalus anubis*) is intermediate between that in rats and man (Chowdhury *et al*, 1977). Therefore, findings from studies done using the male baboon reproductive tissues may easily be extrapolated for human as compared to other species. Since ERVs have formed part of vertebrate genome for millions of years (Van der Kuyl *et al*, 1995a), it's likely that some of them have acquired some biological functions in the cells in which they exist. As part of the effort directed towards elucidating the control of spermatogenesis, analysis of endogenous retroviral elements expressed in primate testicular tissues including germ cells is necessary. Such gaps in knowledge regarding the possible roles of ERVs in male reproductive tissues necessitate further work using appropriate animal models.

1.2. LITERATURE REVIEW

1.2.1 Endogenous retroviral genome organization and distribution

The flow of genetic information usually involves transcription of DNA into RNA, which is later translated to specific proteins (De Robertis and De Robertis Jr, 1988). However, there is an exception to this flow of genetic information, in which RNA can sometimes be copied into DNA (Lewin, 1983). This occurs through the activity of reverse transcriptase (RTase), which is an RNA-dependent DNA polymerase that is able to synthesize DNA on an RNA template (De Robertis and De Robertis Jr, 1988; Huang *et al,* 1989; Johnson *et al,* 1990). This has been demonstrated to be a characteristic feature of all retroviruses (Varmus and Swanstrom, 1984; Huang *et al,* 1989). Endogenous retroviruses (ERVs) have been identified in the genomes of most vertebrate species, among them chickens (Dunwiddie *et al,* 1986), mice, rats (Gross *et al,* 1975), rhesus monkeys (Stromberg and Benveniste, 1983), chimpanzees, baboons (Cohen *et al,* 1981; Van der Kuyl *et al,* 1997) and humans (Martin *et al,* 1981; Lower *et al,* 1996). ERVs show a genomic organization basically analogous to exogenous retroviruses, stably integrated in the genome of their hosts (Benveniste and Todaro, 1974; Jaenisch, 1983; O'Connell *et al,* 1984; Johnson *et al,* 1990; Taruscio and Mantovani, 1998). Their genome (RNA) is replicated within the host cells by reverse flow of genetic information through a DNA intermediary (Urnovitz and Murphy, 1996). The resultant double-stranded DNA is integrated into the genome of the host cell to form a provirus (Varmus and Swanstrom, 1984; Huang *et al,* 1989). In this way, retroviruses can colonize the host germ line and be inherited as endogenous retroviruses (Stoye and Coffin, 2000).

Full-length ERVs possess three major genes; *gag, pol* and *env,* located between two long terminal repeats (LTRs) (Magin *et al,* 1999). These genes may encode the proteins essential for virus assembly, genome replication and integration, and processing of viral proteins (Larsson *et al,* 1989). In addition, the LTRs comprise important signals that participate in initiation, regulation, and termination of transcription (Johnson *et al,* 1990). Solitary LTRs also occur in some cases (Leib-Mosch *et al,* 1993; Knossl *et al,* 1999). These probably originate from excision of the retroviral genome (Ono, 1986; Leib-Mosch *et al,* 1993).

Most ERVs have morphological features related to type C retrovirus particles and have been demonstrated in primate reproductive tissues (Leib-Mosch *et al,* 1990; Larsson *et al,* 1994; Herbst *et al,* 1996; Roelofs *et al,* 1998; Lin *et al,* 1999; Mi *et al,* 2000). The origin of ERVs is not very clear, and numerous experiments have addressed the possibility of retroviruses having co-evolved with their host species. However, some scientists argue that horizontal transmission and eventual insertion of exogenous retroviruses into the germ-line cells of the hosts might have occurred in the ancestors of the present hosts (Jaenisch, 1983; Lower *et al,* 1996). Other investigators suggest that, viruses may have evolved from ancestral retroelements through transcription and recombination including addition of *env* genes (Taruscio and Mantovani, 1998). Some retroelements (intracisternal A-type particles) have been identified in mice (Dupressoir and Heidmann, 1996) and are thought probably to derive from an ancient retrovirus infection that later became compatible and beneficial to the host (Mc Donald, 1993).

Human endogenous retrovirus-R (ERV3) *env* and p30 *gag* expressions have been detected in human testicular carcinoma (Larsson *et al,* 1994). Other studies using several lines of transgenic mice have revealed intracisternal type A particle (IAP) expression, which were consistently restricted to undifferentiated premeiotic type A spermatogonia. ERV-like particles have also been demonstrated in mouse epididymis (Kiessling *et al,* 1987) and it is suggested that they may interact with memory lymphocytes to provide a genetic feedback loop i.e. a transfer of somatically selected genes to the germ line (Rothenfluh, 1995). In a sample of histologically normal human testis, ERV3 *env* showed a pattern comparable to that of IAPs, with expression restricted to the first phases of spermatogenesis but not in the Sertoli or Leydig cells (Larsson *et al,* 1994). However the role of these particles in human and mice testes is not clear.

In a related study on transgenic mice lines, IAP expression as determined by reverse transcriptase-polymerase chain reaction (RT-PCR), was essentially restricted to the male germ line (Dupressoir and Heidman, 1996; Casau *et al,* 1999). Electron microscopy on cauda epididymis in mice revealed aggregates of virus within the epididymal lumen (Kiessling *et al,* 1987), some clustered around sperm head, and others associated with sperm in contact with lymphocyte-like cells. Virus clusters, either associated with cells or free, have also been reported in murine vas deferens (Kiessling *et al,* 1989). Earlier studies in mice indicated a higher expression of ERVs proteins in reproductive tissues especially in the epididymis (Kiessling *et al,* 1989). Reverse transcriptase activity has also been demonstrated in mice (Kiessling, 1984). Despite their expression, it is not

known whether the ERVs in these tissues have any function in cellular differentiation or they are expressed as a consequence of differentiation.

1.2.2. Studies on expression of endogenous retroviruses in human tissues

Endogenous retroviral sequences (ERVs) are now well known genetic components of human DNA (Casau *et al,* 1999). Most of them seem to be defective due to multiple termination codons, deletions, or the lack of a 5' long terminal repeat (Larsson *et al,* 1989; Leib-Mosch *et al,* 1990). Human endogenous retroviruses are grouped into single and multiple copy number families and their classification remains an unresolved problem in virus research. A tentative systematic nomenclature is based on tRNA specificity of the primer binding site, using the one-letter code for the specific amino acid as a suffix to the acronym HERV (Larsson *et al,* 1989). Limitations of this approach arise when several distantly related families have a very similar primer binding site or when cloned proviruses are either devoid of a 5' end or have been only partially sequenced. Furthermore, frequent point mutations due to the lack of selective pressure on defective genomes are blurring the exact sequence of the primer- binding site. Endogenous retroviruses have also been classified according to their homologies to animal retroviruses (Wilkinson *et al*, 1994). Class I families have sequence similarities to mammalian type C retroviruses. Three families sharing substantial homologies not only in the well-conserved *pol* region but also in the *gag* and *env* genes have been grouped into a superfamily, the ER I family (Lower *et al*, 1996). Their closest infectious relatives are murine leukemia virus (MuLV) and baboon endogenous virus (BaEV). Class II

families exhibit homology to mammalian type B and D retrovirus strains. All class II elements identified so far have a lysine primer-binding site, reflecting their derivation from B- and D-type viruses.

Human endogenous retroviral-K (HERV-K) and ERV3 *env* have been shown to be the most biologically active and are specifically activated in steroid-dependent tissues (trophoblast, testes and also sebaceous glands (Lower *et al,* 1996; Taruscio and Mantovani, 1998). Lower *et al,* (1993) and Herbst *et al,* (1996; 1998) have shown that expression of high levels of HERV-K members seem to be restricted to germ cell tumors. They also found out that many other tissues including non-germ cell tumor types, and cell lines do not demonstrate detectable levels of HERV-K expression. However, a low level of expression has been detected by other workers in chronic myeloid leukemia (Brodsky *et al,* 1993a), leukocytes (Brodsky *et al,* 1993b), placenta (Simon *et al,* 1994), peripheral blood mononuclear cells, and brain tissues of healthy persons, and in multiple sclerosis patients (Rasmussen *et al,* 1995). The significance of these sporadic and peculiar expression patterns remains an enigma. Based on the present literature, among HERVs studied so far, HERV-K has been reported as the most biologically active family with regard to the coding of viral proteins and particles, and as earlier noted, it has been closely associated with germ cell tumors (Boller *et al,* 1983, 1993; Bronson *et al,* 1984; Sauter *et al,* 1995; 1996; Lower *et al,* 1996; Simpson *et al,* 1996; Berkhout *et al,* 1999; Casau *et al,* 1999). It is present in about 30 to 50 copies in the human genome (Ono, 1986) with an estimated 10,000 solitary long terminal repeats (Leib-Mosch *et al,* 1993).

In recent studies, sequence analysis of expressed HERV-K genomes has revealed non-defective *gag* genes, a pre-requisite for particle formation (Sauter *et al,* 1995; Herbst *et al,* 1996; Roelofs *et al,* 1998). Antiserum raised against recombinant *gag* proteins of HERV-K cross-reacted with human teratocarcinoma derived virus (HTDV) particles in immunoelectron microscopy linking them to the HERV-K family (Lower *et al,* 1993).

Antibodies against HERV-K10 *gag* protein have been detected in 70-80% of a group of patients with seminomas , the striking observation being the expression of *gag* protein in germcell tumor cells, and not in surrounding testicular tissue (Sauter *et al,* 1995). In a detailed study of patients with different disease conditions (neoplasm, autoimmune diseases, immunosuppression) immune responses in 0-5% of individuals within each disease group was shown; one out of 233 (0.4%) healthy controls had low titer antibodies (Sauter *et al,* 1995). The study showed preferential expression of HERV-K *gag* in active seminomas suggesting a possible involvement in the pathogenesis of this tumor. Further, expression of HERV-K *gag* and *env* has been reported in 100% of a series of germ cell tumor specimens (Herbst *et al,* 1996). HERV-K expression has also been reported in cytotrophoblast-like cells (Herbst *et al,* 1996), and also its *gag* sequences detected in primary germ cell testicular tumors (Varmus, 1988; Sauter *et al,* 1995). HERV-K association with germ cell tumors were confirmed by Roelofs and co-workers (1998), both in adolescents and adult testicular germ cell tumors. However, they also demonstrated presence of these sequences in most normal testicular parenchyma biopsies.

Despite some sporadic expression, there is enough evidence that the presence of HERV-K in testicular germ cell tumors has been recognized by many scientists.

In man, ERV3 expression has been reported on spermatogonia and primary spermatocytes but not detectable in seminomas. However, it has been demonstrated in syncytiotrophoblast-like cells associated with choriocarcinomas (Larsson *et al,* 1994) occurring in testicular tumors. The presence of ERVs in both the testis and placental tissues suggests that the viruses have a biological role associated with reproduction. Additional information on the pattern of expression of virus particles present in the testis, epididymis or associated with spermatozoa is necessary in order to understand their role in male human reproductive process.

1.2.3. Current state of knowledge on human retroviruses

It is estimated that about 10% of the human genome comprises of retroviral sequences. This ranges from short sequences to almost intact retrovirus particles (Lower *et al,* 1996; Urnovitz and Murphy, 1996; Lin *et al,* 2000; Stoye and Coffin, 2000), and that up to 1% of these sequences are of endogenous retroviruses (Baltimore, 1985; Wilkinson *et al,* 1994) similar to exogenous retroviruses both in sequence and structure. This has stimulated investigations concerning the possible pathologic effects that human endogenous retroviruses (HERVs) may have on several reproductive organ systems. Studies on HIV-1/2 and human T-cell leukemia virus type 1 (HTLV-1) have provided knowledge to test whether and under what circumstances, HERVs may be implicated in

the pathogenesis of some diseases. Some associations have been accorded little recognition even in cases where type C retrovirus particles are expressed, these include expression in human reproductive tissues (Jerabek *et al,* 1984; Larsson *et al,* 1989; Urnovitz and Murphy, 1996).

Most endogenous retroviruses appear to be less active than other retroelements; however they may cause genomic changes through rearrangement of genetic material, as well as acting as insertion mutagens (Morse *et al,* 1988). They may also act as sources with which exogenous retroviruses can exchange sequence information and in this manner, they may contribute substantially to expression of exogenous viral activity (Van der Kuyl *et al,* 1995b). A role in enhancing genomic plasticity has been suggested for the ERV-like IAPs in mouse testes (Dupressoir and Heidmann, 1996). Some HERVs have been shown to be transcriptionally active in both normal and neoplastic cells, and in such cases they may regulate adjacent cellular genes (Feuchter and Mager, 1992; Schutle *et al,* 1996). Both the prevalence and maintenance of these endogenous retroviral elements suggest that they may play a role in the biology of the host species (Casau *et al,* 1999). Despite a great deal of investigation, the role of ERVs in human development is still unknown (Lin *et al,* 2000). Therefore, the biological significance of ERVs needs further investigation using an appropriate model in order to gather more knowledge regarding their role in both normal and disease conditions.

1.2.4. Studies on expression of endogenous retroviruses in non-human primate tissues

Endogenous type C viruses have been reported in non-human primates (Sherr *et al,* 1974), and isolated from old world monkeys: MAC-1 from the stump-tailed macaque (*Macaca arctoides*), MMC-1 from the rhesus monkey (*Macaca mulatta*) (Todaro *et al,* 1978; Rabin *et al,* 1979). MAC-1 and MMC-1 are closely related, and have been said to be different isolates of the same virus. CPC-1 was isolated from a *Colobus polykomos* (Sherwin and Todaro, 1979). One family, Baboon endogenous virus (BaEV), is the best-characterised type C virus in non-human primates (Benveniste and Todaro, 1976). It was originally isolated from baboon tissue (*Papio cynocephalus cynocephalus*) by cocultivation with permissive cell lines (Benveniste *et al,* 1974; Van der Kuyl *et al,* 1995b). However, it is said to be chimeric, containing a type D *env* gene (Benveniste *et al,* 1974; Todaro *et al,* 1976). DNA hybridisation, Southern blotting, and PCR amplification have pointed that BaEV is present in many old world monkey species (Shih *et al,* 1991), but many of the integrated genomes are likely to be defective (Cohen *et al,* 1981). Amplification of a small fragment of the BaEV reverse transcriptase (RTase) gene by PCR indicated limited sequence variation both in and between species (Shih *et al,* 1991) pointing to a common ancestry. BaEV has not been detected in new world monkeys (Benveniste *et al,* 1974; Todaro *et al,* 1978) suggesting that the virus entered the germ line after the split between the old world-new world species split (approximated to be 30 to 40 million years ago). Hence, BaEV is present probably as an intact provirus in some old world monkey species. The viral LTR and *env* trees show that BaEV from

mandrills and mangabeys form a separate cluster, while the virus from baboons and African green monkeys (*Cercopithecus aethiops*) are more related to each other resulting in two strains of BaEV (Van der Kuyl *et al,* 1995a). Full-length HERV-K *env* genes and open reading frames for HERV-K *gag* have been reported in DNA of almost all old world primate species (Steinhuber *et al*, 1995; Van der Kuyl *et al,* 1995a). Open reading frames for *env* protein have only been demonstrated in chimpanzee and gorilla (Mayer *et al,* 1999).

There is no conclusive evidence however, showing that BaEV integration into the humans and apes genomes exists. A complete BaEV probe gave some low level of hybridization with human, gibbon, and orang-utan genomic DNA (Benveniste and Todaro, 1976), with more significant values for chimpanzee and gorilla DNA (Shih *et al,* 1991) demonstrating the presence of this viral sequences. However, other studies indicate that BaEV can be found in all species of African monkeys (Van der Kuyl *et al,* 1995a) and many species of Asian non-human primates (Benveniste and Todaro, 1976; Shih *et al,* 1991). This viral sequence distribution demonstrates existence of random cross-species transmissions among primates sharing habitats. BaEV has been shown to bear moderate homology with human ERV1 and ERV3 sequences. These are known defective single-copy sequences located on human chromosomes 18 and 7 respectively (Cohen *et al,* 1985). This homology is of great significance in understanding the possible roles of ERVs in human using non-human primates. In addition, analysis of BaEV proviral structures in the genomic DNA of the yellow baboon (*Papio cynocephalus*) showed lack

of large deletions in the clones studied (Van der Kuyl *et al.* 1995a; 1995b) signifying possible particle formation and biological activity. Analysis of the nucleotide sequence of the ERV3 LTRs revealed a region of close nucleotide homology with the BaEV LTR (O'Connell and Cohen, 1984). The LTRs contain all the sequences necessary for transcription of the viral genome. In addition to providing promoter functions for viral genes, the presence of the LTR sequences at both the 5' and 3' ends of the integrated provirus may lead to activation of host genes adjacent to the viral integration site. Solitary LTRs also occur in some cases (Leib-Mosch *et al.* 1993; Knossl *et al.* 1999). These probably originate from excision of the retroviral genome (Ono, 1986; Leib-Mosch *et al.* 1993) either spontaneously or due to effects of some external factors.

1.2.5. Hypotheses on the potential roles of endogenous retroviruses (ERVs)

The presence of ERVs in the vertebrate genome dates back for more than 30 million years (Benveniste and Todaro, 1976). Their presence may induce a variety of interactions with the host cell or exogenous retroviruses, and such interactions can have multiple consequences on the hosts. In effect, several roles have been suggested for ERVs, both in normal and disease conditions. These include the following:

i) Conferring protection against infections.

It has been shown that expression of endogenous virus genes protects the individual host from infection with a related exogenous retrovirus (Best *et al.* 1997) most likely interfering with shared receptors effects protection. Endogenous retroviruses that bear the

envelope protein homologous to that of infectious exogenous retroviruses may block receptors on target cells obscuring them from the infectious virus (Best *et al,* 1997; Rasmussen, 1997). This has been observed in mice where Fv4 gene products have been adapted and shown to provide resistance to retroviral infection (Ikeda and Sugimura, 1989; Van der Kuyl *et al,* 1995a). Such retroviral restriction has also been reported for the cellular Fv1 gene in mice. The gene encodes resistance *in vivo* to Friend virus-induced disease. The gene encodes *gag*-like protein (Best *et al,* 1996; Coffin, 1996; Goff, 1996) that binds directly to the viral capsid protein of the infecting virion, blocking subsequent integration of the exogenous virus into the host DNA. Similarly, ERV expression may play a beneficial role by making an individual's immune system to become sensitized to antigens that are shared between the ERV and a potential exogenous retroviral pathogen, thus providing the individual with protection against later infection (Stoye and Coffin, 2000). This is converse to embryonic ERV expression and the resulting immunological tolerance, which would be harmful if it allowed a pathogen sharing antigenic determinants with the ERV to escape the body's immune surveillance.

ii). Role of ERVs in the pathogenesis of diseases.

There is accumulating evidence that ERV expression can modulate the immune response (Hara *et al,* 1982; Krieg *et al,* 1989; Krieg and Steinberg, 1990; Abraham and Khan, 1990; Nelson, 1995). ERVs may also be involved in immune or autoimmune processes (Nakagawa and Harrison, 1996) through mechanisms of molecular mimicry, i.e. immune responses against ERV expression products that cross-react with normal cellular proteins

resulting in autoimmune diseases such as glomerulonephritis (Talal *et al*, 1992; Rasmussen *et al*, 1993; Urnovitz and Murphy, 1996) and rheumatoid arthritis (Takeuchi *et al*, 1995; Seidl *et al*, 1999). Also ERV gene products might act as super antigens eliciting destruction of immune cells (Indraccolo *et al*, 1995; Conrad *et al*, 1997) as demonstrated in insulin-dependent diabetes mellitus (Benoist and Malthis, 1997). Endogenous retroviruses may also cause neoplastic transformations as a result of uncorrected DNA errors, blocking of apoptosis, activation of oncogenes and inactivation of tumor suppressor genes (Gross, 1997).

iii) Mutagenic effects.

Integration of proviruses into the genome, either as new infections or after retrotransposition, is a potentially mutagenic event that can affect cellular gene expression (Onions, 1991). Once integrated in the genome, they may result in genomic changes through rearrangements caused by recombination events (Steinhuber *et al*, 1995). They may also promote genomic plasticity as well as contribute to allelic variations (Tassabehji *et al*, 1994; Zhu *et al*, 1992; Lower *et al*, 1996; Casau *et al*, 1999). As stable genetic components of host DNA, they may influence the expression of adjacent cellular genes (Larsson *et al*, 1993). Endogenous retroviruses may also produce somatic or germ line mutations (Morse *et al*, 1988), resulting in the loss of gene function, which may be important, for example tumor suppresser genes (Miki *et al*, 1992). The expression of ERVs in male gonads has been associated with germ cell tumors (Herbst *et al*, 1996, 1998; Roelofs *et al*, 1998), and this may affect adversely the reproductive

capacity of the affected host. Similarly, ERVs expressed in normal gonads may affect the progression of vital reproductive events (Lower *et al.* 1996; Taruscio and Mantovani, 1998), hence it's important to understand the basic course of spermatogenesis in mammals.

1.2.6.1. Spermatogenesis

The testis, as a male organ of reproduction has two main functions: spermatogenesis and steroidogenesis (Dym, 1983; Fawcett, 1986; Callard, 1991). Spermatogenesis has been defined as a complex process that comprises all the events leading to and involving proliferation, differentiation, and development of male germ cells (Johnson and Everitt, 1980; Focko, 1992; Wrobel and Pawar, 1992; Sharpe, 1994). Spermatozoa, which are the end product of this process are released first at puberty as a culmination of events that begin early in foetal life (Roosen-Runge, 1977; Setchel, 1982). These events are complex and involve the migration of primordial germ cells from the yolk sac to the gonadal ridge during foetal life where they occupy the centre of the sex cords. At puberty they are activated into re-entry to mitotic proliferation resulting in spermatogonia cells (Kretser, 1990). Some cells become committed to differentiate, enter meiotic divisions and eventually into spermiogenesis (Roosen-Runge, 1977; Setchel 1982; Focko, 1992). The release of spermatozoa marks the final stage of the process of spermatogenesis.

Briefly, mitotic divisions of spermatogonia take place within the basal compartment of the seminiferous tubules. The last daughter cell synthesizes DNA and divides resulting in

two primary spermatocytes (Johnson and Everitt, 1980). Following the division, homologous chromosomes pair-up (zygotene), shorten and condense. The pairing eventually results in formation of synaptenemal complexes and crossing over of autosomal chromosomes (pachytene), and hence exchanges of genetic material. The chromosomes later pull apart breaking the chiasmatic contacts (diplotene) to yield two secondary spermatocytes (Courot *et al,* 1970; Johnson and Everitt, 1980; Setchel, 1982). At this particular stage the members of each clone are still connected by cytoplasmic bridges (Oduor, 1972; Dym, 1983; Pawar and Wrobel, 1991; Wrobel and Pawar, 1992) (Figure 1) resulting in synchronous development that only terminate at the release of spermatozoa into the lumen of the seminiferous tubule (Roosen-Runge, 1977).

Each secondary spermatocyte carries a single set of chromosomes, and rapidly enter a second phase of meiotic division. The chromatids separate at the centromere resulting in haploid spermatids that eventually enter a packaging stage (spermiogenesis). This phase involves among other changes, nuclear condensation, cytoplasmic remodelling of the spermatid, generation of the tail, formation of midpiece containing the mitochondria, formation of the acrosome and the shedding of the residual body (Johnson and Everrit, 1980; Pawar and Wrobel, 1991).

The process of spermatogenesis involves close association between spermatogenic cells and Sertoli's cells (Griswold *et al,* 1988; Ritzen *et al,* 1989; Focko, 1992). The spermatogenic cells of the same clone are connected by cytoplasmic intercellular bridges

(Dym, 1983; Fawcett, 1986; Pawar and Wrobel, 1991; Wrobel and Pawar, 1992 [Figure 1]) and as the process advances members of each clone of cells undergoing spermatogenesis are moved towards the lumen of the tubule. The shapes of the membranes of Sertoli's cells also vary accordingly to accommodate the changing shapes and size of progressing spermatogenic cells. Sertoli's cells are thought to mediate the influence of hormones (follicle stimulating hormone and testosterone) in this process (Dym, 1983; Fawcett, 1986; Sharpe, 1994). At the completion of spermatid elongation, contacts between Sertoli cells and spermatids are severed and spermatozoa are released into the lumen of the tubules in a process called spermiation.

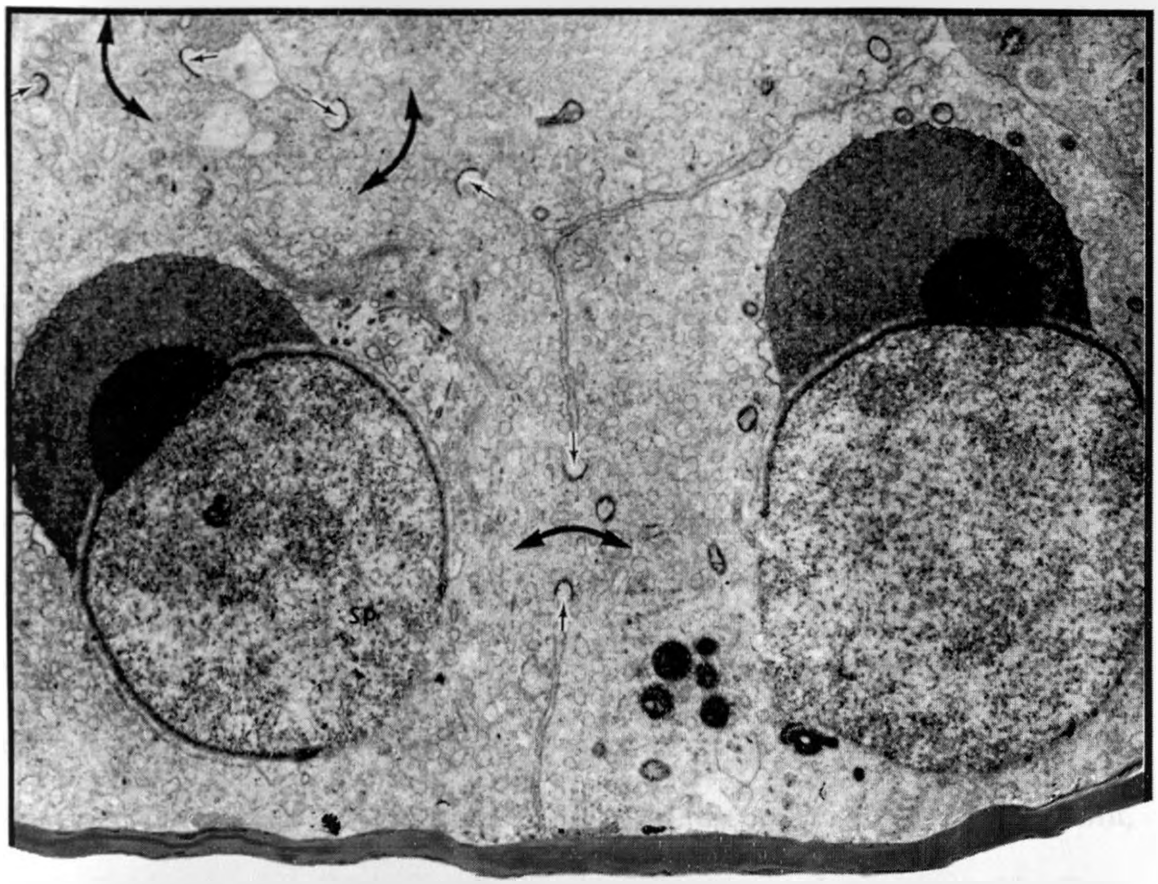


Figure 1. Cytoplasmic bridges between round spermatogenic cells within seminiferous epithelium.

Double edged arrows-Intercellular bridges

Sp -Spermatids

Adapted and modified from Bloom and Fawcett. A textbook of Histology, 11th edition; Saunders (publ.) 1986.

1.2.6.2. Spermatogenic cycle

The process of spermatogenesis proceeds in a cyclic manner (Wrobel and Pawar, 1992) referred to as the cycle of the seminiferous epithelium (spermatogenic cycle). It consists of a series of events beginning with the stem cell spermatogonium to the formation of the spermatozoa with spermiation demarcating two cycles (Courot *et al,* 1970; Wrobel and Pawar, 1992). During the process of spermatogenesis the various generations of spermatogenic cells form associations of defined composition (stages), and the period of time between two successive appearances of similar cellular associations in a given point of the testicular tubule is referred to as a cycle of the seminiferous epithelium (Pawar and Wrobel, 1991; Ojoo, 1995; Onyango *et al,* 2000). This in essence denotes a cyclic re-entry into spermatogenesis by stem cell spermatogonia. One spermatogenic cycle lasts about one quarter of the time required to complete spermatogenesis (Johnson and Everrit, 1980; Smithwick *et al,* 1996), and the duration is constant and species-specific (Dym, 1983), and lasts for about 11 days in the olive baboon (Barr, 1973; Chowdhurry and Steinberger, 1976; Sharpe, 1994), 14 days in chimpanzee (Smithwick *et al,* 1996) and 16 days in man (Schulze and Rehder, 1984). Advanced cells in the spermatogenic clones which were initiated earliest, are displaced progressively from the basal towards the lumen of the seminiferous epithelium by subsequent clones (Onyango *et al,* 2000). The adluminal compartment is separated from the basal compartment by the blood-testis barrier. The blood-testis barrier is important because it normally prevents proteins, including protein hormones and antibodies from coming into contact with the germ cells from spermatocytes onwards, and, in the reverse direction it prevents the proteins of the

haploid spermatogenic cells from causing an immune reaction. The barrier also regulates entry into the testis tubules of many small hydrophilic compounds, with important metabolic and toxicological consequences. However, its primary function is probably to create inside the tubules the appropriate conditions for meiosis (Austin and Short, 1990). The cycle is segmental in most mammals but helical in the baboon and man (Barr, 1973; Chowdhurry and Steinberger, 1976; Schulze and Rehder, 1984) and can be viewed when a transverse section through the tubule is made. This will show each cell type in separate and distinctive points, but successive cycles towards spermatozoa, terminating with spermiation (Sharpe, 1994).

1.2.7. Baboon as a model for studies in reproductive biology.

Baboons are large terrestrial primates that always use either trees or rocks and cliffs as safe sleeping sites to which they return at night (Napier and Napier, 1985). The olive baboon (*Papio cynocephalus anubis*) selected for this study is very adaptable and has been found to do well in captivity. It is also one of the two species available in Kenya and has been used in biomedical research for over two decades. In addition, information regarding the reproductive physiology and anatomy of both male and female is largely documented (Hendrickx, 1971; Coelho, 1985; Watts, 1985; Eley and Bambra, 1993). Anatomical similarity to human, coupled with physiologically similar response to stimuli make this animal a suitable model for studies on human reproduction. It can therefore be used in the studies involving development of germ cells and for understanding the biology of reproduction as it applies to man.

Infancy lasts between one to two years and the young infant is completely dependent upon it's mother (Watts, 1985). A juvenile is independent but not yet fully mature, while an adult is a fully mature individual. Males reach sexual maturity at about eight years of age and attain full body size one or two years later (Coelho, 1985; Napier and Napier, 1985).

Studies demonstrating expression of ERVs in baboon tissues have been done and a full-length BaEV proviral have been analysed in a baboon genomic library (Van der Kuyl *et al,* 1995a; 1995b). Large deletions commonly observed in endogenous virus genomes from other mammalian species was not evident in BaEV. This signified a possibility for particle formation and biological activity. Some level of homology has been demonstrated between BaEV probe, with other non-human primates and with human ERV3 (Benveniste and Todaro, 1976). This strengthens the choice of this species for use in such studies aiming at elucidating the role of ERVs in male primate reproductive tissues.

1.3. RATIONALE AND OBJECTIVES

1.3.1. Rationale

From the foregoing review, it can be inferred that retroviral-like elements such as ERVs that can integrate in host genome may affect normal cellular functions. Their integration may increase the rate of mutations and/or chromosomal aberrations. These may lead to the risk of conditions such as birth defects, early abortions, or other reproductive failures. A general problem has been the biological extrapolation of findings obtained when laboratory animals other than primates are used to study the role of HERVs in the human reproductive cycle. This is due to the fact that there may be significant differences between HERVs and laboratory animal ERVs in the structure, genomic distribution, and biological behaviour. As regards *in vitro* studies, they may not reflect the complex interactions that occur in the living system. In this respect elucidation of the biological activity associated with ERVs expression in non-human primates will be of great importance in the investigation of human diseases (Shih *et al,* 1991). For instance, it has been speculated that the activation of the germ line "endogenous" viral sequences represents an important mechanism of carcinogenesis in many species (Weinberg, 1980). More so, endogenous retroviruses (ERVs) are able to reintegrate into the host genome through reverse transcription, inserting new genomic material into that of the host (Emerman *et al,* 1985). The available evidence suggests several possible roles of ERVs in genomic mapping as they may be involved in chromosomal aberrations and hence mutations (Lower *et al,* 1996). Similarly, the control of spermatogenesis in mammals is not fully understood. Androgens have been shown to be essential for the process of

spermatogenesis, but the molecular mechanisms responsible for cell differentiation during this vital process are not yet known. The intimate association between spermatogenic cells and Sertoli cells, has also been demonstrated as an essential feature in providing the microenvironment for the success of this process. However, the nature of the environment, apart from mediating hormone action, is also not very clear. Such gaps necessitate further investigations in the control of spermatogenesis. Therefore, in this study, an attempt to localise and characterise ERVs and retroviral-like particles in male baboons' reproductive tissues (testicular, epididymal and vasa deferentia) and those associated with spermatozoa (both epididymal and in ejaculum) was made.

In this investigation, the baboon was selected because of its big size, close phylogenetic, anatomic and physiological relationship to man. Its reproductive system has been shown to be comparable to that of human, and it has been previously used in reproductive studies including male immunocontraceptive vaccine development. For this reason, the findings from this study can be extrapolated for HERVs expressed in human reproductive tissues and may facilitate further studies in the efforts to answer a number of basic questions concerning ERVs. These problems include: the nature of ERVs expressed in male reproductive tissues; the factors that appear to regulate their expression; the possible pathogenic consequences associated with their expression, and possibly the clinical diagnostic tests that can detect HERVs or their gene products. In addition, if an intimate association between ERVs and the spermatogenic cells is established to be critical for spermatogenesis to occur, then the particular ERV antigen expressed will form an

important candidate for male immunocontraceptive vaccine develpment. More so, the association of ERVs with spermatozoa may help to understand further the role of semen in the transmission of infectious retroviruses that cause human diseases. In this respect, it is essential to define the pattern of ERVs expression in healthy individuals in order to correlate the possible association between ERV expression and critical reproductive processes, and human diseases of male reproductive organs such as testicular germ cell tumors.

1.3.2.The aim of the study

To determine the expression of ERVs and retrovirus-like particles in the baboon testes, epididymides and vasa deferentia, and relate the findings to a possible role in spermatogenesis and sperm maturation.

1.3.2.1.Specific objectives

1. To localize the endogenous retroviral antigens in testicular, epididymides and vasa deferentia tissues using immunohistochemical techniques.
2. To characterise the endogenous retroviral proteins associated with spermatozoa from the epididymis and ejaculum using sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), western blot and by assaying for reverse transcriptase activity.
3. To determine the relationship between viral particle expression in the normal testes, epididymides and vasa deferentia with sexual maturity of the animals.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Animals used in this study

Male olive baboons (*Papio cynocephalus anubis*) used in this study were obtained from the wild and housed at the Institute of Primate Research (IPR), and all the manipulations done following IPR safety operating procedures. All animals were kept in quarantine for three months and screened for antibodies against SIV and STLV-1 as part of the institute routine screening procedure, and the virus-antibody free animals were used in this study. A total of six male olive baboons were selected for the study as follows: Sexually mature (n=4) and juvenile (n=2) healthy males were allocated to adult and juvenile categories respectively. The animals were housed in two group cages, one of juveniles and another of adults. The cages were constructed of iron sheet roofing and with wire mesh for the enclosure. Hence, they could receive natural lighting. The animals were fed with commercial monkey cubes (Unga Feeds Ltd, Kenya), with fruits and vegetable supplements, three times a week.

2.1.2. Age Estimation for Baboons

The baboons were categorised based on the most behaviorally and biologically meaningful physical changes according to Altmann *et al,* (1981) that assisted in approximating their ages. These characteristics included the following:

Reproductively mature animals were selected from newly acquired animals from the wild whose testicular descend was complete and had attained a length size of at least 40mm, and very visible scrotal bulge (rounding of the scrotum). In addition, extended canines, large size, weight of above 17kg and a total of 32 teeth were considered for adults. Scrotal colour change from pink to grey, loss of facial wrinkling and presence of canine ridge was used to qualify for juvenile category.

These physical characteristics enabled as to distinguish between reproductively immature and mature animal groups (Appendix V).

2.2. Methodology

2.2.1. Semen and tissue collection.

Each adult baboon (pan 1669, 2222, 2377 and 2473) was sedated by administering ketamine/xylazine mixture intramuscularly (i.m) at 10mg/kg - and 0.5mg/kg- body weight for ketamine and xylazine respectively (Suleman, 1993). Semen was obtained by rectal probe stimulation using the spaceage ejaculator (Standard electronics, Inc. Littleton 080160-0670). Electroejaculation was performed ones per week. The semen was collected in sterile pre-warmed 15ml centrifuge tubes and allowed to liquefy at room temperature by allowing to stand for 10-15 minutes.

The volume of the ejaculum was determined by estimation using a calibrated collecting tube or a pippet where volumes were too low. The semen was then centrifuged at 500g for 10 minutes to separate seminal plasma from the sperm pellet. The seminal fluid was

aliquoted then frozen at $-20^{o}C$, and spermatozoa were frozen at $-70^{0}C$ until the time of analysis. After obtaining sufficient semen required for the study (three ejaculations per animal), the animals were euthanised using 20% pentobarbitone (Euthatal®, Rhone Poulenc), and testicular and epididymal tissues surgically removed (Table 1, Plate 1). The tissues were stored and later analysed as described in various procedures below. Juvenile animals (pan 2242 and 2273) were also killed in a similar manner and the tissues listed in table 1 collected through surgical means as mentioned for mature animals above.

Table 1. Tissues obtained for analysis after euthanasia of animals.

Testes
Caput epididymides
Corpus epididymides
Cauda epididymides
Vasa deferentia

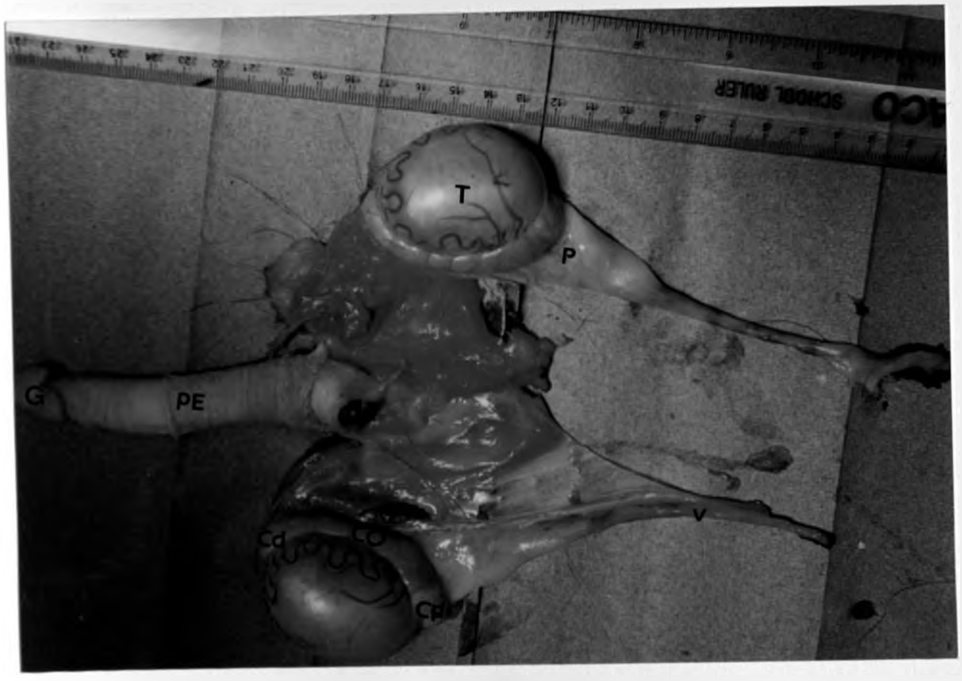
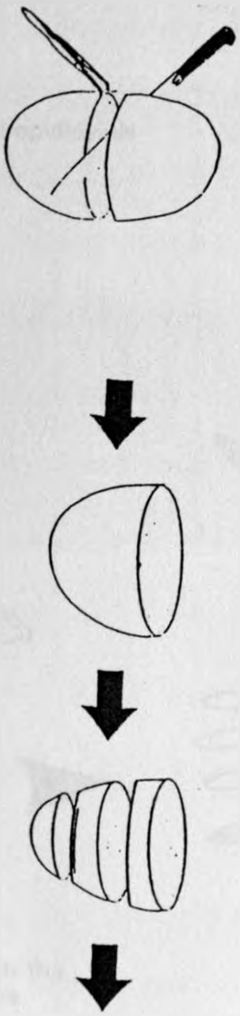


Plate 1. Male reproductive tissues from mature baboon (*Papio cynocephalus anubis*) analyzed in this study.

T-Testis, P-Pampiniform plexus, PE-Penis, G-Glans penis, CP-Caput epididymis, CO-Corpus epididymis, Cd-Cauda epididymis, V-Vas deferens

The tissues were then cut into smaller pieces (Figure 2.), washed in PBS (pH 7.2-7.4), some were fixed in formaldehyde (at room temperature) and Bouin's solution (at 4 ^{0}C), while others were fixed in Tissue Tek.® O.C.T compound (Miles Inc. Diagnostics division Elkhart, IN 46 515 USA), snap-frozen in liquid nitrogen and stored at –70 ^{0}C until the time of sectioning for histology. The epididymides spermatozoa were squeezed out using forceps into 15ml tubes containing PBS (Figure 3) and stored at –70 ^{0}C until the time of analysis.

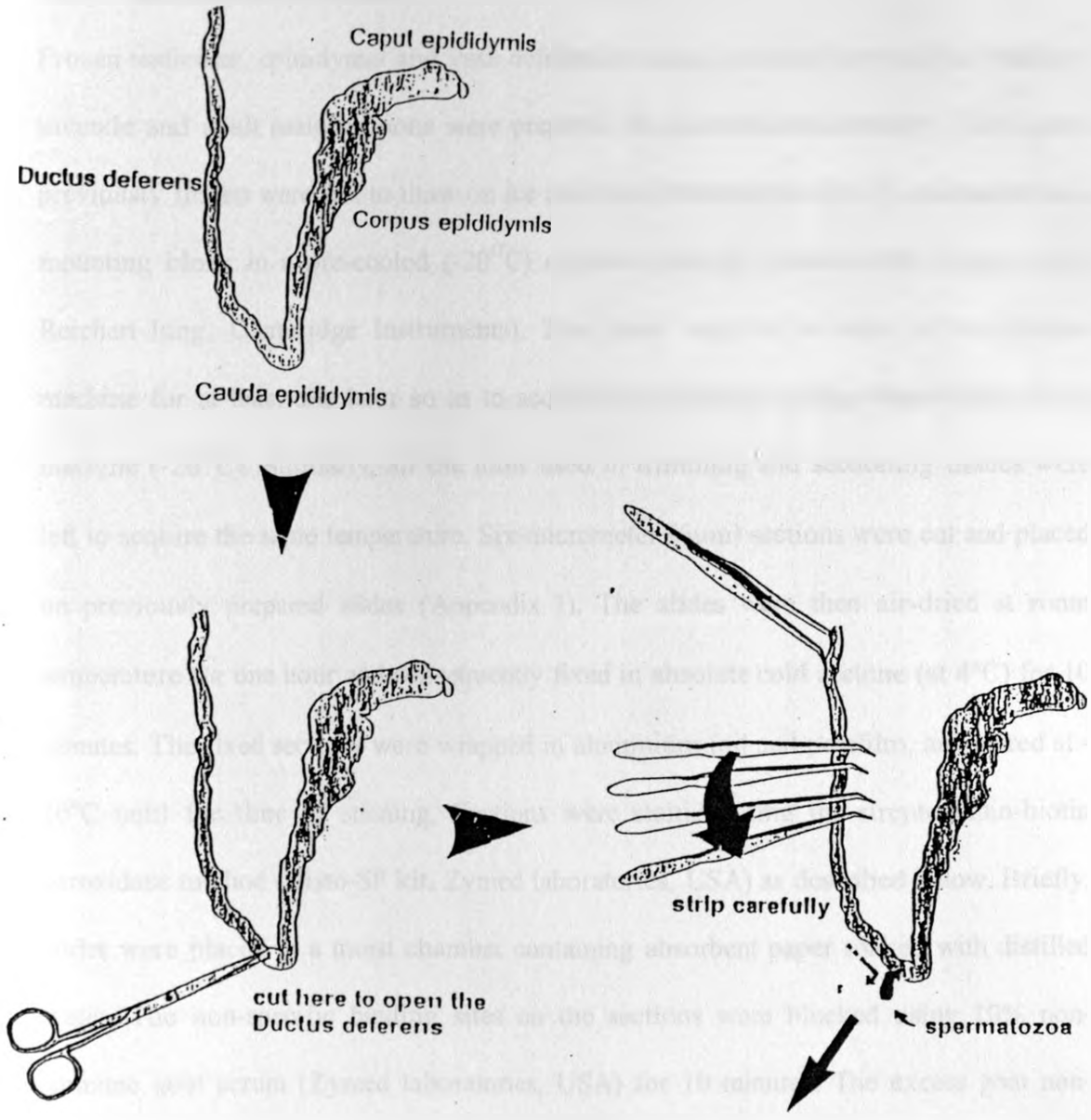
Figure 2. Procedure used when sectioning baboon testes



Some tissues were fixed in O.C.T compound, snap frozen in liquid nitrogen and frozen at –70 °C. Other tissues were fixed in Bouin's solution and formaldehyde.

Adapted with permission from; Alex Schneiders, German Primate Centre, Department of Reproductive Biology, Kellnerweg 4, D-37077 Gottingen, Germany.

Figure 3. Procedure used when collecting epididymal spermatozoa.



Epididymal spermatozoa were suspended in phosphate buffered saline and frozen at –70 °C.

Adapted with permission from; Alex Schneiders, German Primate Centre, Department of Reproductive Biology, Kellnerweg 4, D-37077 Gottingen, Germany.

2.2.2. Immunohistochemistry

Frozen testicular, epididymal and vasa deferentia tissues obtained from normal (healthy) juvenile and adult male baboons were prepared for immunohistochemistry. The tissues previously frozen were left to thaw on ice and then embedded in O.C.T. compound on a mounting block in a pre-cooled (-20^{O}C) cryostat machine (Model-2800 Frigocut (N), Reichert-Jung, Cambridge Instruments). The tissue was left to stay in the cryostat machine for at least one hour so as to acquire the optimum cutting temperature of the machine (-20^{O}C). Similarly, all the tools used in trimming and sectioning tissues were left to acquire the same temperature. Six-micrometer (6μm) sections were cut and placed on previously prepared slides (Appendix I). The slides were then air-dried at room temperature for one hour and subsequently fixed in absolute cold acetone (at 4°C) for 10 minutes. The fixed sections were wrapped in aluminium foil and parafilm, and stored at -20^{o}C until the time of staining. Sections were stained using the streptoavidin-biotin peroxidase method (Histo-SP kit, Zymed laboratories, USA) as described below. Briefly, slides were placed in a moist chamber containing absorbent paper soaked with distilled water. The non-specific binding sites on the sections were blocked using 10% non-immune goat serum (Zymed laboratories, USA) for 10 minutes. The excess goat non-immune serum was then blotted off using tissue paper. The mouse and rabbit primary antibodies diluted using 3% fat-free skimmed milk in PBS (Tables 2 and 3) were added to the sections at a volume of 100μl of the primary antibody per section and incubated for

60 minutes at room temperature in the moist chamber. Unbound antibody were washed off using PBS, pH 7.2-7.4 (3 times) for 2 minutes each.

Volumes of 100μl of appropriate biotinylated secondary antibodies (Zymed laboratories, USA) was added to the sections and incubated for 10 minutes, washed as above and followed by incubation for another 10 minutes with streptoavidin-peroxidase-conjugate (Zymed laboratories, USA). On the other hand, sections incubated with human primary antibodies were incubated with anti-human horse-radish-peroxidase (HRP)-conjugated secondary antibodies (Sigma immunochemicals, USA) for 20 minutes. The unbound antibodies were then washed with PBS, and all the sections were incubated for 10 minutes with 3% hydrogen peroxide as enzyme substrate and 3-amino-9-ethyl carbazole as chromogen. The sections were counterstained with haematoxylin, rinsed in tap water and mounted with a coverslip using glyceryl vinyl alcohol (GVA) then left to dry. The sections were then examined using a light microscope (Model: Olympus Bx 40 FA, Olympus Optical Co. Ltd, Japan).

A monoclonal antibody (W6/32) raised against human HLA class I antigen was used as a positive control. Phosphate buffered saline was used instead of primary antibody as a reagent control. Other controls included isotype controls for monoclonal antibodies (Dako Corp; Carpintena, VA; Table 4) and non-immune sera for polyclonal antibodies.

Table 2. The polyclonal antibodies used in immunohistochemical staining.

ANTIBODY	HOST	DILUTION	SOURCE
Anti-HIV-2ST gp120	Rabbit	1:200	NIH
Anti-ERV3 *env*	Rabbit	1:200	Gift from Prof. P. Venables (Kennedy Institute of Rheumatology, U K).
Anti-HIV-1 p24/25 SF2	Rabbit	1:200	NIH

Table 3. The monoclonal antibodies used in immunohistochemical staining.

ANTIBODY	ISOTYPE	HOST	DILUTION	SOURCE
Anti-SIV mac p27(55-2F12)	IgG_{2b}	Mouse	1:200	NIH
Anti-HIV-1 RT	IgG	Mouse	1:200	NIH
Anti-HIV-1 gp120 (ID6)	IgG	Mouse	1:200	NIH
Anti-HIV-2 CP	IgG	Mouse	1:100	NIH
Anti-HIV-1 gp41(md-1)	IgG	Human	1:200	NIH
Anti-HERV-K *env*	IgG	Mouse	1:10	Gift from W. Vogetseder (Univ. of Innsbruck, Austria)
Anti-HIV-1 gp120 (Chessie)	IgG_1	Mouse	1:20	NIH
Anti-HIV-1 gp41	IgG_3	Mouse	1:200	NIH
Anti-SIV mac 251 *gag* (kk59)	IgG_1	Mouse	1:200	NIH
Anti-SIV mac 251 *gag* (kk64)	IgG_1	Mouse	1:200	NIH
Anti-HIV-1 p24	IgG	Mouse	1:400	NIH
Anti-HIV-1 p24/25 *gag*	IgG_{2b}	Mouse	1:200	NIH

Table 4. The control antibodies used in immunohistochemical staining

ANTIBODY	HOST	DILUTION	SOURCE
W6/32	Mouse	1:10	Sera Lab, UK
IgG_{2a}	Mouse	1:1000	Dako
IgG_{2b}	Mouse	1:1000	Dako
IgG_1	Mouse	1:1000	Dako

Note: For isotype controls, the antibody specificity is directed towards *Aspergillus niger* glucose oxidase, an enzyme that is neither present nor inducible in mammalian tissues.

KEY

Gag : Group specific antigen
CP : Core protein
gp : Glycoprotein
HIV : Human-immunodeficiency virus
env : Envelope protein
mac : Macaque monkey *(Macaca mulatta*)
RT : Reverse transcriptase
SIV : Simian-immunodeficiency virus
ERV : Endogenous retrovirus
HERV : Human endogenous retrovirus
NIH : National Institute of Health, USA.
W6/32 : A monoclonal antibody against monomorphic class I MHC determinant.

2.2.3. Reverse Transcriptase assay

Reverse transcriptase (RTase) activity in the spermatozoa from the ejaculum and epididymides, and in seminal plasma was tested using two kits optimised for semi-quantitative screening of retroviruses (Cavidi HS-kit Mn^{2+} RT and Cavidi HS-kit Mg^{2+} RT [Cavidi Tech AB, Uppsala Science Park, SE-751 83 Uppsala Sweden]). This mode of determining RTase activity enabled a comparative analysis by varying the co-enzyme used in each kit. Human immunodeficiency virus (HIV) clones were used as positive controls while sample buffers were used as reagent blanks to determine background absorbance. The RTase standards from each kit were prepared as indicated in the tables below.

Table 5. Preparation of MmuLV-rRT standard (Cavidi HS-kit Mn^{2+} RT)

The stock solution was prepared by adding 2.1 ml of sample dilution buffer to MmuLV-rRT standard

Test tube	Dilution buffer (µl)	MmuLV-rRT standard (µl)	Concentration (µU/ml)
1	220	50	42 900
2	250	200 of tube 1	19 000
3	250	200 of tube 2	8 460
4	250	200 of tube 3	3 760
5	250	200 of tube 4	1 670
6	250	200 of tube 5	743
7	250	200 of tube 6	330
8	250	200 of tube 7	147
9	250	200 of tube 8	65.2
10	250	200 of tube 9	29.0
11	250	200 of tube 10	12.9
12	250	200 of tube 11	5.73
Measuring range: 0.05µU-250µU (1U=10-20ng)			

Table 6. Preparation of HIV-1 rRT standard (Cavidi HS-kit, Mg^{2+} RT)

The stock solution was prepared by adding 1.5ml of sample dilution buffer to HIV-1 rRT standard provided.

Tube	Dilution buffer (µl)	HIV-1 rRT standard (µl)	Concentration (pg/ml)
1	250	100	1 158
2	250	200 of tube 1	515
3	250	200 of tube 2	229
4	250	200 of tube 3	102
5	250	200 of tube 4	45.2
6	250	200 of tube 5	20.1
7	250	200 of tube 6	8.93
8	250	200 of tube 7	3.97
9	250	200 of tube 8	1.76
10	250	200 of tube 9	0.784
11	250	200 of tube 10	0.348
12	250	200 of tube 11	0.155

The kits used in this assay basically followed the same procedure, only with minimum differences as outlined in the summary below. The reaction mixtures were prepared by mixing the reaction buffer and reaction components [20mM HEPES pH 7.6, 4mM Mg Cl_2, 4mM spermine, 1% (v/v) Triton X-100, 0.4 mM EGTA, 1mg/ml BSA, 0.1 g/l dextran sulphate, 1mM GTP, 40 ng/ml oligo $(dT)_{22}$, and 32 µM BrdUTP] for the kit

optimized for HIV-1rRT (Cavidi HS kit, Mg^{2+} RT), and modified as follows [20mM HEPES pH 7.6, 6 mM Mn, 24mM spermine, 1% (v/v) Triton X-100, 0.4 mM EDTA, 1mg/ml BSA, 6mM β-mercaptoethanol, 60 mM KCl, 1mM GTP, 40 ng/ml oligo $(dT)_{22}$, and 32 μM BrdUTP] for the kit optimized for Moloney murine leukemia virus (MmuLV) RT- like activities (Cavidi HS-kit, Mn^{2+} RT).

From each prepared reaction mixture, 140 μm (for HIV-1 RT kit) and 200μm (for MmuLV-rRT kit) was added to each well of two poly (A) plates from each respective kit, and incubated at 33°C for 60 minutes. After this incubation, 10μl of each undiluted sample was added to corresponding wells of both poly (A) plates from each kit, but leaving the last 16 wells on each plate for background controls and standards. Out of the 16 wells left, 10μl of sample dilution buffer was added to wells E12-H12 and the twelve standards previously prepared as indicated in the tables above, added to wells A11-D12 of both plates from each respective kit.

The plates were firmly sealed with adhesive tapes and incubated at 33°C on an orbital shaker set on gentle agitation. One of the two plates from each kit was incubated for three hours and the other one for overnight. The RTase reaction of the plate incubated for 3 hours was stopped by washing with buffer (8.9 l dH_2O, 75ml Triton X-100, 25ml concentrated washing buffer, Cavidi Tech). The residual fluid in the wells was removed by tapping the plate upside down on absorbing paper and left in that position for 5-10 minutes. The plate was returned to its foil pouch and stored at –20°C. On day two the

RTase reaction of the second plate was stopped by washing as above. Following this, 100µl of RTase product tracer (BrdU binding antibody, conjugated to alkaline phosphatase in 12 ml 1% Triton X-100) was added to each well, sealed firmly with adhesive tape and incubated for 90 minutes at 33°C on an orbital shaker set on gentle agitation. The excess tracer was removed by washing the plates with wash fluid as above for four cycles each plate. To each well of both plates, 100µl (for both Cavidi HS-kit Mg^{2+} RT and Cavidi HS kit, Mn^{2+} RT kit) of alkaline phosphatase substrate (*para*-nitrophenyl phosphate) was added, covered with plastic lids and incubated at room temperature under dark cover with gentle agitation.

The optical density of both plates was read after 30 minutes of incubation at 405nm, repeated after 2 hours, and on the following day after 24 hours of incubation. Data from each reading time was processed separately by determining the RTase activity of each well with the aid of the regression line obtained from the HIV-1-rRT and MmuLV-rRT standards. The data from each reading time was processed differently with values for standard curve and samples being from the same reading time. The absorbance at 405 nm of each standard dilution was plotted against its concentration of standard present. Only RTase activity for wells giving an absorbance within the linear measuring range of the plate reader, and having signals greater than two times the mean of the background controls were determined, according to the manufacturer.

2.2.4. Sodium Dodecyl sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.2.4.1. Protein extraction from spermatozoa

Spermatozoa obtained by rectal probe electroejaculation and excised epididymides (Figure 3) were recovered by centrifugation at 500g for 10 minutes. The sperm pellet was resuspended in lysis buffer [(134mM NaCl, 5mM KCl, 7.5mM $MgCl_2$-$6H_2O$, 5mM glucose, 1% SDS, 5mM Hepes (N-2-hydroxy ethyl piperazine-N'-2-ethanesulphonic acid)] and sonicated using a laboratory mixer emulsifier (Soniprep 150 MSE) for 1-2 minutes at medium power. After sonication, the contents were centrifuged at 4000g for 10 minutes and the supernatant containing extract recovered. The protein concentration of the supernatant was determined according to the procedure of the Bio-Rad Protein assay kit (Bio-rad laboratories, appendix VI). The supernatant was divided into aliquots of 250-500µl and stored at -20^{O}C until the time of fractionation.

2.2.4.2. Electrophoresis

Proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Extracted protein was reduced by boiling in 2% SDS and 2% β-mercaptoethanol for 5 minutes. Bromophenol blue was added to colour and enable to track the protein front. Between 5µg and 6µg (25µl) of each sample and 5µl of a wide range molecular weights (MW) marker (Bio Rad) were loaded in appropriate wells on a discontinuous gel system containing a stacking gel and 10% resolving gel. The loaded proteins were electrophoresed at 150 mA while monitoring the front. The gel was gently removed from the slabs and stained with Coomassie brilliant

blue 250 (Sigma immunochemicals, appendix IV) to identify the protein bands. The MW of the samples were determined using the marker protein of known MW included in the run as a reference. A standard curve was generated by plotting the distance migrated by the protein against it's molecular weight. The molecular weights of the unknowns were extrapolated from this curve.

2.2.5. Western Blot

The proteins were separated using sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as above. Two sets of samples were fractionated; one set was stained with Coomassie brilliant blue 250 as described in section 2.2.4.2. while the second set had it's proteins transfered onto nitrocellulose membranes (Hybond® C) using a Biorad transblot apparatus at 150mA constant current applied for 2 hours. The membranes were stained with Ponceau S stain to determine if there was successful transfer. The stain was removed by washing with TBS. The nitrocellulose membranes were cut appropriately into strips. The strips were then incubated at room temperature for 1 hour in blocking buffer (TBS pH 7.6 [30mM Tris HCL, 137mM Sodium Chloride] containing 1% v/v Tween 20, and 5% w/v dried milk powder) to block non specific binding. The blocking buffer was removed and the strips washed twice (5 times each) in TBS/T (30 mM Tris-HCL, 137mM sodium chloride containing 1% v/v Tween 20) at room temperature. The strips were then incubated overnight (16-18hrs) with primary antibody. The unbound antibody was removed by rinsing once, followed by a three times (5 minutes each) wash of the membranes in TBS/T. This was followed by another

incubation with secondary antibody conjugated to horseradish peroxidase for one hour at room temperature. The membranes were then washed twice (20 minutes each) in TBS and colour developed with substrate buffer (TBS containing 4-chloro-1-naphthol, dissolved in ice-cold methanol containing 0.1% hydrogen peroxide). The membranes were allowed to develop in this solution for 30 minutes. Water was added to terminate the reaction, the membranes dried at room temperature, then photographed.

2.2.6. Production of polyclonal antibodies.

Four healthy female Balb/C mice were obtained from the department of animal resources, of the Institute of Primate Research (IPR). All the mice were approximately 10 weeks of age. They were housed in cages, in rodent houses, fed on cubes and monitored routinely by the technical staff of animal resources department. After acquiring the animals, they were separated into two groups, A and B, each consisting of two mice. All mice were bled to obtain whole blood from which pre-immune sera was extracted. Following this, mice of group A were immunised with whole protein extracted from ejaculate spermatozoa obtained from olive baboon by electroejaculation. On the other hand, group B mice were immunised with protein extracted from spermatozoa obtained from olive baboon epididymides.

Equal volumes of the immunogen and Freund's complete adjuvant (FCA) were mixed in a small beaker using a 5ml syringe. The mixing was done such that a drop from the mixture could not spread on the surface of clean water when released from the syringe.

From this mixture, 200µl(60µg) was inoculated intraperitoneally to each mouse. After one month, the animals were boosted with 200µl(60µg) of the same immunogen in Freund's adjuvant incomplete (FIA). Two weeks after the booster injection, the animals were bled to obtain blood for extraction of sera. The sera were screened for antibody presence by determining their reactivity on mature baboon testes and epididymides using immunohistochemical technique. The procedure followed in immunostaining was as described in section 2.2.2. A monoclonal antibody against human MHC class 1 molecules (W6/32) was used as positive control while preimmune mouse serum was used as negative control, and phosphate buffered saline were used as negative control.

CHAPTER THREE

3. RESULTS

3.1. Immunohistochemistry

3.1.1. Adult baboon tissue immunostaining

Frozen sections (6μm) previously adhered on slides were stained using the Labeled-(strept) Avidin-Biotin (LAB-SA) method (Histostain™ SP Kit, Zymed) for mouse and rabbit primary antibodies. Human primary antibodies were used with anti-human peroxidase-conjugated secondary antibody without Avidin-biotin bridging.

3.1.1.1. Immunoreactivity on testes

A monoclonal antibody (W6/32) that binds human monomorphic class I MHC molecules (HLA-A, B and C), reacted with the peritubular cells forming part of the basement membrane surrounding the seminiferous tubules (Plate 2, Table 7). This antibody was used as a positive control in all subsequent immunohistochemical staining.

A polyclonal antibody against human endogenous retrovirus-R (ERV3) envelope protein reacted with spermatogenic cells (spermatogonia/primary spermatocytes) within the peripheral compartment of seminiferous tubule epithelium from all mature animals (Plate 3, Table 7). However, this antiserum did not react with spermatozoa within the same tubules. This staining demonstrated the expression of ERV3 envelope protein by germ cells during early phases of spermatogenesis in baboons. Anti-HIV-1 p24/25 gag, a polyclonal antibody produced against an exogenous human retrovirus showed weak non-

specific staining on all mature testes tested in this study (Table 7). Similarly, anti-HIV-2 ST gp120, a polyclonal antibody against a human HIV-2 envelope protein, non-specifically bound to testicular cells, both within the seminiferous tubules and interstitium (Table 7). The rest of the antibodies tested did not show any reactivity.

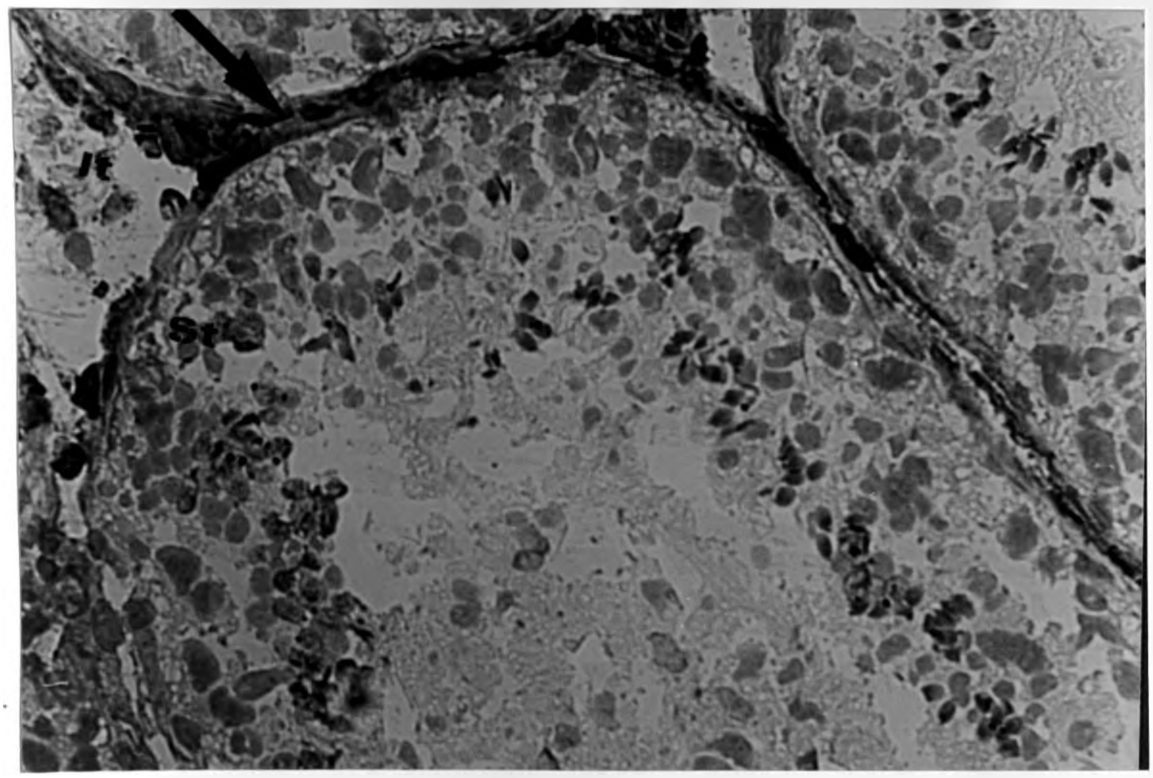


Plate 2: W6/32 (a monoclonal antibody against HLA-A, B and C) reactivity on mature baboon (pan 2222) testis. Strong staining on myoid peritubular cells was observed (shown by an arrow), Mag x200. **St-** seminiferous tubule epithelium,

It- Interstitial tissue

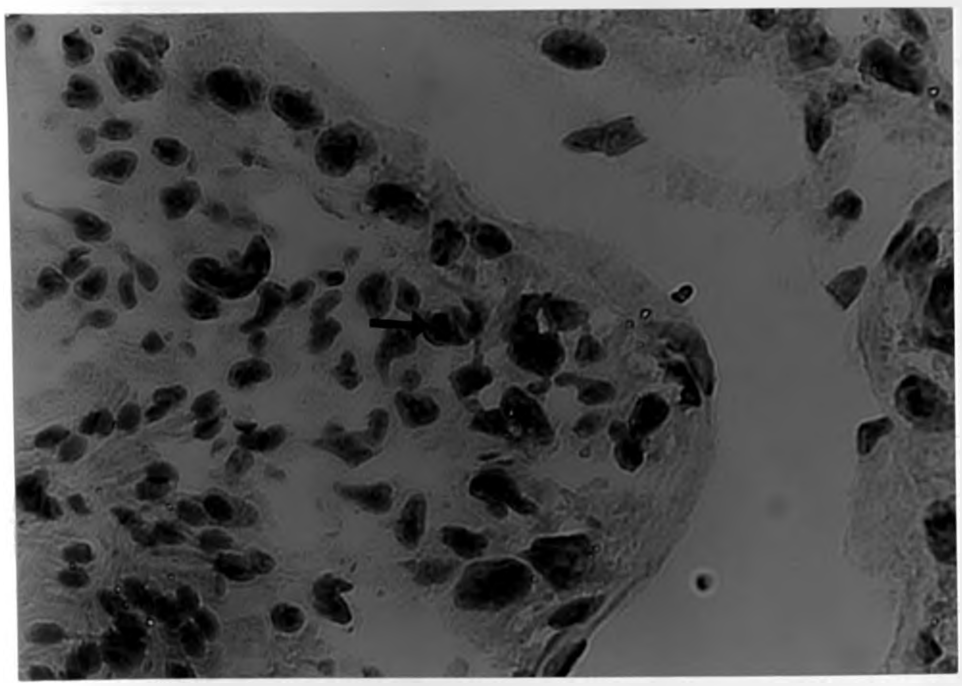


Plate 3: Anti-ERV3 *env* (a polyclonal antibody against a human endogenous retrovirus-R envelope) reactivity on mature baboon (pan 2377) testis. Early phases of spermatogenic cells (spermatogonia/primary spermatocytes) were stained (Arrow), Mag x400).

Table 7. Summary of immunostaining on Mature Baboon Testes

Antibody	Peritubular cells	Spermatogonia/ spermatocytes	Spermatozoa	Sertoli cells	Leydig cells
W6/32	++	-	-	-	-
Anti-HIV-1 gp 120 (ID6)	-	-	-	-	-
Anti-HIV-1 RT	-	-	-	-	-
Anti-HIV-2 CP	-	-	-	-	-
Anti-HIV-2 ST gp120	+/-	+/-	-	-	-
Anti-ERV3 *env*	-	+	-	-	-
Anti-HIV-1 p24/25 SF2	-	-	-	-	-
Anti-SIV mac p27 ($55\text{-}2F_{12}$)	-	-	-	-	-
Anti-HIV-1 gp41(md-1)	-	-	-	-	-
Anti-HIV-1p 24/25 *gag*	+/-	+/-	-	-	-
Anti-SIV mac 251 p17 *gag*	-	-	-	-	-
Anti-HERV-K *env*	-	-	-	-	-

Key

++ High intensity staining.

+ Low intensity staining.

-/+ Indeterminate generalised staining.

- No staining.

3.1.1.2. Immunoreactivity on caput epididymides

Similarly, W6/32 reacted with basement membrane, smooth muscle cell fibers surrounding the epididymal duct and those within the connective tissue stroma (Plate 4, Table 8). The antibody against ERV3 envelope protein (pAb) reacted weakly with spermatozoa within the epididymal lumen. The head of the spermatozoa, particularly the acrosome region strongly bound this antibody (Plate 5, Table 8). This reaction was consistent in all the four mature animals. The remaining antibodies tested were negative.

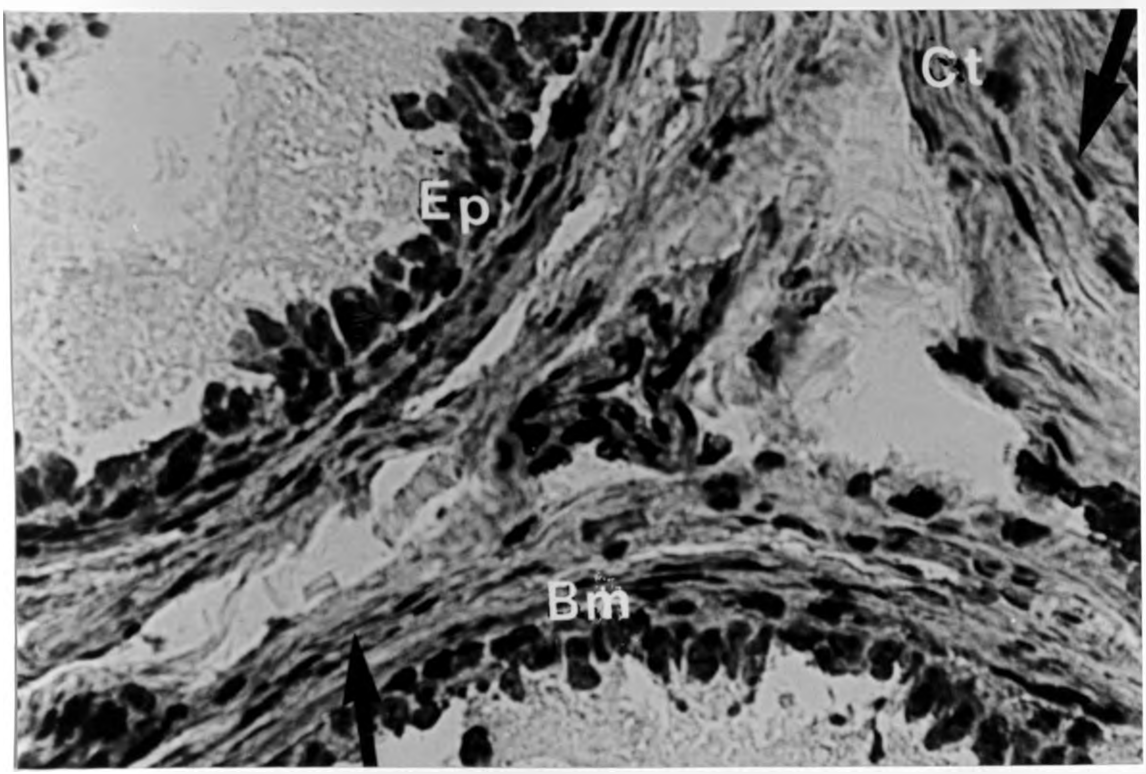


Plate 4: W6/32 (a monoclonal antibody against HLA-A, B and C) reactivity on connective tissue of mature baboon (pan 2377) caput epididymis (Arrow), mag x100.

Ep- epithelium, **Bm-** basement membrane, **Ct-** connective tissue

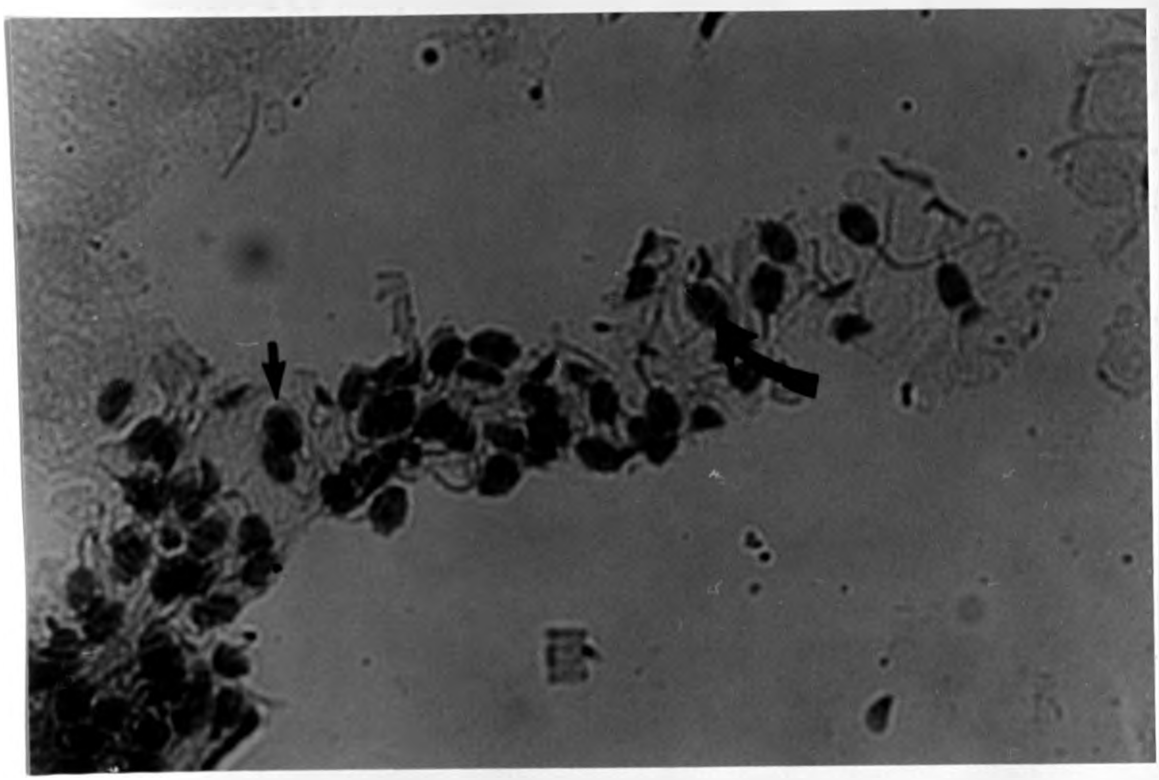


Plate 5: Anti-ERV3 *env* (a polyclonal antibody against a human endogenous retrovirus-R envelope) reactivity on spermatozoa within mature baboon (pan 2222) caput epididymis. Strong staining of the acrosomal cap was observed (Arrow), mag x400.

Table 8. Summary of immunostaining on Mature Baboon Caput Epididymis

Antibody	Spermatozoa	Principal cells	Basal cells	Smooth muscle cells
W6/32	-	-	+/-	++
Anti-HIV-1 gp 120 (ID6)	-	-	-	-
Anti-HIV-1 RT	-	-	-	-
Anti-HIV-2 CP	-	-	-	-
Anti-HIV-2 ST gp120	-	-	-	-
Anti-ERV3 *env*	+ (acrosome)	-	-	-
Anti-HIV-1 p24/25 SF2	-	-	-	-
Anti-SIV mac p27 (55-$2F_{12}$)	-	-	-	-
Anti-HIV-1gp41(md-1)	-	-	-	-
Anti-HIV-1 p24/25 *gag*	-	-	-	-
Anti-SIV mac 251 p17 *gag*	-	-	-	-
Anti-HERV-K *env*	-	-	-	-

3.1.1.3. Immunoreactivity on corpus epididymides

Using the same procedure, the corpus epididymis showed weak staining on basal lamina for W6/32 (Table 9). While all other antibodies tested did not react with any cells/tissues associated with corpus epididymis.

Table 9. Summary of immunostaining on Mature Baboon Corpus Epididymis

Antibody	Spermatozoa	Principal cells	Basal cells	Smooth muscle cells
W6/32	-	-	-/+	-/+
Anti-HIV-1 gp 120 (ID6)	-	-	-	-
Anti-HIV-1 RT	-	-	-	-
Anti-HIV-2 CP	-	-	-	-
Anti-HIV-2 ST gp120	-	-	-	-
Anti-ERV3 *env*	-	-	-	-
Anti-HIV-1 p24/25 SF2	-	-	-	-
Anti-SIV mac p27 (55-2F_{12})	-	-	-	-
Anti-HIV-1 gp41(md-1)	-	-	-	-
Anti-HIV-1 p 24/25 *gag*	-	-	-	-
Anti-SIV mac 251 p17 *gag*	-	-	-	-
Anti-HERV-K *env*	-	-	-	-

3.1.1.4. Immunoreactivity on cauda epididymides

A monoclonal antibody (W6/32) that binds human monomorphic class I MHC molecules (HLA-A, B and C), showed strong reaction on basal lamina and a few fibers within the abundant connective tissue stroma (Plate 6). In addition, an antibody against human endogenous retrovirus-R (ERV 3) envelope reacted weakly with the entire epithelial cells of the cauda epididymal duct (Plate 7, Table 10). However, the spermatozoa within the lumen did not bind any of the antibodies. The rest of the antibodies tested did not show any reactivity.

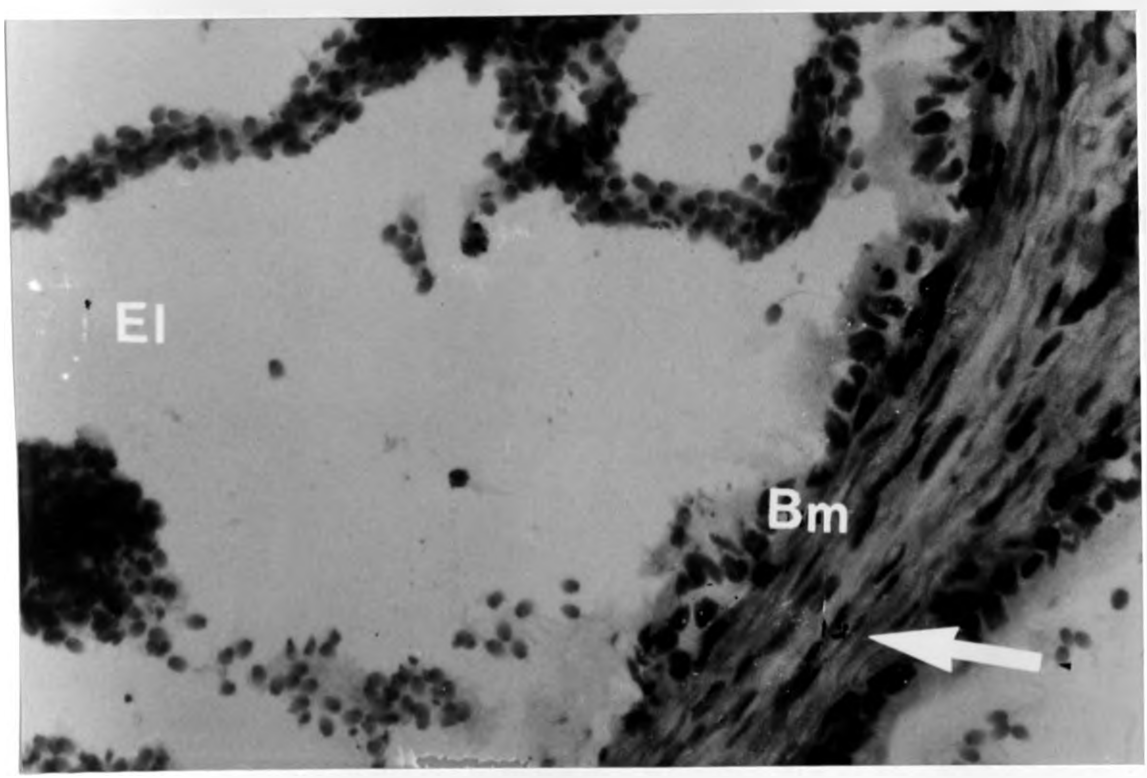


Plate 6: W6/32 (a monoclonal antibody against HLA-A, B and C) reactivity on basal membrane and fibres in the connective tissue of mature baboon (pan 2473) cauda epididymis (Arrow), mag x200. **Bm-** Basement membrane, **El-** Epididymal lumen

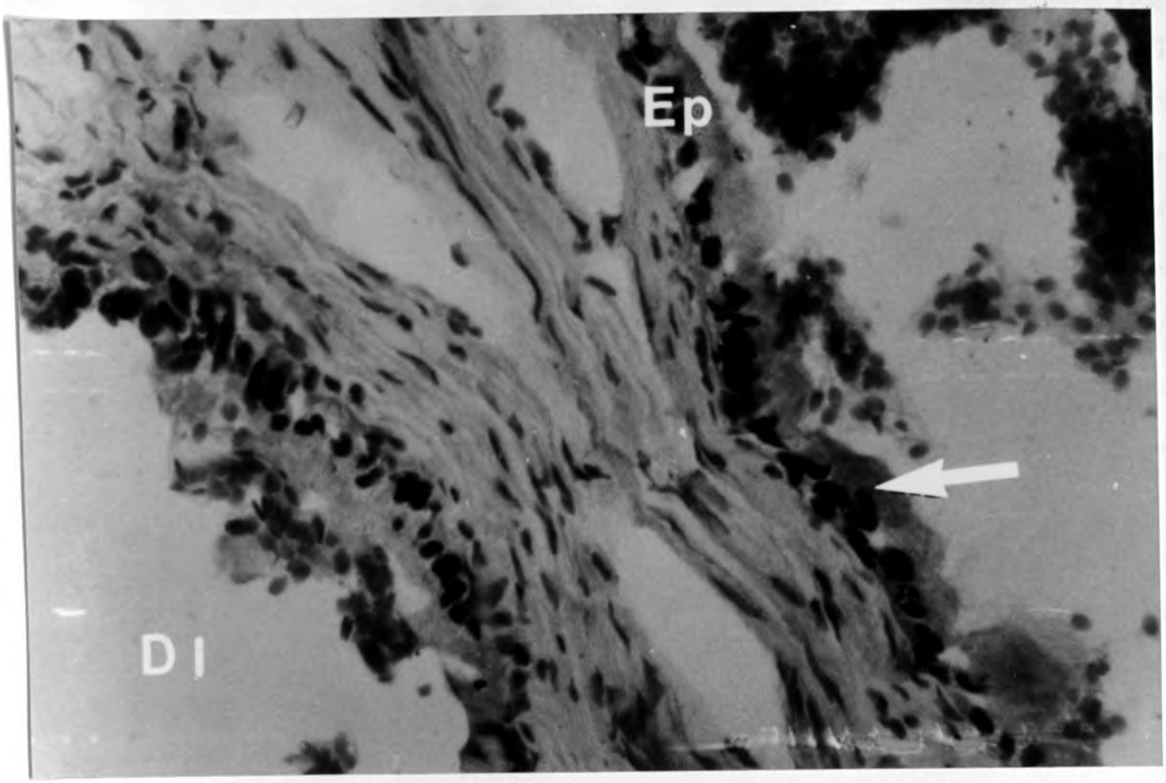


Plate 7: Anti-ERV3 *env* (a polyclonal antibody against a human endogenous retrovirus-R envelope) reactivity on mature baboon (pan 1669) cauda epididymis. The entire epithelia of the ducts was stained (Arrow), mag x200. **Ep**- epithelium, **Dl**- Lumen of the duct

Table 10. Summary of mmunostaining on Mature Baboon Cauda Epididymis

Antibody	Spermatozoa	Principal cells	Basal cells	Smooth muscle cells
W6/32	-	+	+	++
Anti-HIV-1 gp 120 (ID6)	-	-	-	-
Anti-HIV-1 RT	-	-	-	-
Anti-HIV-2 CP	-	-	-	-
Anti-HIV-2 ST gp120	-	-	-	-
Anti-ERV3 *env*	-	+	+	-
Anti-HIV-1 p24/25 SF2	-	-	-	-
Anti-SIV mac p27 (55-2F_{12})	-	-	-	-
Anti-HIV-1 p24/25 *gag*	-	-	-	-
Anti-SIV mac 251 p17 *gag*	-	-	-	-
Anti-HIV-1 gp41(md-1)	-	-	-	-
Anti-HERV-K *env*	-	-	-	-

3.1.1.5. Immunoreactivity on vas deferens

A monoclonal antibody (W6/32) that binds human monomorphic class I MHC molecules (HLA-A, B and C), stained the three layers of the tunica muscularis. However, the staining was weak compared to the rest of the tissues (Plate 8, Table 11). None of the other antibodies tested showed immunoreactivity.

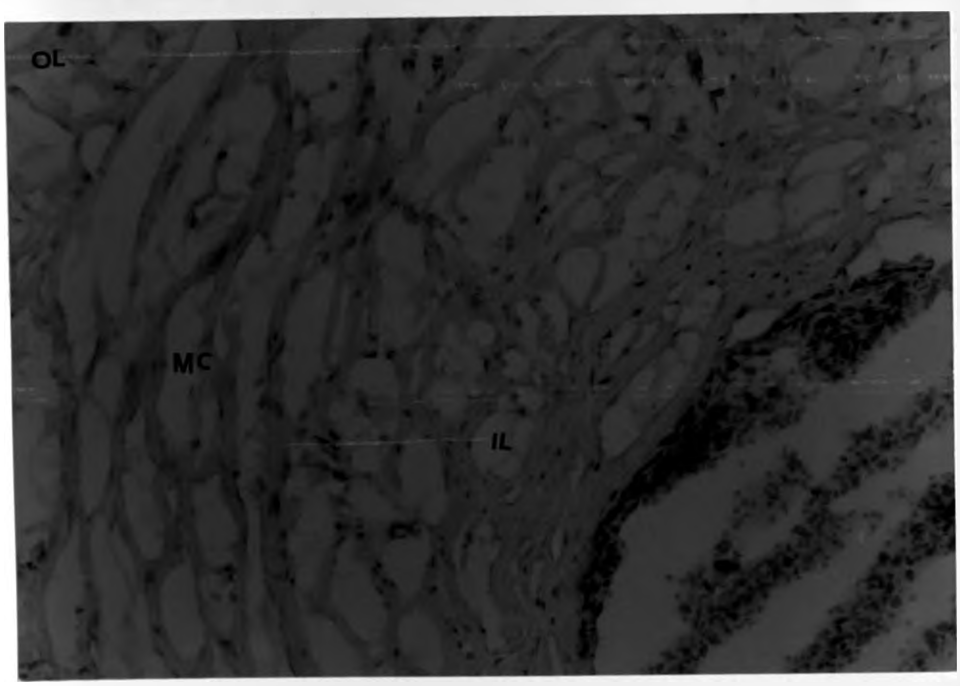


Plate 8: W6/32 (a monoclonal antibody against HLA-A, B and C) reactivity on mature baboon (pan 2222) vas deferens (outer and inner longitudinal muscles, and middle circular layer were weakly stained, Arrow), mag x100. **IL**- Inner longitudinal muscle layer, **MC**- Middle circular layer, **OL**- outer longitudinal muscle layer.

Table 11. Summary of immunostaining on Mature Baboon Vas deferens

Antibody	Spermatozoa	Epithelial cells	Smooth muscle cells
W6/32	-	-	+
Anti-HIV-1 gp 120 (ID6)	-	-	-
Anti-HIV-1 RT	-	-	-
Anti-HIV-2 CP	-	-	-
Anti-HIV-2 ST gp120	-	-	-
Anti-ERV3 *env*	-	-	-
Anti-HIV-1 p24/25 SF2	-	-	-
Anti-SIV mac p27 ($55\text{-}2F_{12}$)	-	-	-
Anti-HIV-1 gp41(md-1)	-	-	-
Anti-HIV-1 p24/25 *gag*	-	-	-
Anti-SIV mac 251 p17 *gag*	-	-	-
Anti-HERV-K *env*	-	-	-

3.1.2. Juvenile baboon reproductive tissue immunostaining

3.1.2.1. Immunoreactivity on testes, epididymides and vasa deferentia

The tissues were processed in a similar manner as indicated for the adult baboons. W6/32 (a monoclonal antibody against HLA-A, B and C) stained interstitial tissue of the testis (Plate 9, Table 12), but weakly as compared to the staining observed in adult baboon testes. This antibody was used as a positive control when immunostaining in subsequent procedures. Anti-HIV-2 ST gp120 (a polyclonal antibody against a human immunodeficiency virus-2 envelope protein) showed some weak staining on juvenile baboon testis (Plate 10, Table 12). Similarly, anti-HIV-1 p25/24 *gag* (a polyclonal antibody against a human immunodeficiency virus type 1 core protein) showed some weak non-specific staining on juvenile baboon testes. Weak non-specific staining of spermatogonia and interstitial tissue was demonstrated (Plate 11, Table 12). An antibody against ERV3 envelope protein found to stain strongly in mature testes did not stain any cells in juvenile testes. A monoclonal antibody against HLA-A, B and C (W6/32) stained the smooth muscle cells of the caput (Plate 12, Table 13), corpus (Table 14) and cauda (Plate 13, Table 15) epididymides of juvenile baboons. The smooth muscle cells surrounding the ducts were stained including part of the parenchyma cells within the ducts. The vas deferens of the young baboon displayed a weaker staining for the same antibody (W6/32, Table 16). The rest of the antibodies used in the study did not show any reactivity.

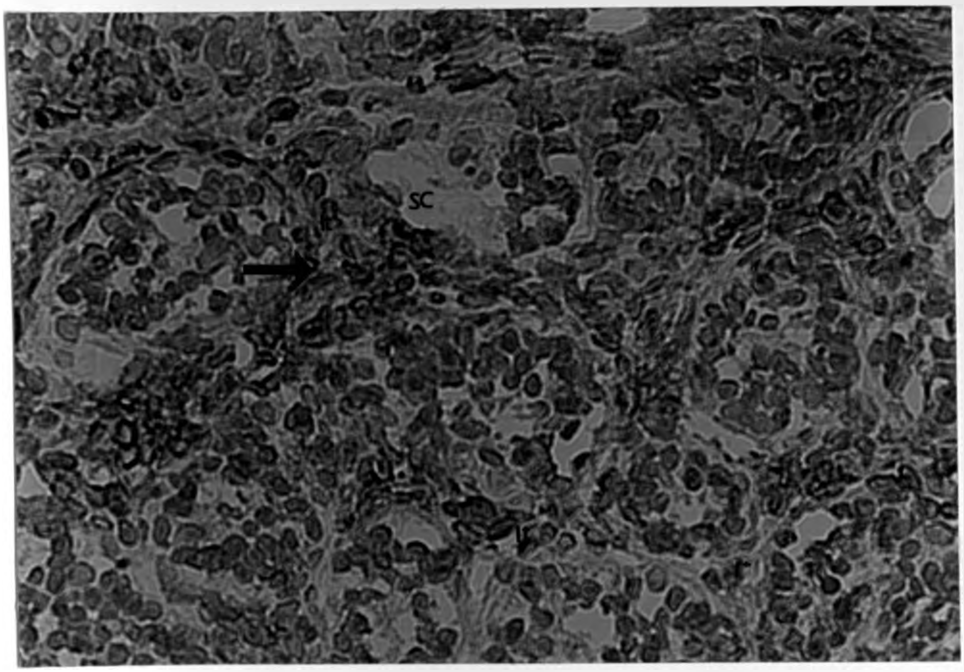


Plate 9: W6/32 (a monoclonal antibody against HLA-A, B and C) reactivity on juvenile baboon (pan 2242) testis. Interstitial cells between seminiferous tubules were weakly stained (Arrow), mag x100, **Sc-** seminiferous tubule, **It-** Interstitium.

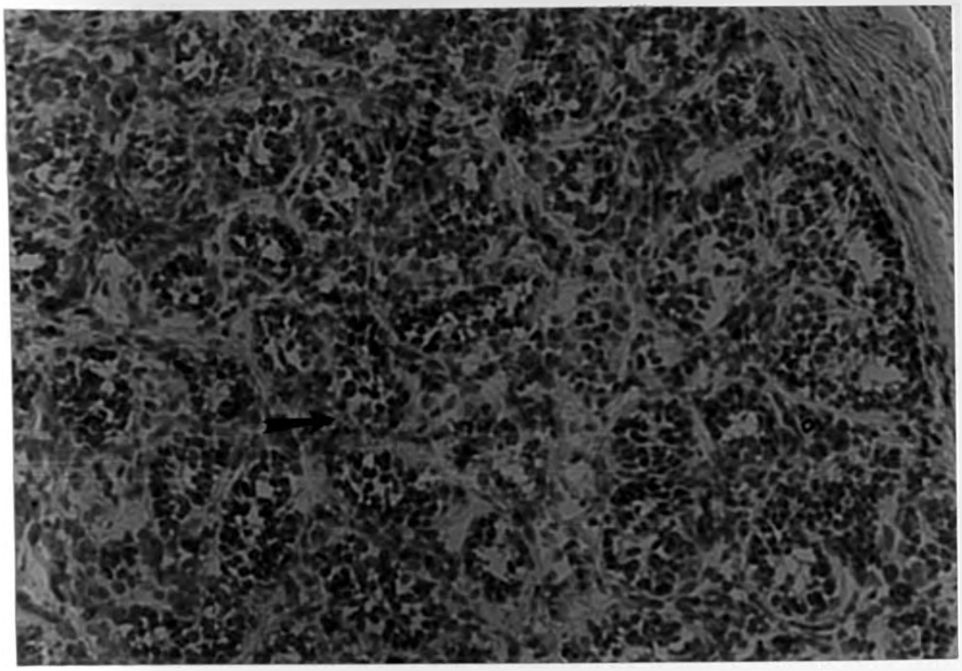


Plate 10: Anti-HIV-2 ST gp120 (a polyclonal antibody against a human immunodeficiency virus type-2 envelope protein) reactivity on juvenile baboon (pan 2273) testis. Non-specific weak staining of spermatogenic cells and interstitium was observed (Arrow) mag x100.

Plate 11: Anti-HIV-1 p25/24 *gag* (a monoclonal antibody against a human immunodeficiency virus type- 1 core protein) reactivity on juvenile baboon (pan 2242) testes. Generalised weak staining of spermatogonia and interstitial tissue was demonstrated (Arrow), mag x100.

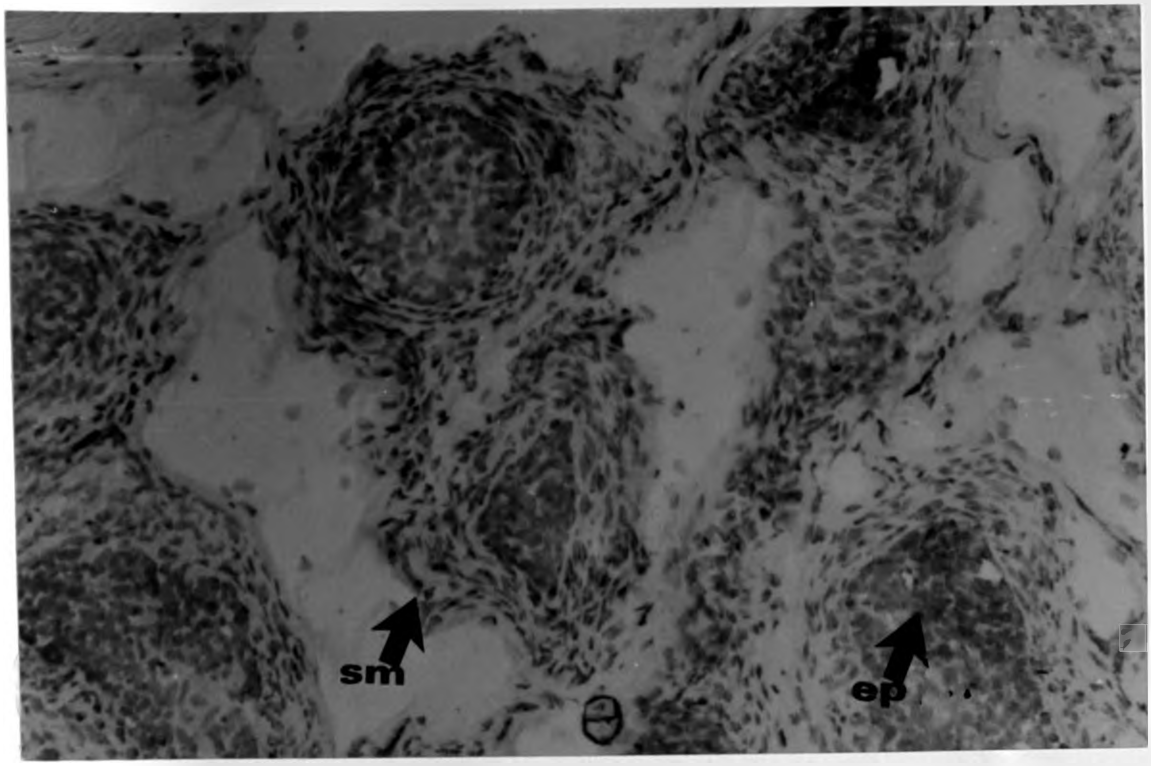


Plate 12: W6/32 (a monoclonal antibody against HLA-A, B and C) reactivity on juvenile baboon (pan 2242) caput epididymis (smooth muscle cells and ducts were stained, Arrow), mag x100. **Sm-** smooth muscle cells, **ep-** epididymal duct parenchyma.

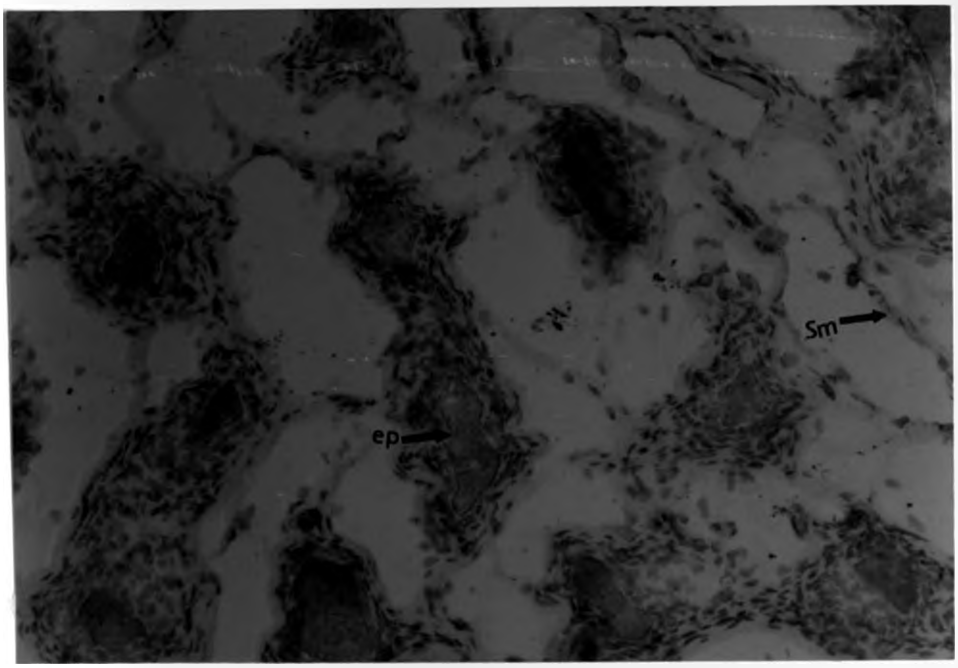


Plate 13: W6/32 (a monoclonal antibody against HLA-A, B and C) reactivity on smooth muscle cells and ducts of juvenile baboon (pan 2242) cauda epididymis (Arrow), mag x100.

Table 12. Summary of immunostaining of Juvenile Baboon Testis

Antibody	Peritubular cells	Spermatogonia	Sertoli cells	Leydig cells
W6/32	+	-	-	+/-
Anti-HIV-1 gp 120 (ID6)	-	-	-	-
Anti-HIV-1 RT	-	-	-	-
Anti-HIV-2 CP	-	-	-	-
Anti-HIV-2 ST gp120	-	+/-	+/-	+/-
Anti-ERV3 *env*	-	-	-	-
Anti-HIV-1 p24/25 SF2	-	-	-	-
Anti-SIV mac p27 (55-2F_{12})	-	-	-	-
Anti-HIV-1 gp41(md-1)	-	-	-	-
Anti-HIV-1 p24/25 *gag*	-	+/-	+/-	+/-
Anti-SIV mac 251 p17 *gag*	-	-	-	-
Anti-HERV-K *env*	-	-	-	-

Table 13. Summary of immunostaining on Juvenile Baboon Caput Epididymis

Antibody	Duct parenchyma cells	Smooth muscle cells
W6/32	+	+
Anti-HIV-1 gp 120 (ID6)	-	-
Anti-HIV-1 RT	-	-
Anti-HIV-2 CP	-	-
Anti-HIV-2 ST gp120	-	-
Anti-ERV3 env	-	-
Anti-HIV-1 p24/25 SF2	-	-
Anti-SIV mac p27 (55-2F_{12})	-	-
Anti-HIV-1gp41(md-1)	-	-
Anti-HIV-1 p24/25 *gag*	-	-
Anti-SIV mac 251 p17 *gag*	-	-
Anti-HERV-K *env*	-	-

Table 14. Summary of immunostaining on Juvenile Baboon Corpus Epididymis

Antibody	Duct parenchyma cells	Smooth muscle cells
W6/32	+	+
Anti-HIV-1 gp 120 (ID6)	-	-
Anti-HIV-1 RT	-	-
Anti-HIV-2 CP	-	-
Anti-HIV-2 ST gp120	-	-
Anti-ERV3 env	-	-
Anti-HIV-1 p24/25 SF2	-	-
Anti-SIV mac p27 (55-2F_{12})	-	-
Anti-HIV-1 gp41(md-1)	-	-
Anti-HIV-1 p24/25 *gag*	-	-
Anti-SIV mac 251 p17 *gag*	-	-
Anti-HERV-K *env*	-	-

Table 15. Summary of immunostaining on Juvenile Baboon Cauda Epididymis

Antibody	Duct parenchyma cells	Smooth muscle cells
W6/32	+	+
Anti-HIV-1 gp 120 (ID6)	-	-
Anti-HIV-1 RT	-	-
Anti-HIV-2 CP	-	-
Anti-HIV-2 ST gp120	-	-
Anti-ERV3 env	-	-
Anti-HIV-1 p24/25 SF2	-	-
Anti-SIV mac p27 (55-2F_{12})	-	-
Anti-HIV-1gp41(md-1)	-	-
Anti-HIV-1 p24/25 *gag*	-	-
Anti-SIV mac 251 p17 *gag*	-	-
Anti-HERV-K *env*	-	-

Table 16. Summary of immunostaining on Juvenile Baboon Vas deferens

Antibody	Epithelial cells	Smooth muscle cells
W6/32	-	+
Anti-HIV-1 gp 120 (ID6)	-	-
Anti-HIV-1 RT	-	-
Anti-HIV-2 CP	-	-
Anti-HIV-2 ST gp120	-	-
Anti-ERV3 env	-	-
Anti-HIV-1 p24/25 SF2	-	-
Anti-SIV mac p27 (55-2F_{12})	-	-
Anti-HIV-1 gp41(md-1)	-	-
Anti-HIV-1 p24/25 *gag*	-	-
Anti-SIV mac 251 p17 *gag*	-	-
Anti-HERV-K *env*	-	-

3.2. Results of Reverse Transcriptase (RTase) Assay

The absorbance at 405nm of each standard dilution was plotted against its HIV-rRT (Cavidi, HS-kit, Mg^{2+} RT) and MmuLV-rRT (Cavidi HS-kit, Mn^{2+} RT) concentration. The best regression line was calculated utilizing the mean absorbance at 405 nm of the background controls as y-axis intercept, only readings within the linear range of the microtitre plate reader were used.

The RTase activity of samples was extrapolated from the regression line according to the manufacturer's instructions. Only sample wells giving absorbance at 405 nm within the linear range of the reader and having signals greater than two times the mean of the background controls had their RTase activity determined. If a higher value was obtained for the shorter or longer RTase reaction time, then this was considered as the RTase value of the sample.

RTase activity was detected in the ejaculate and epididymal spermatozoa, including the seminal plasma of ejaculum (Table 40, Figure 15 and Table 41, Figure 16). The two HIV-1 clones (HIV-1 MN and HIV-1 KS) used as positive controls had the highest RTase activity among the samples tested. The values were 1371 and 1356 pg/ml (Mg^{2+} dependent kit), and 79195 and 29100 µU/ml (Mn^{2+} dependent kit) for HIV-1 MN and HIV-1 KS clones respectively (Table 42). This observation demonstrated magnesium as a better cation (cofactor) for RTase activity associated with HIV-1 and semen than manganese.

Reverse transcriptase in baboon semen samples using Cavidi HS-Kit (Mg^{2+} RT).

Table 17. Results for standard curve from the plate incubated for 3 hours and read after 30 minutes (405nm).

Sample	Concentration (pg/ml)	Optical density (405nm)
S1	515	3.138
S2	229	1.467
S3	102	0.642
S4	45.2	0.22
S5	20.1	0.092
S6	8.93	0.036
S7	3.97	0.002

S- standard

Figure 4. Standard curve for the plate incubated for 3 hours and read after 30 minutes (405nm). (Mg^{2+} RT Assay)

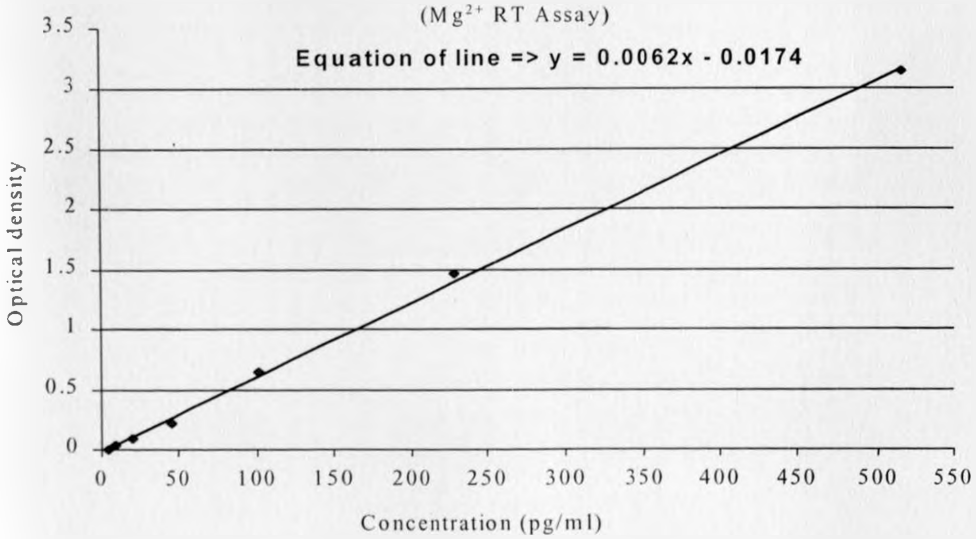


Table 18. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (pg/ml)
HIV-1 SUP (KS Clone)	Over
HIV-1 SUP (MN Clone)	Over
Pan 1669 Seminal plasma	311
Pan 1669 sperm pellet (lysed)	80

Over: The optical density was beyond the reading range of the spectrophotometer
Pan *Papio anubis* (Olive baboon)

Table 19. Results for standard curve from the plate incubated for 24 hours and read after 30 minutes (405nm).

Sample	Concentration (pg/ml)	Optical density (405nm)
S1	102	2.518
S2	45.2	0.797
S3	20.1	0.298
S4	8.93	0.074
S5	3.97	0.034

Figure 5. Standard curve for the plate incubated for 24 hours and read after 30 minutes (405nm). (Mg^{2+} RT Assay)

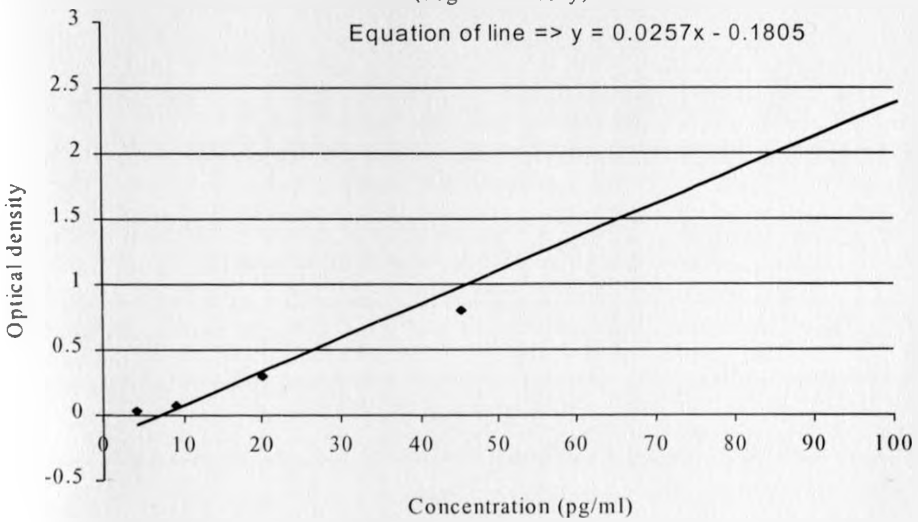


Table 20. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (pg/ml)
HIV-1 SUP (KS Clone)	Over
HIV-1 SUP (MN Clone)	Over
Pan 1669 Seminal plasma	42
Pan 1669 Sperm pellet (lysed)	29

Table 21. Results for standard curve from the plate incubated for 3 hours and read after 2 hours (405nm).

Sample	Concentration (pg/ml)	Optical density (405nm)
S1	1 158	2.249
S2	515	1.523
S3	229	0.87
S4	102	0.439
S5	45.2	0.16
S6	20.1	0.075
S7	8.93	0.025

Figure 6. Standard curve for the plate incubated for 3 hours and read after 2 hours (Mg^{2+} RT Assay)

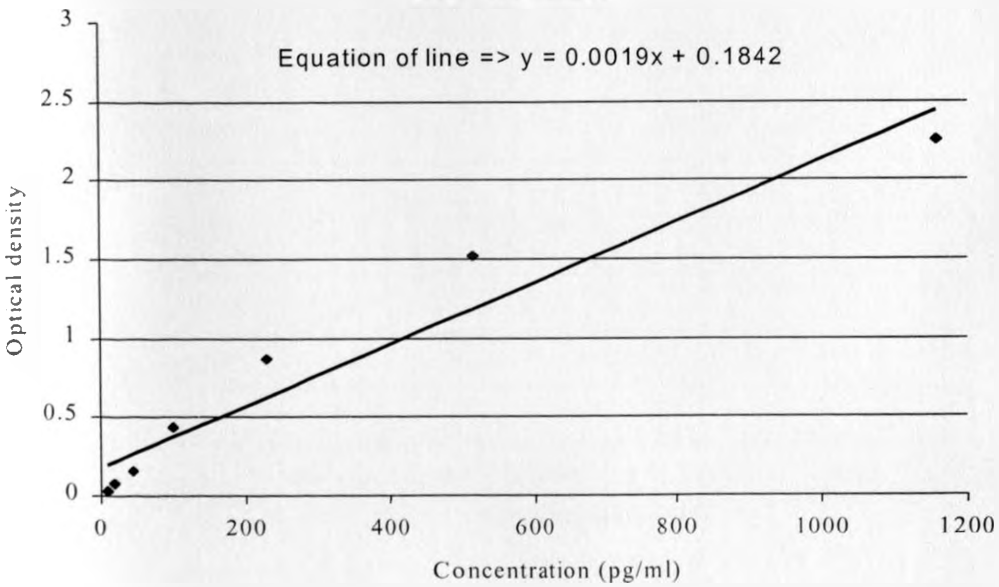


Table 22. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (pg/ml)
HIV-1 SUP (KS Clone)	1,356
HIV-1 SUP (MN Clone)	1,371
Pan 1669 Seminal plasma	861
Pan 1669 sperm pellet (lysed)	231

Table 23. Results for standard curve from the plate incubated for 24 hours and read after 2 hours (405nm).

Sample	Concentration (pg/ml)	Optical density (405nm)
S1	1 158	2.778
S2	515	2.648
S3	229	1.978
S4	102	1.232
S5	45.2	0.472
S6	20.1	0.188
S7	8.93	0.46
S8	3.97	0.026

Figure 7. Standard curve for the plate incubated for 24 hours and read after 2 hours (Mg^{2+} RT Assay)

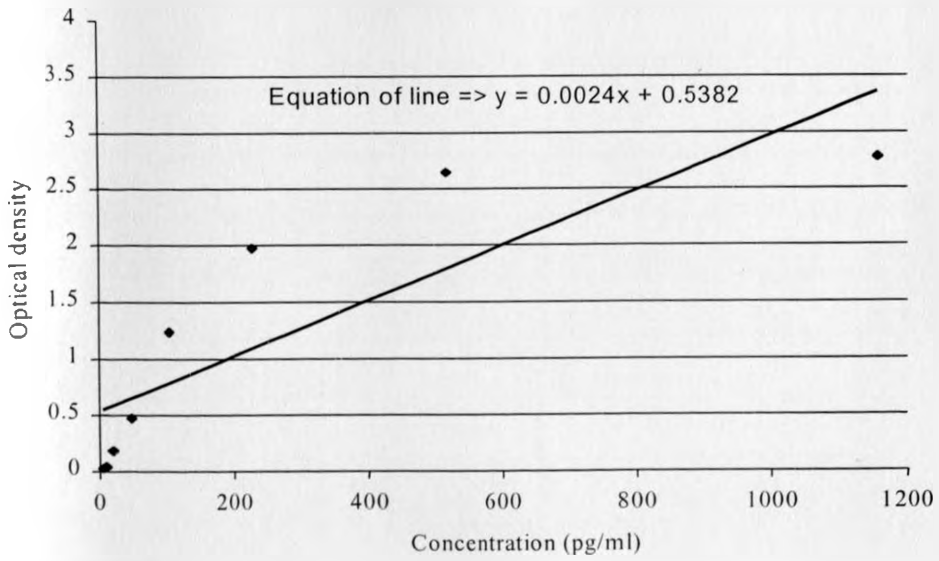


Table 24. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (pg/ml)
HIV-1 SUP (KS Clone)	948
HIV-1 SUP (MN Clone)	925
Pan 1669 Seminal plasma	9
Pan 1669 sperm pellet (lysed)	-

Table 25. Results for standard curve from the plate incubated for 3 hours and read after 24 hours (405nm).

Sample	Concentration (pg/ml)	Optical density (405nm)
S1	1 158	2.628
S2	515	2.357
S3	229	1.749
S4	102	1.015
S5	45.2	0.454
S6	20.1	0.161
S7	8.93	0.049

Figure 8. Standard curve for the plate incubated for 3 hours and read after 24 hours (Mg^{2+} RT Assay)

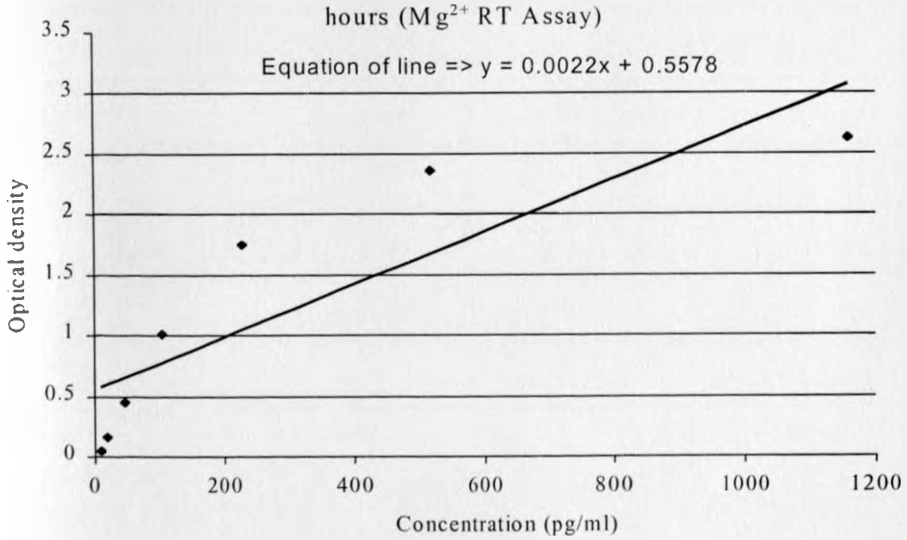


Table 26. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (pg/ml)
HIV-1 SUP (KS Clone)	976
HIV-1 SUP (MN Clone)	960
Pan 1669 Seminal plasma	861
Pan 1669 sperm pellet (lysed)	113

Table 27. Results for standard curve from the plate incubated for 24 hours and read after 24 hours (405nm).

Sample	Concentration (pg/ml)	Optical density (405nm)
S1	1 158	2.632
S2	515	2.623
S3	229	2.562
S4	102	2.08
S5	45.2	1.128
S6	20.1	0.482
S7	8.93	0.123
S8	3.97	0.077

Figure 9. Standard curve for the plate incubated for 24 hours and read after 24 hours (Mg^{2+} RT Assay)

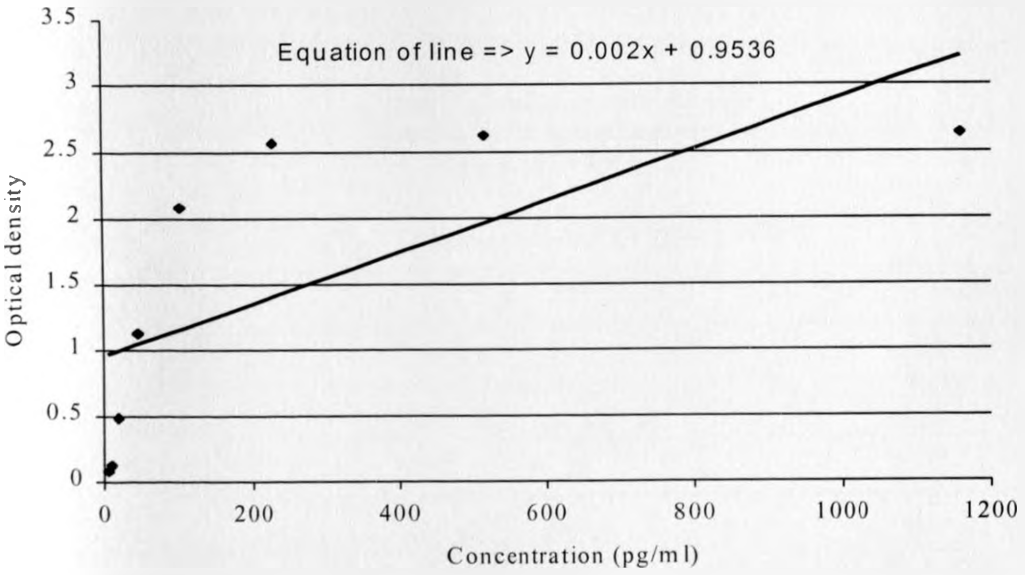


Table 28. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (pg/ml)
HIV-1 SUP (KS Clone)	810
HIV-1 SUP (MN Clone)	821
Pan 1669 Seminal plasma	137
Pan 1669 sperm pellet (lysed)	-

Reverse transcriptase activity in baboon semen samples using Cavidi HS-Kit (Mn^{2+} RT).

Table 29. Results for standard curve from the plate incubated for 3 hours and read after 30 minutes.

Sample	Concentration (µU/ml)	Optical density (405nm)
S1	42 900	0.828
S2	19 000	0.391
S3	8 460	0.247
S4	3 760	0.134
S5	1 670	0.07
S6	743	0.041
S7	330	0.035
S8	147	0.021
S9	65.2	0.014
S10	29	0.025

Figure 10. Standard curve for the plate incubated for 3 hours and read after 30 minutes (Mn^{2+} RT Assay)

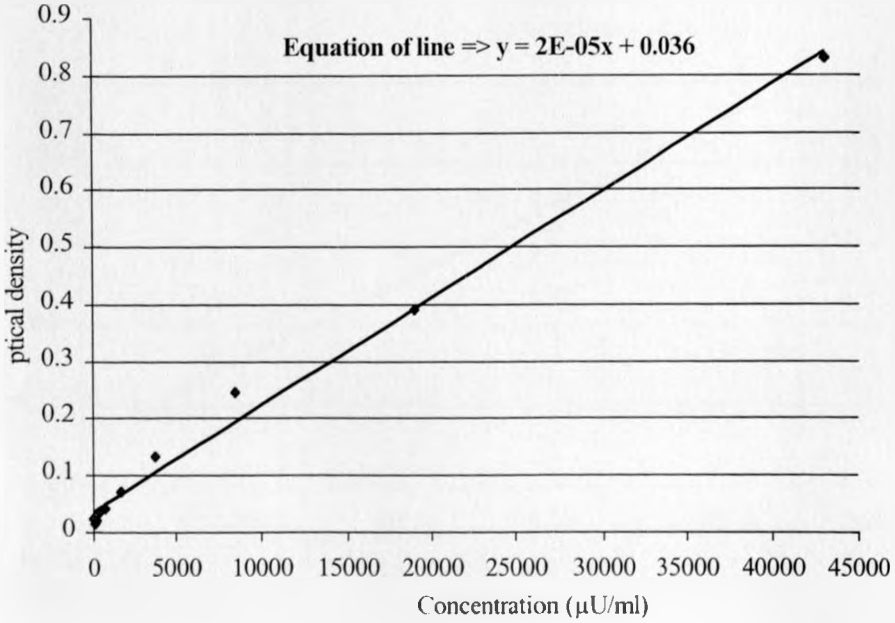


Table 30. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (μu/ml)
HIV-1 SUP (KS Clone)	24 195
HIV-1 SUP (MN Clone)	79 195
Pan 1669 Seminal plasma	-
Pan 1669 sperm pellet (lysed)	-
Pan 1669 epididymal sperm pellet (lysed)	-

Table 31. Results for standard curve from the plate incubated for 24 hours and read after 30 minutes.

Sample	Concentration (µU/ml)	Optical density (405nm)
S1	8 460	2.577
S2	3 760	0.942
S3	1 670	0.493
S4	743	0.326
S5	330	0.195
S6	147	0.114
S7	65.2	0.166
S8	29	0.060

Figure 11. Standard curve for the plate incubated for 24 hours and read after 30 minutes (Mn2+ RT)

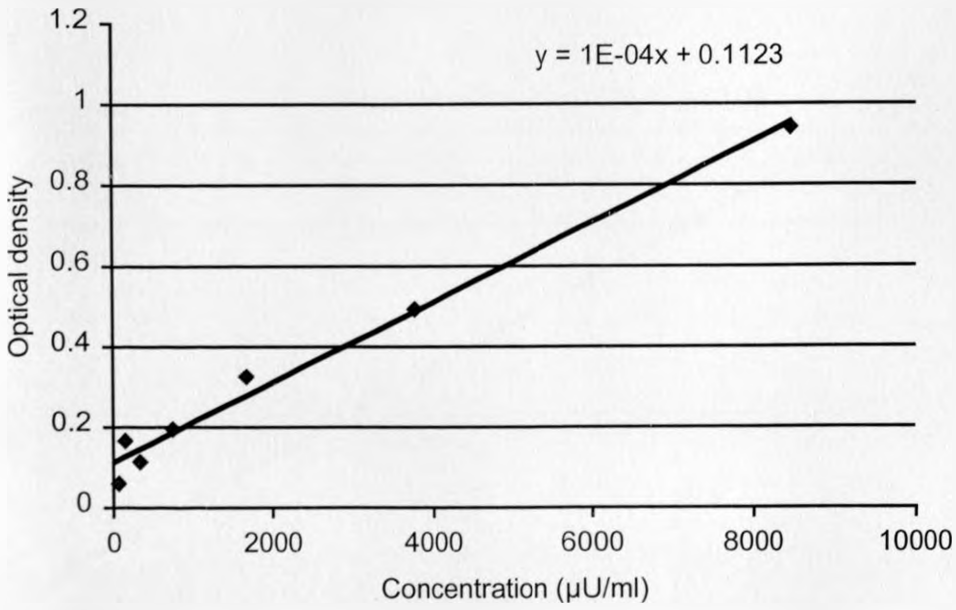


Table 32. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (µU/ml)
HIV-1 SUP (KS Clone)	2 725
HIV-1 SUP (MN Clone)	9 808
Pan 1669 Seminal plasma	-
Pan 1669 sperm pellet (lysed)	-
Pan 1669 epididymal sperm pellet (lysed)	-

Table 33. Results for standard curve from the plate incubated for 3 hours and read after 2 hours

Sample	Concentration (µU/ml)	Optical density (405nm)
S1	42 900	2.349
S2	19 000	1.173
S3	8 460	0.734
S4	3 760	0.401
S5	1 670	0.22
S6	743	0.099
S7	330	0.056
S8	147	0.001
S9	65.2	0.003
S10	29	0.011

Figure 12. Standard curve for the plate incubated for 3 hours and read after 2 hours (Mn^{2+} RT Assay)

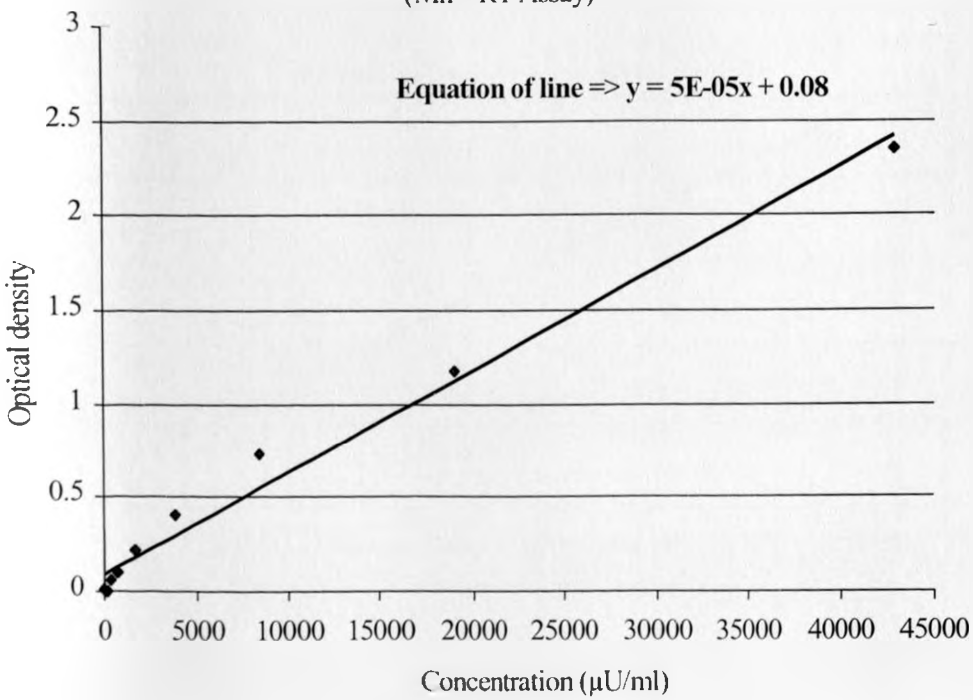


Table 34. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (µU/ml)
HIV-1 SUP (KS Clone)	29 100
HIV-1 SUP (MN Clone)	71 240
Pan 1669 Seminal plasma	-
Pan 1669 sperm pellet (lysed)	-
Pan 1669 epididymal sperm pellet (lysed)	-

Table 35. Results for standard curve from the plate incubated for 24 hours and read after 2 hours

Sample	Concentration (µU/ml)	Optical density (405nm)
S1	3 760	2.555
S2	1 670	1.397
S3	743	0.901
S4	330	0.615
S5	147	0.34
S6	65.2	0.503
S7	29	0.15

Figure 13. Standard curve for the plate incubated for 24 hours and read after 2 hours (Mn^{2+} RT Assay)

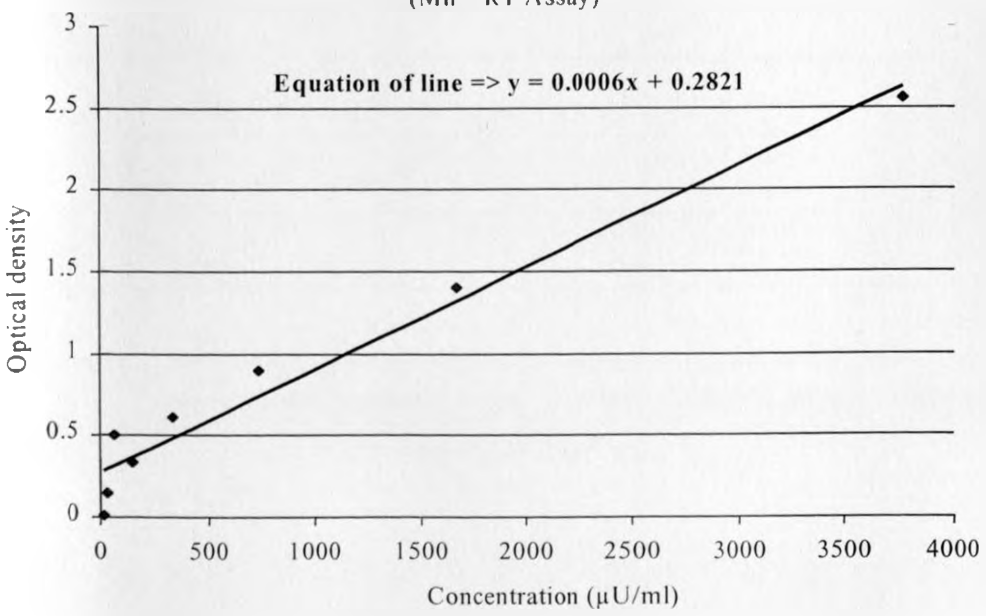


Table 36. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (µU/ml)
HIV-1 SUP (KS Clone)	3 378
HIV-1 SUP (MN Clone)	Over
Pan 1669 Seminal plasma	-
Pan 1669 sperm pellet (lysed)	-
Pan 1669 epididymal sperm pellet (lysed)	-

Table 37. Results for standard curve from the plate incubated for 3 hours and read after 24 hours

Sample	Concentration (µU/ml)	Optical density (405nm)
S1	3 760	2.746
S2	1 670	1.668
S3	743	0.628
S4	330	0.383
S5	147	0.033
S6	65.2	0.026

Figure 14. Standard curve for the plate incubated for 3 hours and read after 24 hours (Mn^{2+} RT Assay)

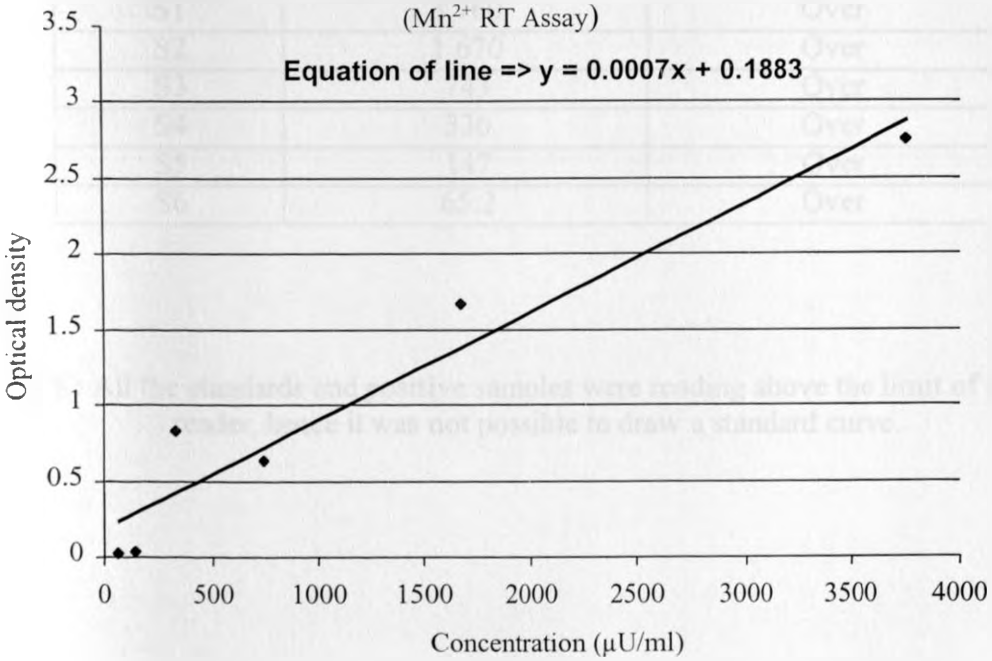


Table 38. RTase concentration in HIV (controls) and baboon semen samples

Sample	Concentration (µU/ml)
HIV-1 SUP (KS Clone)	Over
HIV-1 SUP (MN Clone)	Over
Pan 1669 Seminal plasma	3 287
Pan 1669 ejaculate sperm pellet (lysed)	3 110
Pan 1669 epididymal sperm pellet (lysed)	2 917

39. Results for standard curve from the plate incubated for overnight and read after 24 hours

Sample	Concentration (µU/ml)	Optical density (405nm)
S1	3 760	Over
S2	1 670	Over
S3	743	Over
S4	330	Over
S5	147	Over
S6	65.2	Over

NOTE: All the standards and positive samples were reading above the limit of the plate reader, hence it was not possible to draw a standard curve.

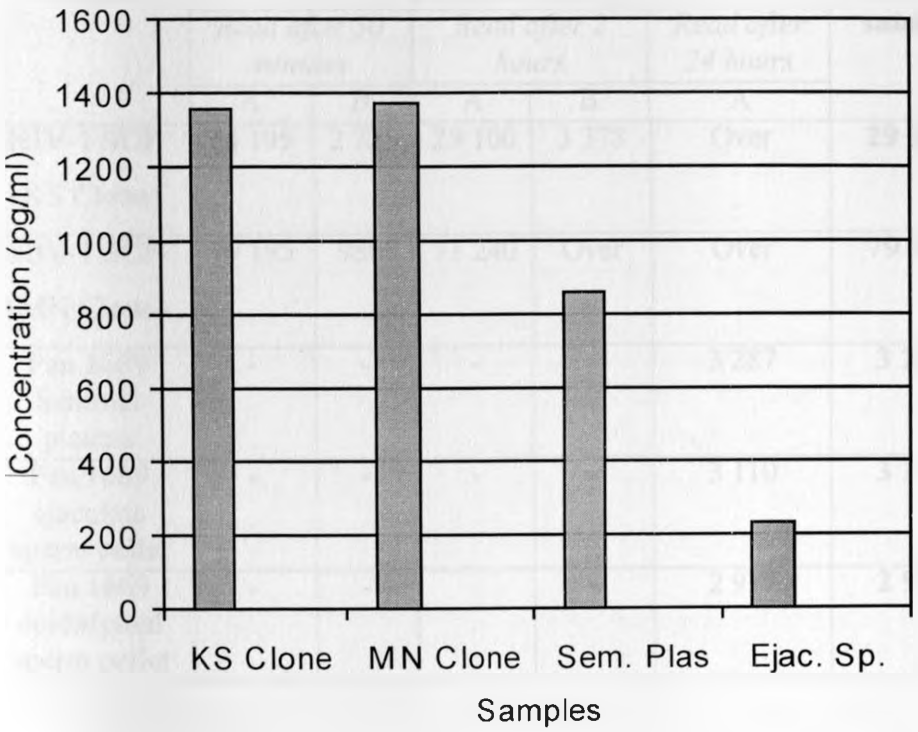
Table 40. A summary of RTase result using Magnesium dependent Kit, RTase concentration in HIV (controls) and baboon semen samples (pg/ml)

Sample	Concentration						Conc. of sample
	Read after 30 min		*Read after 2 hours*		*Read after 24 hours*		
	A	B	A	B	A	B	
HIV-1 SUP (KS Clone)	Over	Over	1 356	948	976	810	**1 356**
HIV-1 SUP (MN Clone)	Over	Over	1 371	925	960	821	**1 371**
Pan 1669 Seminal plasma	311	42	861	9	861	137	**861**
Pan 1669 ejaculate sperm pellet (lysed)	80	29	231	-	113	-	**231**

A: Plate incubated for 3 hours,

B: Plate incubated overnight

Figure 15. A summary of RTase concentration using magnesium dependent kit



Key

KS clone	HIV-1 clone
MN clone	HIV-1 clone
Sem. Plas	Seminal plasma
Ejac. Sp.	Ejaculated spermatozoa

Table 41. A summary of results on reverse transcriptase activity using Mn^{2+} dependent kit.

RTase concentration in HIV (controls) and baboon semen samples (μU/ml)

Sample	Concentration					Conc. of sample
	Read after 30 minutes		*Read after 2 hours*		*Read after 24 hours*	
	A	B	A	B	A	
HIV-1 SUP (KS Clone)	24 195	2 725	29 100	3 378	Over	**29 100**
HIV-1 SUP (MN Clone)	79 195	9808	71 240	Over	Over	**79 195**
Pan 1669 Seminal plasma	-	-	-	-	3 287	**3 287**
Pan 1669 ejaculate sperm pellet	-	-	-	-	3 110	**3 110**
Pan 1669 epididymal sperm pellet	-	-	-	-	2 917	**2 917**

A: Plate incubated for 3 hours,

B: Plate incubated overnight

Figure 16. A summary of RTase concentration using manganese dependent kit

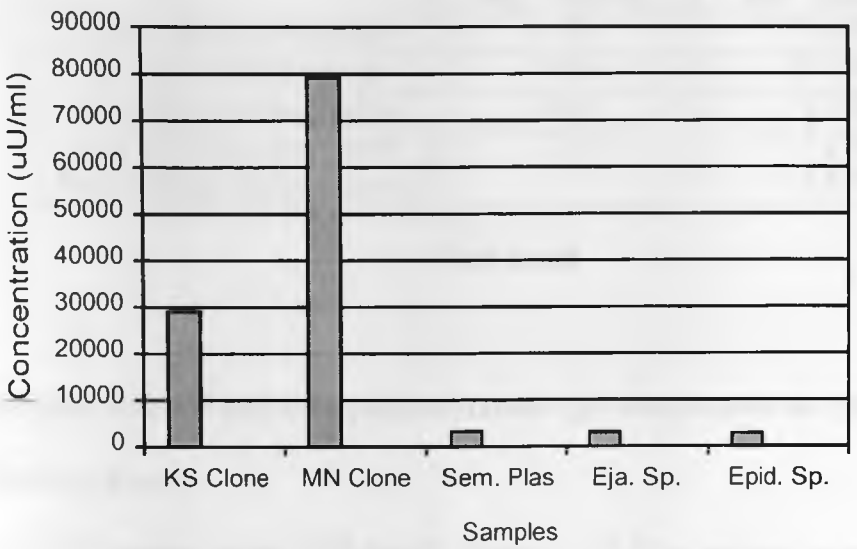


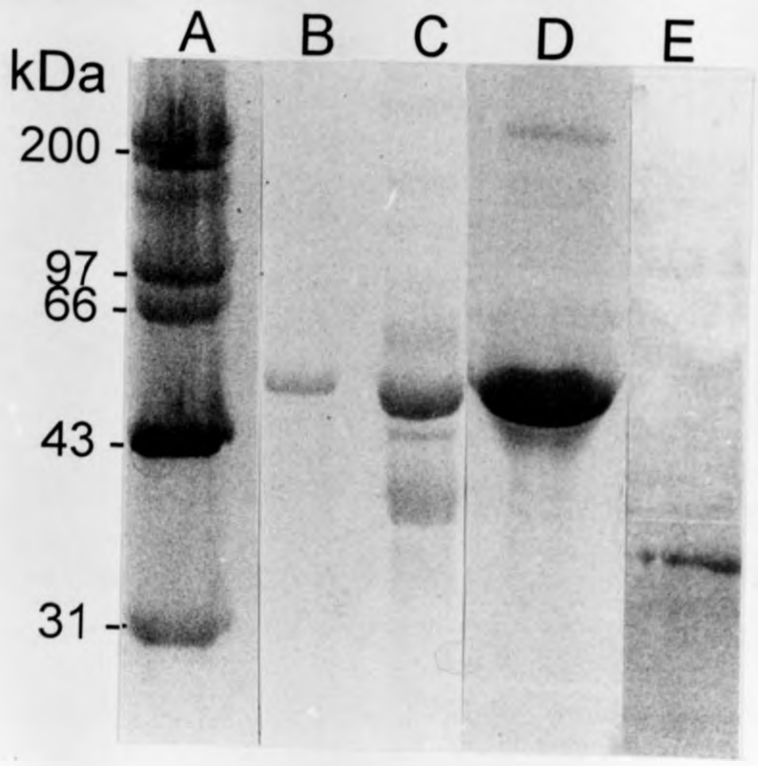
Table 42. Comparative RTase activity between magnesium and manganese dependent kits

Sample type	RTase concentration	
	Mg^{2+} (pg/ml)	Mn^{2+} (µU/ml)
HIV-1 SUP (MN clone)	1 371	79 195
HIV-1 SUP (KS clone)	1 356	29 100
Pan 1669 seminal plasma	861	3 287
Pan 1669 ejaculate sperm	231	3 110
Pan 1669 epididymal sperm	-	2 917

- Not tested

3.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot.

Analysis of proteins using SDS-PAGE showed a 58 kDa protein in sperm pellet from ejaculum (plate 15, lane B), proteins of approximately 49, 58 and 80 kDa in seminal fluid (plate 15, lane C), and an estimated 32 kDa protein in spermatozoa from epididymides (plate 15, lane E). HIV-1 supernatant analysed together with these samples demonstrated a 58 kDa protein (plate 14, lane D) similar to that identified in seminal plasma and spermatozoa from ejaculum. However, the antibodies reactive with the testes and epididymides spermatozoa could not cross-react with any of the proteins from semen samples including epididymal spermatozoa on immunoblot. An antibody against HIV-1 p25/24 *gag* bound to a 58 kDa protein in HIV-1 supernatant (plate 15).



A- High molecular weight standard (Bio-Rad)

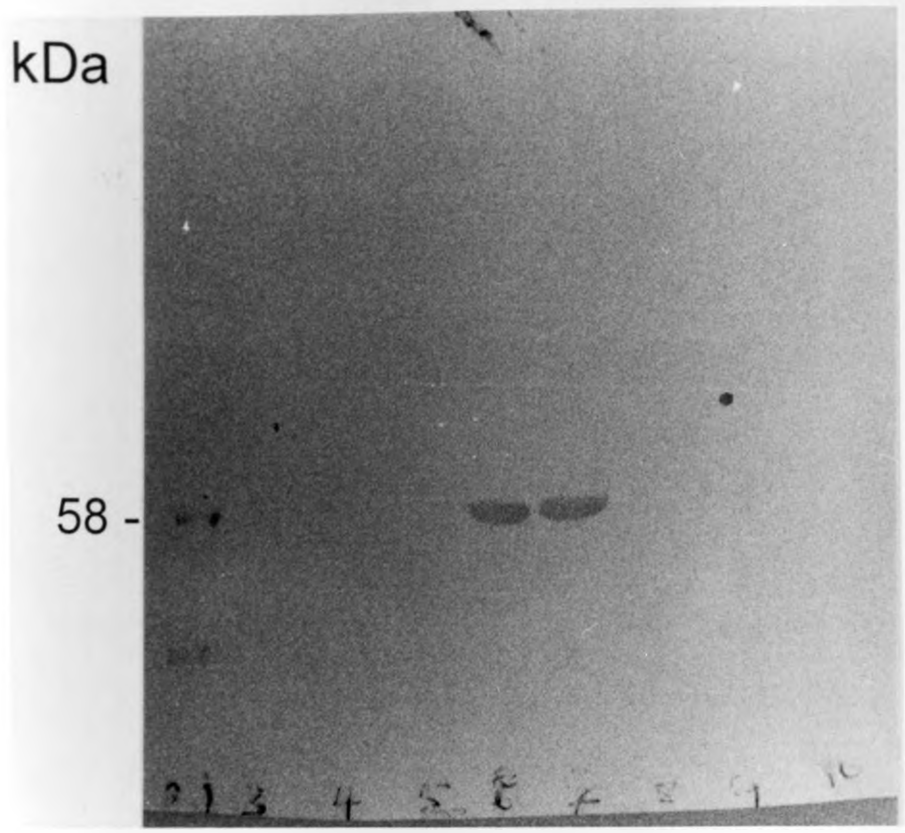
B- Sperm pellet from ejaculum

C- Seminal fluid

D- HIV-1 supernatant

E- Sperm pellet from epididymis

Plate 14. Electrophoresis profile of samples ran on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)



2- High molecular weight standard

3- Seminal fluid

4- Sperm pellet from ejaculum

5- Sperm pellet from epididymis

6- HIV-1 clone MN supernatant

7- HIV-1 clone KS supernatant

8- Solubilisation buffer

9- Skipped

10- Skipped

Plate 15. Results of immunoblot using anti HIV-1 p24/25 *gag* antibody.

3.4. Polyclonal antibody production

Polyclonal antibodies were produced as described in materials and methods (section 2.2.6). The antibody reactivity against baboon testes and epididymidis was tested to determine the level of antibody produced. An antibody against epididymal spermatozoa, raised in mouse 2 (EPS-2) and that against spermatozoa from ejaculum, raised in mouse 4 (EJS-2) reacted strongly with the testes and cauda epididymidis. These two antibodies could not react with caput epididymidis. Antiserum against epididymal spermatozoa raised in mouse 1 (EPS-1) and that against spermatozoa from ejaculum raised in mouse 3 (EJS-3) were weak in their reactivity on the testes, caput epididymidis and cauda epididymidis.

CHAPTER FOUR

4. DISCUSSION, CONCLUSION AND RECOMENDATION

4.1. Discussion

Retroviral sequences have been identified in most mammalian species. In addition to exogenous strains transmitted horizontally, sequences of endogenous retroviruses transmitted through the germ line have been demonstrated. They show structural and sequence similarities to exogenous retroviruses (Coffin, 1984). A pathogenic potential for non-defective endogenous retroviruses has so far only been demonstrated in mice, in which they have been associated with tumors and immunological disorders (Coffin, 1984; Marrack *et al,* 1991). Endogenous retroviruses also have the potential to produce mutations (Morse *et al,* 1988). In humans, genomic DNA contain numerous endogenous retroviral sequences with partial relatedness to animal retroviruses (Knossl *et al,* 1999; Casau *et al,* 1999). However, most of them have been found to be defective and their role still remains unknown (Larsson *et al,* 1989; Leib-Mosch *et al,* 1990). The expression of such ERV-like particles in the testis and epididymis has been demonstrated in human (Larsson *et al,* 1994) and mice (Kiessling *et al*, 1987,1989). Despite this demonstration, their role in male reproductive tissues including the testis is still unknown.

Cell-to-cell interaction in the testes have been demonstrated to be essential for normal testicular function (Skinner, 1991; Focko, 1992). The germ cells develop in a microenvironment supported by the Sertoli's cells. It is possible that the Sertoli's cells mediate the control of germ-cell differentiation. This is through support and provision of

nutrients, release of late spermatids into the tubule lumen, mediating steroidogenic function, phagocytosis, secretory function, cell-to-cell communication, and formation of blood-testis barrier among other functions (Dym, 1983; Fawcett, 1986; Skinner, 1991; Focko, 1992). All spermatogenic cells of the same clone form a syncytium via intercellular bridges (Fawcett, 1986; Pawar and Wrobel, 1991), synchronizing activities at each level of the seminiferous tubule epithelia through intercellular communication. In addition, there is also Sertoli-Sertoli, and Sertoli-germ cell interaction (Russel and Peterson, 1985), where gap junctions exist between spermatogenic cells and Sertoli cells. However, the nature of the actual molecules involved in syncytium formation among the germ cells has not been fully established.

In this study, ERV 3 *env*-like antigens were demonstrated on the early phase spermatogenic cells (spermatogonia and/or primary spermatocytes) within the basal compartment of seminiferous tubules. The association of ERV3 *env* expression with spermatogonia/primary spermatocytes may suggest a physiologic role for ERV3 *env* in spermatogenesis. Envelope glycoproteins of many retroviruses are known to induce formation of multinucleated giant cells (syncytia) by cell fusion (Owens *et al,* 1990). Several studies have also shown a possible role for ERV3 in placental development. Lin *et al,* (1999) demonstrated a decreased cellular proliferation, increased differentiation-related changes in cellular morphology and a significant increase in intercellular fusion in BeWo cell transfected with ERV3 *env*. In a related study, these investigators, Lin *et al,* (2000) showed using a similar placental trophoblast model (BeWo) that ERV3 *env*

decreases cyclic B (a positive cell cycle regulator) and increases P21 (a negative cell cycle regulator) expression, which in turn inhibits cell growth. This mechanism can be inferred to the role of ERV3 *env* in the incomplete cellular division (proliferation) among the spermatocytes and subsequent clones within the seminiferous tubule, which result in syncytium formation (Dym, 1983; Fawcett, 1986; Pawar and Wrobel, 1991; Sharpe, 1994). The point of ERV3 *env* induced P21 effect could be at the intercellular bridges between members of the same clone before spermiogenesis (Figure 1). This hypothesis is plausible only when the inhibitory effect by the viral envelope on cellular proliferation is considered (Lin *et al,* 1999). In addition, we postulate that ERV3 *env* enhances cellular differentiation in early phase spermatogenic cells. Other researchers have proposed that ERV particles may be involved in the formation of syncytia by virtue of the fusogenic properties of the retroviral glycoproteins (Wilkinson *et al,* 1994). For instance, ERV3 *env* transcripts have been found to contain sequences that encode the envelope glycoprotein gp70 that has fusogenic capacity (O' Conell *et al,* 1984). This protein has also been shown to result in increased cellular differentiation among trophoblast cells (Lin *et al,* 1999; 2000). We therefore postulate that ERV3 *env*-like antigens may play a role in early phases of germ cell differentiation. More so, ERV3 has been shown to carry some domains within its envelope that are homologous to that of HERV-W (Mi *et al,* 2000), and both are mapped on the long arm of chromosome 7. A recently identified HERV family in human placenta, HERV-W, (Blond *et al,* 1999; Mi *et al,* 2000) has been demonstrated to play a role in placental morphogenesis by enhancing cytotrophoblast fusion to form syncytiotrophoblast (Mi *et al,* 2000; Stoye and Coffin, 2000). However, a

similar role cannot be postulated for ERV 3 envelope localized among the spermatogenic cells in this study. This is because the nature of syncytia formation is different in the case of spermatogenic cells as compared to that of syncytiotrophoblast in the sense that whereas in the former there is incomplete cell division (cytokinesis), in the latter it's a result of cell fusion. However, ERV3 *env* protein could be inhibiting cellular proliferation among the spermatogenic cells at the level of cytokinesis resulting in syncytia formation.

Studies done using a trophoblast model (BeWo) have indicated that a protein termed syncytin is responsible for cell fusion (Mi *et al,* 2000). This protein was found expressed at high levels in the human placenta, specifically in the syncytiotrophoblast layer and at lower levels in the human testis, but not in any other tissues tested (Blond *et al,* 1999; Stoye and Coffin, 2000). The protein has also been shown to share a 34% identity with BaEV envelope protein (Stoye and Coffin, 2000). DNA nucleotide analysis has also revealed sequences on human chromosome 7 that contained an intact open reading frame (ORF) that posses 100% identity to syncytin cDNA. This observation concurs with the fact that ERV3 gene sequences are located on human chromosome 7 and share homology to BaEV (Cohen *et al,* 1985; Van der Kuyl *et al,* 1995b). These previous studies strengthen the postulate that ERV3 *env*-like proteins demonstrated in this study may play a role in syncytia formation among spermatogenic cells, and or spermatogenic cellular differentiation. It's also possible that the ERV3 *env*- like proteins expressed share homology with BaEV gene products leading to cross-reactivity with anti-ERV3 *env* antibodies.

The expression of ERV3 *env* antigen on the epididymal spermatozoa demonstrated in this study is also striking. This molecule may play a role in sperm-zona binding during fertilization. This argument is based on virus envelope fusogenic properties, as demonstration of such properties in recent studies have shown (Mi *et al,* 2000). In addition, possibilities for existence of ERV 3 *env* receptors on the oocyte cannot be ruled out. The viral envelope protein has properties that enable the virus to bind a specific receptor on a target cell, and so mediate viral entry. The same properties could mediate in spermatozoon-oocyte fusion, entry of the sperm nucleus into the oocyte and eventual fertilization.

Fertilization is a complex process requiring the spermatozoon to undergo a cascade of events including capacitation, acrosome reaction, binding to zona pellucida (ZP), penetration through ZP, and fusion with plasma membrane of the oocyte. The sperm-zona pellucida binding is a species-specific step of the fertilization process, and the molecules involved in this event constitute attractive candidates for the contraceptive vaccine development (Naz, 2000). Although the glycoprotein composition of ZP from several mammalian species has been well elucidated (Wassarman, 1988; Chamberlain and Dean, 1990), there is a limited information regarding molecular identities and biochemical characteristics of the sperm surface molecules involved in ZP binding (Naz and Ahmad, 1994; Naz *et al,* 1995; Naz, 1996). A few of the sperm antigens have been isolated and characterized. Notable among these are lactate dehydrogenase C_4 (LDH-C_4) (Goldberg,

1986), PH-20 (Primakoff *et al,* 1988), SP-10 (Herr *et al,* 1990), fertilization antigen 1 and 2 (Naz *et al,* 1993) among others. ZP3 seems to be the primary molecule involved in sperm recognition and binding (Naz, 2000). Endogenous retroviral sequences expressed in the testes could be having a reproductive role of synchronizing activities of the seminiferous tubule epithelia through syncytia formation, while those expressed on spermatozoa could be having a role in fertilization on the basis of viral envelope fusiogenic properties. This may form part of the molecules to be investigated for possible immunocontraceptive vaccine development.

In this study, reverse transcriptase activity was demonstrated in baboon seminal plasma. In addition baboon spermatozoa from ejaculum and epididymis demonstrated some RTase activity, implying an association between sperm cells and some retrovirus-like RTase activity. Earlier studies have also demonstrated reverse transcriptase activity within the reproductive tract of mice (Kiessling, 1984). These observations suggest activation of ERV sequences in mouse and baboon epididymidis. But whether RTase activity is ubiquitous in the baboon epididymis or is accompanied by retroviral particle formation cannot be established from this finding. However, we can attribute the RTase activity to a possible existence of ERVs sequences since the animals were screened and found negative for SIV and STLV prior to this study. In addition, this study demonstrated ERV3 *env*-like proteins on spermatozoa within the epididymis, which can be associated with other viral genes such as the *gag,* and *pol*, although these were not demonstrated in this study. Reverse transcriptase enzyme is a product of *pol* gene expression, and hence it

is possible that ERV-associated endogenous RTase exists in the epididymis and spermatozoa.

Retroviral particles have most often been detected in humans and other species in an environment associated with steroid hormones such as placenta, testis, adrenal and sebaceous glands (Taruscio and Mantovani, 1998). It is tempting to speculate that ERV expression is regulated through changes in the levels of steroid hormones. In fact, it has been shown that the LTRs of HERV-R (ERV3) contain two potential hormone-responsive elements (Larsson *et al,* 1989). Similarly, the LTRs of HERV-K contain one of these elements, and steroid hormones have been shown to regulate HERV-K mRNA expression (Larsson *et al,* 1989). Since the testis is an active site of androgen hormone synthesis and action, and ERV3 *env* has been demonstrated in it, the association between androgen activity and ERV expression is likely. Also, the expression of ERV3 *env* as demonstrated in this study appears to be associated with sexually mature baboons but not with juveniles. This leads to speculation for a steroid-hormone dependency in expression pattern, and restriction to sexually mature animals.

Another observation was that the cauda epididymis express ERV3 *env*-like particles within its epithelia. The cauda epididymis has a vital role in concentrating and storing spermatozoa awaiting coitus and eventual ejaculation (Dym, 1983; Fawcett, 1986; Sharpe, 1994). The role of retroviral antigens has not been documented, but a possible role in genetic feedback mechanism from somatic to germ cells has been speculated

(Rothenfluh, 1995; Kiessling *et al,* 1989; Taruscio and Mantovani, 1998). The cauda epididymis could be a site for ERV3 *env* synthesis. But whether this is ubiquitous or has a role in enhancing fertilizing ability of spermatozoa or concentration of the same cannot be concluded from this data.

The observation made in this study support previous data showing ERV-like activity in mouse epididymis (Kiessling, 1984) where RTase activity was demonstrated in fluid from the epididymis and in which viral aggregates were observed (Kiessling *et al,* 1987,1989). In this study RTase activity associated with seminal plasma has been demonstrated, and a relationship with the expression of ERV 3 in the cauda epididymis is possible.

Molecular characterization demonstrated some estimated 49, 58 and 80 kDa proteins in seminal fluid, a 58 kDa protein in sperm pellet from ejaculum and a 32 kDa protein from epididymal sperm. Spermatozoa acquire maturity as they transit the epididymis. Changes in the composition of the sperm membrane during transit through the epididymis are induced by exposure of the sperm to specific proteins in the intraluminal microenvironment. It is known that luminal proteins bind to the surface of sperm during epididymal transit (Orgebin-Crist *et al,* 1981; Young *et al,* 1985, 1987) and these proteins effect sperm maturation (Eddy, 1988; Cooper, 1990).

Polypeptide variances in luminal content in different regions of the monkey (*Macaca mulatta*) epididymis have been reported (Arova *et al,* 1975; Arslan *et al,* 1986). Previous independent studies by Isahakia (1989) and Young *et al*, (1994) identified an 82 kDa secretory protein in caput epididymis of the yellow baboon (*Papio cynocephalus*) and Chimpanzee *(Pan troglotydes)* respectively. Androgen induced proteins have also been described in isolated human epididymal tissue (Tezon *et al,* 1985; Ross *et al,* 1990). Evidence in the chimpanzee suggests that the 27 kDa component secreted into the epididymal lumen may affect sperm motility and fertilizing capacity (Young *et al,* 1987; Gould and Young, 1990). In human, a major protein of molecular weight of approximately 27 kDa has been identified in cauda but not in the caput epididymis (Ross *et al,* 1990). Wango (1983), and Wango and Gombe (1995) have also demonstrated the presence of an androgen binding protein in the cauda epididymis but not in the caput and corpus epididymides of dogs. In the monkey (*Macaca mulatta*), an androgen-dependent protein of molecular weight of 27 kDa has been identified in caput, corpus and cauda epididymal fluids (Arslan *et al,* 1986). Whether the accumulation of 49, 58 and 80 kDa protein in baboon seminal fluid result from its secretion in the caput, corpus and/or cauda epididymis cannot be concluded from this study. However, It is speculated that the 80kDa protein is secreted in the epididymis, and could be related to the 82kDa protein identified in baboon caput epididymis (Isahakia, 1989). Previous studies have demonstrated an 80kDa protein on the head region of human and rat spermatozoa, in the testes and epididymis (Bandivdekar *et al,* 2001). This protein is documented to have caused total infertility in male and female rats on immunization. The 58 kDa protein

identified in ejaculate spermatozoa may belong to the major sperm surface proteins that bind to the zona pellucida. This protein is of molecular weight 51+-2kDa, referred to as fertilization antigen 1 identified in the bull (Coonrod *et al,* 1994), rhesus monkey and humans (Naz *et al,* 1984; 1986). A 32 kDa protein isolated from epididymal spermatozoa could play a role in enhancing sperm motility during transit through the epididymis, and may be related to the 27 kDa polypeptide identified as a prominent component of cauda luminal fluid from a variety of species. However, its possible function in initiation of motility during sperm maturation has been suggested only for the mouse (Flickinger *et al,* 1988) and for the chimpanzee (Young *et al,* 1987; Gould and Young, 1990; Young *et al,* 1994). The molecular structure and function of other sperm molecules in primates need to be investigated. This will aid in identifying a unique molecule of high potency and efficacy, leading to a sure prevention of fertilization. Development of a vaccine based on sperm antigens represents a promising approach to contraception (Naz *et al,* 1995). However, only those antigens that are sperm-specific can be employed for immunocontraception.

Conclusion

The expression of virus-like gene products in male baboon testis and epididymis has been demonstrated in this study, and it has been shown that there is association between spermatogenic cells undergoing differentiation and some endogenous virus proteins (ERV3 *env*). We therefore postulate that the virus genome demonstrated in this study could be having a role in maintaining the syncytia that exists between spermatogenic cells that originate from the same stem spermatogonium. This in effect synchronizes the physiological and chemical changes occurring in the cells from the same stem cell. Endogenous retroviral-like molecules in cauda epididymis epithelium could be important in spermatozoa maturation before ejaculation. For epididymal spermatozoa, the expression of ERV3 *env*-like molecules on the acrosome could be important in sperm-egg binding that leads to penetration of zona pellucida and/or egg cell membrane. However, more data still need to be generated from further studies.

Recommendation for future studies

In this study, a limited number of antibodies against HIV, SIV and ERVs were available. Concerted efforts should be made to generate specific antibodies against retroviral-like proteins isolated from baboon spermatozoa. This approach will enhance further understanding of biochemical properties and functions of sperm-associated retroviral-like proteins. Such information will be valuable in immunocontraceptive development as vaccines for male contraception provide an attractive method for family planning.

CHAPTER FIVE

5. REFERENCE AND APPENDICES

5.1.Reference

Abraham, G. N. and Khan, A.S. (1990). Human endogenous retroviruses and immune disease (Review). *Clinical immunology and immunopathology,* **56(1):**1-8.

Altmann, J; Altmann, S. and Hausfater, G.(1981). Physical maturation and age estimates of yellow baboons, *Papio cynocephalus,* in Amboseli National Park, Kenya. *American journal of primatology,***1:**389-399.

Arova, R; Dinaker, N. and Prasad, M. R. N. (1975). Biochemical changes in the spermatozoa and luminal contents of different regions of the epididymis of the rhesus monkey, *Macaca mulatta. Contraception,* **11:**689-700.

Arslan, M; Haider, M. Z. and Qazi, M. H. (1986). Characterization and androgen dependence of specific proteins in the epididymis of adult rhesus monkey. *Archives of Andrology,* **16:**67-74.

Baltimore, D. (1985). Retroviruses and retrotransposons: the role of reverse transcription in shaping the eukaryotic genome. *Cell,* **40:**481-482.

Bandivdekar, A. H; Vernekar, V. J; Moodbidri, S. B. and Koide, S. S. (2001). Characterization of 80 kDa Human Sperm Antigen Responsible for Immunoinfertility. *American Journal of Reproductive Immunology*, **45:**28-34.

Barr, A. B. (1973). Timing of spermatogenesis in four non-human primate species. *Fertility and Sterility*, **24:**381-389.

Benit, L; Lallemand, J; Casella, J; Philippe, H. and Heidmann, T. (1999). ERV-L elements: a family of endogenous retrovirus-like elements active throughout the evolution of mammals. *Journal of Virology*, **73 (4):**3301-3308.

Benoist, C. and Malthis, D. (1997). Autoimmune diabetes. Retrovirus as trigger, precipitator or marker? *Nature*, **388(6645):**833-834.

Benveniste, R. E; Lieber, M. M; Livingston, D. M; Sher, C. J. and Todaro, G. J. (1974). Infectious C-type virus isolated from a baboon placenta. *Nature*, **248:**17-20.

Benveniste, R. E. and Todaro, G. J. (1974). Evolution of type-C viral genes: inheritance of exogenously acquired viral genes. *Nature*, **252:**456-459.

Benveniste, R. E. and Todaro, G. J. (1976). Evolution of type-C viral genes: evidence for an Asian origin of man. *Nature*, **261:**101-108.

Berkhout, B; Jebbink, M. and Zsiros, J. (1999). Identification of an active reverse transcriptase enzyme encoded by a human endogenous HERV-K retrovirus. *Journal of Virology,* **73(3):**2365-2375.

Best, S; Le Tissier, P. R. and Stoye, J. P. (1997). Endogenous retroviruses and the evolution of resistance to retroviral infection. *Trends in microbiology,* **5:**313-318.

Best, S; Le Tissier, P; Towers, G. and Stoye, J. P. (1996). Positional cloning of the mouse retrovirus restriction gene Fv 1. *Nature,* **382(6594):**826-829.

Blond, J. L; Beseme, F; Duret, L; Borton, O; Bedin, F; Perron, H; Mandrand, B. and Mallet, F. (1999). Molecular characterization and placental expression of HERV-W, a new human endogenous retrovirus family. *Journal of Virology,* **73:**1175-1185.

Boller, K; Frank, H; Lower, J; Lower, R. and Kurth, R. (1983). Structural organization of unique retrovirus-like particles budding from human teratocarcinoma cell-lines. *Journal of General Virology*, **64:**2549-2559.

Boller, K; Konig, H; Sauter, M; Mueller-Lantzsch, N; Lower, R; Lower, J. and Kurth, R. (1993). Evidence that HERV-K is the endogenous retrovirus sequence that codes for the human teratocarcinoma-derived retrovirus HTDV. *Virology,* **196:**349-353.

Brodsky, I; Foley, B. and Gillespie, D. (1993a). Expression of endogenous human retroviruses (HERV)-K in chronic myeloid leukemia. *Leukemia and lymphoma,* **11:** 119-123.

Brodsky, I; Foley, B; Haires, D; Johnston, J; Cuddy, K. and Gillespie, D. (1993b). Expression of HERV-K proviruses in human leukocytes. *Blood,* **81:** 2369-2374.

Bronson, D. L; Saxinger, W. C; Ritzi, D. M. and Fraley, E. E. (1984). Production of Virions with retroviral morphology by human embryonal carcinoma cell *in vitro. Journal of General Virology,* **65:**1043-1051.

Callard, G. V.(1991). Testicular functions. In, Kinne R. K. H. (ed). Oogenesis, spermatogenesis and reproduction. Publ. Karger A. G, Switzerland, **Vol. 10**, pp105-12.

Casau, A. E; Vaughan, J. E; Lozano, G. and Levine, A. J. (1999). Germ cell expression of an isolated human endogenous retroviral long terminal repeats of the HERV-K/HTDV family in transgenic mice. *Journal of Virology,* **73(2):** 9976-9983.

Chamberlain, M. E. and Dean, J. (1990). Human homologue of the mouse sperm receptor. *Proceeding of the National Academy of Science, U.S.A,* **87:** 6014 –6018.

Chowdhury, A. K. and Steinberger, E. (1976). A study of germ cell morphology and duration of spermatogenic cycle in the baboon, *Papio anubis*. *Anatomical Records,* **185;**155-169.

Chowdhury, A.K; Marshall, G. and Steinberger, E. (1977). Comparative aspects of spermatogenesis in man and baboon (*Papio anubis)*. *Proceedings of the Fourth International Symposium on Comparative Biology of Reproduction: Reproduction and Evolution,* Australian academy of Sciences, Canberra, Australia, 143-150.

Coelho, A. (1985). Baboon dimorphism: Growth in weight, length and adiposity from birth to 8 years of age. In, Watts, E. (ed)*Non Human Primate Models for Human Growth and Development*. Liss, Inc. A. R; pages, 125-259.

Coffin, J. (1984). Endogenous viruses. In: Weiss, R; Teich, N; Vermus, H; Coffin, J. (eds): RNA tumor viruses (second ed.) *New York: Cold Habour Press,* 1109-1203.

Coffin, J. M. (1996). Retrovirus restriction revealed. *Nature,* **382(6594):**762-763.

Cohen, M; Rein, A; Stephens, R.M; O'Connell, C; Gilden, R.V; Shure, M; Nicolson, M.O; Mc Allister, R.M. and Davidson, N. (1981). Baboon endogenous virus genome: Molecular Cloning and Structural characterization of non-defective viral genomes from DNA of a baboon cell strain. *Proceedings of the National Academy of Science, USA*, **78:**5207-5211.

Cohen, M; Powers, M; O'Connell, C. and Kato, N. (1985). The nucleotide sequence of the *env* gene from the human provirus ERV3, and isolation and characterization of an ERV3-specific cDNA. *Virology*, **147:**449-458.

Conrad, B; Weissmahr, R. N; Boni, J; Arcari, R; Schupbach, J. and Mach, B. (1997). A human endogenous retroviral superantigen as candidate autoimmune gene in type 1 diabetes. *Cell*, **90:**303-313.

Coonrod, S. A; Westhusin, M. and Naz, R. K. (1994). Monoclonal antibody to human fertilization antigen 1 (FA-1) inhibits bovine fertilization *in vitro* : Application in immunocontraception. *Biology of Reproduction*,**51:**14-23.

Cooper, T. G. (1990). Secretory proteins from the epididymis and their clinical relevance. *Andrologia*, **22:**155-165.

Courot, M; Hochereau-de-Reviers, M. and Ortavant, R. (1970). Spermatogenesis. In, Johnson, Gomes, Vandemark (eds). *The testes: Volume I, development, anatomy and physiology*. Pp 339-441.

De Robertis, E. D. P. and De Robertis ,Jr. E. M. F. (1988). Nucleic acids. In, De Robertis, E. D. P. and De Robertis, Jr. E. M. F.(eds). Cell and molecular biology, eighth edition. Pp 29.

Doolittle, R. F; Feng, D. F; Mc Clure, M. A. and Johnson, M. S. (1990). Retrovirus phylogeny and evolution. *Current Topics in Microbiology and Immunology,* **157:**3-4.

Dunwiddie, C.T; Resnick, R; Boyce-Jacino, M; Alegre, J.N. and Faras, A.J. (1986). Molecular Cloning and characterization of *gag-*, *pol-*, and *env* - related gene sequences in the chicken. *Journal of Virology*, **59:** 669-675.

Dupressoir, A. and Heidmann, T. (1996). Germ line-specific expression of intracisternal A-particle retrotransposons in transgenic mice. *Molecular and Cellular Biology*, **16(8):**4495-4503.

Dym, M. (1983). The male reproductive system. In, Weiss Leon, *Histology: Cell and Tissue Biology,* fifth edition, Elsevier Science publishing co. (1983), Pp 1000-1053.

Eddy, E. M. (1988). The spermatozoon. In, Knobil, E; Neil, J. *et al,* (eds). The physiology of Reproduction. New York, Raven press.

Eley, R. M. and Bambra, C. S. (1993). Basic reproductive biology of the baboon. In, Bambra, C. S.(Ed) *Veterinary management and research techniques for reproductive studies in the baboon: A practical approach,* Pp. 29-39.

Emerman, M; Panganiban, A.T. and Temin, H.H. (1985). Insertion of genes into retrovirus genomes and of retrovirus DNA into cell genomes. *Progress in Medical Virology*, **32:** 174-188.

Fawcett, D. W. (1986). Male reproductive system. In, Bloom and Fawcett, (eds): *A textbook of Histology, eleventh edition,* W. B. Saunders company (Publ.) 1986, pp. 796-850.

Feuchter, A. and Mager, D. (1992). SV 40 large T antigen transactivates the long terminal repeats of a large family of human endogenous retrovirus-like sequences. *Virology,* **187:**242-250.

Flickinger, C. J; Herr, J. C. and Klotz, K. L. (1988). Immunocytochemical localization of the major glycoprotein of epididymal fluid from the cauda in the epithelium of the mouse epididymis. *Cell and Tissue Research,* **251:** 603-610.

Focko, F. G. (1992). Cell surface actions of steroids: A complementary mechanism for regulation of spermatogenesis? In, Nieschlag, E. and Habenicht, U. (Ed.) Spermatogenesis, fertilization-Contraception. Molecular, cellular and endocrine events in male reproduction. *Proceedings of Schering Foundation Workshop-4.* Springer-Verlag, Pp-4.

Goff, S. P. (1996). Operating under a *Gag,* order: A block against incoming virus by the Fv 1 gene (Review). *Cell,* **86(5):**691-693.

Goldberg, E. (1986). Sperm specific lactate dehydrogenase and development of a contraceptive vaccine. *In, Reproductive Immunology,* D. A. Clark, B. A. Croy (eds). New York, Elsevier Press, **Pp.** 137-142.

Gould, K. G. and Young, L. G. (1990). Acquisition of fertilizing capacity by chimpanzee sperm. *Folia Primatologia*, **54:**105-108.

Griswold, M. D; Morales, C. and Sylvester, S. R. (1988). Molecular biology of the Sertoli cell. *Oxford Review in Reproductive Biology,* **10:** 124-161.

Gross, L. (1997). The role of viruses in the etiology of cancer and leukemia in animals and in humans. *Proceedings of the National Academy of Science, USA,* **94(9):**4237-4238.

Gross, L; Schidlovsky, G; Feldman, D; Dreyfuss, Y. and Moore, L.A. (1975). C-type virus particles in placenta of normal healthy Sprague-Dawley rats. *Proceedings of the National Academy of Science, USA,* **72:**3240-3244.

Hara, I; Izui, S. and Dixion, F. J. (1982). Murine serum glycoprotein gp70 behaves as an acute phase reactant. *Journal of Experimental Medicine,* **155(2):**345-357.

Hendrickx, A. G. (1971). Embryology of the baboon. University of Chicago press, Chicago, USA.

Herbst, H; Sauter, M. and Mueller-Lartzsch, N. (1996). Expression of endogenous retrovirus-k elements in germ cells and trophoblastic tumors. *American Journal of Pathology,* **149:**1727-1735.

Herbst, H; Sauter, M; Kuhler Obbarius, C; Loning, T. and Mueller-Lartzsch, N. (1998). Human endogenous retrovirus (HERV)-K transcripts in germ cell and trophoblastic tumors. *APMIS* **106:** 216-220.

Herr, J. C; Flickinger, C. J; Homyk, M; Klotz, K. and John, E. C. (1990). Biochemical and morphological characterization of intra-acrosomal antigen SP-10 from human sperm. *Biology of Reproduction*, **42:**181-189.

Huang, L; Silberman, J; Rothschild, H. and Cohen, J. C. (1989). Replication of endogenous virus in human cells (kinetics of DNA synthesis and integration). *Journal of Biological Chemistry,* **261(15):** 8811-8814.

Ikeda, H. and Sugimura, H. (1989). Fv-4 resistance gene: a truncated endogenous murine leukemia virus with ecotropic interference properties. *Journal of Virology*, **63:**5405-5412.

Indraccolo, S; Gunzburg, W. H; Leib-Mosch, C; Erfle, V. and Salmons, B. (1995). Identification of three human sequences with viral superantigen–specific primers. *Mammalian Genome,* **6(5):**339-344.

Isahakia, M. A. (1989). Monoclonal antibody localization of sperm surface antigen secreted by the epididymis of the baboon (*Papio cynocephalus*). *Journal of Reproduction and Fertility,* **86:**51-58.

Jaenisch, R. (1983). Endogenous retroviruses. *Cell,* **32:**5-6.

Jerabek, L.B; Mellors, R.C; Elkon, K.B. and Mellors, J.W. (1984). Detection and immunochemical characterization of a primate type C retrovirus related p30 protein in normal human placentas. *Proceedings of the National Academy of Science, USA*, **81:**6901-6505.

Johnson, M. and Everitt, B. (eds) (1980). Cytodifferentiation of spermatozoa. In, essential reproduction, Blackwell scientific publications. Pp44.

Johnson, P. M; Lyden, T.W. and Mwenda, J..M.(1990). Endogenous retroviral expression in the human placenta. *American Journal of Reproductive Immunology*, **23:**115-120.

Kalter, S. S; Heberling, R. L; Smith, G. C. and Helmke, R. J. (1975). C-type viruses in chimpanzee (*Pan troglodytes*) placentas. *Journal of National Cancer Institute,* **55:**735

Kato, N; Pfeifer-Ohlsson, S; Kato, M; Larsson, E; Rydnert, J; Ohlsson, R. and Cohen, M. (1987). Tissue specific expression of human provirus ERV3 mRNA in human placenta: Two of the three ERV3 mRNAs contain human cellular sequences. *Journal of Virology,* **61:**2182-2191.

Kiessling, A.A (1984). Evidence that reverse transcriptase is a component of murine epididymal fluid (41859). *Proceedings for the Society of experimental Biology and Medicine,* **176:**175-182.

Kiessling, A. A; Crowell, R. and O'Connell R. S. (1987). Sperm-associated retroviruses in the mouse epididymis. *Proceedings of the National Academy of Science, USA,* **84:**8667-8671.

Kiessling, A. A; Crowell, R. and Fox, C. (1989). Epididymis is the principal site of retroviral expression in the mouse. *Proceedings of the National Academy of Science, USA*, **86:**5109-5114.

Knossl, M; Lower, R; and Lower, J. (1999). Expression of the human endogenous retrovirus HTDV/HERV-K is enhanced by cellular transcription factor YY1. *Journal of virology*, **73(2):**1254-1261.

Kretser, D. M. (1990). Hormonal control of spermatogenesis. In, Austin, C. R. and Short, R. V. (eds). *Reproduction in mammals, Book 3: Hormonal control of reproduction.* Second edition, Cambridge university press, pp76.

Krieg, A. M; Gause, W. C; Gourley, M. F. and Steinberg, A. D. (1989). A role for endogenous retrovirus sequences in the regulation of lymphocyte activation. *Journal of Immunology*, **143(8):**2448-2451.

Krieg, A. M. and Steinberg, A. D. (1990). Retroviruses and autoimmunity (Review). *Journal of Autoimmunity*, **3 (2):**137-166.

Laemmli, E. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227:**680-685.

Langat, D. K; Johnson, P. M; Rote, N. S; Wango, E. O; Owiti, G. O. and Mwenda, J. M. (1998). Immunohistochemical localization of retroviral-related antigens expressed in normal baboon placental villous tissue. *Journal of Medical Primatology,* **27:**278-386.

Langat, D.K; Johnson, P. M; Rote, N. S; Wango, E. O; Owiti, G. O; Isahakia, M. A. and Mwenda, J. M. (1999). Characterization of antigens expressed in normal baboon trophoblast and cross-reactive with HIV/SIV antibodies. *Journal of Reproductive Immunology,* **42:**41-58.

Larsson, E; Kato, N. and Cohen, M. (1989). Human endogenous proviruses. *Current Topics in Microbiology and Immunology,* **148:**115-132.

Larsson, E; Anderson, A; Holmberg, L. and Cohen, M. (1993). Expression of endogenous retrovirus (HERV-R) in human tissues. *Journal of Cancer Research and Clinical Oncology,* **199:** 56.

Larsson, E; Anderson, A. and Nilsson, O. (1994). Expression of endogenous retrovirus (ERV3, HERV-R) in human reproductive and embryonic tissues-evidence for a function for envelope gene products. *Uppsala Journal of Medical Science,* **99:** 113-120.

Leib-Mosch, C; Brack-Werner, R; Werner, T; Bachmann, M; Faff, O; Erfle, V. and Hehlmann, R. (1990). Endogenous retroviral elements in human DNA. *Cancer Research (Supplement)* **50:**5636s – 5650s.

Leib-Mosch, C; Haltmeier, M; Werner,T; Geigl, E; Brack-Werner, R; Francke, U; Erfle, V. and Hehlmann, R. (1993). Genomic distribution and transcription of solitary HERV-K LTRs. *Genomics*, **18:**261-269.

Lewin, R. (1983). How mammalian RNA returns to its genome. Work with certain small nuclear RNAs is giving clues about the apparently common flow of information from RNA back to DNA. *Science,* **219:**1052-1054.

Lin, L; Xu, B. and Rote, N. S. (1999). Expression of endogenous retrovirus ERV 3 induces differentiation in BeWo, a choriocarcinoma model of human placental trophoblast. *Placenta,* **20:**109-118.

Lin, L; Xu, B. and Rote, N. S. (2000). The cellular mechanism by which the human endogenous retrovirus ERV-3 *env* gene affects proliferation and differentiation in a human placental trophoblast model, BeWo. *Placenta,* **21:**73-78.

Lower, R; Boller, K; Hasenmaier, B; Korbmacher, C; Muller-Lantzsch, N; Lower, J. and Kurth, R. (1993). Identification of endogenous retroviruses with complex mRNA expression and particle formation. *Proceedings of the National Academy of Science, USA*, **90:**4480-4484.

Lower, R; Lower, J. and Kurth, R. (1996). The viruses in all of us: Characteristics and biological significance of human endogenous retrovirus sequences. *Proceedings of the National Academy of Science, USA*, **93:**5177-5184.

Magin, C; Lower, R. and Lower, J.(1999). cORF and RcRE, the Rev/Rex and RRE/RxRE homologous of the human endogenous retrovirus family HTDV/HERV-K. *Journal of Virology*, **73(11):** 9496-9507.

Marrack, P; Kushnir, E. and Kappler, J. (1991). A maternally inherited superantigen encoded by a mammary tumor virus. *Nature,* **349:**524-526.

Martin, M.A; Bryan, T; Rasheed, S. and Khan, A.S. (1981). Identification and cloning of endogenous retroviral sequences present in human DNA. *Proceedings of the National Academy of Science,USA;* **78:** 4892-4896.

Mayer, J; Sauter, M; Racz,, A; Scherer, D; Mueller-Lantzsch, N. and Meese, E. C. (1999). An almost–intact human endogenous retrovirus K on human chromosome 7. *Nature and Genetics,* **21:**257-258.

Mc Donald, J.F. (1993). Evolution and consequences of transposable elements. *Current Opinions in Genetic Development,* **3:**855-864.

Mi, S; Lee, X; Li, X; Veldman, G. M; Finnerty, H; Racie, L; Lavallie, E; Tang, X; Edouard, P; Howes, S; Keith, J. C. and Mc Coy, J. M. (2000). Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature,* **403:**785-789.

Miki, Y; Nishisho, I; Horii, A; Miyoshi, Y; Utsunomiya, J; Kinzler, K; Vogelstein, B. and Nakamura, Y.(1992). Disruption of the APC gene by a retrotransposal insertion of L1 sequence in a colon cancer. *Cancer Research,* **52:** 643-645.

Morse, B; Rotherg, P; Spandorfer, J. and Astrin, S. (1988). Insertion mutagenesis of the *myc* locus by a LINE-1 sequence in a human breast carcinoma. *Nature*, **333:**87-90.

Nakagawa, K. and Harrison, L.C. (1996). The potential roles of endogenous retroviruses in autoimmunity. *Immunological Reviews*, **152:**193-426.

Napier, J. R. and Napier, P. H. (1985). Baboons. *In, The Natural History of the Primates*. MIT press, Cambridge, Massachusetts. Pp 132-135.

Naz, R. K. (1996). Involvement of protein tyrosine phosphorylation of human sperm in capacitation/acrosome reaction and zona pellucida binding. *Frontiers in Bioscience,* **1:**206-213.

Naz, R. K. (2000). Fertilization-related sperm antigens and their immunocontraceptive potentials. *American Journal of Reproductive Immunology,* **44:**41-46.

Naz, R. K; Alexander, N. J; Isahakia, M. and Hamilton, M.D. (1984). Monoclonal antibody to a human sperm membrane glycoprotein that inhibits fertilization. *Science,* **225:**342-344.

Naz, R. K; Philips, T. M. and Rosenblum, B. B. (1986). Characterization of the fertilization antigen 1 for the development of a contraceptive vaccine. *Proceedings of the National Academy of Scince, USA;* **83:**5713-5717.

Naz, R. K; Morte, C; Garcia-Framiz, V; Kaplan, P. and Marinez, P. (1993). Characterization of a sperm specific monoclonal antibody and isolation of 95 kilodalton fertilization antigens from human sperm. *Biology of Reproduction,* **49:**1236-1244.

Naz, R. K. and Ahmad, K. (1994). Molecular identities of human sperm proteins that bind human zona pellucida: Nature of sperm-zona interaction tyrosine kinase activity and involvement of FA-1. *Molecular Biology of Reproduction and Development,* **39:**397-408.

Naz, R. K; Sacco, A; Singh, O; Pal, R. and Talwar, G. P. (1995). Development of contraceptive vaccines for humans using antigens derived from gametes (spermatozoa and zona pellucida) and hormones (human chorionic gonadotrophin): Current status. *Human Reproduction Update,* **1:**1-18.

Nelson, P. N. (1995). Retroviruses in rheumatic diseases. *Annals of the Rheumatic Diseases,* **54 (6):**441-442.

O'Connell, C. and Cohen, M. (1984). The long terminal repeat sequences of a novel human endogenous retrovirus. *Science*, **226:**1204-1206.

O'Connell, C; O'Brien, S; Nash, W.G. and Cohen, M (1984). ERV-3, A full-length human endogenous provirus: chromosomal localization and evolutionary relationships. *Virology,* **138:**225-235.

Oduor, O. D. (1972). The lymphatic system of the testis of the goat. MSc. Thesis, University of Nairobi.

Ojoo, R. O. (1995). A morphological and endocrinological study of the male reproductive system in the thick-tailed bush baby (*Galago garnetti*). M.Sc. Thesis, University of Nairobi.

Onions, D. (1991). Integration of Viruses into Chromosomal DNA (Review). *Journal of Pathology*, **163(3):**191-197.

Ono, M. (1986). Molecular cloning and long terminal repeat sequences of human endogenous retrovirus genes related to types A and B retrovirus genes. *Journal of Virology*, **58:**937-944.

Onyango, D. W; Wango, E. O; Otiang'a-Owiti, G; Oduor-Okello, D. and Werner, G. (2000). Morphological characterization of the seminiferous cycle in the goat (*Capra hircus*): A histological and ultrastructural study. *Annals of Anatomy,* **182:**1-7.

Orgebin-Crist, M. C; Olson, G. E. and Danzo, B. J. (1981). Factors influencing maturation of spermatozoa in the epididymis. In, Intragonadal regulation of Reproduction, Franchmont, P; Channing, C, (eds). New York Academic press.

Owens, R. J; Turner, C. C; Mulligon, M. J; Srinivas, R. V. and Compans, R. W. (1990). Oligopeptide inhibitors of HIV-induced syncytium formation. *AIDS Research and Human Retroviruses,* **6:**1289-1296.

Pawar, H. S. and Wrobel, K. (1991). Quantitative aspects of Water buffalo (*Bubalus bubalis*) spermatogenesis. *Archives of Histology and cytology,* **54(5):**491-501.

Primakoff, P; Lathropo, W; Woolman, L; Cowan, A. and Myles, D. (1988). Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20. *Nature,* **335:**543-547.

Rabin, H; Benton, C. V; Tainsky, M. A; Rice, N. R. and Gilden, R. V. (1979). Isolation and characterization of an endogenous type C virus of rhesus monkey. *Science,* **204:**841-842.

Rasmussen, H. B. C. (1997). Interactions between exogenous and endogenous retroviruses. *Journal of Biomedical Science,* **4(1):**1-8.

Rasmussen, H. H; Perron, H. and Clausen, J. (1993). Do endogenous retroviruses have etiological implications in inflammatory and degenerative nervous disease?. *Acta Neurological Society of Scandinavia.* **88:** 190-198.

Rasmussen, H. B; Geny, C; Deforges, L; Perron, H; Tourtelotte, W; Heltberg, A. and Claussen, J. (1995). Expression of endogenous retroviruses in blood mononuclear cells and brain tissue from multiple sclerosis patients. *Multiple Sclerosis*, **1:**82-87.

Ritzen, E. M; Hansson, V. and French, F. S. (1989). The Sertoli cell; In Burger, de Kretser (eds). *The Testis*, 2nd edition, Pp. 269-302 (Raven Press, New York, 1989).

Roelofs, H; Ruud, J. H. M. van Gurp; Oosterhuis, J. W. and Looijenga, H. J. (1998). Detection of Human endogenous retrovirus type K-specific transcripts in testicular parenchyma and testicular germ cell tumors of adolescents and adults. *American Journal of Pathology*, **153:**1277-1282.

Roosen-Runge, E. C. (1977). The process of spermatogenesis in animals. Testis I, Pp. 1-214 (Cambridge University Press)

Ross, P; Kan, F. W; Antake, P; Vigneault, N; Chapdelaine, A. and Roberts, K. D. (1990). Protein synthesis and secretion in human epididymis and immunoreactivity with sperm antibodies. *Molecular Biology of Reproduction and Development*, **26:**12-23.

Rothenfluh, H. S. (1995). Hypothesis: a memory lymphocyte-specific soma-to-germ line genetic feedback loop. *Immunology and Cell Biology*, **73:**174-180.

Russel, L. D. and Peterson, R. N. (1985). Sertoli cell junctions: morphological and functional correlates. *International Review of Cytology*. **94:**177-211.

Sauter, M; Schommer, S; Kremmer, E; Remberger, K; Dolken, G. and Lemm, I. (1995). Human endogenous retrovirus K10: Expression of *gag* protein and detection of antibodies in patients with seminomas. *Journal of Virology* ,**69:**414-421.

Sauter, M; Roemer, K; Best, B; Afting, M; Schommer, S; Seitz, G; Hartmann, M. and Mueller-Lantsch, N.(1996). Specificity of antibodies directed against *env* protein of human endogenous retroviruses in patients with germ cell tumors. *Cancer Research,* **56:**4362-4365.

Schulze, W. and Rehder, U. (1984). Organization and Morphogenesis of the human seminiferous epithelium. *Cell and Tissue Research,* **237:**395-407.

Schutle, A.M; Lai, S; Kurtz, A; Czubayko, F; Riegel, A.T. and Wellstein, A. (1996). Human trophoblast and choriocarcinoma expression of the growth factor pleiotrophin attributable to germ line insertion of an endogenous retrovirus. *Proceedings of the National Academy of Science, USA,* **93:**14759-14764.

Seidl, C; Donner, H; Peterslofen, E; Usadel, K. H; Seifried, E; Kaltwasser, J.P. and Badenhoop, K. (1999). An endogenous retroviral long terminal repeat at the HLA-DQB1 gene locus confers susceptibility to rheumatoid arthritis. *Human Immunology,* **60 (1):**63-68.

Setchell, B. P. (1982). Spermatogenesis and spermatozoa. In, Austin, C. R. and Short (eds). *Reproduction in mammals*: Book 1, Germ cells and fertilization, pp63.

Sharpe, R. M. (1994). Regulation of spermatogenesis. In, Knobil, E. and Neill, J. D. (editors), *The physiology of reproduction, Vol. 1, second edition, Raven press Ltd,* **Pp.** 1363-1434.

Sherr, C. J; Lieber, M. M; Benveniste, R. E. and Todaro, G. J. (1974). Endogenous baboon type C virus (M7): biochemical and immunologic characterization. *Virology,* **58:**492-502.

Sherwin, S. A. and Todaro, G. J. (1979). A new endogenous primate type C virus isolated from the old world monkey, *Colobus polykomos. Proceedings of the National Academy of Science, USA,* **76:**5041-5045.

Shih, A; Coutavas, E.E. and Rush, M. G. (1991). Evolutionary implications of primate endogenous retroviruses. *Virology,* **182:** 495-502.

Simon, M; Haltmeier, M; Papakonstantinou, G; Werner, T; Hehlmann, R. and Leib-Mosch, C. (1994). Transcription of HERV-K related LTRs in human placenta and leukemic cells. *Leukemia* **(Supplement 1):** S12-S17.

Simpson, G. R; Patience, C; Lower, R; Tonjes, R. R; Moore, M. D. H; Weiss, R. A. and Boyd, M. T. (1996). Endogenous D-type (HERV-K) related sequences are packaged into retroviral particles in the placenta and possess open reading frames for reverse transcriptase. *Virology*, **222:** 451-456.

Skinner, M. K. (1991). Cell-cell interactions in the testis. *Endocrine Reviews,* **12(1):** 45-61.

Smithwick, E. B; Young, L. G. and Gould, K. G. (1996). Duration of spermatogenesis and relative frequency of each stage in the seminiferous epithelial cycle of the chimpanzee. *Tissue and Cell,* **28(3):**357-66.

Steinhuber, S; Brack, M; Hunsmann, G; Schwelberger, H; Dierich, M. and Vogesteder, W. (1995). Distribution of human endogenous retrovirus HERV-K genomes in humans and different primates. *Human Genetics,* **96:** 188-192.

Stoye, J. P. and Coffin, J. M. (2000). A provirus put to work. *Nature,* **403:**715-717.

Stromberg, K. and Benveniste, R. (1983). Efficient isolation of endogenous rhesus retroviruses from trophoblast. *Virology,* **128:** 518-523.

Suleman, M. A. (1993). Anesthesia and restraint. In, Bambra, C. S.(Ed), *Veterinary management and research techniques for reproductive studies in the baboon: A practical approach,* Pp15-17.

Takeuchi, K; Katsumata, K; Ikeda, H; Minami, M; Wakisaka, A. and Yoshiki, T. (1995). Expression of endogenous retroviruses, ERV3 and lambda 4-1, in synovial tissues from patients with rheumatoid arthritis. *Clinical and Experimental Immunology,* **99(3):**338-344.

Talal, N; Flescher, E. and Dang H. (1992). Are endogenous retroviruses involved in human autoimmune disease? *Journal of Autoimmunity,* 5 **(Supplement A):** 61-66.

Taruscio, T. and Mantovani, A. (1998). Human endogenous retroviral sequences: Possible roles in reproductive physiopathology. *Biology of Reproduction*, **59:**713-724.

Tassabehji, M; Strachan, T; Anderson, M; Campbell, R. D; Collier, S. and Laka, M. (1994). Identification of a novel family of human endogenous retroviruses and characterization of one family member, HERV-K (C4), located in the complete C4 gene cluster. *Nucleic Acid Research*, **22:**5211-5217.

Tezon, J. E; Vazquez, M. H; Pineiro, L; De Leriment, M. A. and Blaquier, J. A. (1985). Identification of androgen-induced proteins in human epididymis. *Biology of Reproduction*, **32:**584-590.

Todaro, G. J; Sher, C. J. and Benveniste, R. E. (1976). Baboons and their close relatives are unusual among primates in their ability to release non-defective type C viruses. *Virology,* **72:** 278-282.

Todaro, G. J; Sher, C. J; Benveniste, R. E; Sherwin, S. A. and Sherr, C. J. (1978). MAC-1, a new genetically transmitted type C virus of primates: "Low frequency activation from stump tail monkey cell cultures". *Cell,* **13:**775-782.

Urnovitz, H. B. and Murphy, W. H. (1996). Human endogenous retroviruses: Nature, occurrence and clinical implications in human diseases. *Clinical Microbiology Reviews,* **9:**72-99.

Van der Kuyl, A. C; Dekker, J. T. and Goudsmit, J. (1995a). Distribution of baboon endogenous virus among species of African monkeys suggests multiple ancient cross-species transmission in shared habitats. *Journal of Virology,* **69 (12):** 7877-7887.

Van der Kuyl, A. C; Dekker, J. T. and Goudsmit, J. (1995b). Full-length proviruses of baboon endogenous virus (BaEV) and dispersed BaEV reverse transcriptase retroelements in the genome of baboon species. *Journal of virology,* **69:**5917-5924.

Van der Kuyl, A.C; Mang, R.; Dekker, J.T. and Goudsmit, J. (1997). Complete nucleotide sequence of simian endogenous type D retrovirus with intact genome organization. Evidence for ancestry to simian retrovirus and baboon endogenous virus. *Journal of Virology,* **71:**3666-3676.

Varmus, H. (1988) Retroviruses. *Science,* **240:**1427-1435.

Varmus, H. and Swanstrom, R. (1984). In, RNA tumor viruses (Weiss, R; Teich, N; Varmus, H; Coffin, J. eds) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Wango, E. O. (1983). Immunological block to spermatogenesis and disruption of cauda epididymal epithelium by antibodies to cellular androgen binding antigens from the cauda epididymis of the dog. *MSc. Thesis University of Nairobi.*

Wango, E. O. and Gombe, S. (1995). The effect of immunizing dogs against an androgen binding cauda epididymal antigen (CABA). *Discoveries and Innovations,* **7:**265-275.

Wassarman, P. M. (1988). Zona pellucida glycoproteins. *Annual Reviews in Biochemistry*, **57:** 415-442.

Watts, E. S. (1985). Adolescent growth and development of monkeys, apes and humans. In Watts (ed.) *Non Human Primate Models for Human Growth and Development*. Liss, A. R; Inc; Pp. 41-65.

Weinberg, R. A. (1980). Origin and roles of endogenous retroviruses. *Cell*, **22:**643-644.

Wilkinson, D. A; Mager, D. L. and Leong, J. C. (1994). Endogenous human retroviruses. In: Levy, J (ed). *Retroviridae*. Plenum press, volume III. New York, N. Y.

Wrobel, K. H. and Pawar, H. S. (1992). Quantitative morphology of the testicular tubular epithelium in water buffalo (*Bubalus bubalis*). *Andrologia*, **24**:63-68.

Young, L. G; Hinton, B. T; Smithwick, E. B. and Gould, K. G. (1994). Sodium, potassium and protein concentrations and 2D-SDS-page of epididymal luminal and ejaculated seminal fluids of the adult chimpanzee (*Pan troglodytes*). *American Journal of Primatology*, **34(3):** 249-259.

Young, L. G; Hinton, B. T. and Gould, K. G. (1985). Surface changes in chimpanzee sperm during epididymal transit. *Biology of Reproduction*, **32**:399-412.

Young, L. G; Hinton, B. T. and Gould, K. G. (1987). Changes in the binding of a 27 kilodalton chimpanzee cauda epididymal glycoprotein component to chimpanzee sperm. *Gamete Research*, **18:**163-178.

Zhu, Z; Hsieh, S; Bentley, D. R; Campbell, D. R. and Volanakis, J. E. (1992). A variable number of tandem repeats locus within the human complement C2 gene is associated with a retroposon derived from a human endogenous retrovirus. *Journal of Experimental Medicine*, **175:**1783-1787.

5.2. APPENDICES

Appendix I

Preparation of slides for frozen sections

1. Slides were placed on the carriage (rack) and washed in xylene bath. The carriage was then shook well onto paper towels to blot of the excess xylene.

2. This was followed by dipping the slides in alcohol bath and the carriage shook well onto paper towels.

3. The carriage was then dipped in distilled water and slides left to dry in 37 ^{o}C cabinet

4. A 0.5% solution of gelatin in distilled water was prepared with gentle heating to then the dried slides in 3. a bove were dipped in this solution.

5. The slides were left to dry then dipped in formal saline (in chemical hood)

Recipe: Formaldehyde (40% technical) 10 ml, NaCl 0.85g, add distilled water to 100ml.

6. Then dried.

NOTE

Xylene and formalin are toxic. Steps 1-3 and 5 were performed in the chemical hood. After shaking off excess liquid onto the paper towels, the towels were left to dry in the hood before disposing of.

Appendix II

Ingredients of Tissue- Tek ® O. C. T. compound

10.24% w/w polyvinyl alcohol

4.26% w/w polyethylene glycol

85.50% w/w non-reactive ingredients

(From Miles Inc. Diagnostics Division Elkhart, IN 46515 USA)

Note:

The Tissue- Tek ® O. C. T. compound is used to bind tissue to the specimen block. It is frozen to optimum cutting temperature in the cryostat before sectioning.

Appendix III

Preparation of 10x PBS

NaCl	80gm
KCl	2gm
$Na_2HPO_4.12H_2O$	29gm (11.5 anhydrous)
KH_2PO_4	2gm
DDH_2O	Make to 1 liter

Appendix IV

Stock solutions for SDS-PAGE

Running gel buffer (L-buffer)

1.5 M Tris Base 18.2g

10% SDS 4mls

Add distilled water to 100mls and pH 8.8

Stacking gel buffer (M-buffer)

0.5M Tris Base 6g

10% SDS 4mls

Add distilled water to 100mls and pH 6.8

Acrylamide/Bis acrylamide stock

30% acryl amide

0.8% Bis acryl amide

Distilled water

Solubilisation buffer

2% SDS

0.0625M Tris Base

10% glycerol

Distilled water, sterilize and filter

Add 2-5% β-mercaptoethanol (add fresh to small aliquots) and Bromophenol blue to colour.

Running (Tank) buffer

0.025M Tris Base

0.192M glycine

0.1% SDS

Distilled water

Coomassie Brilliant Blue 250 stain

0.5% w/v Coomassie Brilliant Blue 250

45% v/v Ethanol

10% v/v Glacial acetic acid

Distilled water (filter through No. 2 Whatman paper)

Destaining solution	**Transfer buffer**
45% v/v ethanol	0.3% w/v Tris Base
10% v/v glacial acetic acid	1.4% w/v Glycine
Distilled water	20% v/v Methanol
	Distilled water.

Appendix V

Features used in estimating the age of animals

Identification number----------

1. Weight----------
2. Hair colour----------
3. Facial skin texture: smooth/ wrinkled----------
4. Facial colour----------
5. Dental pattern: number of teeth----------

Canine ridge eruption----------

6. Testicular descend----------
7. Scrotal colour----------
8. Testis size: length/ breadth----------
9. Date of euthanasia----------

Appendix VI

Bio- rad protein assay

The bio-rad protein assay is a dye-binding assay based on the differential colour change of a dye in response to various concentration of proteins. The kit reagents included:

Dye reagent concentrate (Coomassie brilliant blue G250, phosphoric acid and methanol).

Protein standard (lyophilized preparations of bovine plasma albumin [BSA]).

Micro assay procedure

(1-20µg protein, <25µg/ml)

Several dilutions of protein standard (BSA) containing from 1-25µg/ml were prepared. A standard curve was prepared each time the assay was performed. 0.8ml of standards, appropriately diluted were placed in clean dry test-tubes, and 0.8ml of sample buffer placed in a 'blank' test-tube. To each tube 0.2ml of dye reagent concentrate was added and vortexed gently to mix. After a period of 5 minutes to one hour, the optical density was measured at 595nm versus the reagent blank. The optical density was plotted against the concentration of standards. The unknowns were read from the standard curve.

Appendix VII

Data for standard curve of molecular weight standards used in determining the molecular weights of samples.

Mr. (kDa) of band (standard)	**Distance migrated (cm)**	**Relative mobility (Rf value)**
200	1.6	0.232
116.25	2.2	0.319
97.4	2.9	0.420
66.2	3.5	0.507
42.699	4.3	0.623
31	4.7	0.681
21.5	6.3	0.913
Dye front	6.9	

Appendix VIII

Olive Baboons (*Papio anubis*) used in this study

Animal number	**Weight (kg)**
Pan 2222	22.5
Pan 2377	24.8
Pan 1669	21.7
Pan 2473	22.2
Pan 2273	5.7
Pan 2242	5.7