IMMUNOHISTOCHEMICAL AND BIOCHEMICAL CHARACTERIZATION OF RETROVIRAL-RELATED ANTIGENS EXPRESSED IN NORMAL BABOON PLACENTA AND OTHER TISSUES.

BY

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DECLARATION.

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

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DEDICATION.

This thesis is dedicated to my Parents.

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TABLE OF CONTENTS. **PAGE**

LIST OF FIGURES. **PAGE**

LIST OF TABLES. **PAGE**

ABSTRACT.

The genomes of all eukaryotes contain multiple copies of DNA sequences that are related to sequences found in infectious retroviruses . Endogenous retroviruses (ERVs) are generally non-pathogenic except in mice where they have been found to induce tumours and immunological disorders. The ERVs have morphological features consistent with type-C retroviral particles and are expressed in normal placental tissues in most mammals. They have antigenic similarity with exogenous retroviruses such as HIV-1 and may have a role in the regulation of cellular gene expression, syncytiotrophoblast formation or pregnancy-related immunosuppression.

Baboon endogenous virus (BaEV) is the only endogenous retrovirus so far which has been shown to be infective *in vitro*. The entire nucleotide sequence of BaEV has been determined. It has been shown to have a chimeric genomic structure of about 8 kb long. BaEV particle expression in placental tissues has been demonstrated using electron microscopy. However, to date, very little work has been done to evaluate the expression of retroviral-related antigens in normal baboon tissues. In this project, a panel of antibodies (polyclonal and monoclonal antibodies) against viral proteins (anti-HIV and anti-SIV) were assessed for their cross-reactivity (using immunohistochemistry) with normal placental and other baboon tissues. Retroviral particles were further characterized by immunoblotting and ELISA. Mouse polyclonal antibodies were produced against isolated baboon placental ERV particles and were also characterized as described above.

Some of the antibodies (anti-HIV-1 gp41, anti-HIV-2 gp120, anti-HIV-1 RT, and anti-BERV) displayed specific immunoperoxidase staining on placental syncytiotrophoblast and cross-reacted with exogenous retroviral proteins on immunoblot analyses. Four monoclonal antibodies (anti-ERV3 *env*, anti-HERV-K RT, and HIV-1 gp120) consistently recognized three retroviral cross-reactive proteins of molecular weights 38, 58 and 64 kDa present on placental tissues. Minimal reverse transcriptase (RTase) activity associated with sucrose gradient-purified placental retroviral-like particles was also demonstrated. These studies confirm the specific expression of endogenous retroviral particles in placental villous tissue and suggest retroviral proteins may play an immunomodulatory role at the maternal-fetal interface.

CHAPTER ONE.

INTRODUCTION AND REVIEW OF LITERATURE.

1.1. INTRODUCTION.

Endogenous retroviral sequences have been identified in the genomes of most vertebrate species including rodents, humans and non-human primates such as the baboons and monkeys (Kalter *et al*, 1975; Coffin *et al*, 1984). The expression of retroviruses and/or retroviral-related sequences in humans has been detected in reproductive tissues particularly in the placenta (Larsson *et al*, 1989; Leib-Mosch *et al*, 1990; Wilkinson *et al*, 1994). Electron microscopic studies have described the presence of retroviral-like particles in thin sections of human and non-human primate placental chorionic villous tissues (Kalter *et al*, 1973; Schidlovsky & Ahmed, 1973; Benveniste *et al*, 1974; Kalter *et al*, 1975; Seman *et al*, 1975). These reports suggest ERV particle expression in placental syncytiotrophoblast maybe associated with cytotrophoblast depletion. Recent evidence suggest that ERVs could be involved not only in important normal cellular processes like differentiation and specialization but also in tumorigenesis and immunomodulation (Kato *et al*, 1987). However, correlation between the expression of ERVs and any biological function is yet to be elucidated.

To date, very little work has been done to evaluate the expression of retroviral-related antigens in normal baboon placental tissues. The baboon endogenous virus (BaEV) offers a good opportunity to gain an insight in endogenous virus evolution, and ERV virus expression in primate tissues, and possibly study the possible role of ERVs in primates. BaEV is a type C retrovirus originally isolated from baboon placenta by co-cultivation with a human rhabdomyosarcoma cell line (A204). It is unique in that it is the only endogenous retrovirus so far that has been shown to be infectious (Benveniste *et al*, 1974) and able to replicate in cultured cell lines of several human species. The entire nucleotide sequence of BaEV has been determined. It was shown to be about 8 kb long, and the genomic structure is chimeric, the *gag-pol* region closely related to that of the simian type C retrovirus and the *env* gene of a type D retrovirus (Tamura *et al*, 1981; Kato *et al*, 1987). Intact virus genomes of BaEV are present in *Papionini* monkeys (except the macaques) and in four sub-species of *Cercopithecus aethiops* (Van der Kuyl *et al*, 1995a) and

have been reported to be widespread in all species of African primates (Van der Kuyl *et al*, 1995b) and many species of Asian primates (Shih *et al*, 1991) and even in mediterranean cat species (Benveniste & Todaro, 1974), suggesting the virus is ancient. However, evaluation of tissue expression of BaEV and other ERV gene products in primate tissues has not been performed. In this project, a panel of antibodies (polyclonal and monoclonal antibodies against retroviral proteins (anti-HIV and anti-SIV) and human endogenous retroviral particles (anti-HERVs)) were used to characterize the expression of ERV particles in the baboon placental and other tissues. Murine polyclonal antibodies were raised against isolated ERV particles from baboon placental villous tissues and assessed for their reactivity using immunohistochemistry, western blot and ELISA. The reagents will aid in elucidating the possible role of these ERV particles in prevention of fetal allograft rejection.

1.2. LITERATURE REVIEW.

1.2.1. *Origin of endogenous retroviral particles.*

Retroviridae is a family of retroviruses which have ribonucleic acid (RNA) as their genetic material. The term *retrovirus* refers to the unique replicative strategy of this RNA virus family, a feature of which is the characteristic enzyme, reverse transcriptase (RTase, RNA-directed DNA polymerase). This enzyme functions to back-transcribe the viral RNA genome into a double-stranded DNA insertion element that can then integrate into the host cell DNA. Once integrated, the viral DNA genome (provirus) may be expressed immediately or be carried unexpressed in the host cell through the normal cellular growth and division.

A retrovirus is termed *exogenous* when viral integration in somatic cells is followed by the expression and production of infectious viral particles that may be transmitted horizontally. When a latent infection occurs in the germ cells of an organism, the virus can subsequently be passed benignly to every cell in the next generation. Viral elements carried vertically in the germ line are termed *endogenous*, and may produce infectious, non-infectious, or most commonly no particles at all (Levy, 1986).

The genomes of all eukaryotes contain multiple copies of DNA sequences that are related to sequences found in infectious retroviruses (Coffin, 1984). These elements are transmitted through the germline as stable mendelian genes, yet they

exhibit structural and sequence similarities to infectious exogenous retroviruses. It is these similarities that have led investigators to speculate that endogenous retroviruses are remnants of prior infections with exogenous retroviral agents and, with evolutionary time, changes have occurred to make them no longer infectious or pathogenic. These speculations have been supported with experimental studies that show that the genomes of infectious exogenous retroviruses can integrate into the host chromosome and be inherited through the germ line (Wilkinson *et al*, 1994).

1.2.2. *Structure and classification of retroviruses.*

The basic retrovirus genome organization consists of a tight array of genes that can be sub-divided into the *gag*, *pol* and *env*, as well as the 3' and 5' long terminal repeat (LTR) regions.

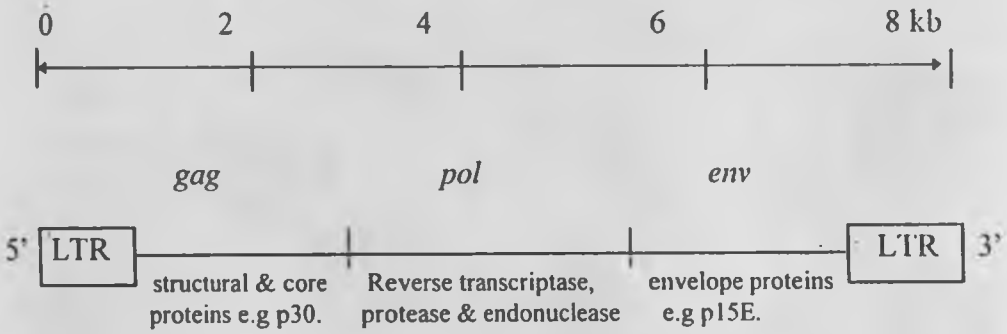


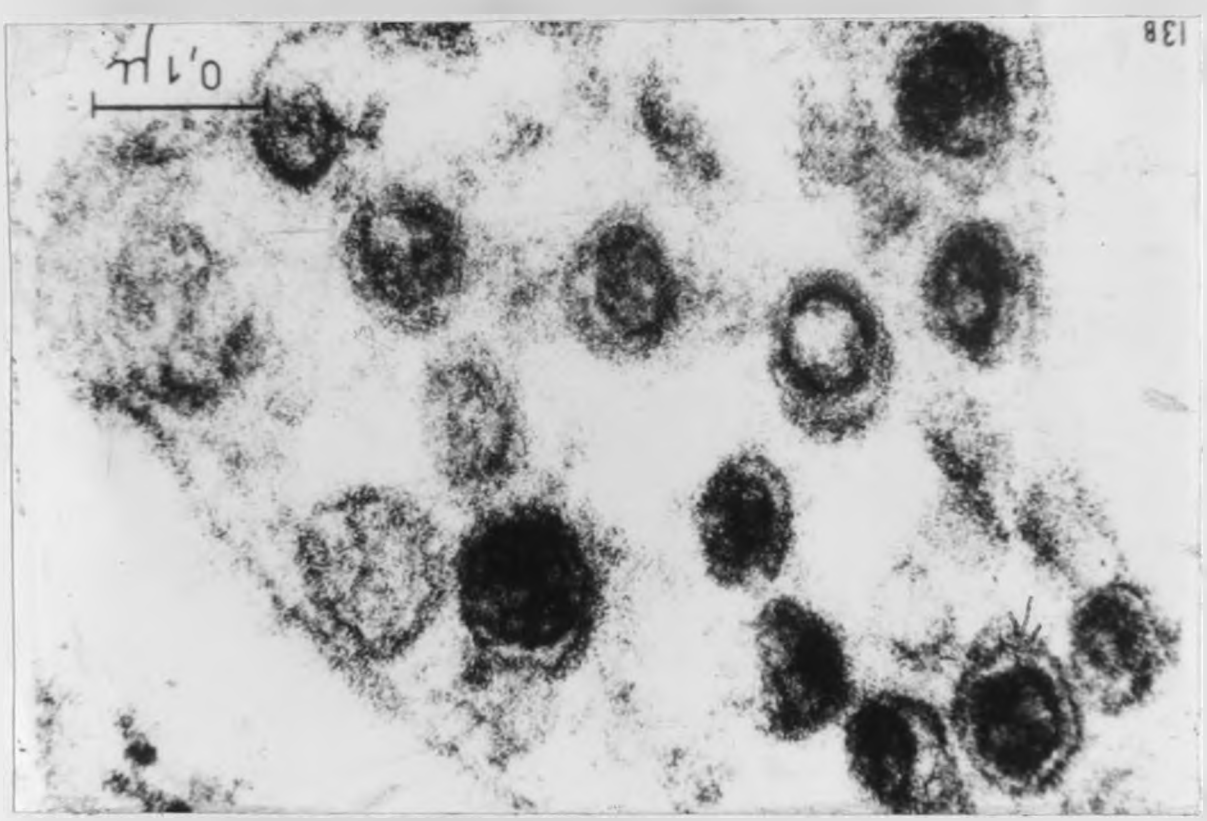
Figure 1:- Schematic representation of a typical integrated endogenous provirus.

The LTRs contain signal and regulatory sequences, many of which may be specific to the particular virus family, and are essential for viral expression and integration. The *gag* region includes genes for structural and matrix proteins that function in the packaging of the viral genome into the final nucleocapsid particle. The *env* region encodes one or more surface glycoproteins and transmembrane proteins that may have viral cell recognition or other biological roles. The *pol* region contains sequences encoding the replication-related enzymes, notably RTase as well as an endonuclease and a protease.

Viral particles can be classified on the basis of morphological characteristics observed on the electron microscope. There are four general classes of viral

particles: types A-D (Bernard, 1960; Fine & Schochetman, 1978). Type A particles are non-budding immature intra-cellular or intra-cisternal forms of type B which themselves are budding or free particles with an eccentric nucleoid and a double membrane. Type C are also budding particles, but with a centered nucleoid, whereas type D particles have a cylindrical nucleoid. Retroviruses that have type C ultrastructural features represent the largest group, where assembly and budding occur simultaneously at the plasma membrane. Both type B and type D retroviruses assemble immature intra-cytoplasmic A-type particles (ICAPs) in the cytoplasm which then migrate to the plasma membrane and are released by budding. In this way, the processes of assembly and budding are unlinked (Sommerfelt *et al*, 1993).

Figure 2 :- Ultra-thin sections of human first trimester syncytiotrophoblast.
Arrow indicates the type-C viral particles expressed.



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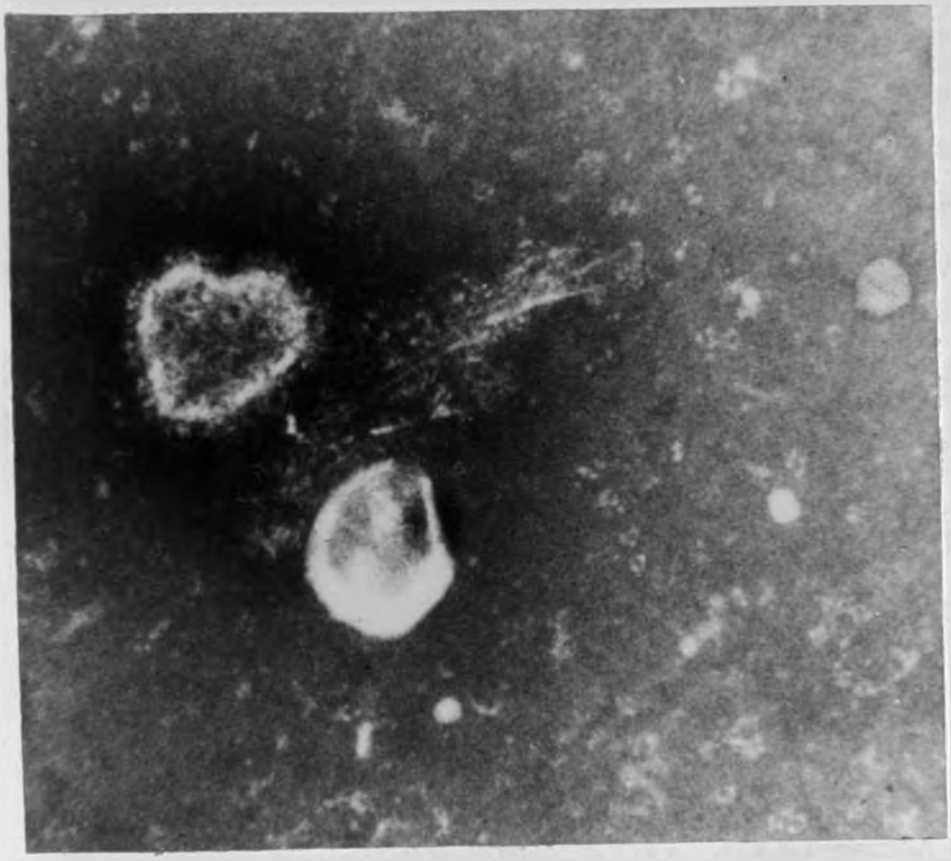
Electron microscopic studies on isolated ERV particles from first and second trimester baboon placental villous tissues have shown presence of particles with features similar to type-C retroviral particles (Figure 3).

Figure 3 : Electron micrograph of type-C virus particle isolated from baboon placental villous tissue.



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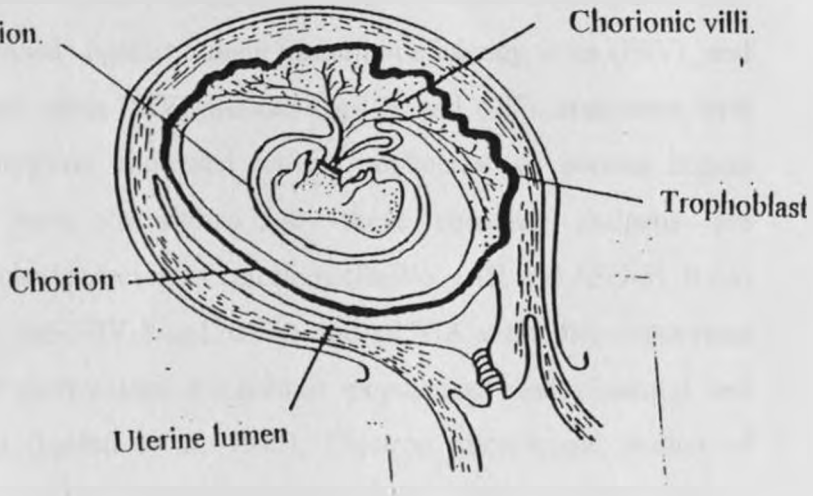
Included among such features is the presence of a surface fringe at the edge of a membrane-bound particle with a diameter of approximately 120 nm. This particle contains an inner core particle that is semi-icosahedral and has a diameter of approximately 90 nm. These features have been found to be consistent between all samples and gestational ages (both first and third trimester) (Lyden *et al*, 1994b).

1.2.3. *Development of the primate placenta.*

The syncytiotrophoblast of the placenta is produced through a process of cellular differentiation in the underlying fetal cytotrophoblast layer. These cells undergo progressive changes during transition to a morphologic and functional intermediate cell which finally undergoes an intercellular fusion event to form a syncytium (Hamilton & Boyd,1960; Yoshido,1964; Enders,1965; Boyd & Hamilton, 1966; Wynn,1972; Wynn,1975; Kurman *et al*, 1984). The structural features of baboon and human early developmental stages are very similar (Hendrickx, 1971; Enders & Schlafke,1986). Although the morphological aspects of primate trophoblast differentiation process have been extensively documented at the ultra-structural level, the exact molecular nature of the cell fusion event or the mechanisms involved have not been clearly defined (Lyden *et al*, 1994a). It has been proposed that ERV particles maybe involved in the formation of the syncytiotrophoblast by virtue of the fusogenic properties of the retroviral glycoproteins (Wilkinson *et al*, 1994).

Figure 4: Schematic representation of the cross-section of the primate pregnant uterus showing the feto-maternal interface.



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1.2.4. ***Expression of ERV particles in the normal placental villous tissues.***

Particles resembling complete retroviruses have been observed in normal human placental and mammary carcinoma tissues. In the human and other primate placental chorionic villi, viral particles have been observed budding from or closely associated with the basal aspect of the placental syncytiotrophoblast (Kalter *et al*, 1973; Dalton *et al*, 1974; Kalter *et al*, 1975; Seman *et al*, 1975; Stromberg & Benveniste, 1983). In addition, endogenous retroviral (ERV) proteins or particles have been observed budding from a variety of human cells including teratocarcinomas, oocytes and sperm (Schidlovsky and Ahmed, 1973; Witkin *et al*, 1975; Witkin & Bendich, 1977; Panem & Reynolds, 1977; Mondel & Hofschneider, 1982; Kiessling *et al*, 1989). Fetal erythroblasts and fibroblasts in primates express type C ERV particles (Smith *et al*, 1977) and these have been isolated from normal human amniotic fluid (Mondel, 1977). Furthermore, these ERV particles, which exhibit ultra-structural features consistent with type C retroviral particles and specific reverse transcriptase (RT) activity have been isolated from normal human placental villous tissues (Mwenda,1994).

Antigens that are cross-reactive with antibodies against exogenous retroviral particles and ERV transcripts have recently been demonstrated in normal human placentas and cell lines (Lyden *et al*, 1994a) These investigators also showed that antibodies raised against human immuno-deficiency virus (HIV) and simian immuno-deficiency virus (SIV) proteins (gp120 and p17) cross-react with endogenous placental antigens expressed within trophoblast of normal human placenta. These studies have also shown that these retroviral antigens are expressed in uninfected choriocarcinoma cell lines (BeWo, JAR and JEG-3). It has also been observed that anti-HIV-1 gp120/160 and p17/18 antibodies cross-react with both mononuclear extravillous trophoblast populations and placental bed trophoblastic giant cells (Lyden *et al*, 1995). Electron microscopic studies of placental ERV particle isolates that displayed retroviral RTase activity and a buoyant density consistent with type C retroviruses (1.17 g/ml on sucrose) indicated the presence of particles with characteristic retroviral ultra-structural features (Lyden *et al*, 1994b). These results suggest that human placental endogenous retroviral proteins may have antigenic similarity with exogenous HIV and that expression of these particles is a normal feature of trophoblast differentiation.

Recent reports have compared exogenous and endogenous retroviral gene regulation and expression in the context of cellular development (Flamant *et al*, 1987; La Mantia *et al*, 1991; Zeichner *et al*, 1992). These studies showed that specific normal developmental cells are selectively permissible to retroviral expression. Retroviral permissiveness is clearly suggested by the developmental context of the ERV particle expression and by a number of previous reports that have indicated various exogenous retroviral and ERV cross-reactivities with placental proteins (Johnson *et al*, 1990; Faulke & Labarrere, 1991). Such cross-reactivity to placental tissues has been observed using antibodies to RD-14 (a feline ERV), BaEV (a baboon ERV), SSAV (a simian sarcoma virus), GaLV (a gippon ape leukemia virus) and HIV-1. Although some of these reports have speculated on the nature of this reactivity, none of the studies have directly addressed this antigen expression in the context of trophoblast development and differentiation (Lyden *et al*, 1994a).

A variety of human endogenous retrovirus sequences (HERVs) have been well-characterized. They all seem to be defective due to multiple termination codons, deletions or the lack of a 5' LTR (Larsson *et al*, 1989; Leib-Mosch *et al*, 1990). Replication-competent human endogenous retrovirus genomes have not yet been isolated. HERVs are grouped into single and multiple copy number families and are classified according to the tRNA they use as a primer for reverse transcription. Thus a retroviral RNA that carries a primer binding site homologous to the 3' end of a lysine tRNA is called HERV-K (Larsson *et al*, 1989; Leib-Mosch *et al*, 1990). Although HERVs were previously only known to be defective genomes unable to code for viral particles, recent reports have shown evidence of HERV-K mRNAs in some cell lines. Lower *et al* (1993), using northern blot analyses, demonstrated the expression of HERV-K mRNAs in human teratocarcinoma cell lines but not in other cell lines. Reverse transcriptase polymerase chain reaction (RT PCR) studies by the same investigators suggest however, that HERV-K may be expressed in many, if not all human cells at levels too low to be detectable in northern blots. It can be speculated that a cellular factor or factors may regulate the synthesis of HERV-K mRNAs depending on the cell type or the state of differentiation. It has also been demonstrated that the human endogenous retroviral element K10 (HERV-K10) gene contains a long open reading frame and encodes a

full length *gag* homologous 73 kDa protein and a functional protease (Mueller-Lantzsch *et al*, 1993). In this context, it should be remembered that other retroid elements [ERV9 (La Mantia *et al*, 1991) and RTVL-H (Wilkinson *et al*, 1990; Lower *et al*, 1993) are also expressed preferentially in human teratocarcinoma cells. Northern blot analysis of RNA from various human cell lines and tissues has demonstrated that elements belonging to the RTVL-H family of human endogenous retroviral sequences (retrovirus-like element with a primer binding site like that of human histidine tRNA, also called HERV-H) are expressed in several cell types (Wilkinson *et al*, 1990). The highest levels of RTVL-H related RNAs were observed in the human teratocarcinoma cell line NTera2DL1, HeL cells (human cervical carcinoma cell line), two bladder carcinoma cell lines and normal amniotic tissues. The same investigators also found that RTVL-H expression varied among the samples of amnion and chorion tissues from different individuals. These findings demonstrate that regulated autonomous expression of RTVL-H sequences occur in human cells.

It has been suggested that HERV-K codes for human terato-carcinoma-derived viruses (HTDVs) (Kurth *et al*, 1980). The HTDV particles have been observed in human teratocarcinoma cells by electron microscopy. The fact that the HTDV particles are stained by an anti-HERV-K *gag* anti-serum in immuno-electron microscopy supports this assumption. Antibodies raised against recombinant *gag* protein of HERV-K react specifically with HTDV particles and HTDV-producing cells (Boller *et al*, 1993). It should be noted that the HERV-K sequence is the only known human endogenous retroviral sequence with no major deletions or stop codons in its structural genes, including the *gag* gene (Lower *et al*, 1993; Mueller-Lantzsch *et al*, 1993). It has also been demonstrated that the *env* gene products are expressed in the human placenta (Kitamura *et al*, 1994). Anti-serum raised against recombinant *env* protein detected substances in syncytiotrophoblasts and vascular endothelia in human term placenta, and immuno-blot analysis revealed that this anti-serum reacted with a protein of 38 kDa in the placenta. These findings suggest that the human placental syncytiotrophoblasts and vascular endothelia preferentially express a molecule encoded by a human endogenous retrovirus sequence.

One of the endogenous retroviral sequences which have been studied in detail is ERV3 (HERV-R). ERV3 is full length provirus that also has a strong

homology to both BaEV and CH2 sequences. It has been mapped on to the long arm of chromosome 7. This retroviral genome contains an open reading frame (ORF) throughout the *env* gene but has in-frame termination codons in the *gag* and *pol* genes, which should preclude the expression of the virus as particles but may produce typical retroviral proteins especially the *env*-encoded p15E and gp70 (O'Conell *et al*, 1984). It has been shown to be expressed in most human tissues except in choriocarcinomas (Kato *et al*, 1987). *In situ* hybridization studies have shown that ERV3 *env* is expressed not only in the placenta but also in other human reproductive tissues such as the testis, embryonic tissues and trophoblastic tumours of ovarian origin (Larsson *et al*, 1994). The expression was mainly associated with syncytiotrophoblasts, and this data demonstrates that it is probably the fusion process itself and not the fertilization which correlates with ERV3 *env* expression.

1.2.5. Possible biological functions of ERV gene products.

i). Formation of syncytiotrophoblast.

As indicated earlier, syncytiotrophoblast is produced through a process of cellular differentiation in the underlying fetal cytotrophoblast layer. It has been postulated that HERV-encoded *env* proteins may play a role in the creation of the syncytiotrophoblast layer in the placenta since retroviral envelope proteins have fusogenic properties (Harris,1991). The recent finding of a putative HERV-R *env* protein expression at this site (Larsson *et al*, 1993) is particularly intriguing in this regard. ERV3 *env* transcripts have been found to contain sequences that encode the trans-membrane protein p15E (which has immunosuppressive properties) and the envelope glycoprotein gp70 (which has fusogenic capacity) (O'Conell *et al*, 1984). The correlation between *env* expression and cell fusion has been shown to exist in the *in vitro* differentiated trophoblast when isolated trophoblasts from term placentas are allowed to mature *in vitro* and fuse to form syncytiotrophoblasts (Boyd *et al*, 1993). In this model systems, *env* expression increases in proportion to the degree of cell fusion. Retroviral cross-reactive antigen expression has also been detected in *in vitro* trophoblastic differentiation (Lyden *et al*, 1995). These investigators found that there was a temporal correlation between the trophoblast differentiation-associated intercellular fusion and the production/movement of the retrovirus cross-reactive structures, and postulated that these could be the fusion-related ERV epitopes.

However, conclusive experimental evidence for the role of ERVs in syncytiotrophoblast formation is still lacking.

ii). Immunomodulatory role at the feto-maternal interface.

The conceptus expresses both maternally and paternally derived histocompatibility leucocyte (HLA) antigens during development (Sutton *et al*, 1983; Hunt *et al*, 1988). Thus the conceptus can be viewed as an allograft, expressing antigens foreign to the mother, yet in most cases there is no rejection of the fetus although a pregnant woman is immunologically competent. It has been suggested that ERV gene products may function in the suppression of the immune reaction at the feto-maternal interface. Recent studies suggest that endogenous proviruses such as ERV9 may encode functional immuno-suppressive proteins (Lindeskog *et al*, 1993). The retroviral trans-membrane envelope protein p15E has been found to be immunosuppressive in that it inhibits the immune responses of lymphocytes, monocytes and macrophages. A region of p15E has been conserved among murine and feline retroviruses. In addition, a homologous region is found in the trans-membrane envelope proteins of the human retroviruses HTLV-I and HTLV-II and in a putative envelope protein encoded by a an endogenous C-type human retroviral DNA. A synthetic peptide (CKS-17) synthesized to correspond to this region of homology was found to inhibit the proliferation of interleukin-2 dependent murine cytotoxic T-cells as well as alloantigen-stimulated proliferation of murine and human lymphocytes (Cianciolo *et al*, 1985). In view of this possible role, it can be postulated that modification of or absence of endogenous retroviral sequences might lead to pregnancy-related pathological conditions such as auto-immunity, early pregnancy loss or gestational trophoblastic disease (Mwenda,1994). The ERV gene products may also function in the suppression of class I MHC gene expression that might assist in the survival of the fetal allograft (Bulmer & Johnson, 1985) .The lack of antigen presentation requirements for MHC class I-restricted T-cell responses prevent generation of paternal antigen-directed cell-mediated immunity (Wood, 1994), although human trophoblasts do express HLA-G, a truncated molecule with limited polymorphism whose antigen-presenting capability has not been established (Hunt & Fishback, 1991). It should be noted that that there are other factors that maybe acting in the same manner to protect the fetus from

immune rejection by the mother. One of these factors is the decay accelerating factor (DAF), which appears to be preferentially expressed at the feto-maternal interface during development and may function specifically to inhibit amplification convertases formed at this site either directly or indirectly as a result of maternal complement activation. This molecule may play an important role in protecting the semi-allogenic human conceptus from maternal complement mediated attack (Holler *et al*, 1990)

iii). Pathological conditions associated with endogenous retroviruses.

a). Auto-immunity.

A pathological potential of non-defective endogenous retroviruses has so far only been demonstrated in mice, where they have been shown to be associated with induction of tumours and immunological disorders (Coffin, 1984). Some gene products of murine endogenous retroviruses, but not intact viruses, are involved in the pathogenesis of malignant and non-malignant diseases (Yoshiki *et al*, 1974; Weiss *et al*, 1982; Takeuchi *et al*, 1989). For example, the envelope glycoprotein gp70 of the murine endogenous retrovirus plays a pivotal role as a major self antigen in the pathogenesis of arteritis and glomerulonephritis in mice. Furthermore, gp70 may be involved in inflammatory processes as an acute phase protein (Shigemoto *et al*, 1992).

Circumstantial evidence suggest that ERV expression or non-expression may have a role in the development of certain pathological conditions in humans. For example, antibodies directed against murine, feline or simian retroviral p30 protein reacted with renal glomeruli of patients with systemic lupus erythematosus (SLE) (Mellors & Mellors, 1976). Rucheton *et al* (1985) further reported the presence of circulating antibodies against *gag* gene products of murine leukemia virus in patients with SLE, rheumatoid arthritis (RA) or mixed connective tissue disease. ERV3 and Lambda-4-1 have been shown to be expressed in synoviocytes and peripheral blood mononuclear cells (PBMC) in patients suffering from RA (Takeuchi, 1994). ERVs have been implicated in the pathology and immune response of Sjogren's syndrome (Shattles *et al*, 1992). Two characteristic antigens recognized by auto-antibodies in SLE and Sjogren's syndrome have been found to share homology and cross-reactivity with retroviral *gag* proteins (Talal *et al*, 1990;

Shattles *et al*, 1992). HERV-K10 *gag* gene expression and antibodies against *gag* proteins have been detected in patients with seminomas (Sauters *et al*, 1995). Some investigators have reported that there is an association between human retroviruses (HTLV-I & II, and HIV) and SLE, RA and other rheumatological disorders (Bailer *et al*, 1994) although they did not rule out other exogenous or endogenous retroviruses that may play a role in the initiation and/or promotion of these diseases. These studies suggest that some human type C endogenous retroviruses or even defective retroviral sequences may be involved in the pathogenesis of auto-immune diseases.

b). Malignancy.

On the other hand, the human choriocarcinoma cell lines (BeWo and JAR) and native malignant placental tissues do not express ERV mRNA transcripts such as ERV3 and ERV9 (Kato *et al*, 1988; Mwenda, personal communication). It is not known whether lack of expression of ERV3 is a characteristic feature of trophoblastic malignancy, although this observation may illustrate how endogenous retroviral sequences could alter human gene expression (Cohen *et al*, 1988). This suggests that ERV expression in embryonic tissues may somehow act to prevent malignancy. However, to date, conclusive evidence for a direct role of ERV sequences in human disease has been lacking and recent reports indicate that endogenous proviral nucleotide sequences show considerable homology with human immuno-deficiency virus (HIV). Hence, it has been suggested that similar or identical transcriptional factor(s) maybe involved in the regulation of ERVs and exogenous retroviruses such as HIV (Horwirtz *et al*, 1992; La Mantia *et al*, 1992)

iv). Perinatal transmission of HIV

Perinatal transmission of HIV occurs in 30 to 50 % of infants born to HIV-infected mothers (Katz & Wilfert, 1989). Some studies have suggested that steroid hormone stimulation could influence proviral activation, and placental expression of HIV long terminal repeats (HIV-LTR) could enhance perinatal transmission of HIV. In addition, the high levels of expression observed in the placenta may contribute to the efficient transmission rate of HIV (Furth *et al*, 1990). Placental trophoblast cells have been shown to be susceptible to infection with exogenous retroviruses such as HIV (Zachar *et al*, 1991; Kesson *et al*, 1993). It has also been

shown that choriocarcinoma cell lines can be infected *in vitro* with HIV-1 (Zachar *et al*, 1991). Using choriocarcinoma cell lines (JAR, BeWo), and FD25 (a trophoblast-derived cell line), David *et al* (1995) have shown that the infection can be prevented either by sCD4 or by antibodies to CD4.

Other investigators (Johnson *et al*, 1990; Mwenda,1994) have suggested that ERV particle expression might infact prevent attachment (in placental tissues) of exogenous infective retroviruses such as HIV by receptor interference, and this may explain the relatively moderate transmission of HIV from mother to child. Studies in mice have shown that ERV gene expression could confer resistance to exogenous retroviral infection by receptor interference, as seen in the case the mouse gene Fv-4 which encodes an ERV *env* protein that confers resistance to retroviral infection (Ikeda & Sugimura, 1989), or by other mechanisms such as target deletion as seen in the case of murine ERV related to mammary mouse tumour virus (MMTV) (Golovkina *et al*, 1992). The ERVs may interfere with exogenous retroviral infection by occupying the receptor sites on the CD4 molecules expressed in the placenta. The placenta expresses CD4 molecule which is the receptor for the external glycoprotein gp120 of the virus. The latter two exhibit a typical ligand and receptor interaction. Inhibitors of this interaction, including antibodies to both gp120 and CD4, and the fusion proteins combining the gp120 binding domains of the CD4 molecule with the Fc portion of the human immunoglobulin (CD4 immuno-adhesins) have proven to be effective in preventing HIV infection at least *in vitro* (Mitsuya *et al*, 1990). It is possible that the endogenous retroviral proteins expressed in the placenta act as the inducing signal for the cell surface IgG-Fc receptor (these are known to be expressed after cellular infection by a variety of viruses of the herpes family) (Johnson & Brown, 1981). If so, then it could be postulated that ERV particle expression may help prevent trans-placental transmission of HIV from a sero-positive mother to the fetus.

1.3. RATIONALE AND OBJECTIVES OF THE STUDY.

1.3.1. Rationale.

Ultrastructural studies have demonstrated expression of retroviral particles in the baboon syncytiotrophoblast. However, very little work has been done on the evaluation of expression of retroviral antigens in normal baboon placental tissues. Definitive characterization of endogenous antigen expression has been hindered by the lack of antibody probes. Recent studies have shown antibodies raised against HIV and SIV retroviral proteins (anti-HIV gp120 and anti-SIV p17) specifically react with normal human placental tissues. This reactivity was often cytoplasmic but in many regions became restricted to the apical aspect of the syncytiotrophoblast (Lyden *et al*, 1994a; Mwenda,1994).

Characterization of retroviral antigens in normal baboon placental tissues and other tissues is critical for the elucidation of the possible immuno-modulatory role of these antigens at the feto-maternal interface. This may lead to identification of candidate trophoblast-specific molecules that may be target antigens for the development of birth control (contra-gestational) vaccines. Finally, this study will also contribute to the understanding of the potential normal function of retroviral proteins in protecting the trophoblast from destruction by maternal immune cells such as cytotoxic natural killer cells.

In the present study, a panel of antibodies (polyclonal and monoclonal antibodies against viral proteins (anti-HIV and anti-SIV)) and antibodies produced against isolated intact retroviral particles from normal baboon placentas were assessed for their cross-reactivity (using immunohistochemistry, western blot and ELISA) on normal baboon placental and other tissues. The aim of this study was to provide information on the tissue localization and distribution of endogenous retroviral proteins in normal (SIV -ve) baboons.

1.3.2. Specific objectives.

The aims of this study were as follows:

a) To localize endogenous retroviral particles within baboon placental tissues by immunohistochemistry.
b) Investigate the expression of ERV proteins in non-placental tissues.
c) Determine the biochemical properties of isolated ERV particles and HIV-related antigens expressed in normal baboon placental villous tissues.
d) To produce and characterize antibodies raised against retroviral particles isolated from normal placental villous tissues.
e) Elucidate the possible role of placental ERVs at the feto-maternal interface.

CHAPTER TWO.

MATERIALS AND METHODS.

2.1. MATERIALS.

2.1.1. Baboon placental tissues.

10 female baboons (*Papio anubis*) maintained at the Institute of Primate Research (IPR) were used in the study. They were housed indoors with natural lighting in group cages, fed on commercial monkey cubes (Unga feeds, Kenya), with fruit and vegetable supplementation thrice weekly. Water was provided *ad libitum*. The baboons used in this study were screened for antibodies against SIV (using Genelavia® Mixt HIV 1 & 2 testing kit, Sanofi Diagnostics, UK) as part of the IPR SIV screening project.

The baboon perineal skin gives an indication of of the stage of the cycle. Dramatic changes in the colour and size occur throughout the cycle. The degree of inflation or tumescence in any individual animal indicates the stage of of the cycle she is in (Eley and Bambra, 1993) (see Appendix I). Various studies have shown that ovulation occurs about one to three days prior to deturgescence at mid-cycle (Hendrickx, 1971;Wildt *et al*, 1977; Shaikh *et al*, 1982). Sex skin readings of the female perineal skin were taken daily to determine the stage of the menstrual cycle. These animals were time-mated and a cesarean section performed to recover placental tissues at various gestational stages (first and second trimester, and term placenta). The gestational period of the baboon is approximately 180 ± 5 days (Eley and Bambra, 1993). This period was divided into 3 as follows:

-1st trimester :- Day 1 to Day 56.

-2nd trimester :- Day 57 to Day 113.

-3rd trimester :- Day 114 to term.

At the time of surgery, the placental tissues obtained were placed in a sterile petri dish, stripped of the umbilical cord and the chorionic membrane and cut into small blocks of approximately 2 cm^3 in volume. Half of the tissues were placed in plastic specimen bottles each containing approximately 5 mls of TissueTek OCT compound (Miles Diagnostics, Elkhart, USA) and snap-frozen in liquid nitrogen. They were then removed, labeled appropriately with the date of surgery, gestational age and code number of the animal, then placed in a plastic bag. The rest of the

placental tissues were placed in sterile plastic bags and all were stored at -70°C until required.

2.1.2. Propagation of SIV-infected cells.

Molt-4 Cl_8 cells (derived from human T-cell lymphocytes) which had been frozen in liquid nitrogen were thawed rapidly (by immersing the vials in warm water) and transferred to 15 ml sterile plastic centrifuge tubes. They were then washed 3 times with sterile RPMI 1640 washing medium (Appendix VI). The cells were then resuspended in 5 mls of the growth medium (RPMI 1640) and transferred to 50 ml tissue culture flasks (Greiner Labortechnik, Germany). They were subsequently incubated in an oven at 37°C in a humidified atmosphere of 95 % O_2:5 % CO_2. The cells were fed with fresh medium every two days and their growth monitored daily using an inverted microscope. When the cells were at their exponential growth phase (4 to 5 days after thawing), non-infected cells were pelletted by centrifuging at 20 000 rpm for two hours at 4°C (SW28 rotor, Beckman Instruments, USA) to obtain a negative control pellet. SIV virus supernatant was pipetted into the medium of the remaining cells. The infection of the cells was monitored by observing the cytolytic effect of the SIV virus on the cells. The infected cells were then pelletted by centrifuging at 20 000 rpm for two hours at 4°C (SW28 rotor, Beckman Instruments, USA). Both the SIV +ve and the SIV -ve control pellets were stored at -20°C until required.

2.2. <u>METHODOLOGY.</u>

2.2.1. Isolation of retroviral particles.

A small section of fresh or frozen placental tissue (approximately 1 cm^3) was rinsed in ice-cold TNE buffer (10 mM Tris-HCl, pH 7.8, containing 150 mM sodium chloride (NaCl) and 1 mM Ethylenediamine N,N-tetra-acetic acid (EDTA)) and homogenized using an Ultra-turrax atomizer (IKA-Labortechnik, Germany). The tissue homogenate was then stirred for 30 minutes using a magnetic stirrer and then centrifuged twice for 30 minutes each time at 2000g, (TH-4 rotor, Beckmann Instruments, USA). The debris was discarded and the supernatant centrifuged at 10,000g for 10 minutes at 4°C using an ultra-centrifuge (SW28 rotor, Beckmann Instruments, USA). The debris was again discarded and the

supernatant layered onto 20 % sucrose (2.3 g sucrose in 10 mls TNE buffer (pH 7.8) containing 1 mM protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) followed by centrifugation at 100,000g for 90 minutes at 4°C (SW41 rotor, Beckmann Instruments, USA). The pellet was then resuspended in 200 µl TNE buffer, aliquoted into cryotubes and stored at -20°C. A protein assay was done on the aliquots to determine the protein concentration, using a BioRad protein assay kit (BioRad Laboratories, USA) (Appendix V).

2.2.2. Reverse transcriptase assay.

The isolated retroviral pellets were assayed in duplicates for specific reverse transcriptase (RTase) activity using reverse transcriptase assay (non-radioactive) kit (Boehringer Mannheim, Germany). Briefly, the pellets were resuspended in 40µl lysis buffer (50 mM Tris buffer, containing 80 mM KCl, 2.5 mM Dithiothreitol (DTT), 0.75 mM EDTA and 0.5 % Triton X-100) and the suspension transferred to a fresh reaction tube. This was then incubated at room temperature for 30 minutes to completely solubilize the viral particles.

HIV-1 reverse transcriptase working dilutions used to prepare a calibration curve were prepared as per manufacturer's instructions. Briefly, 10 µl of HIV-1 reverse transcriptase stock solution (2 ng/µl, provided with the kit) was serially diluted in 1:2 dilution steps as described below:-

STEP	HIV-1 RT	Lysis buffer	HIV-1 RT conc(ng/well)
0	0	150 µl	0
1	10 µl (stock)	390 µl	2.0
2	150 µl of step 1	150 µl	1.0
3	150 µl of step 2	150 µl	0.5
4	150 µl of step 3	150 µl	0.25
5	150 µl of step 4	150 µl	0.125
6	150 µl of step 6	150 µl	0.0625

40 µl of each working dilution was transferred into a sterile reaction tube. The sample containing no HIV-1 RT (step 0) serves as a negative control. The dilution were prepared in duplicates.

20 µl of the reaction mixture (50 mM Tris-HCl, 290 mM/litre KCl, 30 mM $MgCl_2$, 10 mM DTT, 10 µM dUTP, and 750 A260nm/ml template/primer hybrid, poly (A).Oligo$(dT)_{15}$) was then added to each reaction tube containing the viral lysates or HIV-1 RT standards. They were then incubated for 1 hour at 37°C.

After the reverse transcriptase reaction, the samples (60 µl) and the HIV-1 RT working dilutions for establishing the calibration curve (60 µl) were transferred into the wells of the micro-titer plate (MTP) modules pre-coated with streptoavidin and post-coated with blocking reagent. The modules were then covered with cover foil and incubated for 1 hour at 37°C. The solutions were then removed completely from the wells and the wells rinsed 5 times for 30 seconds each time with 250 µl of washing buffer per well. 200 µl of anti-digoxigenin-peroxidase working dilution (200 mU/ml) was added to each well. The MTP modules were then covered again with cover foil and incubated at 37°C for 1 hour.

The solutions were then removed completely and the wells rinsed 5 times with 250 µl of washing buffer per well for 30 seconds each time. The washing buffer was then carefully removed and 200 µl of ABTS® substrate solution was pipetted into each well. The MTP modules were then incubated at room temperature until the colour development (green colour) is sufficient for photometric detection (10 to 30 minutes). The absorbance was then measured at 410 nm using a micro-plate ELISA reader (Dynatech, Germany). A plot of the absorbance of the standard working dilutions as a function of the HIV-1 RT concentrations was constructed to obtain the calibration curve. The RTase activity in the unknowns was estimated by interpolation from the curve and confirmed by linear regression analysis. SIV infected and uninfected Molt-4 Cl_8 cell preparation were used as positive and negative controls, respectively.

2.2.3. Production of antibodies.

Female 8-week old BALB/c mice were tail-bled to obtain control pre-immune sera. They were then immunized intra-peritoneally with 100 µg protein/ml of endogenous retroviral particle (ERV) preparation in an equal volume of Freund's

complete Adjuvant (FCA, Difco, UK) (200 µl per mouse). Four weeks later, the animals were boosted with the same antigen dose in an equal amount of Freund's incomplete adjuvant (FIA, Difco, UK) and test-bled 7 days later. The serum antibody titer was determined by immunohistochemistry using cryostat sections of baboon third trimester placental villous tissue, western blot and ELISA at dilutions of 1:20 to 1:2000. Mice showing strong antibody titer (> 1:500) were given a final antigen dose without adjuvant 4 days before being bled to death from the heart to obtain as much sera as possible. The immune sera and commercial antibodies (both polyclonal and monoclonal antibodies from NIH AIDS Research Reagents Repository and donated by other researchers) were used to characterize retroviral-related antigens expressed in the baboon placenta and other normal baboon tissues as described below. The non-placental tissues used in the study were as follows:-

1. Spleen
2. Brain
3. Heart
4. Liver
5. Ovary
6. Uterus
7. Vagina
8. Adrenal gland
9. Pancreas
10. Skeletal muscle
11. Kidney

2.2.3.1. Immunohistochemistry.

The placental tissues which had been stored at -70°C in TissueTek OCT compound (Miles Diagnostics, Elkhart, USA) were thawed to about -10°C in running water, then fixed onto specimen chucks (stages) using OCT. The specimen chucks were then placed in the cryocabinet of the cryostat (2800 Frigocut N, Cambridge Instruments, UK) for 1 hour so that its temperature decreases to that of the cryostat (-20°C) . The chucks were then fixed onto the microtome and 10 µm thick sections were then cut and removed from the cryostat blade by flash condensation onto previously prepared microscope slides (Appendix II). The sections were air-dried for 1 hour, then fixed in cold acetone (at 4°C) for 10 minutes. The sections were then wrapped in aluminium foil and parafilm and stored at -20°C until required. The other tissues (non-placental tissues) were also prepared, sectioned and stored in the same manner.

The sections were stained using the Zymed Streptoavidin-Biotin peroxidase method (Histostain-SP Kit, Zymed Laboratories, USA). The primary polyclonal antibodies raised in mice as part of this project were diluted 1:500 in 5 % fat-free skimmed milk (Marvel, Cadbury's, UK) in phosphate-buffered saline (PBS), pH 7.4. The commercial antibodies were diluted as per instructions provided.

The slides were arranged in a moisture box and labeled with the name of the antibody to be used using a diamond pencil. The sections were then incubated with 100 µl of the serum blocking solution (10 % non-immune goat serum, provided with the kit) per slide for 10 minutes. The solution was then blotted off and 100 µl of the diluted primary antibodies were added to the corresponding marked slides (enough to cover the section). They were then incubated in the moisture box at room temperature for 30 minutes. The sections were then washed in PBS, (pH 7.4) three times. Subsequently, 100 µl of the relevant biotinylated secondary antibody (goat anti-mouse, anti-monkey, anti-human or anti-rabbit IgG) was added to each section (enough to cover the section). The sections were then incubated at room temperature for 10 minutes in the moisture box, and later rinsed three times with PBS. 100 µl of the enzyme conjugate (streptoavidin peroxidase) was added to each section and incubated for 10 minutes prior to rinsing three times with PBS. 100 µl of the substrate-chromogen mixture (Amino-Ethyl Carbazole (AEC) solution with 0.6 % hydrogen peroxide) was then added to each section and incubated for 10 minutes and subsequently rinsed with distilled water. The sections were counter-stained with 100 µl haematoxylin for 3 minutes, then rinsed with tap water. Two drops of aqueous GVA mountant (Glycerol-polyVinyl Alcohol) were then added to each slide and mounted with a cover slip. The slides were then left to dry overnight, then they were examined under a light microscope.

The positive control monoclonal antibody used in this experiment was W6/32 (SeraLab, UK) , a murine IgG_{2a} monoclonal antibody reactive with a monomorphic class I major histo-compatibility complex (MHC) heavy chain determinant (used at 1:10 dilution of hybridoma culture supernatant). PBS (pH 7.4) and normal mouse serum (NMS) were used as negative controls.

2.2.3.2. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoretic analyses were performed using discontinuous buffer system with 3 % stacking gel and 12.5 % resolving gel as described by Webb *et al* (1985). Homogenates prepared in TNE buffer (pH 7.8) from baboon third trimester placental villous tissues were used. Preparations from SIV-infected and uninfected Molt-4 cells maintained *in vitro* were used as positive and negative controls respectively.

Samples (40 µg protein/ml) were mixed with solubilization buffer (see Appendix III) and heated in a boiling water bath (100°C) for 10 minutes. They were then cooled in ice for 5 minutes. The casting apparatus (Hoefer Scientific Instruments, San Francisco, USA) was assembled as per manufacturer's instructions, and the gels cast as described by Webb *et al* (1985). The samples were subsequently loaded onto the wells (20 µl per well). 10 µl of protein molecular weight calibration markers (BioRad,UK) (Appendix III) was mixed with 5 % β-mercatoethanol and prepared in the same way as the samples, and loaded onto one of the wells. Electrophoresis was performed using an LKB 2050 Midget electrophoresis unit (Hoefer Scientific Instruments, UK) at 150 volts for 2 hours.

When the solvent front reached the bottom of the gel (visualized using Bromophenol Blue), the gel was removed from the casting apparatus and stained with Coomassie Blue staining solution for 20 minutes, with constant shaking. The staining solution was subsequently recovered and the gel placed in the destaining solution (Appendix III) for 20 minutes on a shaker. The destaining solution was then changed and the destaining process continued overnight with constant shaking.

The gel was heat-dried for 2 hours in a slab gel drier (Model 483, BioRad, USA) and cooled in a vacuum for a further 1 hour. The migration distance of each protein (markers and unknowns) was then measured from the top of the resolving gel. The migration distance of each protein was divided by the distance traveled by the tracking dye to obtain the relative mobilities of the proteins (relative to the dye front) and conventionally denoted as Rf. A plot of the logarithm of the molecular weight of the protein standards as a function of the Rf values was constructed. The unknown molecular weights of the protein samples was estimated by interpolation from the curves of log Mr versus Rf, and confirmed by linear regression analysis.

2.2.3.3. Western blot.

SDS-PAGE was performed in the same way using the same samples as described above. However, when the electrophoresis was complete, the protein bands were transferred from the gels onto nitrocellulose sheets (Amersham, UK) using a Trans-Blot SD semi-dry transfer cell (BioRad, UK). The gel was soaked in freshly made protein transfer buffer (Appendix III) for at least 15 minutes. The membrane (9 X 7 cm) was equilibrated by soaking briefly in deionised water, then in the transfer buffer for at least 10 minutes. A transfer cassette was made in the following order:-

2 Whatman blotting papers, nitrocellulose membrane, gel, 2 Whatman blotting papers.

The complete assembly was transferred to the electro-blotting unit and the transfer performed for 1 hour at 0.6 amps and 10 volts, as per manufacturer's instructions.

The nitrocellulose membrane was then separated from the rest and stained with Ponceaus' stain (Appendix III) to show the position of the protein lanes. The sheets were subsequently cut into strips along the lanes and the stain removed using PBS, pH 7.4. The strips were then placed in incubation plates. Non-specific binding was blocked using 5 % fat-free skimmed milk (Marvel, Cadbury's, UK) in PBS, pH 7.4 for 30 minutes. The strips were then incubated with the diluted primary antibodies (3 mls per strip) overnight at 4°C on a shaker.

The strips were then washed 5 times for 5 minutes each time in 5 % fat-free skimmed milk. They were then incubated with the relevant peroxidase-conjugated secondary antibody (anti-mouse, anti-rabbit, anti-monkey or anti-human) diluted 1:500 in 5 % fat-free skimmed milk for 2 hours at room temperature with constant shaking. Antigenic reactivities were detected using 40 mg/ml Diamino-benzedine tetrahydrochloride (DAB) chromogen in PBS, pH 7.4, with 8 % nickel chloride ($NiCl_2$) and 50 % hydrogen peroxide (H_2O_2) as the enzyme substrate (Appendix III). Anti-sera from an SIV-infected vervet monkey (SIVver) and normal monkey sera were used as positive and negative controls respectively.

2.2.3.4. Enzyme-linked immuno-sorbent assay (ELISA).

Cell lysate preparations from SIV-infected cells (positive controls), uninfected Molt-4 Cl_8 cells (negative controls) and placental endogenous retroviral (ERV) particle preparations isolated earlier were used to develop a solid phase ELISA.

An optimum coating antigen concentration (10 µg protein per well) was determined by checker-board titration at different anti-sera dilutions following overnight incubation of microtiter plates (Maxisorp, Nunc, Denmark). Each preparation was diluted in 0.05 M carbonate buffer, pH 9.6 to give a concentration of 100 µg protein/ ml . The 60 inner-most wells of the 96 well plates were coated with the antigen (100 µl of the diluted protein, containing 10 µg of protein). The outermost wells were filled with PBS (pH 7.4) (100 µl per well). The plates were then wrapped in parafilm and stored overnight at 4°C.

The non-attached antigens were removed from the plates which were then washed three times in PBS, pH 7.4, containing 0.05 % Tween-20. The antigens were fixed in freshly prepared 0.1 % glutaraldehyde in PBS for 3 minutes. The plates were washed again 3 times in PBS, then 100 µl of 3 % bovine serum albumin (BSA) in PBS was pipetted into each well to block non-specific binding sites and kept overnight at 4°C, followed by further washing.

The polyclonal antibodies produced as part of this project were diluted 1:500 in 5 % fat-free skimmed milk in PBS. The monoclonal antibodies from NIH and donated by other researchers were diluted as per instructions provided with the antibodies. The antigens in the wells were then incubated with 100 µl of the appropriate anti-sera dilution in duplicates for 2 hours at room temperature, and then, following further washing, incubation for 2 hours with 100 µl of the appropriate peroxidase-conjugated secondary antibody (Sigma, USA) diluted 1:500 in 5 % fat-free milk, with 0.5 % normal baboon serum (NBS) to block any species cross-reactive anti-Ig activity.

The wells were washed with 0.1 % BSA in PBS, and 100 µl of the substrate added (0.4 mg/ml of ortho-phenylenediamine dihydrochloride (OPD) (Sigma, USA) in 0.05 M phosphate-citrate buffer, pH 5.5, with 40 ul of 30 % hydrogen peroxide (H_2O_2) per 100 ml of substrate buffer. When the color intensity has developed for the positive samples, the reaction in all the wells was stopped with 100 µl of 4 M sulphuric acid (H_2SO_4) per well and the optical absorbance (OD) at 492 nm read using a Titertek

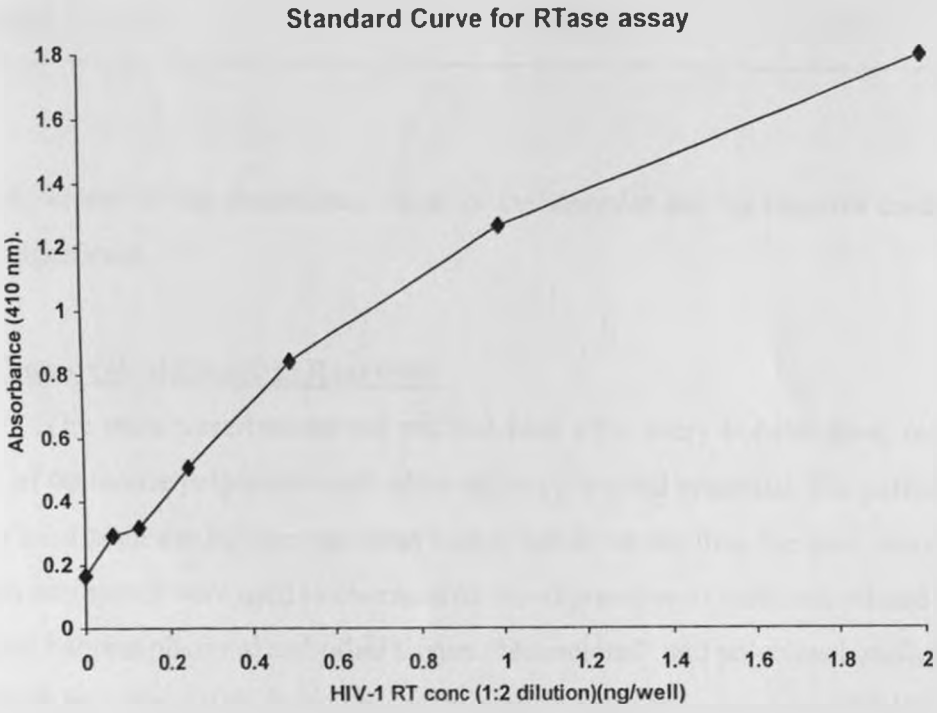
multiskan ELISA reader (Flow Laboratories, Finland). The positive reactivity was taken to be the antibody titers that gave an absorbance value greater than twice that of the negative controls (normal mouse serum (NMS)).

CHAPTER THREE

3. RESULTS

3.1. Reverse transcriptase activity

Minimal reverse transcriptase activity was detected in the baboon third trimester placental villous tissues which were tested. The positive control used was Molt-4 cell preparation infected with SIV. The negative control was uninfected Molt-4 cytosolic cell preparation.



Sample	Gestational age	Mean Absorbance (410 nm)	RT concentration (ng/well).
Baboon placental tissue			
PAN 1476	150 days	0.299	0.0206
PAN 1408	123 days	0.269	-0.016
PAN 1293	170 days	0.309	0.033
Controls.			
Positive control (SIV)		1.736	1.782
Negative control		0.262	-0.011

The difference in the absorbance values of the samples and the negative control was not very significant.

3.2. Immunohistochemical Reactivity

The mice were immunised and test-bled after every booster dose, resulting in a total of 40 mouse polyclonal antibodies against placental retroviral-like particles isolated from third trimester baboon placental villous tissues at the time the mice were sacrificed. These antibodies were used to characterize the expression of retroviral-related antigens in normal baboon placental and other tissues. Monoclonal and polyclonal antibodies kindly donated by the AIDS research and reference reagent programme (NIAID,NIH) and donated by other researchers (Tables 1a and 1b) were also used to confirm the expression of these retroviral-related antigens in the normal baboon placental tissues. These antibodies were mainly produced against various HIV and SIV Proteins, the HIV receptor CD4 and human endogenous retroviral (HERV) particles. The reactivity of these antibodies is summarized below:

3.2.1. Reactivity against placental tissues

Indirect immuno-peroxidase staining on cryostat sections of baboon third trimester placental tissues was performed (Tables 2 & 3). The anti-sera raised in mice as part of this project was code-named BERV (baboon endogenous retrovirus). The positive sera showed heavy focal granular cytoplasmic staining within the syncytiotrophoblast (Figure 5). Only anti-BERV 13 and anti-BERV-16 also showed a weak positive staining on mesenchyme (Table 2a). In contrast, only scattered granular staining was observed for the villous cytotrophoblast.

Of the commercial antibodies tested, anti-HIV-2ST gp120 (an anti-serum to HIV-2 120 kDa glycoprotein, raised in rabbit) showed a strong positive staining on the syncytiotrophoblast, with a little background staining on the mesenchymal tissue (Table 3b). Anti-CD4 (T4-4) (an anti-serum raised against recombinant soluble human CD4 that was produced in CHO cells (Table 3b)) and anti-HIV-1 gp41 (Md-1) (a monoclonal antibody (IgG_2, kappa chain) obtained from EBV-immortalized peripheral blood mononuclear cells from HIV-positive individuals) (Table 3a) also showed positive immunoperoxidase staining with the syncytiotrophoblast. Anti-HIV-1 p25/24 (#381) (a monoclonal antibody (IgG_3, kappa chain) produced by fusion of cloned B-cells from an HIV sero-positive individual with mouse myeloma cells) was also positive (Table 3a).

W6/32 (SeraLab, UK) a murine IgG_{2a} monoclonal antibody produced against monomorphic class I major histo-compatibility complex (MHC) heavy chain determinant (used at 1:10 dilution of hybridoma culture determinant) was used as a positive control. Normal mouse serum (NMS) and phosphate-buffered saline (PBS), pH 7.4 were used as negative controls. In all cases, the positive control antibodies showed a very strong positive staining on the syncytiotrophoblast (Figure 6, Table 2a) while the negative control sera (NMS) and negative control (PBS) sections failed to display any reaction (Figure 7, Table 2a).

3.2.2. Reactivity against non-Placental Tissues

Some of the polyclonal antibodies (anti-BERVs) tested were positive against a variety of non-placental tissues (Table 4). The immuno-peroxidase staining observed on the spleen sections was confined to the red pulp (venous sinusoids and intervening cellular cords, stained reddish-purple) (Figure 8). The staining on the brain cortex

sections was diffuse, with the cells being deeply stained in the inner layers of the cortex. The outer granular layer, which contains a dense population of small pyramidal cells and endothelial cells was also deeply stained (Figure 9). The staining on the liver was confined to the sinusoids and the lining of the central vein (Figure 10). The staining on the heart muscle was confined to the epimycium lining of the muscle fibres (Figure 11). The staining on the pancreas was confined to the exocrine portion of the pancreas while no reaction was evident in the islets of langerhans (Figure 12).

The immunohistochemical reactivity of most of the monoclonal antibodies tested against a variety of non-placental tissues was either negative, or at best, showed a diffuse generalized cytoplasmic pattern (Table 5). Anti-HIV-2ST gp120, anti-CD4 (T4-4) and anti-HIV-1 gp41 showed weak positive reactivity against ovarian sections. Anti-SIVmac p17 (a monoclonal antibody (IgG_{2a}) and anti-HIV-2 CP (a monoclonal antibody (IgG_{2a}, kappa chain) raised against an HIV-2 viral lysate (strain LAV-2)) were positive against the liver sections. The staining was confined to the sinusoids and the lining of the central vein (Figure 10).

W6/32, which was used as a positive control in the placental sections, was strongly positive against the spleen sections, staining the venous sinusoids and the cellular cords of the red pulp (Figure 8), but showed a weak background staining on the rest of the tissues tested. Normal mouse serum (NMS) and PBS, which were used as negative controls in all these sections, failed to display any reaction.

3.3. Enzyme-linked immuno-sorbent assay (ELISA)

Most of the monoclonal antibodies were negative against density-gradient purified ERV particles from third trimester placental villous tissues (Table 8). Anti-SIVmac p27 (an IgG_{2b} monoclonal antibody raised against a 27 kDa SIV protein from macaque monkeys) and anti-CD4 (T4-4) (a polyclonal antibody raised against recombinant soluble human CD4 that was produced in CHO cells) showed a strong ELISA reactivity against ERV particle preparations. Anti-HIV-1 p25/24 (#384) (a polyclonal antibody reactive against viral p25/24 *gag* products) was strongly reactive against antigens expressed in placental ERV particles, infected and uninfected cell preparation. Anti-SIVmac p17 (an IgG_{2a} monoclonal antibody raised against a 17 kDa protein that may correspond to the p16 *gag* precursor protein) was strongly reactive on

placental membrane preparation and uninfected cytosolic cell preparation but did not react on the SIV-infected cell preparation. Anti-HIV-1 gp120 (ID6) (an IgG_{2a} monoclonal antibody raised against recombinant LAV-1 gp120 preparation) showed some moderate reactivity against placental ERV particle preparation. Anti-CD4 gp55 showed positive reactivity on uninfected cells and moderate reactivity on placental membrane preparation. Anti-HIV-1 p25/24 (#383) (a monoclonal antibody raised against HIV-1SF2 virus p25/24 *gag* and cross-reactive with all other HIV-1 isolates) and anti-SIVver (an anti-serum from an SIV-infected vervet monkey in the IPR colony) also showed moderate reactivity on the placental preparation but was negative on the positive and negative controls.

As expected, many of the mouse polyclonal antibodies produced as part of this project reacted positively on the placental ERV particle-coated ELISA plates (Table 9). Anti-BERV-1 and anti-BERV-3 showed a strongly positive reaction with the placental membrane preparation but not on the SIV-infected and uninfected cell preparation. Anti-BERV-9, anti-BERV-10, anti-BERV-12, anti-BERV-13, anti-BERV-15 and anti-BERV-16 recognized antigens expressed in the infected and uninfected cell preparation, but not on the placental membrane preparation. Anti-BERV-11 was specific to a protein expressed in SIV-infected cell preparation. The rest of the polyclonal antibodies were non-specific, showing a positive reaction on the placental membrane preparation, infected and uninfected cell preparation. NMS was used as a negative control.

3.4. Western blot results

3.4.1. Monoclonal antibodies

The monoclonal antibodies tested showed a varied pattern of recognition of proteins on western blot analysis (Table 10, Figure 14). Anti-HIV-1 p25/24 (generated against HIV-1SF2 virus p25/24 *gag* product and cross-reactive with all other HIV-1 isolates), anti-HIV-1 gp41, anti-HIV-1 CP, anti-HIV-2 CP, anti-ERV3 *env* , anti-HERV-K *env*, anti-HERV-K RT, anti-SIVmac p17 (HD5), anti-HIV-1 gp41 (Md-1), anti-HIV-1 p24/55 and anti-CD4 gp55 all recognized a 46 kDa protein expressed on the placental membrane preparation but not on SIV infected cells (Figure 13, Table 10). Anti- ERV3 *env* also recognized a 58 kDa protein expressed on the placental membrane preparation and SIV infected cells but not on uninfected cells. The same protein was also recognized by anti-HERV-K RT. Two proteins, (a 38 kDa and a 64 kDa protein)

expressed by both the placental membrane preparation and SIV-infected cells but not in uninfected cells was recognized by anti-HIV-1 p17 (a polyclonal serum raised against a p17 protein from HTLV-IIIB). Anti-SIVagm (an anti-serum raised in pig-tailed monkeys against SIVagm-155 from African green monkeys) and anti-SIVsmm (an anti-serum raised in rhesus monkeys against SIVsmm-236 from the sooty mangabey monkey) also recognized a 38 kDa protein on placental membrane preparation (Figure 14).

3.4.2. Polyclonal antibodies

Mouse polyclonal antibodies raised against isolated retroviral particles as part of this project were further characterized by immuno-blotting. All polyclonal antibodies recognized proteins of molecular weight of over 30 kDa expressed in placental ERV preparation, SIV-infected and uninfected cells (Table 11, Figures 13 & 15). Anti-SIVver (VER 182, immune sera obtained from an SIV-infected vervet monkey in the IPR colony), used as a positive control, consistently recognized a protein of molecular weight (Mw) 88 kDa on SIV +ve blots, but showed multiple bands on placental membrane preparations (Table 11, Figures 13 & 14). A 40 kDa protein expressed in infected, uninfected and placental ERV preparation was detected by anti-BERV 38 and anti-BERV-39. The same (or similar 40 kDa protein) was detected in the positive and negative controls but not in the placental membrane preparation by anti-BERV-18, anti-BERV-35, anti-BERV-36, and anti-BERV-40. It was also detected by anti-BERV-4 in the negative controls only. Anti-HIV-1 p25/24 *gag* (a polyclonal serum raised against purified p25/24 *gag* products produced in *Escherichia coli*) recognized a 52 kDa protein expressed in placental membrane preparation and SIV-infected cells but not in uninfected cells. Anti-HIV-2ST gp120 recognized a 58 kDa protein also expressed in placental membrane preparation and SIV-infected cells but not in uninfected cells. A 64 kDa protein, similar to the one recognized by the monoclonal antibodies, was detected by anti-BERV-33 on the placental membrane preparation and the positive controls, but not on the negative controls (Table 11).

TABLE 1 (a).

List of polyclonal antibodies used in the project and their source.

ANTIBODY	CODE	HOST	DILUTION	SOURCE
anti-HIV-2ST gp120	1410	Rabbit	1:1000	NIH
anti-HIV-1 p25/24 *gag*	384	Rabbit	1:500	NIH
anti-CD4 gp55	806	Rabbit	1:500	NIH
anti-SIVagm	241	Monkey	1:500	NIH
anti-CD4 (T4-4)	806	Rabbit	1:500	NIH
anti-SIVver	VER 182	Monkey	1:500	IPR
anti-HIV-1 p17	286	sheep	1:500	NIH
anti-HIV-1 gp120	567	Sheep	1:500	NIH
anti-ERV3 *env*		Rabbit	1:500	Prof. P. Venables (Kennedy Inst.itue of Rheum, UK).
Anti-HERV-K RT	3813	Rabbit	1:500	Dr.S.Schommer, Inst med Microbiol & Hyg, Germany
anti-BERV		Mouse	1:500	Polyclonal antibodies produced as part of this project.

KEY.

NIH : National Institutes of Health. USA. These antibodies were from the AIDS Research and Reference reagent Programme, Division of AIDS, NIAID,NIH (NIAID :-National Institute of Allergy and Infectious Diseases).

IPR : Institute of Primate Research.

agm : African green monkey (*Cercopithecus aethiops*)
BERV : Baboon endogenous retrovirus.
CD4 : Cluster determinant-4.
CP : Core Protein
gp : Glycoprotein
HIV : Human immuno-deficiency virus
mac : macaque monkey (*Macaca mulatta*)
RT : Reverse transcriptase.
SIV : Simian immuno-deficiency virus
smm : Sooty mangabey monkey (*Cercocebus atys*)
ver : Vervet monkey (*Cercopithecus aethiops*).

TABLE I (b).

List of monoclonal antibodies used in the project and their source.

ANTIBODY	CODE	DILUTION	HOST	SOURCE
anti-SIVmac p27 (55-2F12)	1610	1:500	Mouse	NIH
anti-HIV-1 RT	769	1:500	Mouse	NIH
anti-HIV-1 gp120 (ID6)	2343	1:500	Mouse	NIH
anti-SIVmac p17 (HD5)	882	1:200	Mouse	NIH
anti-HIV-2 CP	740	1:100	Mouse	NIH
anti-HIV-1 gp160 (Chessie)	1209	1:20	Mouse	NIH
anti-HIV-1 p25/24 *gag*	383	1:500	Mouse	NIH
anti-HIV-1 gp41	531	1:50	Human	NIH
anti-HIV-1 p25/24 SF2	384	1:500	Rabbit	NIH
anti-HIV-1 gp41 (Md-1)	1223	1:500	Human	NIH
anti-SIVsmm	242	1:1000	Monkey	NIH
anti-HIV-1 p24/55	381	1:500	Human	NIH
anti-HIV-1 p25/24	381	1:500	Human	NIH
anti-SIVagm	241	1:1000	Monkey	NIH
anti-HERV-K *env*		1:10	Mouse	Dr. W. Vogetseder (Univ of Innsbruck, Austria)
RV3-27		Neat	Mouse	Dr. Mwenda, IPR.
W6/32		1:10	Mouse	Seralab,UK

NB: The abbreviations are the same as those used in Table 1a.

TABLE 2 (a).

Immunohistochemical reactivity of mouse anti-BERV polyclonal antibodies with baboon placental villous tissues.

ANTIBODY	sT/cT	eVcT	Mesenchyme
anti-BERV 1	+	-	+/-
anti-BERV 2	++	-	-
anti-BERV 3	++	-	+/-
anti-BERV 4	+/-	-	-
anti-BERV 5	++	-	+/-
anti-BERV 6	++	-	-
anti-BERV 7	-	-	-
anti-BERV 8	++	-	-
anti-BERV 9	++	-	+/-
anti-BERV 10	+	-	+/-
anti-BERV 11	++	-	+/-
anti-BERV 12	+	-	+/-
anti-BERV 13	+++	-	+
anti-BERV 14	+++	-	-
anti-BERV 15	++	-	+/-
anti-BERV 16	+	-	+

Control antibodies.

ANTIBODY	sT/cT	eVcT	Mesenchyme
W6/32 (positive control)	+++	-	-
PBS (negative control)	-	-	-
NMS (negative control)	-	-	-

KEY:

sT/cT : Syncytiotrophoblast/cytotrophoblast.
eVcT : extravillous cytotrophoblast.

+++ : Very strong positive staining.
++ : Strong positive staining.
+ : Positive staining.
+/- : Background or weak staining.
- : No staining.

TABLE 2 (b).

Immunohistochemical reactivity of mouse anti-BERV polyclonal antibodies with baboon placental villous tissues.

ANTIBODY	sT/cT	eVcT	Mesenchyme
anti-BERV 17	+++	-	-
anti-BERV 18	++	-	+/-
anti-BERV 19	+	-	-
anti-BERV 20	++	-	-
anti-BERV 21	+/-	-	-
anti-BERV 22	+/-	-	-
anti-BERV 23	++	-	-
anti-BERV 24	+/-	-	-
anti-BERV 25	++	-	-
anti-BERV 26	+	-	-
anti-BERV 27	+	-	-
anti-BERV 28	++	-	-
anti-BERV 29	++	-	-
anti-BERV 30	+/-	-	-
anti-BERV 31	-	-	-
anti-BERV 32	-	-	-

NB: The dilution for all the above mouse polyclonal antibodies was 1:500.

NT : Not tested.

For other codes see table 2a.

TABLE 3 (a).

Immunohistochemical reactivity of commercial monoclonal antibodies with baboon placental villous tissues.

ANTIBODY	sT/cT	eVcT	Mesenchyme
anti-SIVmac p27	-	-	+/-
anti-HIV-1 RT	-	-	+/-
anti-HIV-1 gp120	+/-	-	-
anti-SIVmac p17	+/-	-	-
anti-HIV-2 CP	+/-	-	+/-
RV3-27	-	-	+/-
anti-HIV-1 gp160	-	-	-
anti-HIV-1 p25/24 *gag* (#381)	+	-	-
anti-HERV-K *env*	+/-	-	-
anti-ERV3 *env*	+/-	-	-
anti-HERV-K RT	-	-	+/-
anti-HIV-1 gp41	+/-	-	-
anti-HIV-1 p24/55	+/-	-	+/-
anti-HIV-1 gp41 (Md-1)	+	-	+/-

TABLE 3 (b).

Immunohistochemical reactivity of commercial polyclonal antibodies with baboon placental villous tissues.

ANTIBODY	sT/cT	eVcT	Mesenchyme
anti-HIV-2ST gp120	++	+/-	+/-
anti-HIV-1 p25/24 *gag*	-	-	-
anti-CD4 gp55	-	-	-
anti-SIVagm	-	-	-
anti-SIVver	+/-	-	-
anti-CD4 (T4-4)	+	-	-

NB: The abbreviations are the same as in Table 2a.

TABLE 4a.

Immunohistochemical reactivity of mouse anti-BERV polyclonal antibodies with non-placental baboon tissues.

ANTIBODY	SPLEEN	BRAIN	HEART	LIVER	OVARY
PBS	-	-	-	-	-
NMS	-	-	-	-	-
W6/32	+++	+/-	+/-	+/-	+/-
anti-BERV 1	NT	NT	NT	NT	NT
anti-BERV 2	NT	NT	NT	NT	NT
anti-BERV 3	+	-	+	+	NT
anti-BERV 4	-	-	-	-	NT
anti-BERV 5	+	++	++	++	NT
anti-BERV 6	+/-	+/-	+/-	+/-	NT
anti-BERV 7	-	-	-	-	NT
anti-BERV 8	+/-	+/-	+	NT	NT
anti-BERV 9	+	+/-	+	+	NT
anti-BERV 10	+	+/-	+/-	+	NT
anti-BERV 11	+	+	+	NT	NT
anti-BERV 12	+/-	+/-	+/-	++	NT
anti-BERV 13	++	+++	+	-	+/-
anti-BERV 14	++	++	++	-	+/-
anti-BERV 15	+	++	+	-	+/-
anti-BERV 16	+/-	+/-	+	+	-
anti-BERV 17	++	++	++	++	NT

TABLE 4b.

Immunohistochemical reactivity of mouse anti-BERV polyclonal antibodies with non-placental baboon tissues.

ANTIBODY	UTERUS	VAGINA	ADRENAL	PANCREAS	SK.MUSCE	KIDNEY
anti-BERV 13	+/-	+/-	+/-	+	-	+/-
anti-BERV 14	+/-	-	+/-	+/-	+	+/-
anti-BERV 15	-	-	+/-	+/-	+/-	+/-
anti-BERV 16	+/-	-	+/-	+/-	+/-	-

NB: The abbreviations are the same as in Table 2a.

TABLE 5.

Immunohistochemical reactivity of commercial antibodies with baboon adult (non-placental) tissues.

ANTIBODY	SPLEEN	BRAIN	HEART	LIVER	OVARY
anti-SIVmac p27	-	-	-	-	-
anti-HIV-1 gp120	-	-	-	-	-
anti-SIVmac p17	-	+/-	-	+	-
anti-HIV-2 CP	-	-	-	+	-
W6/32	+++	+/-	+/-	+/-	+/-
anti-HIV-1 RT	-	-	-	+/-	-
anti-ERV3 env	NT	NT	NT	NT	+
anti-HIV-1 p25/24	NT	NT	NT	NT	+/-
anti-HIV-2ST gp120	NT	NT	NT	NT	+
anti-HIV-1 gp41	NT	NT	NT	NT	+
anti-CD4 (T4-4)	NT	NT	NT	NT	+
NMS	-	-	-	-	-

NB: The abbreviations are the same as in Table 2a.

NT: Not tested.

TABLE 6a.

ELISA reactivity of commercial antibodies with SIV-infected and uninfected Molt-4 cells and trophoblast membrane preparation.

ANTIBODY	PL.MEMB. μOD ± SD	SIV +ve μOD ± SD	SIV -ve μOD ± SD
NMS	0.05 ± 0.04	0.08 ± 0.02	0.03 ± 0.01
anti-HIV-1 gp120	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.01
anti-CD4 gp55	0.07 ± 0.01	0.04 ± 0.02	0.09 ± 0.01
anti-HIV-1 gp160	0.01 ± 0.01	0.02 ± 0.01	0.00 ± 0.00
anti-HERV-K *env*	0.01 ± 0.00	0.02 ± 0.01	-0.01± 0.01
anti-ERV3 env	0.04 ± 0.03	0.02 ± 0.00	0.06 ± 0.01
anti-HERV-K RT	0.01 ± 0.01	0.02 ± 0.00	0.05 ± 0.01
anti-HIV-1 p25/24 *gag* (# 384)	0.19 ± 0.07	0.39 ± 0.02	0.40 ± 0.01
anti-SIVmac p17	0.17 ± 0.01	0.12 ± 0.01	0.24 ± 0.04
anti-HIV-1 p25/24 *gag* (# 383)	0.09 ± 0.01	0.08 ± 0.03	0.07 ± 0.01
anti-HIV-2 gp120	0.05 ± 0.02	0.07 ± 0.01	0.02 ± 0.01
anti-HIV CP	0.04 ± 0.02	0.12 ± 0.01	0.02 ± 0.01
anti-HIV-1 gp120 (ID6)	0.11 ± 0.00	NT	NT
anti-SIVmac p27	0.15 ± 0.08	NT	NT
anti-HIV RT	0.02 ± 0.02	NT	NT
W6/32	0.01 ± 0.00	NT	NT
anti-CD4 (T4-4)	0.23 ± 0.02	NT	NT
anti-HIV-1 gp41 (Md-1)	0.03 ± 0.00	NT	NT
anti-HIV-1 gp41	0.05 ± 0.01	NT	NT
Normal monkey serum	0.04 ± 0.02	0.06 ± 0.10	0.02 ± 0.09
anti-RV3-27	0.03 ± 0.01	0.06 ± 0.02	0.01 ± 0.01
anti-SIVver	0.06 ± 0.00	0.08 ± 0.01	0.05 ± 0.02

Optical density (OD) measured at 492 nm

TABLE 6b.

The range of OD values derived from the negative control serum (NMS) used to determine the cut-off point for positive samples (see the text for the details).

		PL.MEMB.	SIV +ve	SIV -ve
-	:	$\mu OD \leq 0.05$	$\mu OD \leq 0.08$	$\mu OD \leq 0.03$
+/-	:	$0.05 < \mu OD \leq 0.09$	$0.08 < \mu OD \leq 0.16$	$0.03 < \mu OD \leq 0.05$
+	:	$0.09 < \mu OD \leq 0.14$	$0.16 < \mu OD \leq 0.23$	$0.05 < \mu OD \leq 0.10$
++	:	$0.14 < \mu OD \leq 0.18$	$0.23 < \mu OD \leq 0.31$	$0.10 < \mu OD \leq 0.15$
+++	:	$\mu OD > 0.18$	$\mu OD > 0.31$	$\mu OD > 0.15$

Note: μ :- mean of optical densities from 3 different experiments

TABLE 7a.

ELISA reactivity of murine anti-BERV polyclonal antibodies against SIV-infected and uninfected Molt-4 Cl_8 cell preparation, and baboon trophoblast membrane preparation .

ANTIBODY	PL.MEMB. μOD ± SD	SIV +ve μOD ± SD	SIV -ve μOD ± SD
NMS	0.03 ± 0.06	0.08 ± 0.02	0.03 ± 0.01
anti-BERV 1	0.20 ± 0.18	0.03 ± 0.01	0.05 ± 0.01
anti-BERV 2	0.66 ± 0.09	0.26 ± 0.02	1.15 ± 0.00
anti-BERV 3	0.24 ± 0.12	0.07 ± 0.01	0.06 ± 0.01
anti-BERV 4	0.60 ± 0.15	0.41 ± 0.01	0.43 ± 0.01
anti-BERV 5	0.43 ± 0.02	0.10 ± 0.00	0.10 ± 0.01
anti-BERV 6	0.36 ± 0.02	0.25 ± 0.01	1.19 ± 0.01
anti-BERV 7	0.06 ± 0.02	0.16 ± 0.01	0.09 ± 0.01
anti-BERV 8	0.71 ± 0.18	0.63 ± 0.03	0.50 ± 0.01
NMS	0.10 ± 0.01	0.09 ± 0.11	0.10 ± 0.01
anti-BERV 9	0.05 ± 0.02	0.20 ± 0.01	0.21 ± 0.01
anti-BERV 10	0.06 ± 0.01	0.35 ± 0.01	1.12 ± 0.01
anti-BERV 11	0.05 ± 0.02	0.28 ± 0.00	0.15 ± 0.00
anti-BERV 12	0.07 ± 0.01	0.77 ± 0.01	0.53 ± 0.03
anti-BERV 13	0.17 ± 0.02	0.31 ± 0.00	0.25 ± 0.00
anti-BERV 14	0.23 ± 0.05	0.43 ± 0.02	1.39 ± 0.01
anti-BERV 15	0.11 ± 0.01	0.43 ± 0.01	0.22 ± 0.01
anti-BERV 16	0.15 ± 0.05	0.77 ± 0.01	0.60 ± 0.00

TABLE 7b.

The range of OD values derived from the negative control serum (NMS) used to determine the cut-off point for positive samples (see the text for the details).

		Pl. MEMB.	SIV +ve	SIV -ve
Anti-BERV 1-8	-	μ OD ≤ 0.03	μ OD ≤ 0.08	μ OD ≤ 0.03
	+/-	0.03<μ OD≤ 0.06	0.08<μ OD≤ 0.16	0.03<μ OD≤ 0.06
	+	0.06<μ OD≤ 0.09	0.16<μ OD≤ 0.24	0.06<μ OD≤ 0.10
	++	0.09<μ OD≤ 0.12	0.24<μ OD≤ 0.32	0.10<μ OD≤ 0.13
	+++	μ OD > 0.12	μ OD > 0.32	μ OD > 0.13
Anti-BERV 9-16	-	μ OD ≤ 0.10	μ OD ≤ 0.09	μ OD ≤ 0.10
	+/-	0.10<μ OD≤ 0.20	0.09<μ OD≤ 0.17	0.10<μ OD≤ 0.20
	+	0.20<μ OD≤ 0.30	0.17<μ OD≤ 0.26	0.20<μ OD≤ 0.30
	++	0.30<μ OD≤ 0.40	0.26<μ OD≤ 0.35	0.30<μ OD≤ 0.40
	+++	μ OD > 0.40	μ OD > 0.35	μ OD > 0.40

Note: μ - mean of optical densities from 3 different experiments

TABLE 8.

Summary of the reactivities of monoclonal and polyclonal commercial antibodies on ELISA.

ANTIBODY	PL. MEMB.	SIV +ve	SIV -ve
anti-HIV-1 gp120	-	-	-
anti-CD4 gp55	+/-	-	+
anti-HIV-1 gp160	-	-	-
anti-HERV-K *env*	-	-	-
anti-ERV3 *env*	-	-	+
anti-HERV-K RT	-	-	+/-
anti-HIV-1 p25/24 *gag* (#384)	+++	+++	+++
anti-SIVmac p17	++	+/-	+++
anti-HIV-1 p25/24 *gag* (#383)	+/-	-	+
anti-HIV-2 gp120	-	-	-
anti-HIV-2 CP	-	+/-	-
anti-HIV-1 gp120 (ID6)	+	NT	NT
anti-SIVmac p27	++	NT	NT
anti-HIV-RT	-	NT	NT
W6/32	-	NT	NT
anti-CD4 (T4-4)	+++	NT	NT
anti-HIV-1 gp41 (Md-1)	-	NT	NT
anti-HIV-1 gp41	-	NT	NT
anti-RV3-27	-	-	-
Normal monkey serum	-	-	-
anti-SIVver	+/-	-	+/-

NB: The abbreviations are the same as in Table 2a.
NT : Not tested.

TABLE 9.

Summary of reactivities of polyclonal antibodies on ELISA.

ANTIBODY	PL. MEMB.	SIV +ve	SIV -ve
anti-BERV 1	+++	-	+/-
anti-BERV 2	+++	++	+++
anti-BERV 3	+++	-	+/-
anti-BERV 4	+++	+++	+++
anti-BERV 5	+++	+/-	+
anti-BERV 6	+++	++	+++
anti-BERV 7	+/-	+/-	+
anti-BERV 8	+++	+++	+++
anti-BERV 9	-	+	+
anti-BERV 10	-	++	+++
anti-BERV 11	-	++	+/-
anti-BERV 12	-	+++	+++
anti-BERV 13	+/-	++	+
anti-BERV 14	+	+++	+++
anti-BERV 15	+/-	+++	+
anti-BERV 16	+/-	+++	+++

NB: The abbreviations are the same as in Table 2a.

PL. MEMB : Trophoblast membrane preparation.
SIV +ve : SIV-infected molt-4 Cl8 cytosolic cell preparation.
SIV -ve : Uninfected molt-4 Cl8 cytosolic cell preparation.

TABLE 10.

Summary of immunoblot reactivities of retroviral monoclonal antibodies with SIV-infected and uninfected cell preparation, and trophoblast membrane preparation.

ANTIBODY	PL.MEMB (Mw in kDa)	SIV +ve (Mw in kDa)	SIV -ve (Mw in kDa)
anti-HIV-1 gp160	-	-	-
anti-SIVsmm	38	-	-
anti-HIV-1 p25/24 (#381)	46	-	-
anti-HIV-1 RT	-	-	-
anti-HIV-1 gp41	46	-	-
anti-HIV-1 gp120	-ve	-	-
anti-HIV-2 CP	46	-	-
anti-ERV3 *env*	46,58,82	38,58	-
anti-HERV-K *env*	46	-	-
anti-HERV-K RT	46,58,82	58,64,88	-
anti-HIV-1 p25/24 *gag* (#383)	82	64	NT
anti-SIVmac p17	46	-	NT
anti-HIV-1 gp41 (Md-1)	46	-	NT
anti-HIV-1 p25/24 SF2	78,82	NT	NT
anti-HIV-1 p24/55	46	-	NT
anti-SIVagm	38	88	-
anti-HIV-1 p17	38,64	38,52,64	-
anti-HIV-1 gp120 (#567)	38,64	38	-
anti-HIV-1 CP	46	-	-
anti-RV3-27	-	-	-
W6/32	-	-	-

-: No bands detected

NT: Not tested

TABLE 11.

Summary of immunoblot reactivities of polyclonal antibodies with SIV-infected and uninfected cell preparation, and trophoblast membrane preparation .

ANTIBODY	PL.MEMB. (Mw kDa)	SIV +ve (Mw kDa)	SIV -ve (Mw kDa)
anti-SIVver	38,45,48,50,78,199.	88	-
anti-HIV-1 p25/24 *gag*(#384)	52	30,52,81	-
anti-HIV-2ST gp120	58,82	25,58	-
anti-CD4 gp55	26,46,49,82,90	-	-
anti-BERV 1	35,52,64,72,75	-	-
anti-BERV 2	34,52,60,64,72,79	34,64	NT
anti-BERV 3	52,72,82	-	NT
anti-BERV 4	28,34,48,64,75,79	34,48,64	40
anti-BERV 13	34,64,69,75	-	NT
anti-BERV 14	31,34,48,60,75,79	33,48	NT
anti-BERV 15	64,69	NT	NT
anti-BERV 16	34,64,69,75	NT	NT
anti-BERV 18	-	40,42	40
anti-BERV 22	64	NT	NT
anti-BERV 23	-	-	-
anti-BERV 25	68,91,95	NT	-
anti-BERV 28	68	NT	NT
anti-BERV 29	31,52,97	NT	NT
anti-BERV 31	64,90,97	NT	-
anti-BERV 33	52,64,78,90,97	34,52,64	-
anti-BERV 34	90,97	NT	NT
anti-BERV 35	-	40	40
anti-BERV 36	90	40	40
anti-BERV 37	64,90,97	NT	-
anti-BERV 38	40,52.64	40	40
anti-BERV 39	40,52,90,97	40	40
anti-BERV 40	-	40,60	40,58
Normal monkey serum	-	-	-

Note: -: No bands detected.

NT: Not tested.

TABLE 12

Summary of the reactivities of the monoclonal antibodies on trophoblast membrane preparation .

ANTIBODY	W. BLOT	HISTOLOGY	ELISA
anti-SIVsmm	+	NT	NT
anti-HIV-1 p25/24 (#381)	+	+	NT
anti-HIV-1 gp41	+	+/-	-
anti-HIV-1 RT	-	-	-
anti-HIV-1 gp160	-	-	-
anti-HIV-1 gp120	-	+/-	-
anti-HIV-2 CP	+	+/-	-
anti-ERV3 *env*	+	+/-	-
anti-HERV-K *env*	+	+/-	-
anti-HERV-K RT	+	-	-
anti-HIV-1 gp120 (#567)	+	NT	NT
anti-HIV-1 CP	+	NT	NT
anti-SIVmac p17	+	+/-	++
anti-HIV-1 p25/24 *gag*	+	-	+/-
anti-HIV-1 gp41 (Md-1)	+	+	-
anti-HIV-1 p24/55	+	+/-	NT
anti-HIV-1 p25/24 SF2	+	NT	NT
anti-SIV p27	NT	-	++
anti-HIV-1 gp120 (ID6)	NT	NT	+
anti-RV3-27	-	-	-
anti-SIVagm	+	-	NT
anti-HIV-2ST gp120	+	++	NT
anti-HIV-2 gp120	NT	-	NT
W6/32	-	+++	-
Normal monkey serum	-	-	-
Normal mouse serum	-	-	-

TABLE 13

Summary of the reactivities of polyclonal antibodies on baboon trophoblast membrane preparation.

ANTIBODY	W. BLOT	HISTOLOGY	ELISA
anti-BERV 1	+	+	+++
anti-BERV 2	+	++	+++
anti-BERV 3	+	++	+++
anti-BERV 4	+	+/-	+++
anti-BERV 5	NT	++	+++
anti-BERV 6	NT	++	+++
anti-BERV 7	NT	-	+/-
anti-BERV 8	NT	++	+++
anti-BERV 9	NT	++	-
anti-BERV 10	NT	+	-
anti-BERV 11	NT	++	-
anti-BERV 12	NT	+	-
anti-BERV 13	+	+++	+/-
anti-BERV 14	+	+++	+
anti-BERV 15	+	++	+/-
anti-BERV 16	+	+	+/-
anti-BERV 17	NT	+++	NT
anti-BERV 18	-	++	NT
anti-BERV 19	NT	+	NT
anti-BERV 20	NT	++	NT
anti-BERV 21	NT	+/-	NT
anti-BERV 22	+	+/-	NT
anti-BERV 23	-	++	NT
anti-BERV 24	NT	+/-	NT
anti-BERV 25	+	++	NT
anti-BERV 26	NT	+	NT
anti-BERV 27	NT	+	NT
anti-BERV 28	+	++	NT
anti-BERV 29	+	++	NT
anti-BERV 30	NT	+/-	NT
anti-BERV 31	+	-	NT
anti-BERV 32	NT	-	NT
anti-BERV 33	+	NT	NT
anti-BERV 34	+	NT	NT

TABLE 13 (continued).

ANTIBODY	W. BLOT	HISTOLOGY	ELISA
Anti-BERV 35	-	NT	NT
anti-BERV 36	+	NT	NT
anti-BERV 37	+	NT	NT
anti-BERV 38	+	NT	NT
anti-BERV 39	+	NT	NT
anti-BERV 40	-	NT	NT
anti-SIVver	+	+	+/-
anti-CD4 gp55	+	-	+/-
anti-HIV-1 p17	+	NT	NT
anti-HIV-1 p25/24 gag (#384)	+	-	+++
anti-HIV-1ST gp120	+	-	-
anti-CD4 (T4-4)	NT	+	+++

Figure 5 :

Immuno-peroxidase staining of cryostat sections of baboon third trimester placental villous tissues with polyclonal antibody (anti-BERV-17) (mag X 400)

sT :- Syncytiotrophoblast

cT :- Cytotrophoblast

IVS :- Intervillous space

Figure 6.

Immuno-peroxidase staining of cryostat sections of baboon third trimester placental villous tissues with monoclonal antibody (W6/32) (mag X 400)

sT :- Syncytiotrophoblast

cT :- Cytotrophoblast

IVS :- Intervillous space

Figure 7.

Placental section stained with haematoxylin to illustrate lack of positive reactivity (blue stain) (mag X100).

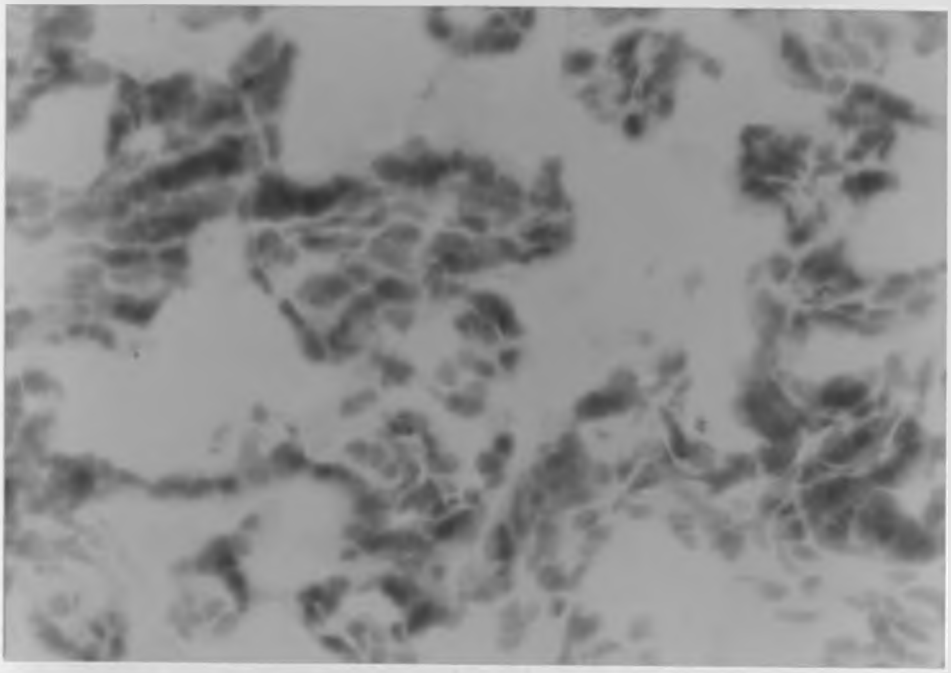


Figure 8.

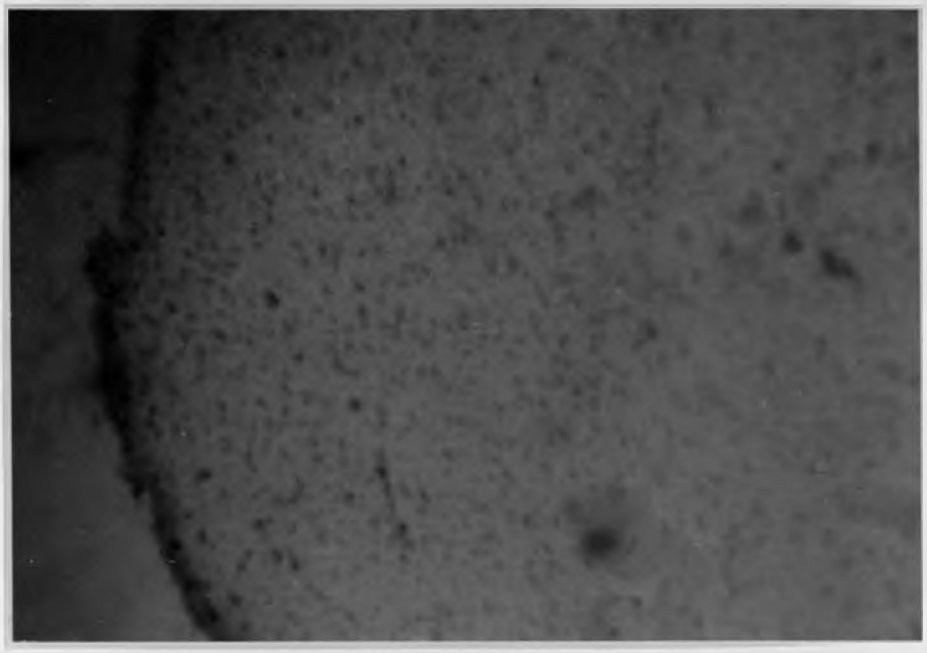
Immunoperoxidase staining of cryostat sections of baboon spleen with anti-BERV-17 antibody (mag X 100)

RP :- Red pulp (venous sinusoids and intervening celular cords)

WP :- White pulp (consists of lymphoid aggregations)

Figure 9.

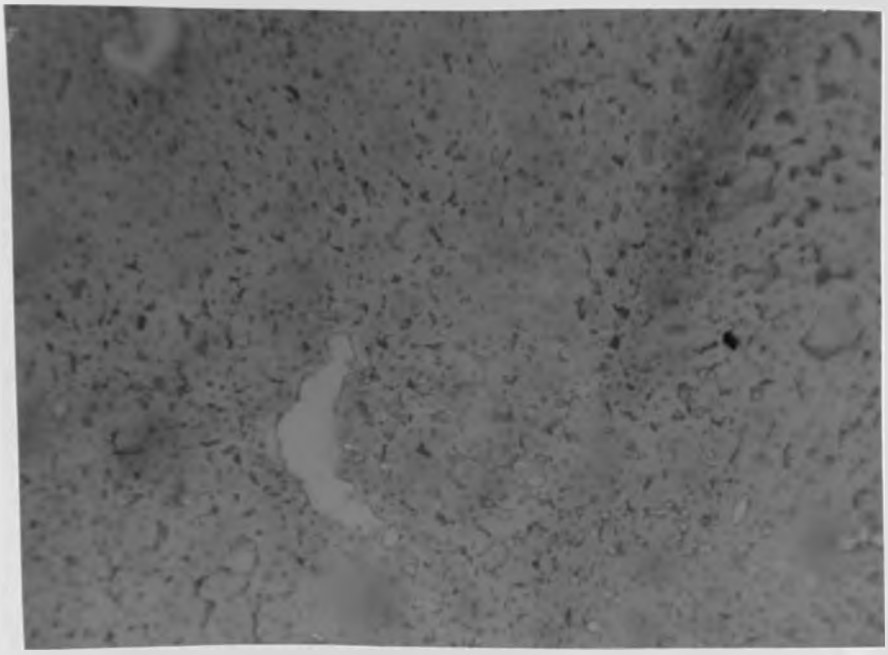
Immuno-peroxidase staining of cryostat sections of the baboon brain (cerebral cortex) with anti-BERV-17 antibody (mag X 100)



GL :- Granular layer of cortex.

Figure 10.

Immunoperoxidase staining of cryostat sections of the baboon liver with anti-BERV-17 antibody (mag X 100).



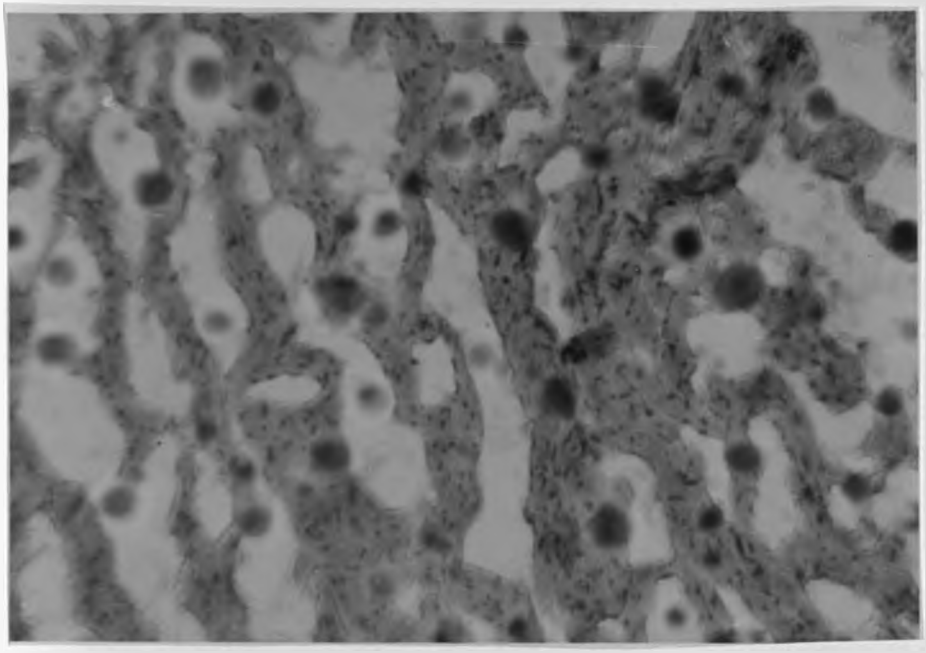
V :- Central vein

H :- Hepatocytes

S :- Sinusoids

Figure 11.

Immunoperoxidase staining of cryostat sections of the baboon heart muscle with anti-BERV-17 antibody (mag X 100).



E :- Epimycium

M :- Cardiac muscle fibres.

Figure 12.

Immuno-peroxidase staining of cryostat sections of the baboon pancreas with anti-BERV-13 antibody (mag X 100).

D :- Ducts

E :- Exocrine tissues.

I :- Islets of Langerhans

Figure 13

Immuno-blot reactivities of polyclonal antibodies with SIV +ve cell preparation and ERV particles isolated from baboon placental villous tissues.

Legend

1. Anti-BERV 22
2. Anti-BERV 39
3. Anti-BERV 38
4. Anti-BERV 31
5. Anti-BERV 34
6. Anti-BERV 33
7. Anti-BERV 36
8. Anti-SIVver (positive control)
9. Anti-BERV 23
10. Anti-SIVver (positive control)
11. Non-immune serum (negative control)

Figure 14.

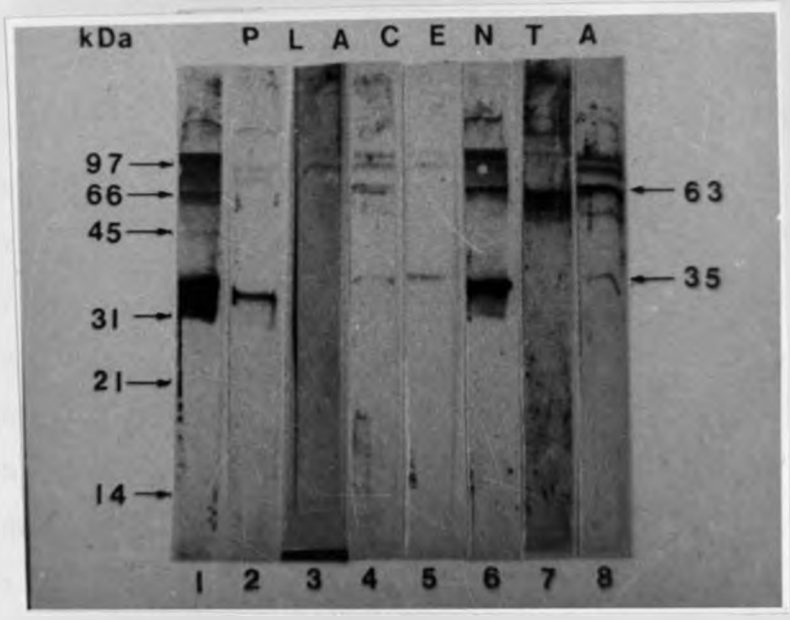
Immuno-blot reactivities of monoclonal and polyclonal antibodies with SIV +ve cell preparation and ERV particles isolated from baboon placental villous tissues.

Legend

1. Anti-HIV-1 gp120
2. Anti-HIV-1 gp41
3. Anti-SIVver (positive control)
4. Anti-HIV-1 CP
5. Anti-SIVagm
6. Anti-SIVsmm
7. Anti-BERV 25
8. Anti-BERV 29
9. Anti-SIVver (positive control)
10. Non-immune serum (negative control)
11. Anti-SIVagm

Figure 15.

Immunoblot reactivities of polyclonal antibodies with baboon placental ERV particles.



Legend

1. Anti-BERV 14
2. Anti-BERV 13
3. Anti-BERV 15
4. Anti-BERV 2
5. Anti-BERV 16
6. Anti-BERV 4
7. Anti-BERV 3
8. Anti-BERV 1

CHAPTER FOUR.

DISCUSSION AND CONCLUSION.

4.1. DISCUSSION.

In man, non-human primates and many other eutherian mammals, the formation of the syncytiotrophoblast during implantation is essential for successful pregnancy. The syncytiotrophoblast is a terminally differentiated multinucleated cell that is derived from an underlying single cell layer, the cytotrophoblast. It possesses no mitotic activity and is essentially formed by cell fusion. Cell fusion is a highly specific and rare event, the most noted cell fusion in animals being that of the oocyte and spermatozoon at fertilization. However, cell fusion does occur in certain pathological conditions. For example, HIV-infected lymphocytes express CD4 differentiation antigen, which mediates cell fusion resulting in multinucleated syncytia. Implantation is a critical stage of early embryonic growth in mammals which requires a controlled growth of rapidly proliferating trophoblast with subsequent formation of placenta and a modulation of the immune system to prevent fetal rejection. It has been proposed that one role of the endogenous type-C retroviral particles expressed by syncytiotrophoblast is to promote viral antigen mediated fusion of cytotrophoblast cells resulting in formation of syncytiotrophoblast (Smith *et al*, 1988).

Viral particles have been observed in many human neoplastic tissues and tumour cell cultures. However, type-C particles have also been recognized in non-neoplastic cells, particularly in normal embryonic tissues and in certain autoimmune conditions (Levy, 1977; Panem & Reynolds, 1979). Kalter *et al* (1973) had earlier observed 100 nm type-C particles in 13 of 13 baboon placentas, ranging from 27 to 170 days gestation as well as in 4 of 6 human placentas. Both budding and free particles were noted primarily at the basal aspect of the syncytiotrophoblast at the interface with the villous cytotrophoblast or trophoblastic basement membrane. This has been confirmed by further reports which have shown type-C particles in human (Vernon *et al*, 1974); chimpanzee (Kalter *et al*, 1975); marmoset (Seman *et al*, 1975) and squirrel monkey (Smith *et al*, 1977) placentas. Another report has described numerous type-C viral particles budding from the basal plasma membrane of the syncytiotrophoblast during early embryo implantation in the

marmoset (Smith & Moore, 1988). Ultrastructural studies on isolated ERV particle samples obtained from normal placental chorionic villous tissue extracts have been done (Lyden *et al*, 1994b). These fractions were found to express RTase activity that was Mg^{2+} dependent and occur at a buoyant density on sucrose gradients characteristic of type-C particles (1.15-1.17 g/ml) (Johnson *et al*, 1990). These studies confirm the presence of discrete particles within isolates from normal first and third trimester human placentas which exhibit ultrastructural features consistent with type-C retrovirus particles (Lyden *et al*, 1994b).

Baboon endogenous retrovirus (BaEV) is a type-C retrovirus originally isolated from baboon placenta by co-cultivation with a human rhabdomyosarcoma cell line (A204). BaEV has been shown to be able to replicate in cultured cells of several mammalian species. The entire nucleotide sequence of BaEV has been determined and the genomic structure was shown to be chimeric, the *gag-pol* region closely related to that of the simian type-D retroviruses (Tamura *et al*, 1981; Kato *et al*, 1987). However, the evaluation of the tissue expression of BaEV and other ERV gene products in baboon tissues has not been performed. Hence the objective of the present study was to localize and characterize retroviral antigens expressed in the normal (SIV-seronegative) baboon placental and other adult tissues. The approach followed involved isolation of retroviral-like particles, production and characterization of antibodies against these particles. These antibodies as well as those from commercial sources and donated by other investigators were used in the present study to assess tissue expression of retroviral antigens.

Minimal reverse transcriptase activity was detected in the third trimester placental tissues tested. This was in contrast with earlier reports indicating the presence of reverse transcriptase activity in baboon placental tissues (Kalter *et al*, 1973). Two conclusions can be made from this:

(i) It is possible baboon placental retroviral isolates possess RTase activity but the inappropriate template was used. The template/primer used in this study was Poly(A).Oligo$(dT)_{15}$, supplied with the kit (non-radioactive RTase assay kit supplied by Boerhinger Mannheim, Germany). It was not possible to repeat the assay using different templates (e.g. Poly(rC).Oligo$(dG)_{12-18}$, which had been used successfully in human placental tissues (Mwenda *et al*, 1994) to investigate whether this was true due to unavailability of the template at the time when the research was going on. The

same investigator also showed that in the human, the placental RTase associated with isolated particles is Mg^{2+} dependent, and is able to utilize Poly(rC).Oligo$(dG)_{12-18}$. It is necessary to examine the template/primer preference of the baboon placental ERV particles before a definite conclusion on RTase activity associated with baboon placental tissues can be drawn. A comparative study of the template/primer preference of the human placental ERV particles indicated that the above-mentioned templates are necessary for specific detection of retroviral RTase activity whereas Poly(dC).Oligo$(dG)_{12-18}$ is essential for assessing contamination with cellular polymerases (Mwenda, 1993). Other investigators (Benveniste *et al*, 1974) had earlier used a Poly(A) template and Oligo$(dT)_{12-18}$ primer to assay RTase activity on M7 virus (an infectious C-type virus isolated from a baboon first trimester placenta and grown on FCf2Th cells (a fetal canine thymus cell line)) successfully. The apparent lack of RTase activity in the placental preparation may also be due to degradation of the template primer by RNase, (there is more RNase activity in placental tissues than in other tissues) which may explain why RTase activity was detected only in the SIV cytosolic preparation. RTase activity may also have been lost during the isolation of retroviral-like particles from the trophoblast.

(ii) It is possible that ERV particle expression may be reduced in third trimester placental tissues than in first trimester tissues. This had earlier been suggested by other investigators (Mondel & Hofschneider, 1982) who detected significant RTase activity in human first trimester placental tissues but hardly any in term placental tissues. The same might true for the baboon placental tissues, but this remains to be investigated. The level of ERV expression would be expected to be low in third trimester placental tissues if ERV expression is involved in syncytiotrophoblast formation since in third trimester tissues, the syncytiotrophoblast is fully formed. There are very few, if any cytotrophoblastic cells still present in the placental villi at term. However, earlier reports indicate that RTase from primate placental tissues is more difficult to purify compared to RTase attributed to exogenous retroviral particles. This is because of widespread existence of normal cellular DNA polymerases in this tissue and the extensive presence of proteinases which degrade RTase during the purification procedure (Vogel & Chandra, 1981). In this study, the positive control (SIV-infected molt-4 Cl_8 cytosolic cell preparation) maintained *in vitro*) always showed significant

RTase activity, while there was no significant difference between the negative control (uninfected molt-4 Cl_8 cell cytosolic cell preparation) and the assay blank. More specific methods of protein isolation and purification such as high performance liquid chromatography (HPLC) may need to be used to isolate sufficient RTase-rich material. This method had earlier been used successfully in the human placental tissues by other investigators (Mwenda *et al*, 1994).

The positive immunoperoxidase staining with monoclonal antibodies raised against exogenous (HIV) retroviral particles suggests that the particles being expressed in the syncytiotrophoblast share antigenic similarity to HIV-1 and HIV-2 glycoproteins (as shown by the positive immunoperoxidase staining of anti-HIV-1 gp41 and HIV-2ST gp120). This was consistent with the results from ultrastructural analyses which indicate the presence of retroviral particles in the syncytiotrophoblast. No cross-reactivity of these monoclonal antibodies with non-placental tissues was detected. This suggests that the retroviral-like particles were localized at the syncytiotrophoblast. The cross-reactivity of the mouse polyclonal antibodies (anti-BERV-13,14 & 17) with non-placental tissues indicate that the syncytiotrophoblast and the non-placental tissues share cross-reactive antigens. However, these antigens seem to be selectively expressed in some of the tissues. The staining of anti-BERVs on the spleen, the liver and the pancreas illustrates this point. The possibility that some of these antigens could be ERV gene products cannot be ruled out since it has been shown that in mice, some murine strains produce *env* gene products constitutively in the liver, kidney and epididymis (Shigemoto *et al*, 1992). The non-specificity of the polyclonal antibodies underlines the necessity for production of placental retroviral-specific monoclonal antibodies for use in ERV particle characterization.

A protein which is antigenically related to the HIV receptor molecule CD4 is also localized in the syncytiotrophoblast. This had been shown earlier to be present in most primate placentas, and its possible role in perinatal transmission of HIV from a sero-positive mother to the fetus postulated (Mitsuya *et al*, 1990). It is possible that the placental ERV particles on the syncytiotrophoblast may help prevent or reduce HIV transmission by receptor interference. The placenta has been shown to be susceptible to exogenous retroviral infection (Zachar *et al*, 1991; Kesson *et al*, 1993) but the perinatal transmission rate of HIV is moderate (30 to 50 %) (Katz & Wilfert, 1989). A retroviral-like antigen, gp120 has been identified in clusters of fibroblast-like cells in the human

mesenchymal stroma of the chorionic villi of normal human placental tissues (Faulk & Labarrere, 1991). In this study, a polyclonal antibody against HIV-2 gp120 protein (anti-HIV-2ST gp120, Table 3b) was strongly reactive on syncytiotrophoblast, with an indeterminate staining on mesenchyme. It is likely that the protein detected was a retroviral protein, identical to the envelope glycoprotein gp120 of HIV-1 and HIV-2 retroviruses, which interacts with the CD4 receptor molecule on CD4+ T-cells during infection. The expression of this glycoprotein on the placental villous tissues may help in preventing transplacental transmission of HIV in humans and SIV in monkeys. The molecule gp120 has been reported to interfere with the interaction of CD4 with class II antigens of the major histocompatibility complex antigen-presenting cells (Gay *et al*, 1987). It is possible that either cell-bound or soluble gp120-like molecules within chorionic villi may function as inhibitors of CD4 receptors on placental tissues, thus interfering with the normal mechanisms of infection by HIV. Another possibility resulting from this interaction is that it may result in inhibition of CD4 molecules on maternal lymphocytes when and if they gain access into the extra-embryonic tissues of the placenta. In this regard, it is significant that soluble gp120 has been shown to inhibit antigen-driven T-cell proliferation, and that this inhibition is blocked by soluble CD4 (Manca *et al*, 1990). The HIV-1 envelope glycoprotein gp120 interaction with the CD4 molecule has also been shown to interfere with the normal function of CD4, thereby contributing to the immunosuppression observed after HIV-1 infection (Diamond *et al*, 1988). Whether this mechanism plays a role in the immunobiology of pregnancy is not yet known.

On immunoblots, anti-ERV3 *env* (a monoclonal antibody reactive against *env* gene products) and anti-HERV-K RT both detected a 58 kDa protein on SIV positive blots and placental membrane preparation. Anti-HIV-2ST gp120 also detected the same protein. This suggests that this protein maybe an ERV gene product since it was not expressed in on SIV negative blots and the two antibodies are specific for *env* and *pol* products (envelope glycoproteins and reverse transcriptase, respectively). Envelope glycoproteins of many infectious retroviruses are known to induce formation of multinucleated giant cells by cell fusion (Owen *et al*, 1990). The mechanism of formation of syncytiotrophoblast have not been elucidated. The expression of ERV gene products in the placental villous tissue may suggest that ERV envelope glycoproteins may be involved in the fusion of the cytotrophoblast cells to form the syncytiotrophoblast in haemochorial

placentas of higher primates. Two other proteins that may have been retroviral proteins (of molecular weights 38 kDa and 64 kDa) were recognized by anti-HIV-1 p17. This antibody was a polyclonal antiserum specific for HIV-1 17 kDa protein as well as its parent and degradation products. This protein (the 38 kDa protein) may be similar to the 38 kDa molecule reported by Kitamura *et al* (1994) in human placental syncytiotrophoblasts. In this report, it was speculated that the 38 kDa molecule maybe a product of the *env* gene or related retroviral sequences because apparently gene products from retroviral sequences are often modified proteolytically just after translation. In addition, the 38 kDa molecule was not detected in the liver, which lacks *env* gene products (Rabson *et al*, 1983). Since other investigators have found retroviral products of differing size in these cells (Suni *et al*, 1981,1984), various types of endogenous retroviral products might be simultaneously expressed in chorionic villi of the placenta. Anti-HIV-1 p25/24 *gag* also detected a 52 kDa protein in placental membrane preparation and SIV positive blots. This antibody cross-reacts with all HIV-1 isolates tested to date. The above results confirmed the immunohistochemical results that show retroviral cross-reactive particles are expressed on the placental villous tissues.

On ELISA, the results were not conclusive. Most of the antibodies tested did not show any specificity for antigens expressed in the placental membrane preparation. Some of the antibodies like anti-HIV-1 p25/24, which is a polyclonal antibody reactive against viral p25/24 *gag* products, showed strong positive reaction on both the negative and the positive controls, and on the placental membrane preparation. Anti-SIVmac p17, a monoclonal antibody raised against a 17 kDa protein that may correspond to the p16 *gag* precursor protein was also strongly reactive on the placental membrane preparation and the uninfected cell preparation, but was negative on the SIV infected cell preparation. These results suggest that molt-4 cells and placental villous tissues express *gag* products or antigens cross-reactive with HIV-1 *gag* proteins. However, further research is needed before this conclusion can be arrived at. Both these antibodies were positive on western blot, detecting bands of 52 kDa and 46 kDa respectively on placental villous tissue preparation. However, they did not react on the placental sections. More investigations are necessary using more specific techniques like northern blots to detect mRNA, to confirm whether these were actually *gag* products. The polyclonal antibodies produced as

part of this project were non-specific in their reaction on ELISA, and no definite conclusion can be drawn as to their specificity to antigens expressed on placental tissues.

W6/32 (SeraLab,UK), a murine IgG_{2a} monoclonal antibody reactive with a monomorphic class I major histocompatibility complex (MHC) heavy chain determinant, used at 1:10 dilution of hybridoma culture supernatant) which was used as a positive control on immunohistochemistry, is known to be unreactive with human placental villous trophoblast, whereas extravillous cytotrophoblast in the placental bed is W6/32-reactive by immunohistochemistry (Redman *et al*, 1984; Bulmer & Johnson, 1985). In contrast, Stern *et al* (1987) have demonstrated that the syncytiotrophoblast is the only cellular component of baboon early placental villous tissue which is reactive with this monoclonal antibody. The same investigators also showed that the W6/32-reactive component has a molecular weight of 41 kDa, and is associated with β_2–microglobulin, whereas baboon peripheral lymphocytes express 45 kDa W6/32-reactive antigens comparable with HLA-A, B and C heavy chains of human lymphocytes. The positive reaction of W6/32 on the baboon placental villous tissue therefore indicated that major histocompatibility complex (MHC) class I-like antigens are expressed in the syncytiotrophoblast. It had earlier been shown that the human trophoblast does not express the classical class I MHC (HLA-A,B, & C) gene products, the usual targets for alloreactive cytolytic T cells, which may indeed be the factor contributing to the protected status of the fetus (Hunt *et al*, 1988). Instead, the human trophoblast expresses a distinctive class I MHC molecule, HLA-G which appears to be monomorphic and thus incapable of stimulating an alloreactive cytotoxic T-cell response (Geraghty *et al*, 1987). The HLA-G protein is expressed exclusively by extravillous cells of the human trophoblast (Schmidt & Orr, 1993). This property suggests that HLA-G may play a part in maintaining immunological tolerance between the mother and the semi-allogenic fetus (Parham, 1995). These antigens have been reported to serve in the development not of a typical immunological response but to the stimulation of pregnancy-specific pathways, where positive signals are transduced by the fetal components and harboured by the maternal immune system to support fetal survival (Athanassakis *et al*, 1995). Furthermore, class-II MHC molecules, which are necessary components for antigen recognition and development of a humoral or cellular immune reaction, are absent from all layers of the placenta (Athanassakis *et al*, 1989). It is possible that this absence supports a mechanism that protects the fetus from a typical

maternal immune response, whereas induction of class-II antigens could be the starting point of the fetal rejection procedure (Athanassakis *et al*, 1990). In view of the differences in immunoperoxidase staining observed in baboon and human placental trophoblast using W6/32 antibody, it would be interesting to evaluate the expression of HLA-G in the non-human primate placental tissues. However, further evaluation of HLA-G expression in baboon placental tissues has been hindered by limited availability of specific reagents (antibody and DNA probes). In this study, it should also be noted that W6/32 only showed a strong immunoperoxidase staining on the baboon syncytiotrophoblast and the adult baboon spleen sections, but was consistently negative on all the other sections tested. It was also negative on immunoblots. The significance of this is not known.

The precise biological functions of endogenous retroviral expression in the placenta of higher primates, including man , remains to be established. There is now clear evidence for the presence of retroviral particles. The presence of such retroviral particles within the normal primate placenta raises a number of questions. Among this is the possibility that ERV particles, or their constituent proteins, could function to provide the placenta with specific "retroviral" characteristics (Levy, 1986; Cohen & Larsson, 1988; Johnson *et al*, 1990). Possible examples would include:

i). Mediation of trophoblastic intercellular fusion by ERV-encoded proteins.

ii). Receptor interference of exogenous retrovirus binding to the trophoblast.

iii). Localized ERV-mediated immunosuppression related to allograft survival.

Identification and characterization of cross-reactive antigens that are expressed by normal placental tissues may lead to the elucidation of their biological roles at the feto-maternal interface. It is possible that endogenous retroviral proteins may down-regulate immune responses at the feto-maternal interface, leading to the survival of the fetal allograft. The baboon is an ideal non-human primate model for research in reproduction. The great apes, which are closest to man in many anatomical and physiological aspects of reproduction, are highly endangered species in the wild, hence are not practical models for research for most studies. The baboon is a suitable substitute. Detailed accounts of its implantation and fetal development are available (Hendrickx, 1971). It is a proven model in applied aspects of endocrinology, teratology and testing of steroidal contraceptive agents. It is a continuous breeder, breeds well in captivity, has a long menstrual cycle (21-34 days) with a reproductive hormone profile similar to that in humans and its

reproductive organs are similar to those of the human (Hendrickx, 1971), making the baboon an ideal model for research in reproduction. A lot of studies in male and female fertility regulation have been successfully conducted (Bambra, 1993 and references therein). It has also been shown to harbor the simian immunodeficiency virus (SIV) and can thus be used as a model for both heterosexual as well as perinatal transmission of SIV/HIV. A question that remains to be answered is whether the endogenous retroviral particles are involved in local pregnancy-related immunobiological events. If so, they could be suitable candidate targets for developing a contra-gestational vaccine to be used to promote immunological rejection of the blastocyst before or soon after implantation.

4.2. CONCLUSION.

Polyclonal and monoclonal antibodies have been produced and characterized using immunological and biochemical techniques. The polyclonal antibodies produced against isolated ERV particles from baboon third trimester placental tissues detected similar antigens to those detected by the anti-HIV and anti-SIV monoclonal antibodies on immunoblots. The imunohistochemical reactivity also indicated the presence of retroviral-like particles in placental villous tissues and their localization within the syncytiotrophoblast. This observation was consistent with the results from ultrastructural studies in the human placenta. The possibility of exogenous infections by SIV was ruled out by SIV testing. Thus it can be concluded that these particles were retroviral-related antigens and their expression in the normal baboon placental tissues may be as a result of endogenous retroviral genome.

4.3. SUGGESTIONS FOR FUTURE RESEARCH.

Available evidence and the results of the present study clearly indicate that more research remains to be done in order to understand the possible biological functions of the ERV particles in pregnancy-related immunosuppression. The following areas maybe considered for further research:

i). In this study, polyclonal antibodies were raised against placental retroviral-enriched material. These antibodies were characterized by various techniques. These approach may be followed to produce specific monoclonal antibodies.

ii). Further biochemical and serologic characterization of retroviral antigens expressed in normal baboon placental tissues in order to understand the possible role in the immunobiology of placental development and differentiation.

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APPENDICES.

Appendix I

Sex Skin evaluation.

Stage 0-Perineal skin is flat, pale pink in colour, and heavy deep wrinkles of sex skin, especially around the rectum, are visible. There is no swelling of the vulval lips. The stage lasts about 8 to 10 days.

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

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

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

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

Stage 5-Colour of the skin is deep red and shiny, and there is an absolute absence of wrinkles. The sex skin is large, and gives the impression of an over-inflated balloon. When viewed from the side, the whole rear end of the Animal appears inflated. Often, if the animal has been cycling frequently, the tautness of the skin is so extreme that cuts appear which bleed. The stage can last up to 10 days but may not be present in all animals.

Ovulation occurs in the last two or three days of the maximum inflation. Stage 1 to beginning with menstruation and ending with deturgescence, are referred to as: Inflation:

stage, pre-ovulatory phase, follicular phase, the period of turgescence, and the period tumescence respectively. The length is about 15 days.

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

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

Stage 8-This is the stage of pregnancy, and is denoted by a completely flat perineum of crimsom colour, and the sex skin is often seen flaking off. This stage lasts approximately 6 months.

Stage 8-This is the period post-partum and prior to resumption of cycling. It is the period of lactational Amenorhoea. The skin is flat and pinkish grey in colour.

(Adapted from Eley and Bambra, 1993).

Appendix II.

Preparation of Slides for frozen tissue sections

Arrange the slides in a carriage well.

1) Wash the slides in a xylene bath, then shake the carriage well on to paper towels.

2) Dip the slides into an alcohol bath, Then shake the carriage onto paper towels.

3) Dip the slides in distilled water.

4) Leave to dry in a 37°C cabinet.

5) Prepare a 0.5 % solution of Gelatin in distilled water (heat gently to dissolve).
Dip the dried slides into this.

6) Leave to dry.

7) Dip the slides in Formal saline (in chemical hood).
(Recipe -Formaldehyde (40 % technical) =10 mls.
-Sodium Chloride (NaCl) = 85 g
Dissolve in distilled water to make 100 mls.)

8) Dry the slides. They can be stored indefinitely at this stage.

Appendix III.

a) Solubilization buffer (3X)

To make 10 mls, take -

-Sodium dodecyl sulfate (SDS)	: 0.6 grams
-1 M Trizma buffer, pH 6.8	: 1.875 mls
-Glycerol	: 3 mls
-Distilled water	: 7 mls

b)(i) Coomassie Blue stain

To make 200 mls, take -

-Coomassie Brilliant Blue250	: 1.0 g
-Ethanol	: 90 mls
-Glacial Acetic Acid	: 20 mls
-Distilled water	: 90 mls.

Filter the solution through a No 2 Whatman filter paper.

(ii) Destain solution

The destain solution contains all the above except the Brilliant Blue powder or solution

c) Ponceaus stain

-0.1 g Ponceaus powder

(3-Hydroxy-4-[2-sulfo-4(4-sulfophenylazo)phenylazo]-2,7-naphthalenedisulfonic acid) ($C_{22}H_{12}N_4O_{13}S_4Na_4$).

-1 ml of 1 % Acetic Acid

Make up to 100 mls with distilled water

d) (i) Stock solution of DAB

40 mg DAB (Diamino-Benzidine Tetrahydrochloride) dissolved in 1 ml distilled water

Aliquot in 100 or 200 µl and store at -20°C in the dark

(ii) Working solution

-Stock DAB : 200 µl

-TBS/PBS, pH 7.2-7.4 : 10 mls

-8 % $NiCl_2$: 50 µl

-30 % H_2O_2 : 30 µl

Acrylamide stock (30 %)

Acrylamide powder : 29.2 grams

Bis-acrylamide : 0.8 grams

Dissolve in 100 mls of distilled water, and filter through 0.45 µ filter.

Running Gel

	10 %	15 %	20 %	12.5 %
L buffer, pH 8.8	2.61 ml	2.61 ml	2.61 ml	2.61 ml
30 % Acrylamide	3.60 ml	5.25 ml	6.99 ml	4.38 ml
Distilled water	4.35 ml	2.60 ml	0.855 ml	3.465 ml
TEMED	4.80 µl	4.80 µl	4.80 µl	4.80 µl
10 % APS	39.8 µl	39.8 µl	39.8 µl	39.8 µl

TEMED : N,N,N'N'-Tetramethyl-ethylenediamine ($C_6H_{16}N_2$)

APS : Ammonium persulfate.

Stacking Gel

M-buffer, pH 6.8	:	1.26 mls
Acrylamide stock	:	0.25 mls
Distilled water	:	3.01 mls
TEMED	:	4.00 µl
10 % APS	:	24.00 µl

Running Gel buffer (L buffer)

1.5 M Trizma base	:	18.2 grams
0.4 % SDS	:	0.4 grams.

Add distilled water to make 100 mls and pH 8.8

Stacking Gel buffer (M-buffer).

0.5 M Trizma base	:	6.0 grams
0.4 % SDS	:	0.4 grams.

Add distilled water to make 100 mls and pH 6.8

Tank buffer (Running buffer).

0.025 M Trizma base	:	3.0 grams
0.192 M Glycine	:	14.4 grams
0.1 % SDS	:	1.0 grams

Add distilled water to make 1 litre.

Transfer buffer.

Methanol	:	20.0 mls
10 % SDS	:	0.75 mls
Glycine	:	0.58 grams.
Trizma base	:	1.16 grams.

Add 180 mls distilled water to make 200 mls.

Appendix IV.

ELISA buffers

a) 0.05 M Carbonate buffer, pH 9.6

- $NaHCO_3$: 2.1 g
- NaOH : 0.25 g

Dissolve in distilled water to make 1 litre and adjust pH to 9.6.

b) Phosphate-buffered Saline (PBS)(10X)

NaCl	: 80 g
KCl	: 2 g
$Na_2HPO_4.12H_2O$	: 29 g (11.5 g anhydrous).
KH_2PO_4	: 2 g

Dissolve in distilled water to make 1 litre, adjust pH to 7.4 and dilute 10 times before use.

c) 0.2 M Phosphate-Citrate buffer, pH 5.5

$Na_2HPO_4.2H_2O$	: 18.3 g
Citric acid H_2O	: 10.22 g

Make up to 1 litre with distilled water and adjust pH to 5.5

d) Others.

⇒ 0.1 % Glutaraldehyde in PBS

⇒ PBS-Tween (0.05 %)

⇒ 3 % BSA in PBS

⇒ Antibody dilution buffer:- 5 % non-fat skimmed milk (Marvel, Cadbury's, UK) in PBS, pH 7.4. (Weigh 5 g and dissolve in 100 mls of 1X PBS, pH 7.4).

⇒ 4 M H_2SO_4

Appendix V

BioRad Protein Assay

The standard protein stock solution provided with the kit contains 1.39 mg/ml of Bovine Serum Albumin (BSA). A serial dilution was made.

Procedure.

6 Eppendorf tubes were labeled std 1 to 6. 200 µl of PBS was pipetted into tubes 2 to 6. 200 µl of the stock solution was pipetted into std 1. 200 µl of the stock was then pipetted into std 2, vortexed to mix with the PBS, then 200 µl of std 2 was transferred to std 3, vortexed and the same procedure repeated. The samples being tested were diluted 1:100 (2 µl made up to 200 µl with distilled water). Clean dry test-tubes were labeled in duplicates for the standards and the samples. 100 µl of each standard and sample solution was pipetted into the corresponding labeled test-tubes. The dye reagent was prepared by diluting 1 part dye reagent with 4 parts distilled water and the resulting solution filtered through # 1 whatman paper. 5 mls of the diluted dye was added to each tube and vortexed. Then the tubes were incubated for 5 minutes. The absorbance was measured at 595 nm using a double beam spectrophotometer (Cecil Instruments, UK). A graph of the absorbance of the standards against their concentration was then drawn. The concentration of the samples was estimated from the graph.

Appendix VI

Culture Media

Preparation of RPMI 1640 (Autoclavable Powder)

1) Prepare sterile 1000 cm^3 of double distilled water in a bottle
2). Weigh out 5.1 grams of RPMI 1640 powder in a sterile beaker
3). Suspend the powder in double distilled water (approximately 20 ml)
4). Pour the above suspension into a 500 ml sterile bottle and make up to 500 mls with double distilled water
5). Autoclave at 121°C for 15 minutes.
6). Cool down the above in a Refrigerator.
7). Add 15 mls of 7.5 % $NaHCO_3$.
8). Add 5 mls of Penicillin/Streptomycin stock (100 IU/ml penicillin and 100 µg/ml streptomycin)
9). Add Glutamine (7.5 mls for 500 mls)
10) Add 55 µl of 10 % Fetal calf serum (FCS).
11).Mix well, aliquot and freeze stock at -20°C until use.